약학박사학위논문

간암 진행과정에서 전이활성인자 MTA1의 전사조절 기전 규명

The mechanism of transcriptional regulation of metastasis-associated protein 1 gene expression during hepatocarcinogenesis

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ABSTRACT

The mechanism of transcriptional regulation of metastasis-associated protein 1 gene expression during hepatocarcinogenesis

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Expression of metastasis-associated protein 1 (MTA1) gene correlates with the degree of invasion and metastasis in hepatocellular carcinoma (HCC); however, little is known about the transcriptional regulation of the MTA1 gene.

The first part of this study identified the MTA1 gene as a target of p53-induced transrepression. The MTA1 promoter contains three putative p53 response elements (p53REs), which were repressed by the p53-inducing drug 5-fluorouracil (5-FU). Notably, 5-FU treatment decreased MTA1 expression only in p53 wild-type cells. p53 and histone deacetylases 1/2 were recruited to the p53REs, but H3K9Ac was released after 5-FU treatment. Proteomics analysis of the p53 repressor complex, which was

pulled down by the MTA1 promoter, revealed that the poly(ADP-ribose) polymerase 1 (PARP-1) was part of the complex. Interestingly, p53 was poly(ADP-ribose)ylated by PARP-1, and the p53-mediated transrepression of the MTA1 gene required poly(ADP-ribose)ylation of p53. In summary, we report a novel function for poly(ADP-ribose)ylation of p53 in the gene-specific regulation of the transcriptional mode of p53 on the promoter of MTA1.

Expression of MTA1 is induced by hepatitis B virus X protein (HBx); however, little is known about the transcriptional regulation of MTA1 gene expression. In second part of this study we report that the 5′-flanking region of the human MTA1 promoter contains two CpG islands. Transient expression of HBx in Chang liver cells increased the methylation of the CpG island1 from 18% to 49% when measured by bisulfite-modified direct sequencing. Chromatin immunoprecipitation showed that HBx recruited DNMT3a and DNMT3b to the CpG island1. In silico analysis of CpG island1 predicted the existence of putative p53-binding sequences. p53 was pulled down by a DNA probe encoding the p53-binding sequences but not by the methylated DNA probe. The mouse MTA1 promoter also contains a CpG island encoding a p53-binding sequence of which p53 binding was decreased in the presence of HBx, and expression of MTA1 and DNMT3

was increased in the liver of HBx-transgenic mice. Comparison of MTA1 and DNMT3a expression in human normal liver and HCC specimens produced a significant correlation coefficient >0.5 (r = 0.5686, P = 0.0001) for DNMT3a, and a marginally significant coefficient (r = 0.3162, P = 0.0103) for DNMT3b. These data show that HBx induces methylation of CpG island in the MTA1 promoter, which interferes with DNA binding of p53 in the specific DNA region. This result may explain the molecular mechanism responsible for the induction of MTA1 gene expression by HBx.

Key words : MTA1; p53; PARP-1; poly(ADP-ribose)ylation; HBx; Hepatocellular carcinoma; DNA methylation

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

1. Metastasis-associated protein 1

Metastasis is a complex disease involving a series of discrete biological processes that move tumor cells from the primary neoplasm to a distant location. Tumor cells invade the tissue surrounding the primary tumor, enter either the lymphatics or the bloodstream, survive and eventually arrest in the circulation, extravasate into a tissue and grow at the new site (Figure 1**).**

Metastasis-associated protein 1 (MTA1) was discovered in a differential hybridization screen conducted to identify the candidate genes selectively expressed between highly metastatic and nonmetastatic 13762 NF rat mammary adenocarcinoma cell lines (Toh *et al*., 1994). A homologous gene was also expressed in human cancer cell lines, and its human cDNA counterpart, MTA1, was cloned by Nawa *et al*. in 2000 (Nawa *et al*., 2000). After, several reports from independent research groups showed well correlated with the degree of invasion and lymphatic metastasis in colorectal, ovarian, prostate, hepatocellular and breast carcinomas (Toh and Nicolson. 2009) and consider strong candidate for a cancer metastasis promoting gene.

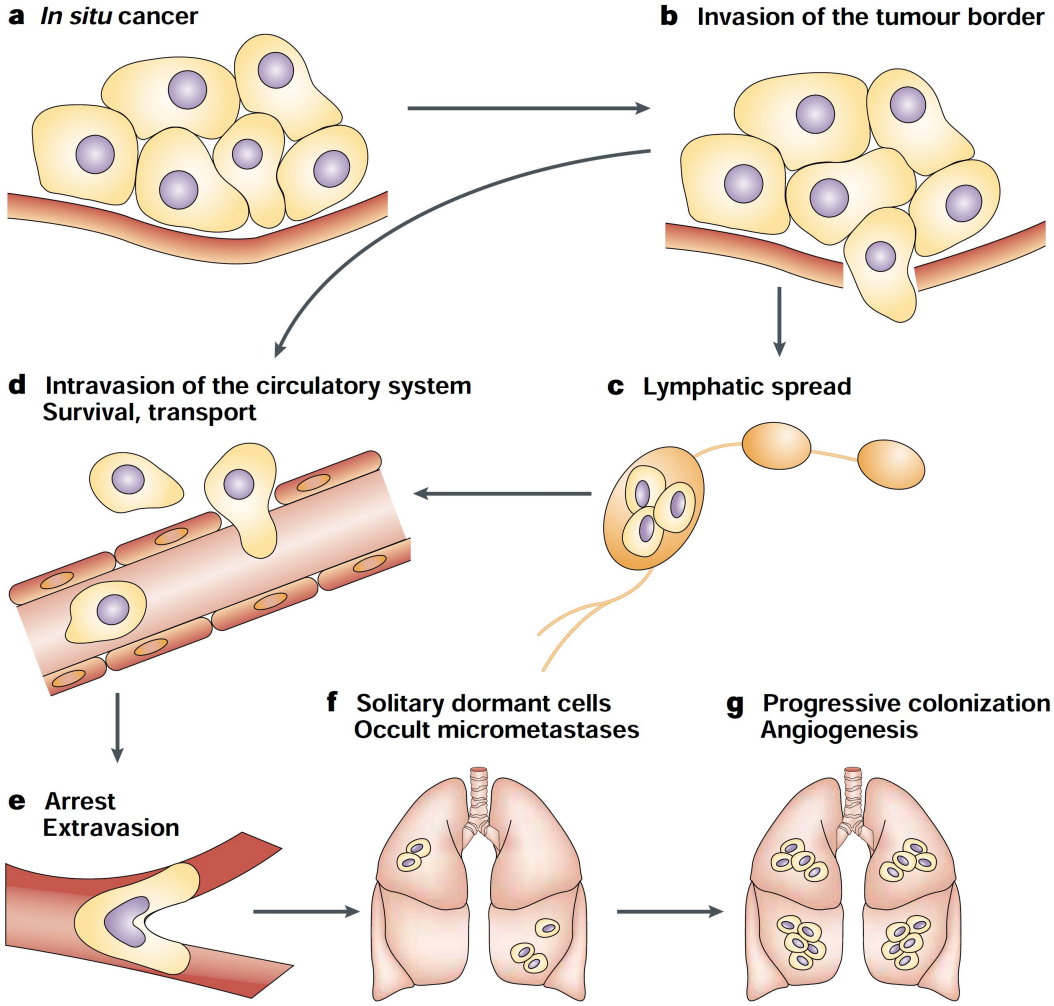

a In situ cancer
b Invasion of the tumour border
c Lymphatic spread
d Intravasion of the circulatory system
Survival, transport
e Arrest
Extravasion
f Solitary dormant cells
Occult micrometastases
g Progressive colonization
Angiogenesis

Figure 1. The tumor metastatic process. (A) Cellular transformation and tumour growth. (B) Invasion requires reversible changes in cell–cell and cell–extracellular-matrix adherence, destruction of proteins in the matrix and stroma, and motility. Metastasizing cells can (C) enter via the lymphatics, or (D) directly enter the circulation. (E) Most circulating tumour cells being rapidly destroyed. After the tumour cells have survived the circulation, they become trapped in the capillary beds of distant organs by adhering either to capillary endothelial cells or to subendothelial basement membrane that might be exposed. (F) Metastatic colonization of the distant site progresses through single cells, which might remain dormant for years, to occult micrometastases and (G) progressively growing, angiogenic metastases. (Steeg, 2003)

1.1. Domain structure of the MTA1 protein

MTA proteins represent a family of gene products encoded by three distinct genes, i.e. MTA1 at 14q32, MTA2 at 11q12-q13.1, and MTA3 at 2p21, and six reported isoforms (MTA1, MTA1s, MTA1-ZG29p, MTA2, MTA3, and MTA3L (Figure. 2A). The molecular weights of the gene products of MTA1, MTA2, and MTA3 are approximately 80, 70, and 65 kDa, respectively. Analysis of MTA proteins shows that human MTA2 and MTA3 are 68 and 73 % identical to human MTA1, respectively, with the highest homology concentrated in the N-terminal half of the proteins. In contrast, the C-terminal halves of MTA proteins are divergent (Manavathi and Kumar. 2007). The MTA1 proteins contain one BAH (bromo-adjacent homology) domain, one ELM (egl-27 and MTA1 homology) domain, and one SANT (SWI, ADA2, N-CoR, TFIIIB-B) domain that is similar to the DNA binding domain of Myb-related proteins. BAH domain is involved in protein-protein interactions, whereas the SANT domain binds to histone tails. The ELM domain has an unknown function. MTA1 contain a GATA zinc finger DNA binding domain (Figure. 2B) (Manavathi and Kumar. 2007).

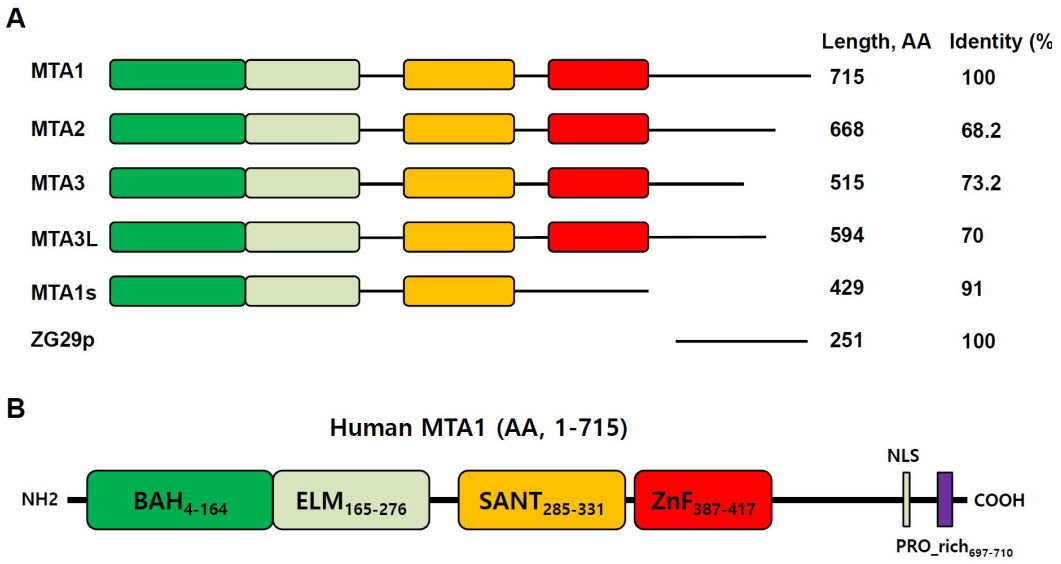


Figure 2. Schematic representation of comparison of structural domains among MTA family members. (A) comparison of various domains among the MTA family proteins. (B) schematic illustration of MTA1 protein. BAH, bromo-adjacent homology; ELM, egl-27 and MTA1 homology; SANT, SWI, ADA2, N-CoR, TFIIIB-B; ZnF, zinc finger; NLS, nuclear localization signal; AA, amino acids; PRO-rich, proline-rich domain.

1.2. Biological role of MTA1

The metastasis-associated protein 1 (MTA1) is a component of the nucleosome remodeling and deacetylation (NuRD) complex, which functions as a transcriptional repressor (Denslow and Wade, 2007) (Figure 3**)**. MTA1 is regarded as a candidate metastasis-associated gene, as it is overexpressed in various human cancers, such as breast, gastrointestinal, prostate, and hepatic cancers, the pathophysiological features of which correlate well with tumorigenesis characterized by invasion and metastasis (Toh and Nicolson, 2009). Recently, we and others provided molecular links that connect the overexpression of MTA1 with progression to malignancy. MTA1 binds and activates the hypoxia-inducible factor-1 alpha (HIF-1α), an important transcriptional regulator under hypoxia, which promotes the metastatic potential of cancer cells (Moon *et al*., 2006; Yoo *et al*., 2006). The MTA1 protein is stabilized by DNA damage induced by ionizing or UV radiation, leading to deregulation of the DNA damage repair system, which comprises p53, p21/WAF, and ATR (Li *et al*., 2009a, 2009b, 2010a, 2010b). Further, MTA1-NuRD complex targets repression of BRCA1 and 15-LOX-1 gene expression, while it activates gene expression of BCAS3 and Pax5, which are important regulators in carcinogenesis of breast, colon and lymphoma (Gururaj *et al*., 2006; Balasenthil *et al*., 2007; Molli *et al*., 2008; Zuo *et*

al., 2009). Importantly, knockdown of MTA1 suppressed cell migration and invasion, which reversed the malignant phenotypes *in vitro*, and delayed tumor development in mouse xenografts (Qian *et al*., 2005; Kai *et al*., 2011).

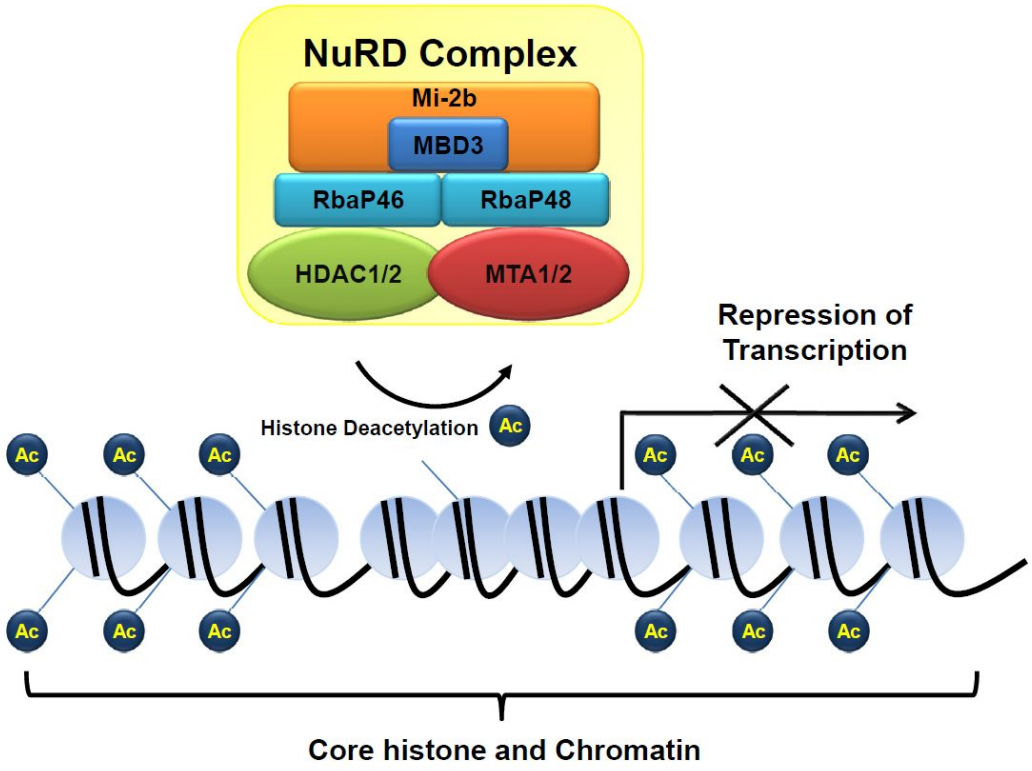


Figure 3. Schematic representation of components of NuRD complexes. The fundamental function of MTA proteins is chromatin remodeling and histone deacetylation, resulting in repression of transcription. MTA proteins are included in the protein complex named NuRD, which also contains histone deacetylases (HDAC1 and 2), Methyl-CpG-binding domain protein (MDB3), histone binding proteins RbAp46/48 and the dermatomyositis-specific autoantigen Mi-2, and has strong transcription repressing activities.

1.3. Regulation of MTA1 Gene Expression

Although various studies has identified a downstream targets of MTA1 proteins, the regulation mechanism of MTA1 gene expression is poorly understood. Previously, hypoxia, Heregulin-β1 and hepatitis B virus X protein (HBx) are known to activate transcriptional expression of MTA1 (Mazumdar *et al*., 2001; Yoo *et al*., 2006; Yoo *et al*., 2008). NF-κB signaling and c-MYC oncoprotein is also reported to upregulate MTA1 expression in human cancers (Zhang *et al*., 2005; Bui-Nguyen et al., 2010a).

2. The role of p53 in cancer

The tumor suppressor p53 is the best-studied transcriptional factor and has a broad spectrum of functions in cellular physiology, including cell-cycle arrest, apoptosis, DNA repair, and autophagy (Vousden and Lane, 2007). Recently, several reports indicated that p53 is also involved in cancer progression by regulating cancer invasion and metastasis specifically. Wild-type p53 induced mouse double-minute-2-mediated degradation of Slug, which is a member of the Snail family of zinc finger transcription factors that represses E-cadherin expression, resulting in the suppression of cancer cell invasion (Wang *et al*., 2009). p53 repressed the transcriptional

expression of CD44, which is a transmembrane cell-surface protein that promotes tumor growth and metastasis (Godar *et al*., 2008). Moreover, loss of p53 function enhanced invasion and metastasis in a number of *in vivo* models of metastatic pancreatic tumors and hepatocellular carcinoma (Lewis *et al*., 2005; Morton *et al*., 2008), which suggests that p53 is involved in the regulation of many metastasis-associated genes, which probably include MTA1.

p53 facilitates transcriptionally positive or negative regulation of gene expression. The well-defined p53-associated activating complexes usually include histone acetyltransferases, such as the CREB-binding protein, p300, and p300/CBP-associated factor, and induce the transcriptional expression of genes such as p21, GADD45a, and BCL2-associated X protein (BAX) (Riley *et al*., 2008). p53 also *trans*-represses the expression of genes associated with oncogenesis and cancer progression, such as survivin and the vascular endothelial growth factor (VEGF). However, functional differences between the *trans*-activating and *trans*-repressing complexes of p53 and their DNA-binding elements are important to understand p53 function fully. Posttranslational modifications (PTMs), such as phosphorylation, acetylation, and ubiquitination, are also considered as important factors that lead to alteration of the transcriptional activity of p53. In addition, p53 is a

substrate for O-linked N-acetylglucosamine glycosylation and prolyl isomerization, which function in the regulation of the stability and transactivation activity of the protein (Meek and Anderson, 2009). PTM of p53 has been extended to include poly(ADP-ribose)ylation (PARylation); however, its role in the transcriptional function of p53 is poorly understood (Wesierska-Gadek *et al*., 1996; Kumari *et al*., 1998; Simbulan-Rosenthal *et al*., 1999).

3. Transcriptional control by PARP-1

Poly(ADP-ribose) polymerase-1 (PARP-1) is an abundant and ubiquitous nuclear enzyme (D'Amours *et al*., 1999). PARP-1, encoded by the ADPRT (ADP-ribosyl transferase) gene, is an abundant and highly conserved chromatin bound enzyme which binds to nicked DNA as a homodimer and mediates protection against DNA damage. Poly(ADP-ribose) polymers have a short half life because of their rapid hydrolysis by the enzyme poly(ADPribose) glycohydrolase (PARG). When DNA is mildly damaged, PARP-1 is activated and participates in the DNA repair process so that the cell survives (Figure 4). However, in the case of extensive DNA damage, PARP-1 is overactivated and induces a depletion of cellular NAD+ and ATP levels leading to cell dysfunction or even to necrotic cell death (Martin *et al*., 2000; Cepeda *et al*., 2006).

Recent studies suggest that poly(ADP-Ribose) polymerase-1 (PARP-1) which plays roles in a variety of cellular processes, including transcription, cell-cycle, and cellular signaling as well as DNA repair, is an important player in PTMs of cellular proteins (Ziegler and Oei, 2001). Originally, PARP-1 is well-known as an abundant nuclear enzyme that poly(ADP-ribosyl)ates (PARylates) both DNA and histones, leading to

modify chromatin structure (Kraus and Hottiger, 2013). Several reports have shown another potential of the enzymatic function of PARP-1. That is, PARP-1 directly PARylates chromatin-associated proteins and transcriptional factors, as well as histones, to alter their own molecular action modules. For example, PARP-1 interacts with and PARylates Smad3/4 complex, consequently controlling Smad-mediated transcription (Lönn *et al*., 2010). In another example, p53 is known to be modified via PARylation by PARP-1 and blocked export to the cytoplasm (Kanai *et al*., 2007). However, alteration of the transcriptional activity of PARylated p53 is not reported.

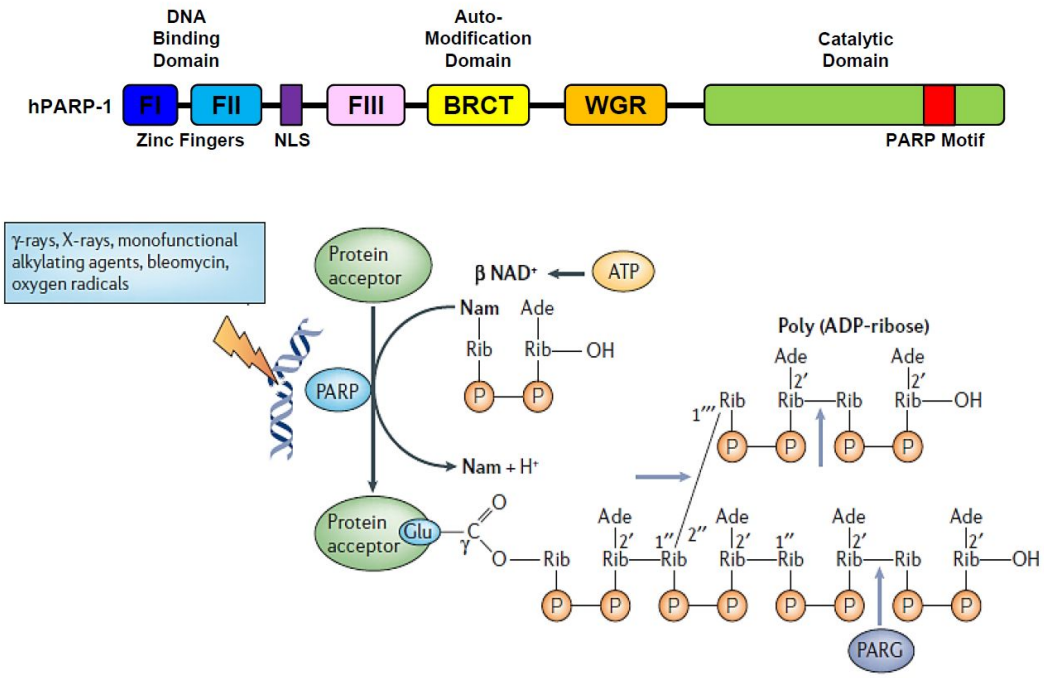


Figure 4. Structural and Functional Organization of PARP-1. The major functional units of PARP-1 are an amino-terminal DNA-binding domain (DBD), a central automodification domain (AMD), and a carboxyterminal catalytic domain (CD). In response to DNA-strand breaks, poly(ADP-ribose) polymerases-1 (PARP-1) hydrolyse NAD+, releasing nicotinamide (Nam) and one proton (H+), and catalyse the successive transfer of the ADP-ribose moiety to nuclear protein acceptors that might be transiently inactivated. The same chemistry is used during the elongation and branching reactions. The degradative nuclear enzyme poly(ADP-ribose) glycohydrolase (PARG) has endo- and exoglycolytic activities that cleave glycosidic bonds between ADP-ribose units (Schreiber *et al*., 2006).

4. Hepatitis B Virus X Protein: Structure, Function and Biology

The worldwide prevalence of hepatitis B virus (HBV) infection, is estimated to be as high as 4–5% of the earth's total population. With more than 350 million chronic HBV carriers, this virus stands as one of the most common human pathogens and it causes significant public health problems (Ganem *et al*., 2004). Chronic infection by hepatitis B virus (HBV) is a major cause of the development and progression of hepatocellular carcinoma (HCC) (Neuveut *et al*., 2010).

HBV is the prototype member of a family of small enveloped DNA virus called hepadnaviruses. The genome of hepadnaviruses is a relaxed circular, partially double-stranded DNA genome that replicates via an RNA intermediate **(**Figure 5**).** The HBV genome is around 3.2 kb in length and presents a highly compact genetic organization with 4 overlapping open reading frames (ORFs) that cover the entire genome. The pre-S/S ORF encodes the three viral surface proteins, the pre-C/C ORF encodes the eantigen (HBeAg) and the core antigen (HBcAg), the P ORF encodes the terminal protein (TP) and the viral polymerase that possesses DNA polymerase, reverse transcriptase and RNaseH activities. The X gene encodes

a small protein that is essential for virus replication **(**Figure 5**)** (Neuveut *et al*., 2010).

Among the viral proteins encoded in the 3.2 kb HBV genome, HBV X (HBx) protein is implicated in hepatocellular carcinogenesis because it leads to modulation of transcriptional expression of many cellular genes involved in oncogenesis, proliferation, inflammation, and immune responses (Neuveut *et al*., 2010; Zhang *et al*., 2006). Overexpression of HBx transforms rodent hepatocytes in vitro (Seife *et al*., 1991) and it enhanced the survival and proliferative potential of liver cells (Yun *et al*., 2002). Decisively, the transgenic mice encoding HBx were shown to either induce HCC or to potentiate c-myc-induced HCC (Kim *et al*., 1991). The HBx transgenic mice were also susceptible to chemical carcinogen-induced liver cancer (Slagle *et al*., 1996). Recently published data support the idea that HBx plays critical roles in the invasive and metastatic properties of intrahepatic and extrahepatic metastases of HCC. The balance between adhesion and de-adhesion in cells is modulated by HBx, which may facilitate integrin-mediated cell migration in primary tumor sites (Lara-Pezzi *et al*., 2001). HBx increases the invasion potential by upregulating the expression of the membrane-type matrix metalloproteinases and miR-143, which contributes to tumor metastasis (Ou *et al*., 2007; Zhang *et al*., 2009).

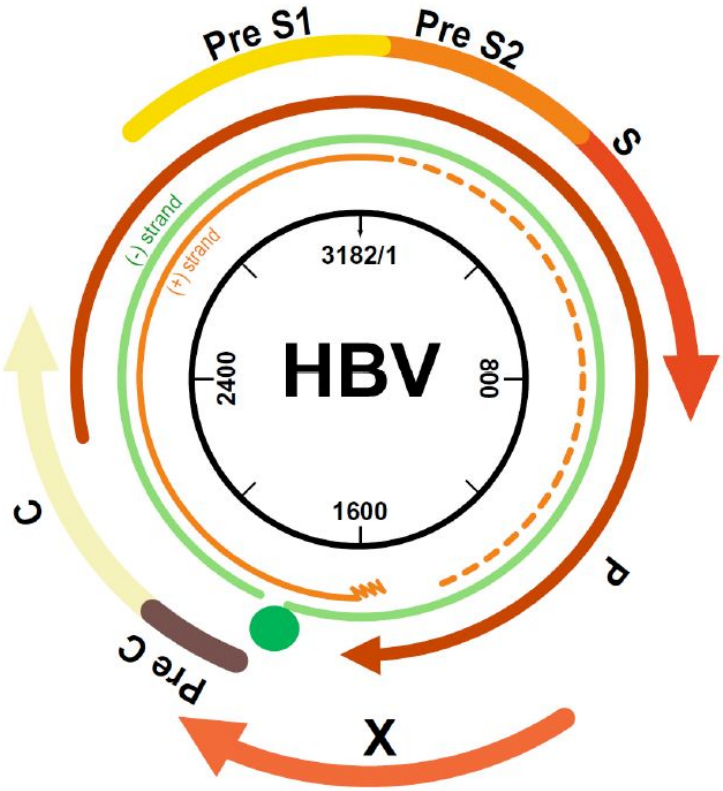


Figure 5. Schematic representation of the circular, partially double-stranded HBV DNA genome, which contains four ORF coding for polymerase (P), surface antigens (PreS1, PreS2, and S), precore (PreC), core (C), and X (Neuveut *et al*., 2010).

5. HBx protein is involved in epigenetic modifications

Epigenetic modifications are defined as heritable changes in gene expression occurring without alteration of underlying DNA sequences and comprise many layers of complexity including DNA methylation, histone modifications, and chromatin remodeling (Taby and Issa, 2010). DNA methylation is a covalent modification of the cytosine ring at the 5′ position of a CpG dinucleotide and is performed by DNA methyltransferases (DNMTs) (Klose and Bird, 2006). Three active DNA methyltransferases (DNMT1, DNMT3a and DNMT3b) have been identified in mammals (Figure 6). The classical maintenance methyltransferase is known as DNMT1. DNMT1 is primarily a maintenance methyltransferase that preserves methylation patterns during cell division. Two other enzyme families, DNMT3a and DNMT3b, participate in establishment of *de novo* methylation patterns during early embryonic development (Denis *et al*., 2011).

In HCC, the epigenetic inactivation caused by DNA hypermethylation has been established for several tumor suppressors and adhesion molecules such as p16INK4A, p14ARF, and cadherins (Tischoff and Tannapfe, 2008). For example, hypermethylation around the E-cadherin promoter region is associated with reduced E-cadherin level in HCC (Wei *et*

al., 2002). Methylation-induced silencing of the M-cadherin gene has significance as a prognostic indicator of poor survival in HCC patients (Yamada *et al*., 2007). The promoter of tissue factor pathway inhibitor-2, a Kunitz-type serine protease inhibitor that represses cellular invasion, is highly methylated and is repressed in human HCC (Wong *et al*., 2007). However, the epigenetic regulation of the MTA1 gene promoter has not been reported.

HBx protein is involved in epigenetic modifications during hepatocarcinogenesis by increasing the recruitment of DNMTs and methyl-binding proteins to the promoters of target genes. The cell-cell adhesion molecule E-cadherin has been assumed to act as a tumor suppressor negatively regulating several critical steps of invasion and metastasis. HBx protein induces transcriptional activation of DNMT1, resulting in DNA hypermethylation of inactivate E-cadherin promoter and repressing its expression (Lee *et al*., 2005). A similar pathway can also inactivate a negative regulator of cell cycle progression gene p16INK4A expression (Zhu *et al*., 2010). Transiently transfected with HBx, hypermethylation of the insulin-like growth factor-binding protein-3 (IGFBP-3) promoter region is observed, mediated by DNMT1 and DNMT3A1 and 3A2 (Park *et al*., 2007). HBx protein-induced hypermethylation of retinoic acid receptor-β2 (RAR-β2) via up-regulation of

DNMT1 and DNMT3A results in down-regulation of its expression in HCC cells (Jung *et al*., 2010). HBx also induces hypermethylation of the promoters of genes such SH3 domain-containing, and proline-rich region-containing protein 1 (ASPP1), and ASPP2 by recruiting DNMT1, DNMT3a1, and DNMT3a2 (Zhao *et al*., 2010).

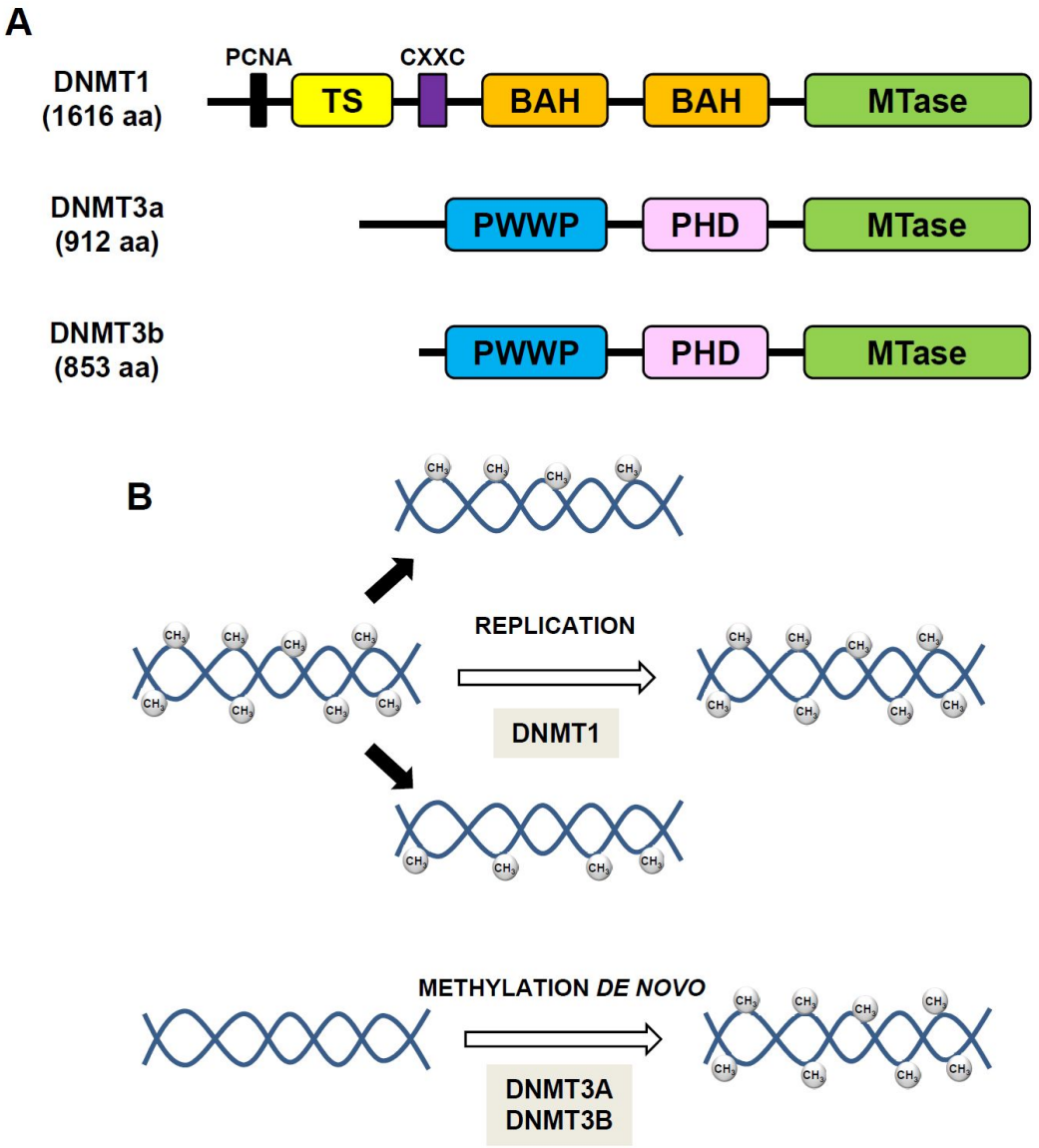

A
PCNA
CXXC
DNMT1
(1616 aa)
TS
BAH
BAH
MTase
DNMT3a
(912 aa)
PWWP
PHD
MTase
DNMT3b
(853 aa)
PWWP
PHD
MTase
B
REPLICATION
DNMT1
METHYLATION DE NOVO
DNMT3A
DNMT3B

Figure 6. Schematic representation of the human DNA methyltransferase. (A) Dnmts share a conserved catalytic domain (MTase), but differ in their N-terminal regulatory regions. Dnmt1 contains a proliferating cell nuclear antigen (PCNA) binding domain, a pericentric heterochromatin targeting sequence (TS), a CXXC domain, and two bromo adjacent homology domains (BAH). Dnmt3a and 3b comprise a PWWP domain named after a conserved Pro-Trp-Trp-Pro motif and an PHD (plant homeodomain) domain. (B) Model for the distinct roles of Dnmt1 and Dnmt3a/3b in de novo and maintenance methylation.

Purpose of the study

MTA1 is normally present only at low levels in various tissues, but there are elevated MTA1 levels in a variety of tumor types such as breast, gastrointestinal, prostate, and hepatic cancers. An elevated expression of MTA1 gene is associated with tumorigenesis characterized by invasion and metastasis. Despite of the important relationship between MTA1 gene expression and cancer progression, the regulation of MTA1 gene expression is little known. Therefore, **First: we studied the identification of transcriptional regulatory mechanisms of MTA1 gene. This study aims to analysis of the potential transcriptional factor binding sites on the promoter region of human MTA1 gene and identification of the transcriptional regulatory mechanisms of the MTA1 gene.**

Previously, we reported that expression of MTA1 was induced by hepatitis B virus X protein (HBx) (Yoo *et al*., 2008). However, the mechanism of HBx induced MTA1 exrpession is still little known. Therefore, **Second, we studied the mechanism of HBx induced MTA1 gene expression. This study aims to analysis of the promoter region of human MTA1 gene and to identify the molecular mechanism of HBx induced MTA1 gene expression.**

II. MATERIALS AND METHODS

1. Cells and Cell Culture

Chang, WRL-68, MCF7, MDA-MB-231, Hep3B, HepG2 and NIH3T3 cell lines were obtained from American Type Culture Collection. HCT116 and p53 isogenic derivative colon cancer cells were provided kindly by Dr. Bert Vogelstein (The Johns Hopkins University School of Medicine, Baltimore, MD). Wild-type and p53-deficient MEFs were provided kindly by Dr. Han-Woong Lee (Yonsei University). MEFs were used at passages 3–5. Cells were maintained in either Dulbecco's Modified Eagle's Medium or McCoy's 5A Medium containing 10% fetal bovine serum in an incubator with 5% CO_2 and 95% air at 37°C. 5-FU and PHEN were purchased from Sigma–Aldrich. Chang X-34 cells, in which HBx gene expression is under the control of a doxycycline-inducible promoter.

2. Plasmids, si-RNA, and Transient Transfection

The p53 eukaryotic expression vector was provided by Dr. Bert Vogelstein (The Johns Hopkins University School of Medicine) and subcloned into the pCMV–Myc vector. The *MTA1* promoter (–860 to +216 relative to the transcription initiation site) was cloned from a human BAC genomic clone

(RZPD) *via* PCR amplification and subsequent insertion into the SacI/NheI site of the pGL2-Basic vector (Promega, Madison, WI, USA). The eukaryotic expression vectors for p3XFLAG7.1-HBx was described previously (Yoo *et al*., 2008). The si-RNA duplexes targeting p53 (5'-GCAUGAACCGGAGGCCCAUTT-3' and 5'-AUGGGCCUCCGGUUCA UGCTT-3), si-DNMT3a (5'-CAACAUCGAAUCCAUGAAAUU-3' and 5'-UUUCAUGGAUUCGAUGUUGUU-3') and si-DNMT3b (5'-ACGCACA GCUGACGACUCAUU-3' and 5'-UGAGUCGUCAGCUGUGCGUUU-3'), and control nonspecific si-RNA were synthesized and purified by ST Pharm. si-PARP-1 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz Biotech). Transient transfection and reporter gene analysis were performed as described previously (Yoo *et al*., 2008). The significance of any differences was determined using Student's *t* tests and was expressed as a probability value. Mean differences were considered significant at $P < 0.05$.

3. Western Blot Analysis, Immunoprecipitation, RT-PCR

Western blotting and immunoprecipitation were carried out as using specific antibodies against MTA1, p53 (DO-1), PARP-1, HDAC-1, HDAC-2 (Santa Cruz Biotech), PAR (Trevigen), or a-tubulin (Calbiochem). RT–PCR was carried out as described previously (Yoo et. al., 2008) Briefly, 1.5 μg RNA

was mixed 45 ng random hexamer (Invitrogen) and incubated for 5min at 65℃ and then added reaction mixture containing 200U M-MLV reverse transcriptase, 10mM dNTP, 0.1 M DTT and 5X first strand buffer (Invitrogen) in a final volume of 20㎕. Incubation 60 min at 42℃ and 5 min at 95℃ heat inactivation. Then, put 20 ㎕ distilled water and the cRNA was stored at -20℃ until further use. PCR were performed with i-taq DNA polymerase (intron) and MJ Research PTC 200 thermal cycler. The primers listed Table 1.

4. Chromatin immunoprecipitation (ChIP)

Cells were fixed for 15 min in 0.75 % formaldehyde, placed in lysis buffer (50 mmol/L HEPES-KOH (pH 7.5), 140 mmol/L NaCl, 1 mmol/L EDTA (pH 8), 1 % Triton X-100, 0.1 % Sodium Deoxycholate, 0.1 % SDS and fresh protease inhibitor cocktail), and then chromatin was sheared into 500 bp to 200 bp fragments by sonication. Equal amounts of sample were immunoprecipitated with, 2 ㎍ of anti-p53 (DO-1), anti-HDAC1, anti-HDAC2, anti-DNMT3a, anti-DNMT3b, anti-PARP-1 (Santa Cruz Biotech), anti-p53 (PAb421) (Calbiochem), anti-FLAG (Sigma), or anti-AcH3K9 (Abcam). Bound target DNA fragments were analyzed for presence of sequences -718 to -348 (ChIP1), +89 to +366 (ChIP2) of

human MTA1 promoter and -2323 to -2017 (mChIP) of mouse MTA1 promoter by PCR amplification. The primers used to amplify DNA fragments including the p53 response elements in the promoter of *p21* and *BAX* were described in Table 2.

5. DNA Pull-down Assays and Proteomics

MTA1 promoter templates biotinylated at the 5' end of the forward strand (–547 to –423 for Pull1 and +89 to +336 for Pull2) were produced using PCR. Methylated PCR products were prepared *in vitro* using SssI (CpG) DNA methyltransferase (New England Biolabs). One hundred micrograms of nuclear extracts were incubated with a 10 nM biotinylated DNA probe in a binding buffer (20 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.01% NP-40, and protease inhibitor cocktail) containing 1 mg/mL of herring-sperm DNA and 20 mL of streptavidin-sepharose at 4°C overnight. The protein–DNA complexes were washed three times with binding buffer and were subjected to SDS-PAGE followed by western blotting using anti-p53, anti-PARP-1, anti-HDAC1, or anti-HDAC2 antibodies (Santa Cruz Biotech).

For LC–MS/MS analysis, pulled down proteins were fractionated using SDS–PAGE. Protein bands were excised, in-gel digested with trypsin, and analyzed further using LC–MS/MS on a ThermoFinnigan LTQ ion-trap

mass spectrometer (Thermo Fisher Scientific) coupled to an Agilent 1200 series capillary chromatographic system. Samples were suspended in 0.4 % acetic acid (v/v) and separated on a reverse-phase C18 column (75 mm id ´ 150 mm) prepared in-house in a 40 min linear gradient containing 5–40 % acetonitrile and 0.1 % formic acid at a flow rate of 0.4 mL/min. The mass spectrum was acquired using data-dependent mass transition with dynamic exclusion for 30 s. Analytical events consisted of four consecutive full MS scans in a range of m/z 300–2000, followed by three MS2 scans of the three most intense peptide ions. LC–MS/MS data were used to search the IPI human protein database using the SEQUEST algorithm incorporated into the BioWorks software (version 3.2) (Thermo Electron).

6. Electrophoretic Mobility Shift Assay (EMSA)

Recombinant p53 proteins (Santa Cruz Biotech) or nuclear extracts obtained from HepG2 cells were incubated in the presence or absence of 100- or 200-fold excess of cold probe at room temperature in a reaction buffer containing 10 ng of poly(dI-dC) and 100 μg/ml of BSA in 20 mM HEPES (pH 7.8), 25 mM KCl, 10% glycerol, 2 mM MgCl2, 0.1 M EDTA, 0.02% NP-40, and 1 mM DTT. After a 10 min of preincubation at room temperature, 80 fmol radioactively labeled DNA probe was added, and the

incubation was continued for another 20 min. Samples were fractionated on a 6 % native polyacrylamide gel. The sequences of oligonucleotides used for EMSA are shown in Table 3.

7. Cell Migration Assay

Wile-type HCT116 (p53+/+) and HCT116 (p53-/-) cells were transfected with si-GFP or si-MTA1. After 24 h of transfection, cultured media were collected for migration assay. The culture insert (Ibidi) was placed into μ-Dish and slightly pressed on the top to ensure tight adhesion. An equal number of human dermal fibroblasts (HDFs) were added into the two reservoirs of the same insert and incubated at 37°C / 5% CO_2. After 24 h, media were changed with the conditioned-media and the insert was gently removed to create a gap of ~500 μm. Cells migrating into the gap region were monitored with a microscope.

8. HCC Samples and qRT-PCR

Six normal liver samples and 30 patients with HBV-associated HCC were described previously (Na *et al*., 2009). All samples were collected anonymously according to Institutional Review Board guidelines, and the histopathological features of the examined HCCs are described previously

(Na *et al*., 2009). For total RNA extraction from formalin-fixed paraffin-embedded tissues, each tissue section was stained with hematoxylin, and nontumor or cancer regions were microdissected with a laser microdissection system or cut directly with a needle from sections. RNA isolation and qRT-PCR was performed as previously described previously (Na *et al*., 2009). The following primers and probes were used for qRT-PCR. DNMT3a: 5′-ACTACATCAGCAAGCGCAAG-3′ (forward primer), 5′-CACAGCATTCATTCCTGCAA-3′ (reverse primer), and Universal ProbeLibrary probe: # 75. DNMT3b: 5′- CCGAGAACAAATGGCTTCAG-3′ (forward primer), 5′- TTCCTGCCACAAGACAAACA-3′ (reverse primer), and Universal ProbeLibrary probe: # 64. MTA1: 5′-GGCAGACA TCACCGACTTG-3′ (forward primer), 5′-ACACCTGGGTCTCCAACCCT-3′ (reverse primer), and Universal ProbeLibrary probe: # 54 (Roche Diagnostics). Five HCC specimens showing high expression of MTA1 in tumor were selected for bisulfite sequencing.

9. Statistics

Experimental values are expressed as the mean ± S.D. of three independent experiments. The significance of any difference was determined by Student's t tests and expressed as a probability value. For clinicopathological studies,

the independent data (normal vs non-tumor region) and the matched data (non-tumor vs cancer) of gene expression was analyzed using Wilcoxon signed rank test and Mann-Whitney test, respectively. Mean differences were considered significant at $P < 0.05$. The relationship between expression of DNMTs and MTA1 was analyzed by calculating Pearson correlation coefficient.

Table 1. The sequences of oligonucleotides used for RT-PCR.

Oligonucleotide name		Nucleotide sequence
p53	Sense	5'-CATCTACAAGCAGTCACAGCA-3'
	Antisense	5'-TTCCGTCCCAGTAGATTACCA-3'
MTA1	Sense	5'-CACGCACATCAGGGGCAA-3'
	Antisense	5'-GTGCGAAGGTGCCCACA-3'
p21	Sense	5'-ATGTCAGAACCGGCTGGGGATGTC-3'
	Antisense	5'-GGGCTTCCTCTTGGAGAAGATC-3'
Bax	Sense	5'-CCCTTTTGCTTCAGGGTTTC-3'
	Antisense	5'-TGTTACTGTCCAGTTCGTCC-3'
survivin	Sense	5'-ATGGGTGCCCCGACGTT-3'
	Antisense	5'-TCAATCCATGGCAGCCAG-3'
b-actin	Sense	5'-CGTGGGCCGCCCTAGGCACCA-3'
	Antisense	5'-TTGGCTTAGGGTTCAGGGGGG-3'

Table 2. The sequences of oligonucleotides used for ChIP.

Oligonucleotide name		Nucleotide sequence
ChIP1	Sense	5'-AAAGAGCACGGCCGCTCCTGGA-3'
	Antisense	5'-GGTCCTCCGGCATTCCTCCCTGA-3'
ChIP2	Sense	5'-ATCGCGCCTCCATTTTCC-3'
	Antisense	5'-ATTTTGGGTTGGGGTGAG-3'
mChIP1	Sense	5'-CGGTCTTCCTCTCGGTTTGC-3'
	Antisense	5'-GGCGCATTCAAGATCTCAGA-3'
p21	Sense	5'-GAAATGCCTGAAAGCAGAGG-3'
	Antisense	5'-GCTCAGAGTCTGGAAATCTC-3'
BAX	Sense	5'-TCAGCACAGATTAGTTTCTG-3'
	Antisense	5'-GGGATTACAGGCATGAGCTA-3'
survivin	Sense	5'-TTGAACTCCAGGACTCAAGTGA-3'
	Antisense	5'-GATGCGGTGGTCCTTGAGA-3'

Table 3. The sequences of oligonucleotides used for EMSA.

Oligonucleotide name		Nucleotide sequence
p53RE1	Sense	5'-GCACGACCACCTGTCCAGAGATGCCACGCG-3'
	Antisense	5'-CGCGTGGCATCTCTGGACAGGTGGTCGTGC-3'
p53RE2	Sense	5'-CGAAGGCCAAGGACACGCCCTGCCTGGTGT-3'
	Antisense	5'-ACACCAGGCAGGGCGTGTCCTTGGCCTTCG-3'
p53RE3	Sense	5'-CGCCGCCGCCGGCCCGGACATGGCCGCCAA-3'
	Antisense	5'-TTGGCGGCCATGTCCGGGCCGGCGGCGGCG-3'
Mut 2,3	Sense	5'-CGAAGGCCGGTGACGGTCCCTGCCTGGTGT-3'
	Antisense	5'-ACACCAGGCAGGGACCGTCACCGGCCTTCG-3'
Mut 4	Sense	5'-CGCCGCCGCCGGCCCGGACGGGGCCGCCAA-3'
	Antisense	5'-TTGGCGGCCCCGTCCGGGCCGGCGGCGGCG-3'
p21 promoter p53RE	Sense	5'-GGAAGAAGACTGGGCATGTCTGGGCAGAGA-3'
	Antisense	5'-TCTCTGCCCAGACATGCCCAGTCTTCTTCC-3'

III. RESULTS

1. Poly(ADP-ribosyl)ation of p53 induces gene-specific transcriptional repression of MTA1

1.1. MTA1 promoter activity is repressed by p53

Although MTA1 is a well-defined metastatic and angiogenic factor, the molecular details of the regulation of its expression are not understood fully. To investigate the transcriptional regulators of MTA1 expression, potential *cis* elements located in the 5′ upstream promoter sequences relative to the transcription initiation site of the MTA1 gene were analyzed using MatInspector (Cartharius *et al*., 2005). Interestingly, three putative p53 response elements, p53RE1, p53RE2, and p53RE3, were located in this promoter region (Figure 7A). We cloned the 1 kb 5′ upstream promoter and constructed an MTA1 promoter reporter gene. 5-Fluorouracil (5-FU), which is an anticancer agent that induces p53, was tested for its potential to modulate the MTA1 promoter. First, deletion of p53RE1 and p53RE2 largely increased basal-level activity, indicating that p53, the p53REs are involved in the regulation of MTA1 expression. Second, the activities of both the 1 kb and 0.5 kb promoter reporters were decreased after 5-FU

treatment, in a dose-dependent manner, indicating that p53RE3 is also associated with the regulation of the MTA1 gene (Figure 7A). Consistently, the expression levels of the MTA1 protein and mRNA were decreased considerably in MCF-7 and HepG2 cells, whereas that of p53 was increased dramatically after 5-FU treatment (Figure 7B). The involvement of p53 in the 5-FU-mediated repression of MTA1 was confirmed by the observation that overexpression of p53 repressed the expression of MTA1 (Figure 7C), whereas knockdown of p53 using si-RNA recovered the levels of the MTA1 protein in the presence of 5-FU (Figure 7D).

1.2. 5-FU treatment decreases MTA1 protein level in wild-type p53-expressing cell line

To confirm the involvement of p53 in the regulation of the expression of the MTA1 gene, various cell lines with different p53 status, i.e., wild type (MCF7 and HepG2), nonfunctional mutant type (MDA-MB-231), and deficient (Hep3B), were examined for MTA1 levels after 5-FU treatment. 5-FU decreased both the protein and the mRNA levels of MTA1 in p53 wild-type cells exclusively (Figure 8A). 5-FU decreased MTA1 expression in the wild-type HCT116 cells, but not in the p53 isogenic derivative HCT116 (p53+/–) or HCT116 (p53–/–) cells. Similarly, 5-FU repressed the level of

MTA1 in the wild-type mouse embryonic fibroblasts (MEF), but not in p53 knockout MEF cells. Interestingly, the basal expression level of MTA1 was upregulated in HCT116 (p53+/–), HCT116 (p53 –/–), and p53 knockout MEF cells (Figure 8B). Taken together, these data indicate that p53 may be an important factor in the regulation of the transcriptional expression of the MTA1 gene.

1.3. The MTA1 promoter contains p53REs that repress the transcriptional expression of MTA1

We characterized the p53REs located in the MTA1 promoter further using various protein-DNA binding analysis (Figure 9A). Chromatin immunoprecipitation (ChIP) assays using specific p53 antibodies demonstrated clearly that p53 bound to a region containing p53RE1 and p53RE2 (ChIP1), and to another region containing p53RE3 (ChIP2). The specificity of these bindings was demonstrated using anti-Myc antibody that detected Myc-tagged p53 introduced exogenously (Figure 9B). Next, we performed DNA pull-down assays using biotin end-labeled double-stranded oligonucleotide probes encoding p53REs and their mutants. Binding of p53 was detected clearly using the two wild-type probes, Pull1 and Pull2, and was diminished by the addition of an anti-p53 antibody, PAb240. p53 binding to the three

probes encoding the mutant types was decreased, indicating the specific binding of p53 to these elements (Figure 9C and 9D). Electrophoretic mobility shift assay (EMSA) demonstrated further that the recombinant p53 protein specifically bound to p53RE2 and p53RE3, but not to p53RE1 (Figure 9E). Finally, we examined whether the 5-FU-induced repression of *MTA1* was associated with the binding of p53 to the MTA1 promoter. ChIP analysis showed that binding of p53 on the p53REs was significantly increased in the presence of 5-FU. Concomitantly, bindings of HDAC1 and HDAC2 were increased transiently, and acetylation of H3K9 was decreased (Figure 9F). These results were confirmed by EMSA and DNA pull-down assays that showed increased binding of p53 complex, HDAC1, and HDAC2 to the p53RE2 and p53RE3 upon 5-FU treatment (Figure 9G and 9H). Together, these results suggest that the binding of a p53 repressor complex including HDACs to the p53RE2 and p53RE3s on the MTA1 promoter induces the transcriptional repression of the MTA1 gene after 5-FU treatment.

1.4. PARylation of p53 induced by PARP-1 is required for repression of MTA1

To characterize further the p53 repressor complex on the MTA1 promoter, the proteins that were pulled down from nuclear extracts of HepG2 cells were subjected to proteomics analysis using a combination of liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Table 4). Among the proteins identified, PARP-1 recorded the most hits with the highest probable score (Figure 10A and Table 4). The binding of PARP-1 to the MTA1 promoter was verified using western blotting (Figure 10B). Interestingly, the binding of PARP-1 to the MTA1 promoter regions ChIP1 and ChIP2 increased dramatically after 5-FU treatment, suggesting that the biological function of 5-FU may involve the binding of PARP-1 to the MTA1 promoter (Figure 10C).

Table 4. Summary of MTA1 promoter (-547 ~ -423) interacting proteins found by LC/MS/MS analysis.

Score	Accession	MW (KDa)	Gene symbol	Gene Name
496.3	156523968	113	PARP1	poly (ADP-ribose) polymerase family, member 1
200.2	71153825	148.9	MYBBP1A	MYB binding protein (P160) 1a
30.2	64085377	66.1	IGF2BP2	insulin-like growth factor 2 mRNA binding protein 2
24.3	28380083	65.5	NCOA5	nuclear receptor coactivator 5
20.2	74712656	39.3	IKIP	IKK interacting protein
20.1	97537467	108.7	TRAP150	thyroid hormone receptor associated protein 3
18.2	2833271	37	USF2	upstream transcription factor 2, c-fos interacting
16.1	30795212	63.7	IGF2BP3	insulin-like growth factor 2 mRNA binding protein 3
10.3	47605556	106.1	BCLAF1	similar to Bcl-2-associated transcription factor 1
10.2	46577705	55.9	Fxr	nuclear receptor subfamily 1, group H, member 4
10.2	209572664	59	RBBP5	retinoblastoma binding protein 5
10.2	229462842	106.9	CAF1B	chromatin assembly factor 1, subunit A (p150)
10.2	13128860	55	Hdac1	histone deacetylase 1
10.2	221316741	115.7	Nat10	N-acetyltransferase 10 (GCN5-related)
10.2	206729942	154.2	KDM6A	lysine (K)-specific demethylase 6A
10.1	55958376	50.3	NF-YC	nuclear transcription factor Y, gamma
10.1	115502553	119.7	PELP1	proline, glutamate and leucine rich protein 1
10.1	20070321	31.9	nmnat1	nicotinamide nucleotide adenylyltransferase 1
10.1	116284376	55.4	Hdac2	histone deacetylase 2
10.1	38257795	132.8	KDM2A	lysine (K)-specific demethylase 2A

Next, we investigated whether PARylation of p53 was involved in the repressive function of p53 on the MTA1 promoter. First, we found that immunoprecipitated p53 was recognized by specific anti-PAR antibodies, demonstrating that the p53 protein was PARylated (lane 4, Figure 11A). 5-FU treatment increased the level of PARylated p53; in contrast, 6-(5*H*)-phenanthridinone (PHEN), which is an inhibitor of PARP-1, decreased the level of PARylated p53 (lanes 5 and 6, Figure 11A). As p53 interacts directly with HDAC1 to exert its repressive function, we examined whether PARylation of p53 affected the binding of p53 to HDAC1 (Juan *et al*., 2000). Binding of p53 to HDAC1 was detected in the presence of 5-FU; however, this binding was abolished in the presence of PHEN (Figure 11B). Similarly, the binding of p53, HDAC1, and HDAC2 to the MTA1 promoter, which was increased after 5-FU treatment, was diminished in the presence of PHEN (Figure 11C). Taken together, these data suggest that p53 is PARylated by PARP-1 in the presence of 5-FU and that PARylated p53 forms a repressor complex with HDAC1 and HDAC2 on the MTA1 promoter, resulting in the repression of the MTA1 gene. A similar increase of the binding of p53 was observed on the *survivin* promoter, which is a target of the repressive function of p53 (Wang *et al*., 2010) (Figure 11D). Interestingly, we found that PARylation of p53 was barely detectable in

MDA-MB-231 which has a mutated p53 (Figure 12A). 5-FU treatment did not increase, or even decrease, the level of p53 PARylation in MDA-MB-231cells, and the mutant p53 did not bind to the MTA1 promoter (Figures 12B and 12C). Furthermore, PHEN decreased the binding of p53 to the survivin promoter, which may represent a transcriptional derepression that is consistent with the recovery of the 5-FU-mediated repression of survivin after PHEN treatment. These observations were largely in contrast with the observation of binding of p53 to the promoters of p21 and BAX, which are targets of the activating function of p53 (Riley *et al*., 2008). The binding of p53 to these promoters was enhanced further by PARP-1 inhibition in cells exhibiting suppression of the induction of mRNA levels by PARP-1 inhibition (Figure 13).

1.5. MTA1-mediated HIF-1α stabilization and VEGF induction are repressed by functional p53 and PARP-1

To confirm the involvement of PARP-1 in the repressive function of p53 on the MTA1 promoter, we examined whether knockdown or chemical inhibition of PARP-1 affected the expression level of MTA1. As expected, si-PARP-1 or PHEN treatment increased the basal expression level of MTA1 and recovered the 5-FU-induced repression of MTA1 (Figure 14A). Finally, we showed that knockdown of either p53 or PARP-1 increased the levels of

the HIF-1α and VEGF, and it further prevented the 5-FU-induced decrease in the level of HIF-1α and VEGF (Figure 14B). Finally, we demonstrated the potential importance of our findings in the MTA1-mediated cell migration. We carried out MTA1 knock-down experiments in the wild-type HCT116 (p53+/+) and isogenic derivative HCT116 (p53–/–) cells. The conditioned-media obtained from the MTA1-knocked down HCT116 (p53+/+) induced a less motility of HDFs (45% wound closure at 12 h) to compare with that from the control HCT116 (p53+/+) (71% wound closure at 12 h), indicating that MTA1 is involved in the cell migration. The migration of HDFs was more prominent with the conditioned-media obtained from HCT116 (p53-/-) (81% wound closure at 12 h), whereas it was inhibited by the media obtained from the knock-down of MTA1 in HCT116 (p53-/-) (46% wound closure at 12 h) (Figure 14C). This result suggests that p53 affects the MTA1-mediated cell motility. Taken together, our results demonstrated that p53 functionally repressed the transcriptional expression of the MTA1 gene via binding to the p53REs, which was facilitated by PARP-1-mediated PARylation of p53 (Figure 14D).

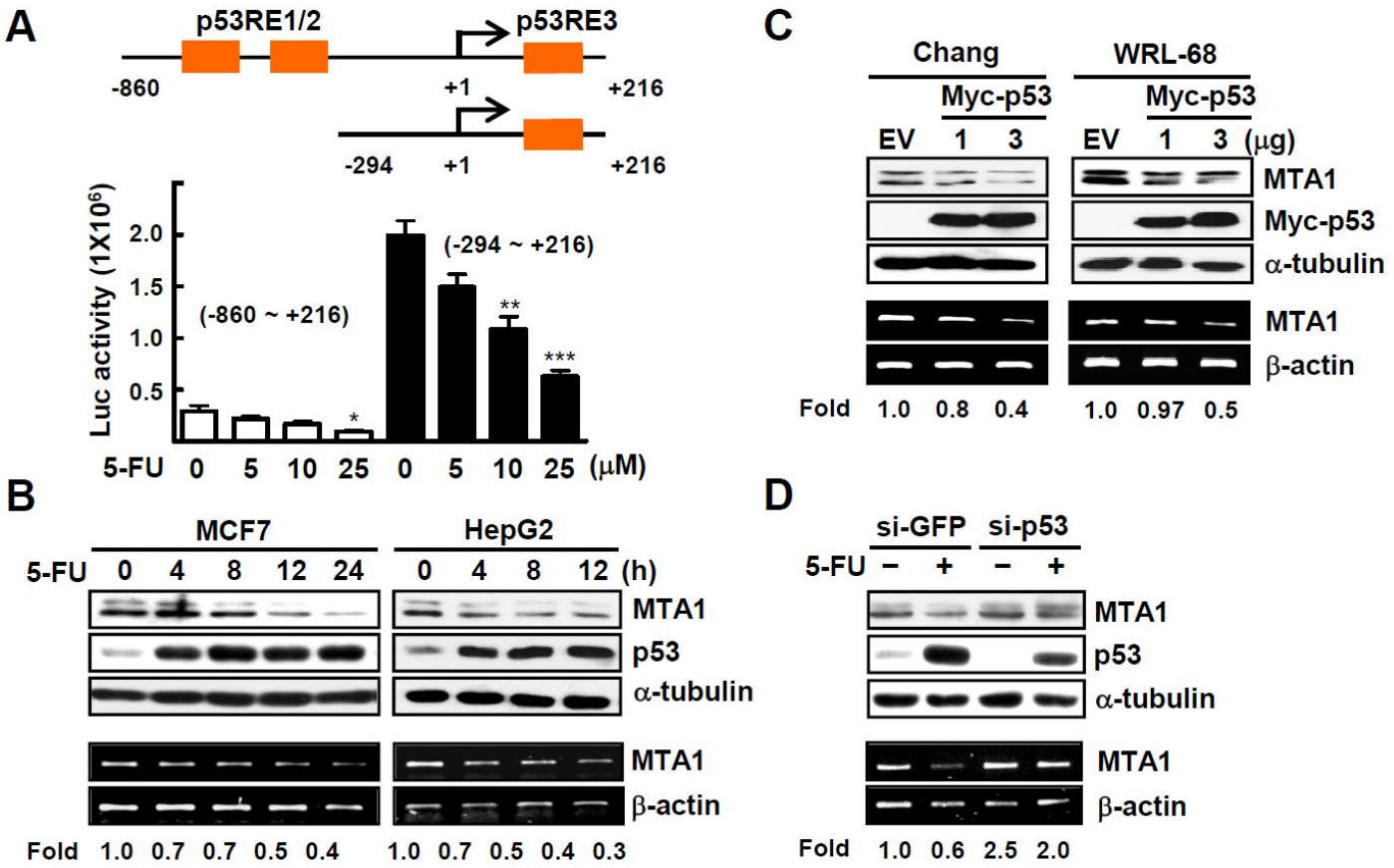
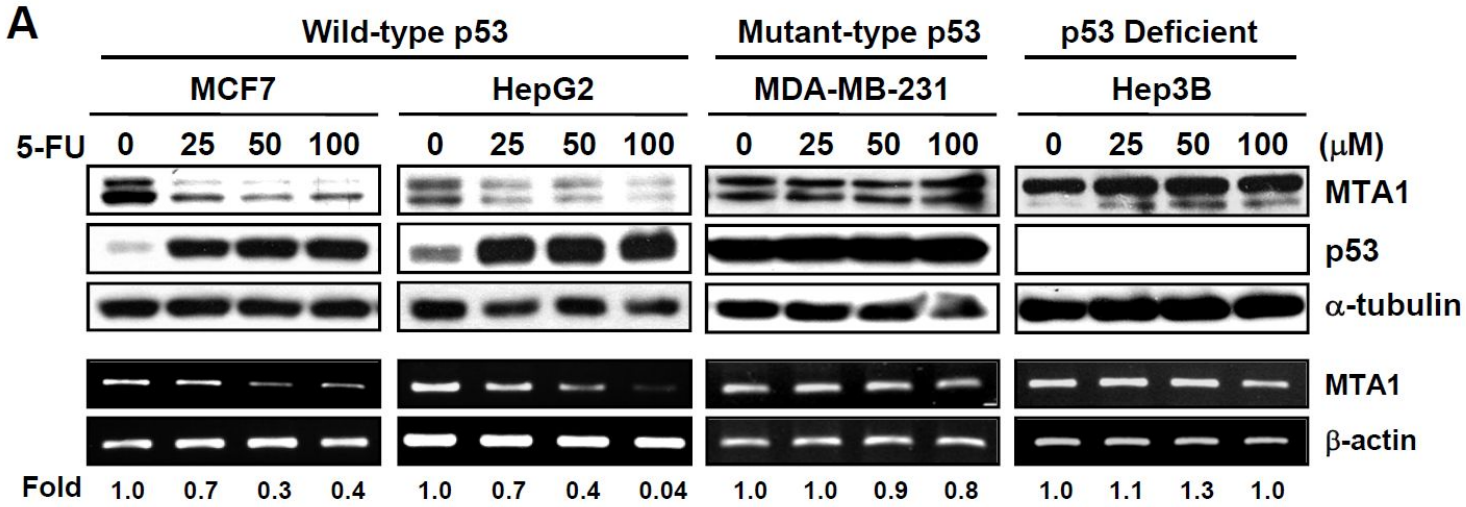

A
p53RE1/2
p53RE3
-860
+1
+216
-294
+1
+216
Luc activity (1X10⁶)
2.0
1.5
1.0
0.5
(-860 ~ +216)
(-294 ~ +216)
*
**
***
5-FU 0 5 10 25 0 5 10 25 (μM)
B
MCF7
HepG2
5-FU 0 4 8 12 24 0 4 8 12 (h)
MTA1
p53
α-tubulin
MTA1
β-actin
Fold 1.0 0.7 0.7 0.5 0.4 1.0 0.7 0.5 0.4 0.3
C
Chang
WRL-68
Myc-p53
Myc-p53
EV 1 3 EV 1 3 (μg)
MTA1
Myc-p53
α-tubulin
MTA1
β-actin
Fold 1.0 0.8 0.4 1.0 0.97 0.5
D
si-GFP
si-p53
5-FU – + – +
MTA1
p53
α-tubulin
MTA1
β-actin
Fold 1.0 0.6 2.5 2.0

Figure 7. Expression of MTA1 is repressed by p53 at transcription level. (A) Schematic representation of the human MTA1 promoter. Predicted p53 binding sites are shown as filled boxes. Numbers indicates the beginning nucleotide number relative to transcription initiation site of MTA1 gene (upper). HepG2 cells were transfected with the MTA1 promoter-Luc reporter and treated with the indicated concentrations of 5-FU for 24 h. Experimental values are expressed as the mean ± SD (n = 3). *P < 0.05, **P < 0.01, and ***P < 0.001 *vs* vehicle-treated control (lower). (B) MCF7 or HepG2 cells were treated with 50 μM 5-FU for the indicated periods. The protein level of MTA1 was analyzed by western blotting (WB). (C) Chang liver or WRL-68 cells were transfected with empty vector (EV) or pCMV-Myc-p53. Whole cell lysates were prepared and protein levels were evaluated by western blotting. (D) HepG2 cells were transfected with si-GFP or si-p53, and then treated with 50 μM 5-FU for 24 h. Expression of the indicated proteins was analyzed by western blotting.


A
Wild-type p53
Mutant-type p53
p53 Deficient
MCF7
HepG2
MDA-MB-231
Hep3B
5-FU 0 25 50 100 0 25 50 100 0 25 50 100 0 25 50 100 (μM)
MTA1
p53
α-tubulin
MTA1
β-actin
Fold 1.0 0.7 0.3 0.4 1.0 0.7 0.4 0.04 1.0 1.0 0.9 0.8 1.0 1.1 1.3 1.0

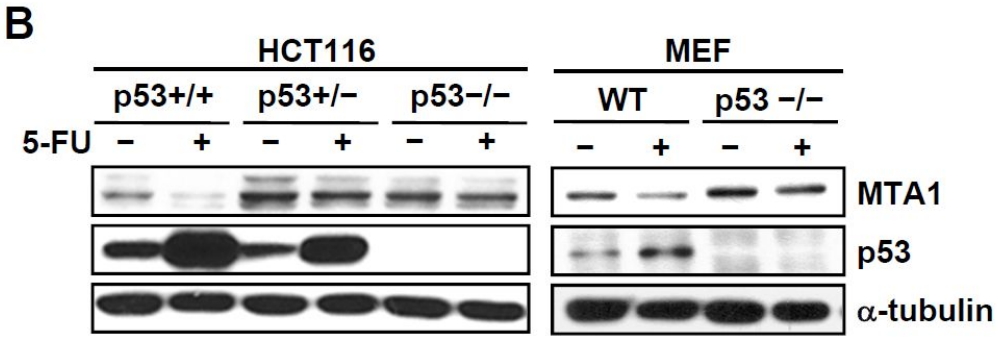
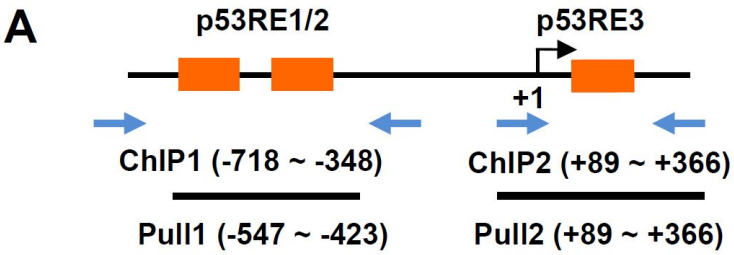
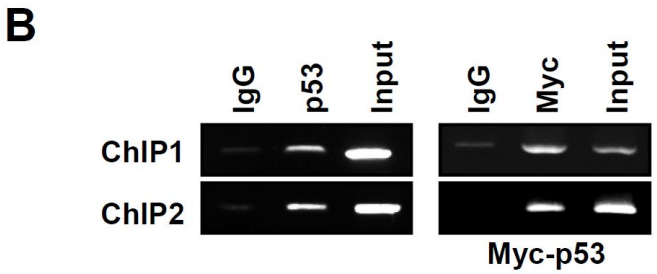

B
HCT116
MEF
p53+/+
p53+/−
p53−/−
WT
p53 −/−
5-FU − + − + − + − + − + − +
MTA1
p53
α-tubulin

Figure 8. 5-FU treatment decreases MTA1 protein level in only cell lines expressing wild-type p53. (A) Cells with differential p53 status were treated with the indicated concentrations of 5-FU for 24 h. The protein level of MTA1 was analyzed by western blotting (WB). (B) Wild-type HCT116 and p53 isogenic derivates cells were treated with 100 μM 5-FU for 24 h. Wild-type MEF and p53 knockout MEF were treated with 25 μM for 24 h. The protein level of MTA1 was analyzed by western blotting.





C

Pull1
ACGACCACCTGTCCAGAGATGCCACGCG : p53RE1
ACGACCACGGGTCCAGAGATGCCACGCG : mut1
CGAAGGCCAAGGACACGCCCTGCCTGGTGT : p53RE2
CGAAGGCCGGTGACACGCCCTGCCTGGTGT : mut2
CGAAGGCCAAGGACGGTCCCTGCCTGGTGT : mut3

Pull2
CGCCGCCGCCGGCCCGGACATGGCCGCCA : p53RE3
CGCCGCCGCCGGCCCGGACGGGGCCGCCA : mut4

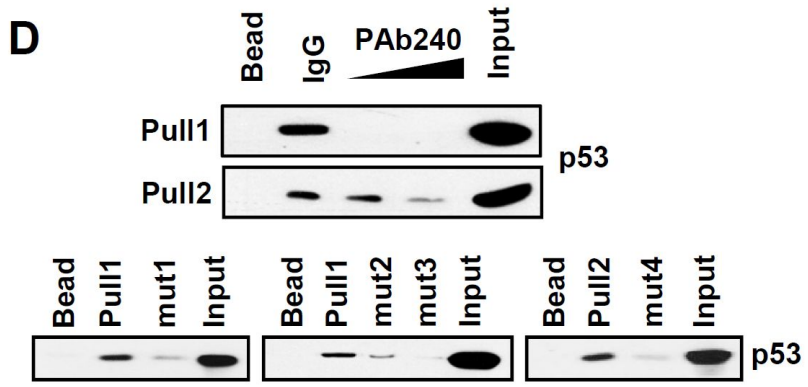


Figure 9. MTA1 promoter contains p53REs that repress transcriptional expression of MTA1. (A) Schematic representation of the human MTA1 promoter with DNA regions for ChIP and pull-down experiments. Predicted p53 binding sites are shown as filled boxes. (B) HepG2 cells that were transfected with pCMV-Myc-p53 were tested for p53 DNA binding by ChIP analysis. DNA fragments that immunoprecipitated by anti-p53 or anti-Myc antibodies were amplified by PCR using primers for ChIP1 and ChIP2. (C) DNA sequences for wild-type and mutant-type p53REs examined for p53 binding by DNA pull-down assays. (D) Nuclear extract obtained from HCT116 was incubated with the PCR amplified double-stranded biotin end-labeled oligonucleotide probe (Pull1 or Pull2) with 1 μg or 2 μg p53 antibody (PAb240) (upper). Nuclear extract obtained from HCT116 was incubated with the biotin end-labeled oligonucleotide probes for Pull1 or Pull2 containing wild-type or mutant-type p53REs (lower). Pull-downed mixtures fractionated by SDS-PAGE were proved by western blotting using an anti-p53 antibody.

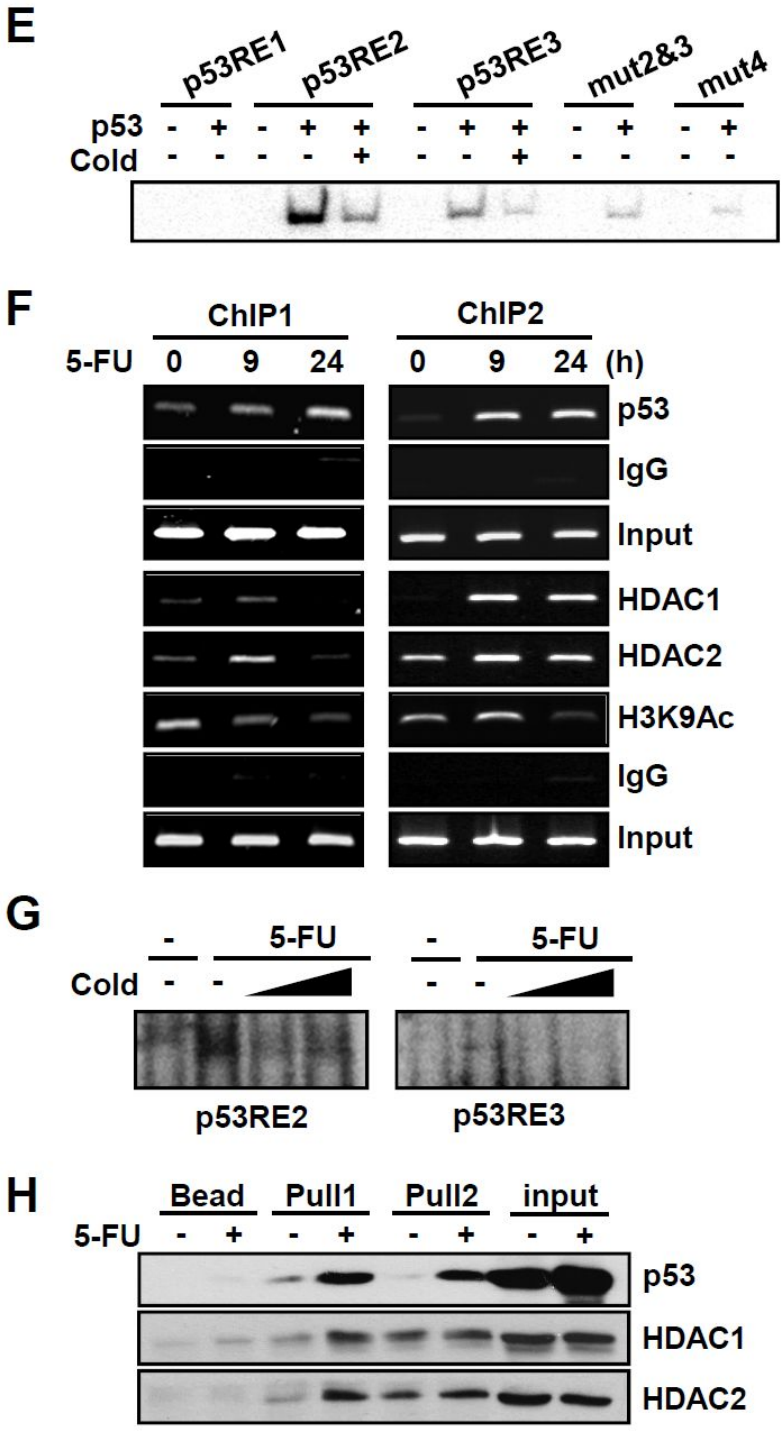

E
p53RE1
p53RE2
p53RE3
mut2&3
mut4
p53 - + - + + - + + - + - +
Cold - - - - + - - + - - - -
F
ChIP1
ChIP2
5-FU 0 9 24 0 9 24 (h)
p53
IgG
Input
HDAC1
HDAC2
H3K9Ac
IgG
Input
G
- 5-FU - 5-FU
Cold - - - -
p53RE2
p53RE3
H
Bead
Pull1
Pull2
input
5-FU - + - + - + - +
p53
HDAC1
HDAC2

Figure 9. continued

(E) EMSA was carried out with the recombinant p53 protein. 4 μg recombinant p53 protein was incubated with [^{32}P]-labeled double-stranded oligonucleotides probes corresponding to the p53RE1, p53RE2, p53RE3, mut2&3 or the synthetic mut4 oligomer. 100-fold excess of cold probe was added as indicated. (F) HepG2 cells were treated with 50 μM 5-FU for 9 h or 24 h. DNA fragments that immunoprecipitated by anti-p53, anti-HDAC1, anti-HDAC2, or anti-H3K9Ac antibodies were amplified by PCR with primers for ChIP1 and ChIP2. (G) EMSA was carried out with the nuclear extracts prepared from HepG2 cells with and without 50 μM 5-FU treatment for 24 h. [^{32}P]-labeled double-stranded oligonucleotides corresponding to the p53RE2 or p53RE3 were used as probe. 100 or 200-fold excess of the p53RE present in the p21 promoter (Cold) were added as indicated. (H) DNA pull-down assay was carried out with the nuclear extracts prepared from HepG2 cells with and without 50 μM 5-FU treatment for 24 h.

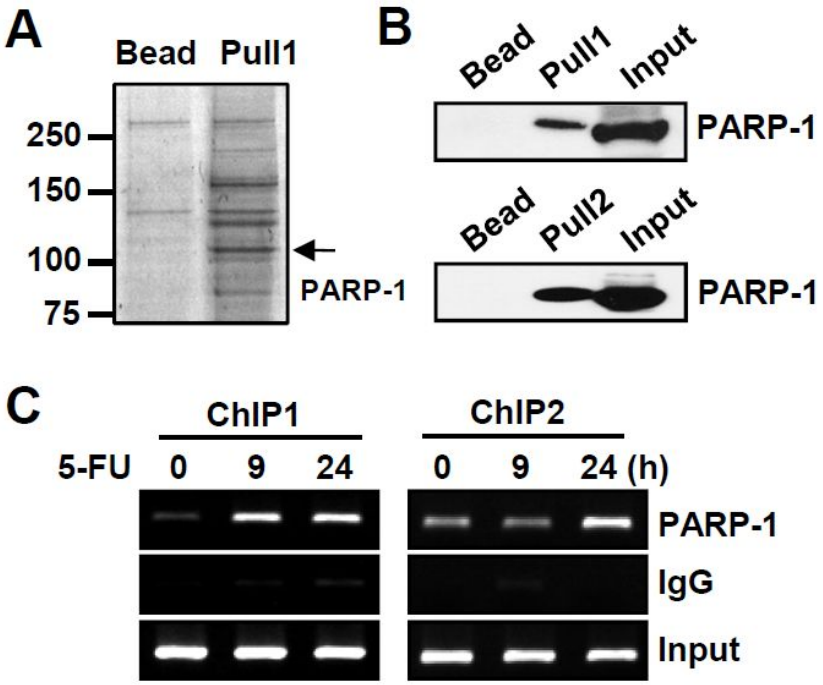
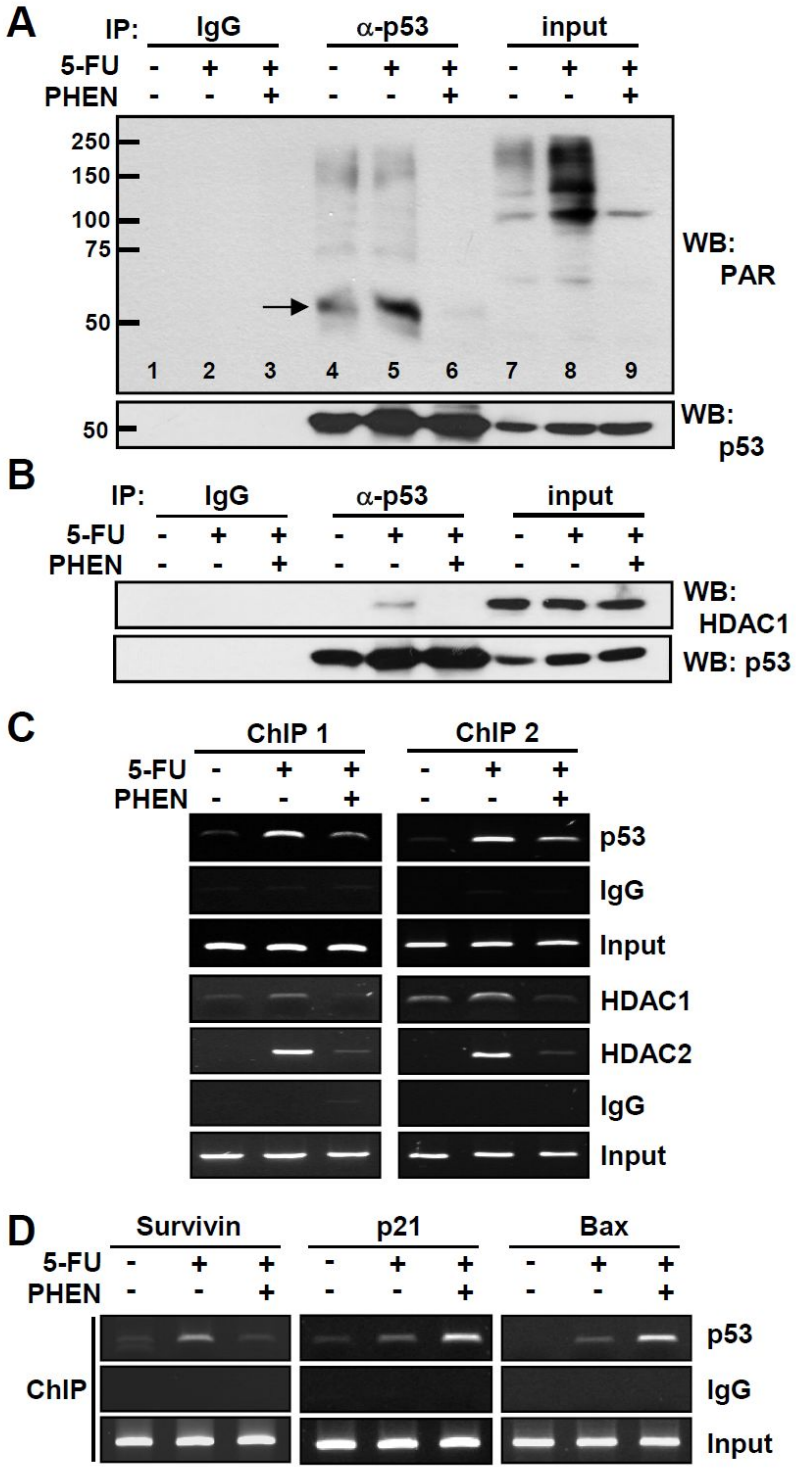


Figure 10. 5-FU treatment increases PARP-1 binding to p53REs on the MTA1 promoter. (A) Nuclear extract obtained from HepG2 cells was incubated with the PCR amplified double-stranded biotin end-labeled oligonucleotide probe (Pull1). No oligonucleotide probe was added to the bead control. The pull-downed proteins were fractionated by SDS-PAGE and stained by Coomassie blue stain. Arrow indicates PARP-1 that was verified by western blotting. (B) Pull-downed mixture was fractionated by SDS-PAGE and proved by western blotting using an anti-PARP-1 antibody. (C) HepG2 cells were treated with 50 μM 5-FU for indicated periods. DNA fragments that immunoprecipitated by an anti-PARP-1 were amplified by PCR with primers for ChIP1 and ChIP2.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IgG
α-p53
input
5-FU
PHEN
250
150
100
75
50
1 2 3 4 5 6 7 8 9
WB: PAR
50
WB: p53
B
IP:
IgG
α-p53
input
5-FU
PHEN
WB: HDAC1
WB: p53
C
ChIP 1
ChIP 2
5-FU
PHEN
p53
IgG
Input
HDAC1
HDAC2
IgG
Input
D
Survivin
p21
Bax
5-FU
PHEN
ChIP
p53
IgG
Input

Figure 11. PARylation mediated by PARP-1 is required for p53 to bind to the MTA1 promoter. HepG2 cells were treated with 100 μM PHEN with or without 50 μM 5-FU for 24 h as indicated. (A) Whole cell lysates were immunoprecipitated (IP) with normal IgG or anti-p53 antibodies, and precipitates were probed by western blotting (WB) using an anti-PAR antibody. Arrow indicates p53 that was verified by WB. The membrane was stripped and reprobed with an anti-p53 antibody as control. (B) Whole cell lysates were immunoprecipitated (IP) with normal IgG or anti-p53 antibodies, and precipitates were probed by WB using an anti-HDAC1 antibody. The membrane was stripped and reprobed with an anti-p53 antibody as control. (C) DNA fragments that immunoprecipitated by anti-p53, anti-HDAC1 or anti-HDAC2 antibodies were amplified by PCR with primers for ChIP1 and ChIP2. (D) DNA fragments that immunoprecipitated by normal IgG or anti-p53 antibodies, were amplified by PCR using primers for the promoter regions containing p53 response elements for survivin (-181 to +81), *p21* (-1688 to -1335), or Bax (-883 to –455).

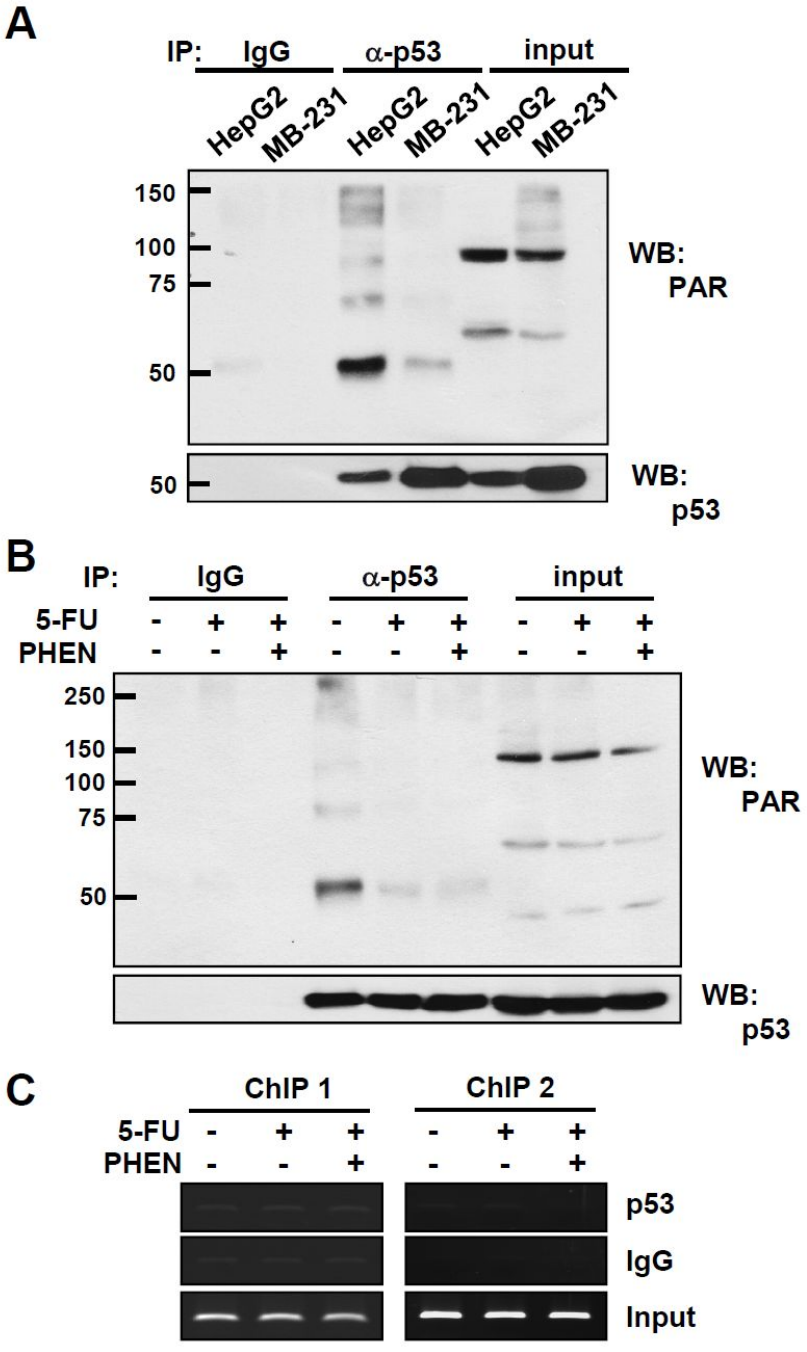

A
IP: IgG
α-p53
input
HepG2
MB-231
HepG2
MB-231
HepG2
MB-231
150
100
75
50
WB: PAR
50
WB: p53
B
IP: IgG
α-p53
input
5-FU - + + - + + - + +
PHEN - - + - - + - - +
250
150
100
75
50
WB: PAR
WB: p53
C
ChIP 1
ChIP 2
5-FU - + + - + +
PHEN - - + - - +
p53
IgG
Input

Figure 12. PARylation of mutant p53 present in MDA-MB-231 cells. (A), (B) Whole cell lysates were immunoprecipitated (IP) with normal IgG or anti-p53 antibodies, and precipitates were probed by western blotting (WB) using anti-PAR antibodies. Arrow indicates p53 that was verified by WB. The membrane was stripped and reprobed with an anti-p53 antibody used as an IP control. (C) MDA-MB-231 cells were treated with 50 μM 5-FU for 24 h. DNA fragments that immunoprecipitated by an anti-p53 antibody were amplified by PCR using primers for ChIP1 and ChIP2.

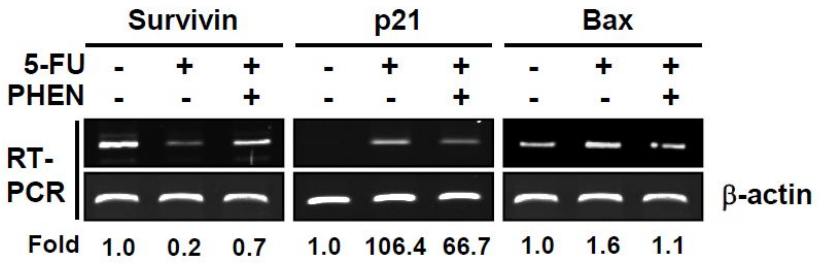


Figure 13. Effects of PHEN on the 5-FU-induced regulation of gene expression for *survivin*, *p21* and *Bax*. HepG2 cells were treated with 100 μ M PHEN with or without 50 μM 5-FU for 24 h as indicated. The mRNA expression of MTA1 was analyzed by RT-PCR. The density of PCR bands was determined by an image analysis system and normalized to the corresponding β-actin band. The fold changes in expression levels are shown at the bottom of the gel illustrations.

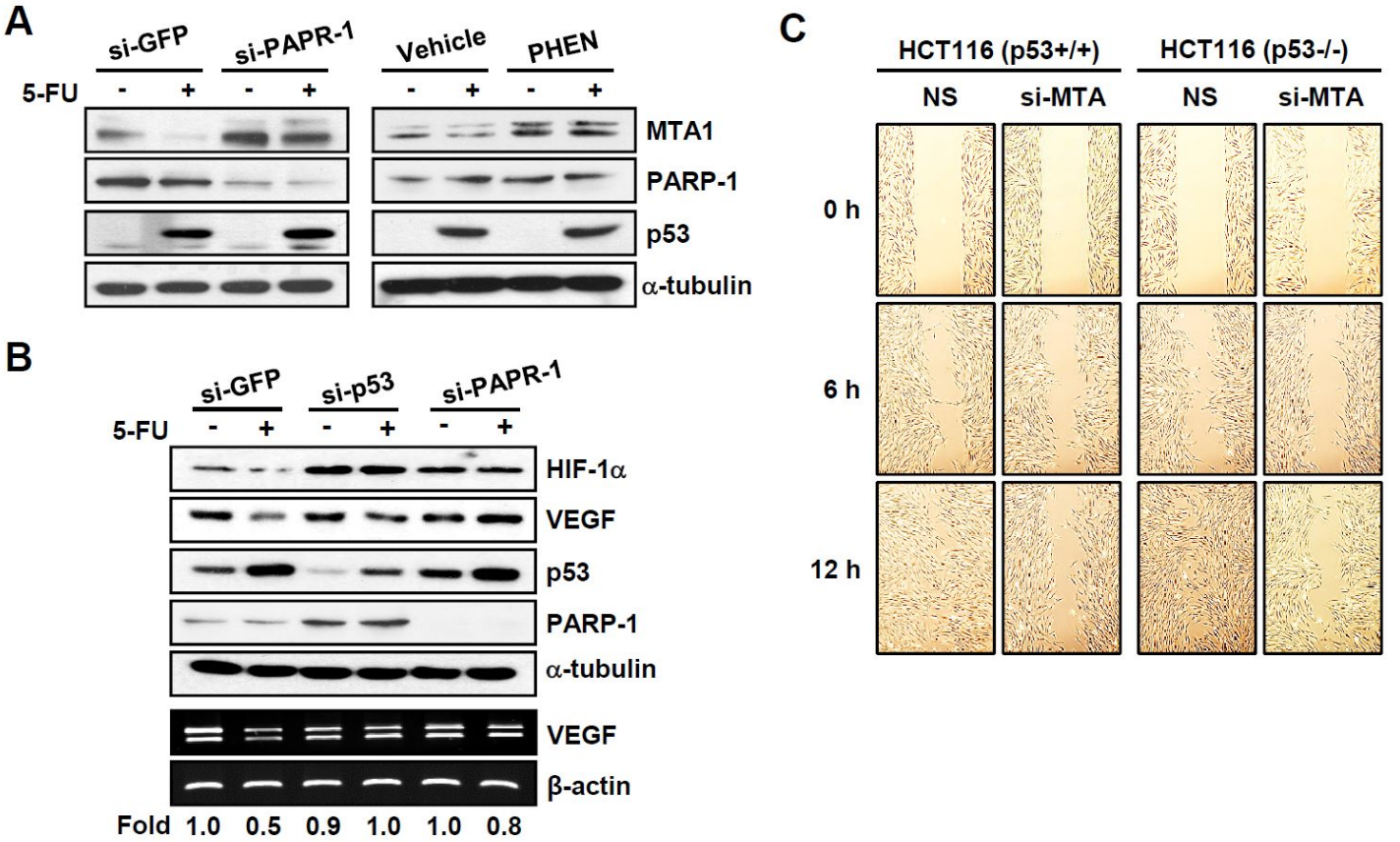

A
si-GFP
si-PAPR-1
Vehicle
PHEN
5-FU
- + - + - + - +
MTA1
PARP-1
p53
α-tubulin
B
si-GFP
si-p53
si-PAPR-1
5-FU
- + - + - +
HIF-1α
VEGF
p53
PARP-1
α-tubulin
VEGF
β-actin
Fold 1.0 0.5 0.9 1.0 1.0 0.8
C
HCT116 (p53+/+)
HCT116 (p53-/-)
NS
si-MTA
NS
si-MTA
0 h
6 h
12 h

Figure 14. p53 and PARP-1 repress the MTA1-mediated HIF-1α and VEGF expression. (A) HepG2 cells were transfected with si-GFP or si-PARP-1. After 48 h of transfection, the cells were treated with 50 μM 5-FU for 24 h. Or HepG2 cells were treated with 100 μM PHEN with or without 50 μM 5-FU for 24 h as indicated. Whole cell lysates were prepared and protein levels were evaluated by western blotting. (B) HepG2 cells were transfected with si-GFP, si-p53 or si-PARP-1. After 48 h of transfection, cells were treated with 50 μM 5-FU for 24 h. Western blotting (WB) was performed to monitor the expression of proteins. (C) Migration of HDFs that were cultured with the conditioned-media obtained from the si-GFP or si-MTA1 transfected HCT116 was monitored. (D) Schematic representation for the transcriptional-repression of MTA1 gene by DNA binding of PARylated p53 repressor complex on the MTA1 promoter.

D

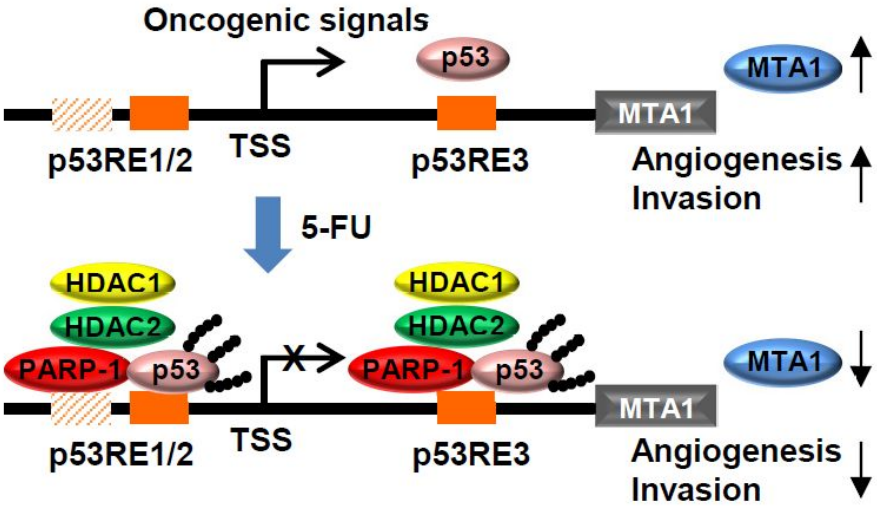


Figure 14. continued

(D) Schematic representation for the transcriptional-repression of MTA1 gene by DNA binding of PARylated p53 repressor complex on the MTA1 promoter.

2. Epigenetic control of metastasis-associated protein 1 gene expression by hepatitis B virus X protein during hepatocarcinogenesis

2.1. HBx increases methylation of the MTA1 promoter

Although HBx is associated with high expression of MTA1 in HCC, the mechanism underlying this regulation of MTA1 expression is largely unknown. To identify the molecular details of HBx-induced MTA1 gene expression, we cloned a potential MTA1 promoter located in the 5` sequence upstream of the coding region of MTA1 (-860 to +216) in the human BAC genomic clone encoding chromosome 14. Cotransfection of the reporter containing the putative human MTA1 promoter with the HBx expression vector dose-dependently increased the reporter activity (Figure 15A), suggesting that the 5` upstream sequence functions as a promoter of MTA1. An analysis using the CpGplot program identified two potential CpG islands that span positions -583 to -459 and -212 to +160 on the MTA1 promoter (http://www.ebi.ac.uk/emboss/cpgplot) (Figure 15B). To study the methylation status of the regions, we performed bisulfite sequencing. The methylation level of CpG island 1 was about 35%, whereas that of CpG island 2 was about 0.1% in Chang liver cells, white blood cells, and normal liver tissues obtained from healthy donors (Figure 15C). Surprisingly,

exogenously introduced HBx significantly increased methylation level of the CpG island1 (span positions -548 to -475) in WRL-68 and Chang liver cells. Doxycycline increased the methylation level of CpG island1 in Chang X-34 cells whose expression of the HBx gene is under the control of an inducible doxycycline promoter (Figure 16A and 16B) (Yoo *et al*., 2008). The increases in methylation were accompanied by increases in the expression level of MTA1 (Figure 16C). Expression of HBx was slightly detectable without doxycyclin treatment in Chang X-34, which probably represented leaky expression of HBx, and a relatively high basal expression level of MTA1 and methylation status of CpG island1 (Yoo *et al*., 2003).

2.2. p53 does not bind to the methylated promoter of the MTA1 gene

We found that CpG island 1 in the MTA1 promoter contains a p53-binding site that is associated with transcriptional repression of MTA1 (Figure 17A) (Lee *et al*., 2012). We hypothesized that the HBx-induced methylation of the CpG island interferes with the p53 binding and that this could rescue transcription of MTA1. To prove that p53 binds to this region, we performed ChIP analysis with primers that amplify the CpG island 1 and the region near the transcription start site. Introduction of exogenous HBx decreased the binding of p53 to CpG island 1 (ChIP1) and increased

acetylation level of the lysin 9 in histone 3 at near the transcription start site (ChIP2) (Figure 17B). It has been reported that p53 does not bind to methylated DNA promoters, resulting in the upregulation of genes such as survivin (Nabilsi et al., 2009). We performed DNA pull-down experiments using nuclear extracts containing p53. A clear binding of p53 was observed with the unmethylated probe, but the binding was reduced with the methylated probe, indicating that p53 binds mainly to the unmethylated MTA1 promoter (Figure 17C). The involvement of p53 in HBx-induced MTA1 was demonstrated further in p53-knocked down WRL-68 cells. Repression of p53 expression by si-RNA markedly increased the protein level of MTA1 (2.9-fold, lane 3). However, coexpression of HBx did not increase MTA1 further (2.6-fold, lane 4), suggesting that MTA1 expression is fully activated by p53 knockdown (Figure 17D).

2.3. HBX recruits DNMT3a and DNMT3b to the CpG island of the human MTA1 promoter

To identify the catalytic enzymes associated with HBx-induced DNA methylation, we examined whether DNMT3a and DNMT3b are recruited in the CpG island of the MTA1 promoter. When HBx was exogenously introduced in Chang cells, expression level of DNMT3a was enhanced as

previously shown (Park *et al*., 2007, Jung *et al*., 2010) ChIP analysis showed that both DNMT3a and DNMT3b bound to CpG island 1 and that the binding increased in the presence of HBx. Interestingly, HBx itself bound to the same region of the promoter (Figure 18A). Further, DNMT3a and DNMT3b knock down by si-RNA reduced expression levels of MTA1 (Figure 18B). To confirm the involvement of DNMT3a and DNMT3b in the methylation of CpG island1, we monitored the DNA methylation status of CpG island1 after DNMT3a and DNMT3b were knocked down by si-RNA. HBx did not significantly alter the methylation status of CpG island1 when expression of DNMT3a and DNMT3b was repressed (Figure 18C). This result demonstrates that HBx increases the transcription of MTA1 by releasing p53 from CpG island1 by increasing promoter methylation through recruitment of DNMT3a and DNMT3b (Figure 18D).

2.4. The expression level of DNMT3 increases in the liver of HBx-expressing transgenic mice

Previously we showed that expression level of MTA1 was elevated markedly in the livers from HBx-transgenic mice compared with wild-type mice (Yoo *et al*., 2008). Therefore, we next examined whether the expression levels of DNMT3a and DNMT3b are associated with the increased expression of

MTA1 in the HBx transgenic mouse livers. In a similar pattern with the MTA1 expression, the levels of DNMT3a and DNMT3b were increased markedly in HBx-transgenic livers, suggesting a potential link between the induction of MTA1 and DNMT3 expression (Figure 19A and 19B). The level of p53 protein was significantly decreased at 9 month-old HBx TG to compare with wild type control mice. This result may be relevant with the previous observation that HBx down regulates p53 promoter activity.25 Interestingly, a p53 binding sequence was found in one of two potential CpG islands in the mouse MTA1 promoter (Figure 19C). When HBx was exogenously introduced, DNA binding of p53 in the CpG island1 was reduced whereas DNA bindings of DNMT3a and DNMT3b were enhanced greatly (Figure 19D). Increases in expression levels of DNMT3a and DNMT3b were also observed in the mouse NIH3T3 cells in the presence of HBx (Figure 19E).

2.5. Expression level of MTA1 is correlated with the MTA1 promoter methylation in HCC specimens

Finally, we performed qRT-PCR analysis to assess the expression levels of MTA1, DNMT3a, and DNMT3b in 30 HBV-associated HCC specimens after microdissection of HCC tissues and the adjacent nontumorous tissues (Na *et*

al., 2009). The expression level of MTA1 did not differ significantly between the HCC tissues and the adjacent nontumorous region, however, the expression levels in both groups were elevated considerably compared with the level in normal human livers (P = 0.001) (Figure 20A). The expression level of DNMT3a in the HCC and nontumorous tissues varied considerably in the groups, and the mean values were higher than normal, but the differences were not significant. The level of DNMT3b was significantly higher in the HCC samples than in the nontumorous tissues (P = 0.004), but the expression levels of DNMT3b in both the HCC and nontumorous tissues did not differ significantly compared with the level in normal human livers. Pearson correlation analysis of MTA1 and DNMT3 expression in human normal livers and the HCC specimens showed a strong correlation (r = 0.5686, P = 0.0001) for DNMT3a and a marginally higher correlation (r = 0.3162, P = 0.0103) for DNMT3b, which may indicate the involvement of DNMT3a and DNMT3b in the regulation of MTA1 expression (Figure 20B).

Bisulfite sequencing was used to analyze the CpG island 1 methylation status of four normal human livers and five HCC tissues with higher MTA1 expression. The methylation level was significantly higher (P = 0.0088) in the HCC tissues. MTA1 mRNA expression and the MTA1

promoter methylation level correlated significantly (r = 0.7201, P = 0.0287), indicating a close association between the DNA methylation status of the MTA1 promoter and MTA1 expression level (Figure 20C). The results obtained from the HBx-transgenic mice and clinicopathological studies strongly support our hypothesis that the HBx-induced methylation of the MTA1 promoter upregulates the transcription of MTA1, which may contribute to the development of HBV-associated HCC.

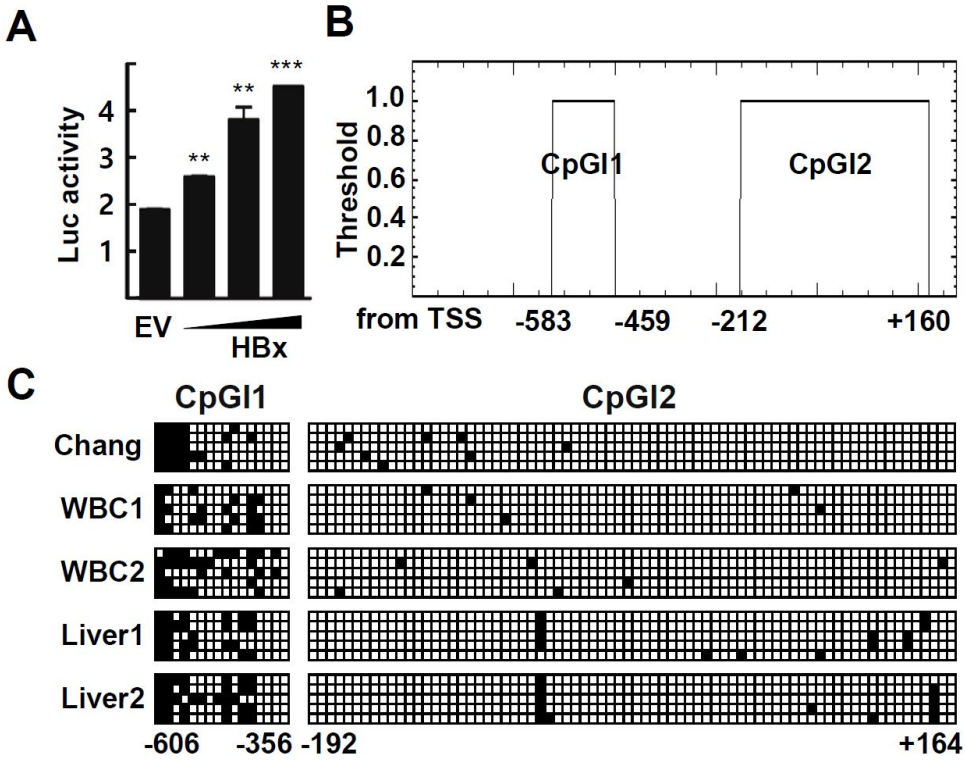


Figure 15. Methylation analysis of the MTA1 promoter. (A) HepG2 cells were transfected with the human MTA1 promoter-Luc reporter and increasing amounts of p3XFLAG7.1-HBx or empty vector (EV). Experimental values are expressed as the mean ± S.D. (n = 3). **P < 0.01, and ***P < 0.001 *vs* EV transfected. (B) Putative CpG islands are located at bases -583 to -459 (CpGI1) and -212 to +160 (CpGI2) in the human MTA1 promoter. (C) Sequencing analysis of the putative MTA1 promoter CpG islands after bisulfite modification. Five clones from Chang liver cells (Chang), human white blood cells (WBC1 and WBC2), and normal liver tissues (Liver1 and Liver2) were analyzed for each PCR fragments. Filled squares, methylated; and open squares, unmethylated.

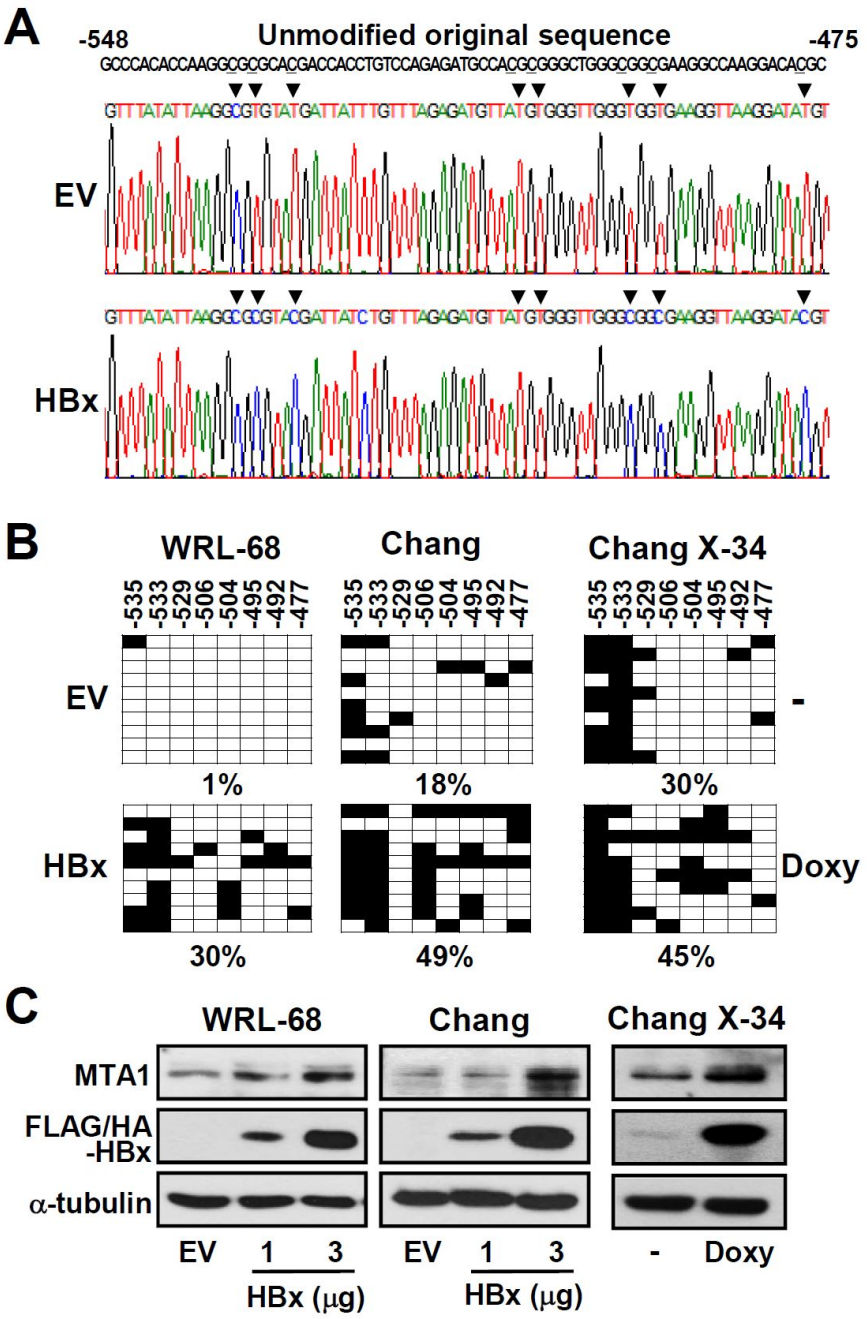

A
-548
Unmodified original sequence
-475
GCCCACACCAAGGCGCGCACGACCACCTGTCCAGAGATGCCACGCGGGCTGGGCGGCGAAGGCCAAGGACACGC
EV
HBx
B
WRL-68
Chang
Chang X-34
-535 -533 -529 -506 -504 -495 -492 -477
EV
HBx
1%
18%
30%
30%
49%
45%
-
Doxy
C
WRL-68
Chang
Chang X-34
MTA1
FLAG/HA -HBx
α-tubulin
EV 1 3
HBx (μg)
EV 1 3
HBx (μg)
- Doxy

Figure 16. HBx increases methylation of the human MTA1 promoter CpG island1. (A) Representative sequencing traces from bisulfite-modified direct sequencing. ▼ denotes methylated or unmethylated cytosines. Seven of unmethylated cytosines were converted to uracil in empty vector (EV)-transfected WRL-68 cells, but two in p3XFLAG7.1-HBx-transfected cells. (B) p3XFLAG7.1-HBx or EV was transfected into the indicated cells or treated with vehicle or 2 mg/ml of doxycycline (Doxy) for 24 h. Results from bisulfite sequencing analysis of the MTA1 promoter CpG island1 were shown. Filled squares, methylated; and open squares, unmethylated. Numbers at the bottom indicate the proportion of methylated CpG sites relative to total CpG sites examined. (C) Indicated amount of p3XFLAG7.1-HBx or EV was transfected into cells, or treated with doxycycline. Whole cell lysates were prepared and protein levels were evaluated by western blotting.

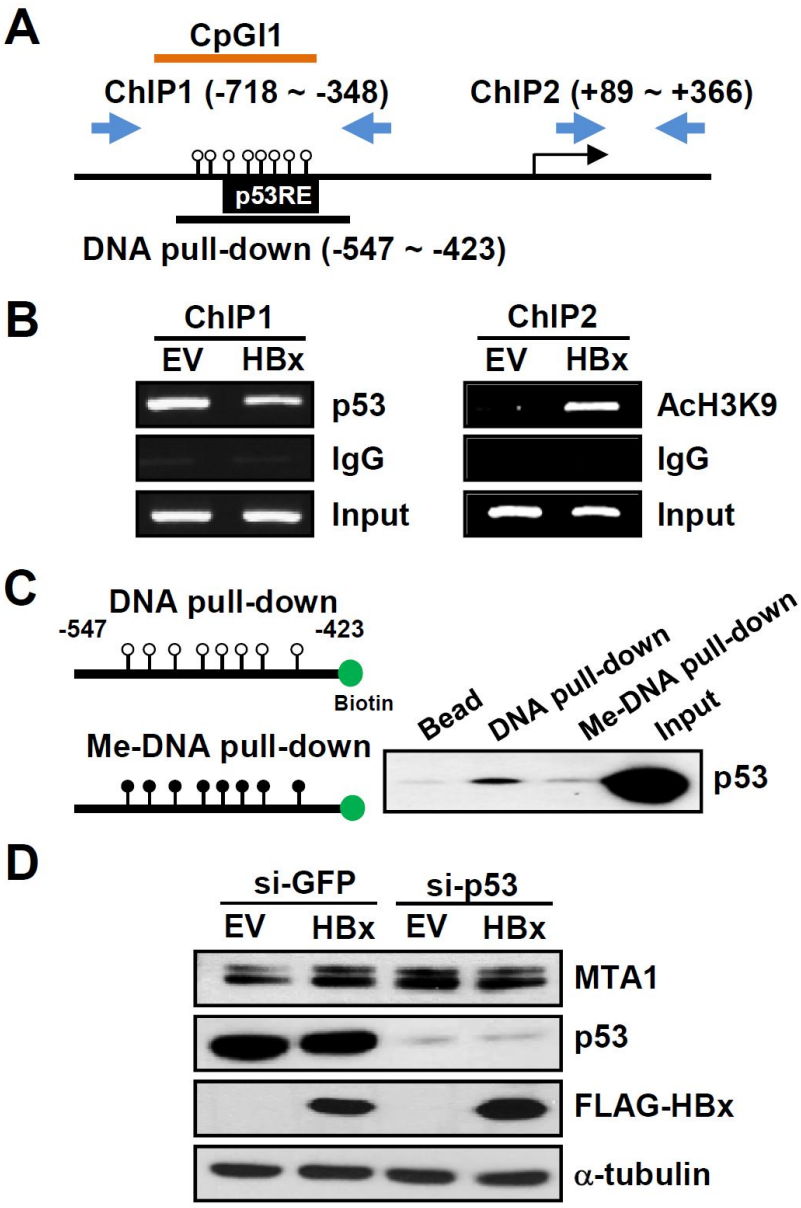

A
CpGI1
ChIP1 (-718 ~ -348)
ChIP2 (+89 ~ +366)
p53RE
DNA pull-down (-547 ~ -423)
B
ChIP1
EV
HBx
p53
IgG
Input
ChIP2
EV
HBx
AcH3K9
IgG
Input
C
DNA pull-down
-547
-423
Biotin
Me-DNA pull-down
Bead
DNA pull-down
Me-DNA pull-down
Input
p53
D
si-GFP
si-p53
EV
HBx
EV
HBx
MTA1
p53
FLAG-HBx
α-tubulin

Figure 17. Methylation of the MTA1 promoter CpG island 1 leads to inhibition of p53 binding. (A) Schematic presentation of the human MTA1 promoter encoding p53 binding sequences (p53RE) and DNA regions for ChIP and pull down experiments. (B) Chang liver cells were transfected with empty vector (EV) or p3XFLAG7.1-HBx. DNA fragments that immunoprecipitated by anti-p53 (left) and anti-acetyl histone 3 lysine 9 (right) were amplified by PCR using primers specific for ChIP1 and ChIP2. (C) Nuclear extracts obtained from HCT116 cells were incubated with unmethylated or methylated double-stranded biotin end-labeled oligonucleotide probes. Precipitates pulled-down by sterptoavidine beads were analyzed for p53 binding by western blotting. (D) WRL-68 cells were transfected with si-GFP or si-p53. After 24 h of transfection, cells were transfected with EV or p3XFLAG7.1-HBx for another 24 h. Expression of the indicated proteins was analyzed by western blotting.

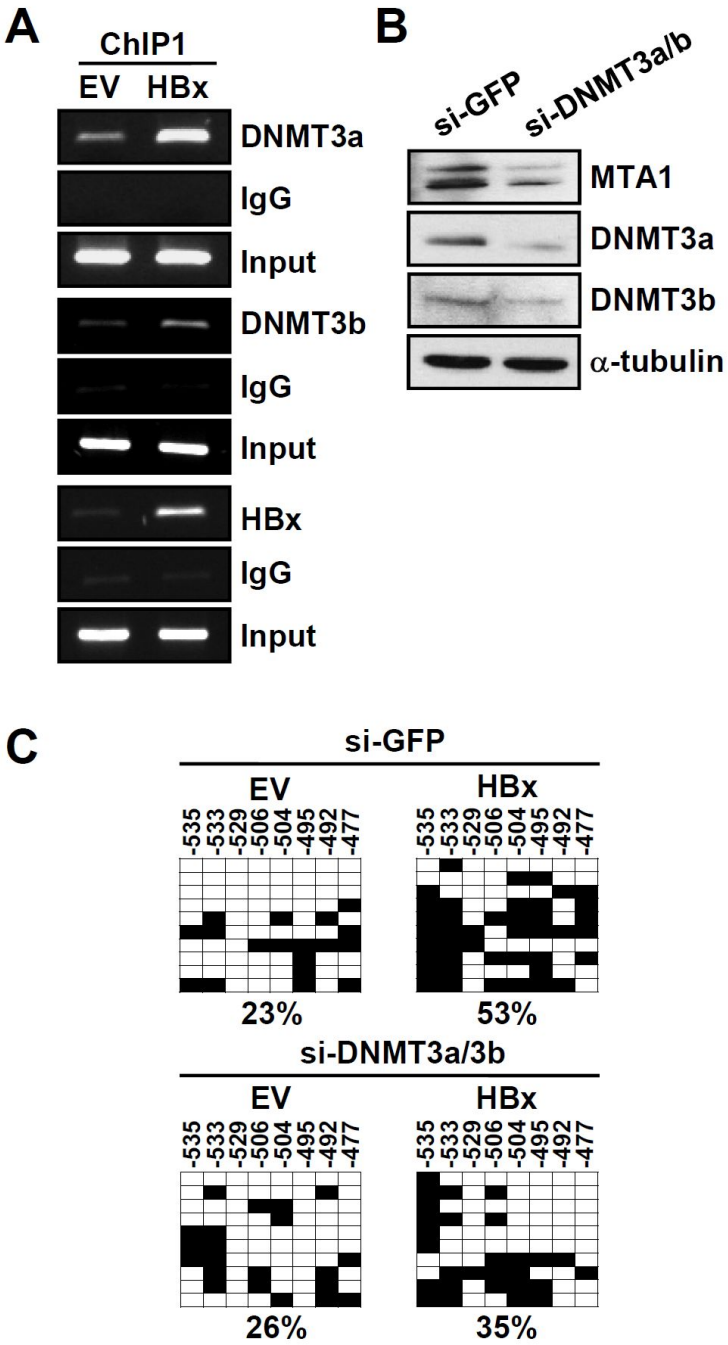

A
ChIP1
EV
HBx
DNMT3a
IgG
Input
DNMT3b
IgG
Input
HBx
IgG
Input
B
si-GFP
si-DNMT3a/b
MTA1
DNMT3a
DNMT3b
α-tubulin
C
si-GFP
EV
HBx
-535 -533 -529 -506 -504 -495 -492 -477
23%
53%
si-DNMT3a/3b
EV
HBx
-535 -533 -529 -506 -504 -495 -492 -477
26%
35%

Figure 18. HBx enhances recruitment of DNMT3a and DNMT3b on the MTA1 promoter CpG island. (A) Chang liver cells were transfected with empty vector (EV) or p3XFLAG7.1-HBx. DNA fragments that immunoprecipitated were amplified by PCR. (B) Chang liver cells were transfected with si-GFP or mixed si-DNMT3 (si-DNMT3a and si-DNMT3b). After 72 h of transfection, whole cell lysates were prepared and protein levels were evaluated by western blotting. (C) Chang liver cells were transfected with si-GFP or mixed si-DNMT3 (si-DNMT3a and si-DNMT3b). After 24 h of transfection, cells were transfected with EV or p3XFLAG7.1-HBx for another 24 h. Results from bisulfite sequencing analysis of the MTA1 promoter CpG island were shown. Filled squares, methylated; and open squares, unmethylated.

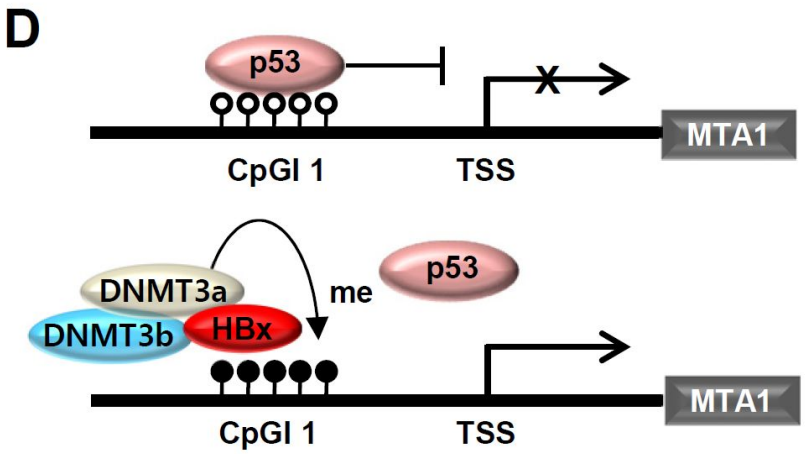


Figure 18. continued

(D) Schematic representation for the HBx-induced derepression of MTA1 gene by methylation of the CpG island on the promoter.

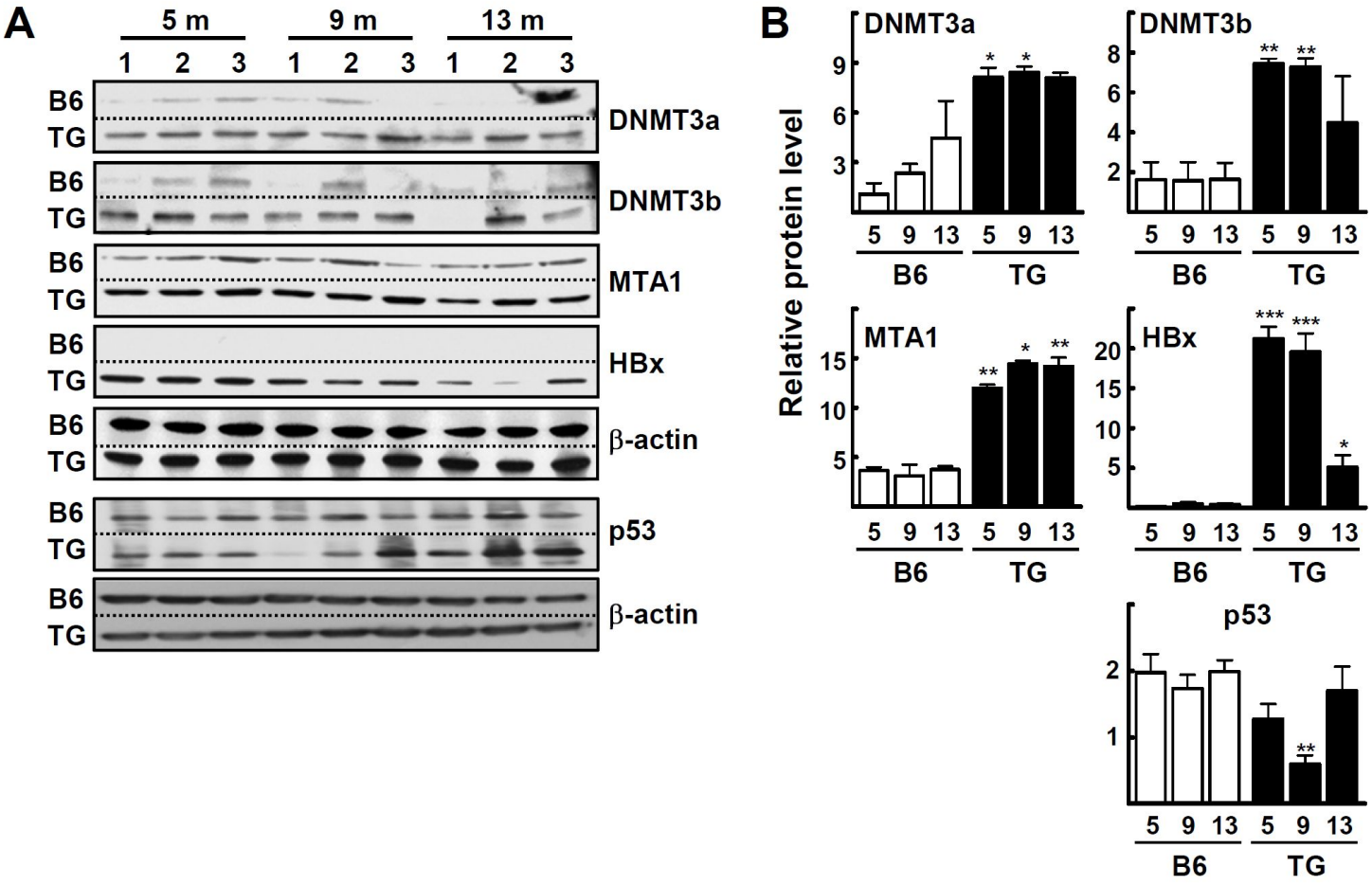
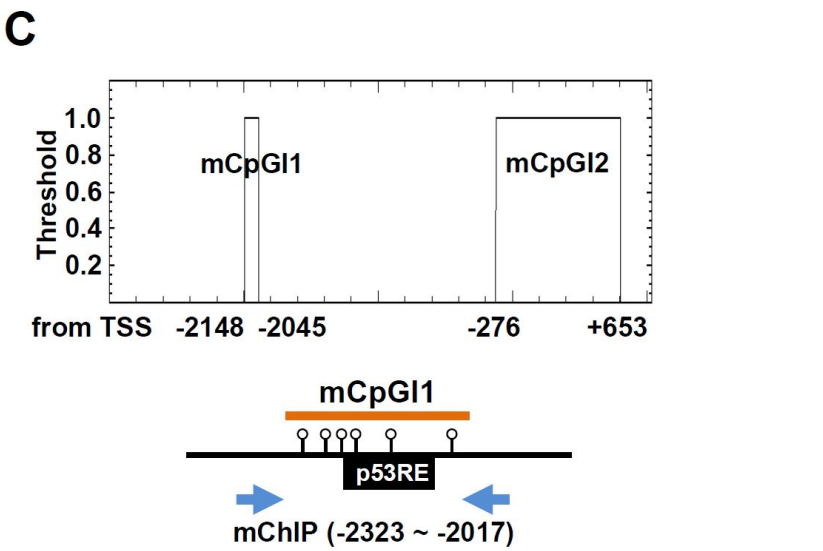

A
5 m
9 m
13 m
1 2 3 1 2 3 1 2 3
B6
TG
DNMT3a
DNMT3b
MTA1
HBx
β-actin
p53
β-actin
B
Relative protein level
DNMT3a
DNMT3b
MTA1
HBx
p53
5 9 13 5 9 13
B6
TG

Figure 19. Increases in expression level of MTA1, DNMT3a, and DNMT3b in the liver of HBx-transgenic mice. (A) Tissue lysates were obtained from the liver of the indicated aged B6 and HBx-TG mice. The tissue lysates were separated on two different polyacrylamide gels, transferred to the same PVDF membranes, and probed with specific antibody by the same procedure. (B) Density of each protein band in (A) was determined using an image analysis system and normalized to that of the corresponding β-actin. Y-axis represents the relative protein level to compare with that of 5 month-old WT mice. *p<0.05, **p<0.005, ***p<0.0001 *vs* age-matched B6 mice (n=3).


C
Threshold
1.0
0.8
0.6
0.4
0.2
mCpGI1
mCpGI2
from TSS
-2148
-2045
-276
+653
mCpGI1
p53RE
mChIP (-2323 ~ -2017)

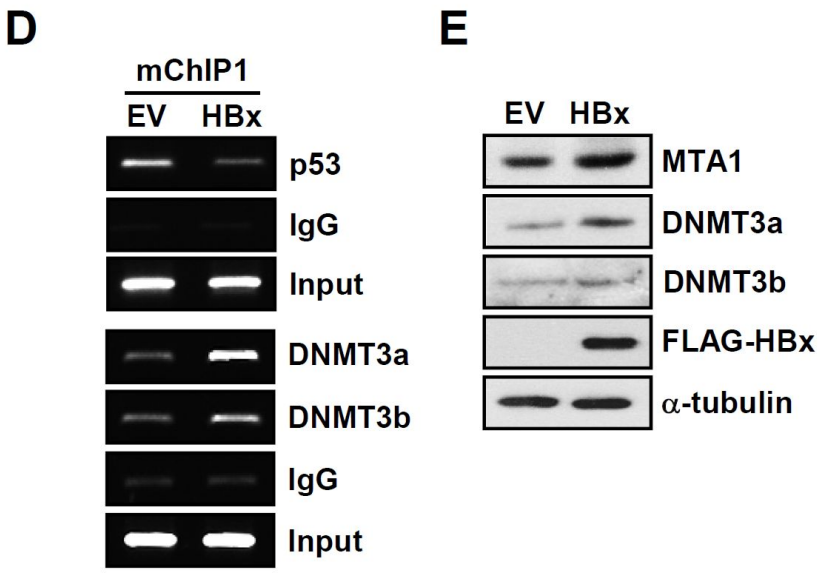

D
mChIP1
EV
HBx
p53
IgG
Input
DNMT3a
DNMT3b
IgG
Input
E
EV
HBx
MTA1
DNMT3a
DNMT3b
FLAG-HBx
α-tubulin

Figure 19. Continued

(C) Putative CpG islands are located at bases -2148 to -2045 (mCpGI1) and -276 to +653 (mCpGI2) in the mouse MTA1 promoter (upper). Schematic presentation of the mCpGI1 encoding p53 binding sequences (p53RE) with DNA region for ChIP (lower). (D) NIH3T3 cells were transfected with empty vector (EV) or p3XFLAG7.1-HBx. DNA fragments that immunoprecipitated by anti-p53, anti-DNMT3a, or anti-DNMT3b, were amplified by PCR using primers specific for mChIP. (E) NIH3T3 cells were transfected with EV or p3XFLAG7.1-HBx. Whole cell lysates were prepared and protein levels were evaluated by western blotting.

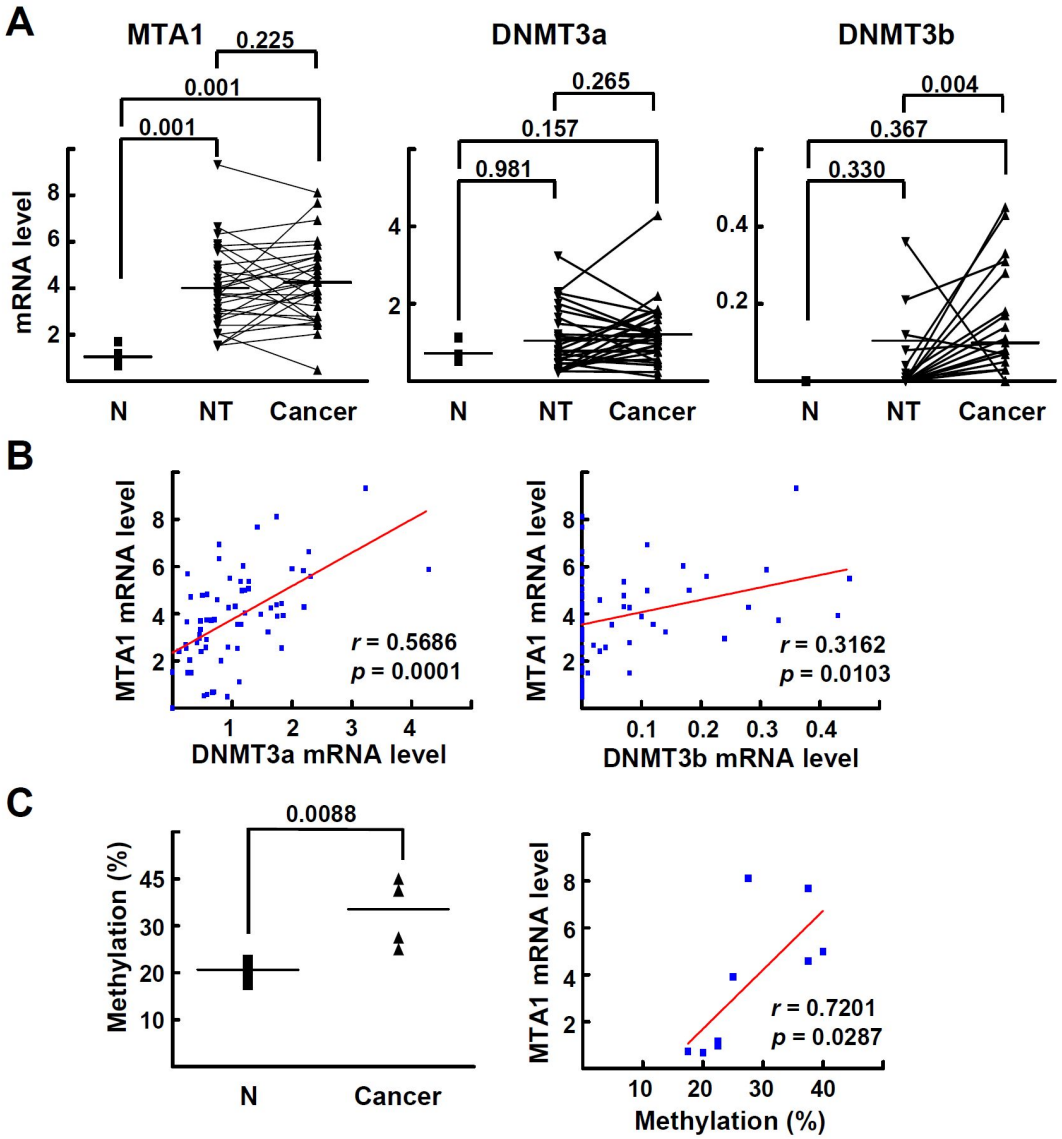

A
MTA1
0.225
0.001
0.001
mRNA level
8
6
4
2
N
NT
Cancer
DNMT3a
0.265
0.157
0.981
4
2
N
NT
Cancer
DNMT3b
0.004
0.367
0.330
0.4
0.2
N
NT
Cancer
B
MTA1 mRNA level
8
6
4
2
r = 0.5686
p = 0.0001
1
2
3
4
DNMT3a mRNA level
MTA1 mRNA level
8
6
4
2
r = 0.3162
p = 0.0103
0.1
0.2
0.3
0.4
DNMT3b mRNA level
C
0.0088
Methylation (%)
45
30
20
10
N
Cancer
MTA1 mRNA level
8
6
4
2
r = 0.7201
p = 0.0287
10
20
30
40
Methylation (%)

Figure 20. Expression level of MTA1 is correlated with the MTA1 promoter methylation in HCC specimens. (A) The HBV-associated HCC tissues that obtained from 30 HCC patients (Na *et al*., 2009). qRT-PCR was performed with cDNA prepared from the microdissected non-tumor or cancer regions. The independent data (normal (N) *vs* nontumor (NT) region and normal *vs* cancer) and the matched data (nontumor *vs* cancer) of gene expression was analyzed using Wilcoxon signed rank test and Mann-Whitney test, respectively. (B) Pearson correlation analysis for comparison of mRNA level of MTA1 and DNMT3a/3b gene in 6 human normal liver and 30 HCC specimens. (C) Methylation status of the CpG island 1 of four normal human livers and five HCC tissues with higher MTA1 expression were analyzed by bisulfite sequencing (left). Pearson correlation analysis for comparison of MTA1 mRNA expression level and % methylation of the CpG island of MTA1 promoter (right).

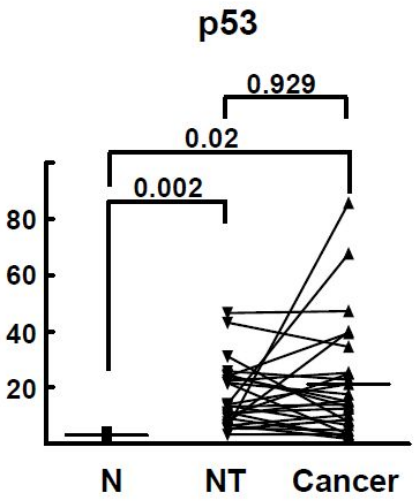


Figure 21. Expression level of p53 in the HCC specimens. qRT-PCR was performed with cDNA prepared from the microdissected non-tumor or cancer regions used to Figure 6. Four HCC specimens and 2 normal liver samples were not analyzed due to RNA quality problem. The independent data (normal (N) vs nontumor (NT) region and normal vs cancer) and the matched data (nontumor vs cancer) of gene expression was analyzed using Wilcoxon signed rank test and Mann-Whitney test, respectively.

IV. DISCUSSION

MTA1 is highly expressed in hepatocellular carcinoma and its elevated expression associated with angiogenesis, metastasis, more advanced stage and poorer prognosis. Chronic infection of HBV is a major cause of development and progression of hepatocellular carcinoma. The MTA1 expression levels were significantly greater in hepatitis B virus-associated HCC compared with hepatitis C virus-associated HCC. MTA1 has been reported to enhance angiogenesis through cross-talk with hypoxia-inducible factor-1alpha, an important angiogenic factor in cancers. Up regulation of MTA1 expression has been demonstrated in tumor and transformed cell hypothesizing its global deregulation in cancer. Thus, the mechanism how expression of MTA1 gene is regulated during carcinogenesis becomes an important issue. Herein, we demonstrated that the tumor suppressor p53 plays a vital role in the repression of the expression of MTA1. We also found that the HBx-induced methylation of MTA1 promoter is linked to a decrease in tumor suppressor function of p53, which further supports the oncogenic potential of HBx in development and progression of the HBV-associated HCC.

1. Poly(ADP-ribosyl)ation of p53 induces gene-specific transcriptional repression of *MTA1*

An elevated expression of MTA1 gene is associated with carcinogenesis characterized by invasion and metastasis, indicating a global deregulation of MTA1 gene expression in cancer. Thus, the mechanism how expression of MTA1 gene is regulated during carcinogenesis becomes an important issue. Here, we demonstrated that the tumor suppressor p53 plays a vital role in transcriptional repression of MTA1 gene. Interestingly, the p53-mediated *trans*-repression of MTA1 gene requires PARylation of p53 that catalyzed by PARP-1. As far as we know, we report for the first time that PARylation of p53 is a new type of PTM that induces *trans*-repression of oncogenic proteins such as MTA1.

Although PARP-1 is an abundant nuclear enzyme that plays important roles in DNA repair, recent studies have demonstrated its function in gene-specific epigenetic regulation. PARP-1 PARylates directly chromatin-associated proteins, including histone variants, chromatin-modifying enzymes, and transcriptional factors (Kraus, 2008). PARP-1 targets the p53 protein, resulting in PARylated p53; however, the effect of PARylation on the biological function of p53 is poorly understood (Wesierska-Gadek *et al*.,

1996; Kumari *et al*., 1998; Simbulan-Rosenthal *et al*., 1999). Limited observations suggest that PARylation of p53 regulates the DNA-binding properties or the localization of p53 (Mendoza-Alvarez and Alvarez-Gonzalez, 2001; Kanai *et al*., 2007). In this study, we report a novel function of PARylated p53: involvement in gene-specific regulation of the transcriptional mode of p53. Surprisingly, PARP-1 inhibition using PHEN resulted in differential perturbation of the binding of p53 to the promoters of target genes (Figure 11). PHEN treatment inhibited p53 binding to the promoters of MTA1 and survivin, which are regulated in the *trans*-repression mode, whereas it increased p53 binding to the promoters of p21 and BAX, which are regulated in the *trans*-activation mode (Figure 11D). Interestingly, we found that PARylation of p53 was barely detectable in MDA-MB-231 which has a mutated p53 (Figure 12A). 5-FU treatment did not increase, or even decrease, the level of p53 PARylation in MDA-MB-231 cells, and the mutant p53 did not bind to the MTA1 promoter (Figures 12B and 12C).

In general, PAR has a higher negative charge density than DNA; therefore, PARylation of proteins may affect protein–protein and protein–DNA interactions (Kim *et al*., 2005). However, the molecular details of the mechanism *via* which PARylation of p53 specifies transcriptional

outcome remain an open question. Recent research may provide several potential answers to this question. First, chromatin dynamics induced by PARylated p53 may promote the recruitment of differential transcriptional coactivators onto target genes. Mehrotra *et al*. (2011) reported that PARP-14 regulates STAT6-dependent transactivation by switching HDACs to HAT coactivators on the promoter of the target gene, Fcer2a. Second, PARylation of p53 may be linked to DNA methylation, which is essential for specific patterns of gene expression during tumor development. Although a link to PARylation was not established, p53 and DNMT1 may cooperate toward the *trans*-repression of the p53 target genes survivin and *CDC25C* (Estève *et al*., 2005). Inhibition of the PARylation process via either the chemical inhibitor 3-aminobenzamide or the ectopic overexpression of poly (ADP-ribose) glycohydrolase altered the pattern of DNA methylation in the promoter of the HTF9 or DNMT1 genes (Zardo and Caiafa, 1998; Zampieri *et al*., 2009). Finally, DNA sequences for p53 binding may distinguish between PARylated and non-PARylated p53. p53 response elements differ largely regarding the number of copies of the consensus motif and in the pattern of the array. Wang *et al*. (2009) demonstrated that activating p53REs are the canonical sequences, whereas repressing p53REs are noncanonical. We found that the p53REs located in the MTA1 promoter are similar to the

noncanonical p53RE of the survivin promoter, which is apparently different from the canonical p53REs of the promoters of BAX or p21 (Riley *et al*., 2008; Wang *et al*., 2009). Further experimental evidences are necessary to clarify the mechanisms proposed and will promote a better understanding of the differential transcriptional functions of p53 and PARP-1.

5-FU has been used widely in the treatment of a broad spectrum of cancers, including colorectal and breast cancers. 5-FU induces a shortage of pyrimidine nucleotides, and, more importantly, induces p53 (Longley *et al*., 2003). Here, we demonstrated that MTA1 is a novel target of 5-FU-induced p53. 5-FU repressed the expression of MTA1 efficiently via induction of p53, thereby abolishing the stability of the MTA1-induced HIF-1α protein and downregulating VEGF (Figure 14). These findings are also observed in a clinical setting, as the global transcript profiles of 5-FU-based chemoradiotherapy colorectal adenocarcinoma samples exhibited more repressed expression of the MTA1, VEGF, and survivin genes compared with that observed in nontreated or short-course radiotherapy samples (Petty *et al*., 2009). Taken together, these observations indicate that the repression of MTA1 may represent an important mechanism underlying the anticancer effects of 5-FU treatment.

As PARPs are activated by DNA-damaging chemotherapy and irradiative radiation, these molecules are attractive therapeutic targets for cancer treatment (Krishnakumar and Kraus, 2010). Therefore, PARP inhibitors are developed to target a wide spectrum of cancers or to sensitize cancer cells to anticancer therapy. A variety of PARP-1 inhibitors, including Iniparib, Olaparib, and Veliparib, are currently in clinical trials (Yap *et al*., 2011). However, much attention should be given to our observation that knockdown or chemical inhibition of PARP-1 decreased the 5-FU-induced p53 binding on the promoter of MTA1, and subsequently recovered the expression level of the *MTA1* gene (Figure 11C and 14A). Likewise, Wang *et al*. demonstrated that 3-aminobenzamide, a PARP inhibitor suppressed radiation-induced p53 and the expression of the p53 target genes (Wang *et al*., 1998) and PARP-1 knockout MEF cells were slightly increased p21 expression when exposure g-irradiation. In contrast to radiation, single alkylating agent 2'-methyl-2'-nitrose-urea exposure produced a strong up-regulated of p21 in PARP-1 deficient cells (Valenzuela *et al*., 2002). These data indicate that different type of DNA damage is determinant of the role of PARP-1 in the p53-mediated response to genotoxic stress (Valenzuela *et al*., 2002). Similarly, PARP-1 interacts with and PARylates other transcription factors, such as SMAD3/4 and the Kruppel-like factor 8,

thereby regulating their target genes (Lönn *et al*., 2010; Lu *et al*., 2011). These observations indicate that inhibition of PARP-1 may cause disturbances in gene expression that may drive the stimulation of a genetic network that enhances oncogenic potential. More intriguingly, several preclinical studies have reported that PARP inhibitors are more effective in BRCA-deficient cells than in wild-type BRCA cells (Yap *et al*., 2011). Furthermore, a recent trial showed that iniparib failed to prolong survival in metastatic, triple-negative breast cancer (O'Shaughnessy *et al*., 2011). The study enrolled 519 women with metastatic triple negative breast cancer from 109 sites in the United States. Patients were randomized to receive a standard chemotherapy regimen (gemcitabine and carboplatin) with or without iniparib. These findings may raise issues regarding the spectrum of clinical application of PARP-1 inhibitors, which may be related to our finding of gene-specific regulation mediated by PARylated p53.

2. Epigenetic control of metastasis-associated protein 1 gene expression by hepatitis B virus X protein during hepatocarcinogenesis

Because of the strong connection between MTA1 gene expression and progression of HBV-associated HCC, identifying the molecular mechanism responsible for the effect of HBx on the transcriptional activation of the MTA1 gene is an important task. It was shown recently that HBx recruits p65 to the NF-κB consensus motif on the relaxed MTA1 gene chromatin (Bui-Nguyen *et al*., 2010a) HBx controls the expression of the MTA1 gene by suppressing miR-661 and subsequently activates inducible nitric oxide synthase in liver cancer cells (Bui-Nguyen *et al*., 2010b). However, the mechanisms responsible for the increased expression of MTA1 in HCC remain largely unknown. In the present study, we demonstrated the existence of an epigenetic mechanism for MTA1 gene regulation during hepatocarcinogenesis, which was mediated by HBx protein. We observed that HBx induced the methylation of human MTA1 gene promoter CpG island1, which contains the specific DNA-binding sequences for p53 (Figure 16B). The MTA1 gene promoter also contains a CpG island2 region; however, this

region did not respond to HBx-mediated methylation, probably because of the presence of DNA-binding sites for transcription factors that confer resistance to de novo methylation in cancer cells.

Epigenetic inactivation caused by hypermethylation of a promoter is well established for genes involved in the initiation and progression of HCCs (Tischoff and Tannapfe, 2008) The levels of DNMT1, DNMT3a, and DNMT3b were increased significantly in HCC tissues compared with nonneoplastic liver tissues (Figure 20A) (Saito *et al*., 2003; Park *et al*., 2006). In many cases, the aberrant DNA methylation is associated with gene silencing. For example, the expression levels of tumor suppressor genes, such as p16INK4A, correlates inversely with the expression of DNMT3a (Tischoff and Tannapfe, 2008). However, we observed a significant positive correlation between the mRNA expression levels of MTA1, DNMT3a and DNMT3b in the present study (Figure 20B). Similarly to our observation, DNA methylation-mediated derepression was reported for several genes with oncogenic potential. The methylation of the hTERT promoter at the CCCTC-binding factor-binding site inhibits the DNA binding of CCCTC-binding factor, which increases hTERT expression, especially in

human tumors (Renaud *et al*., 2007). Methylation of the survivin gene promoter inhibits the binding of p53 and causes derepression of survivin gene expression (Nabilsi *et al*., 2009). Interestingly, the observation that HBx recruited DNMTs to the MTA1 promoter in our investigation (Figure 18A and 19D), raises the possibility that the function of HBx is linked to specific targeting of promoters of both tumor suppressor genes and genes with oncogenic potential. Indeed, HBx induces hypermethylation of the IGFBP-3 promoter by recruiting DNMT1, DNMT3A1, and DNMT3A2, which suppress IGFBP-3 expression (Zhu *et al*., 2010). By contrast, HBx suppresses the expression of p16INK4A, RAR-β2, ASPP1, and ASPP2, in HCC tissues by upregulating or recruiting DNMT1 and DNMT3A (Zhu *et al*.,2010; Jung *et al*., 2010; Zhao *et al*., 2010). HBx induces the transcriptional activation of DNMT1, which causes subsequent DNA hypermethylation of the promoter of E-cadherin (Lee *et al*., 2005). Therefore, HBx may be one of the most potent and efficient epigenetic regulators that control cellular gene expression and may have beneficial effects for viral survival and propagation through immortalization of host cells.

In this study, we found that DNA methylation-induced derepression of the MTA1 gene was closely associated with the function of p53. This observation may be related to the previous observation that the loss of p53 function increases invasion and metastasis in several in vivo models of HCC (Lang *et al*., 2004; Lewis *et al*., 2005). However, mRNA level of p53 was significantly higher in the non-tumorous and tumor tissues compared with the p53 level in normal human livers (Figure 15). However, the mRNA level of p53 may not represent the functional p53, since inactivation of p53 by mutations is frequently found in tumors associated with HBV infection.32 Further the negative cross-talk between HBx and p53 protein has been addressed in context of HBV-associated hepatocarcinogenesis. HBx binds to the wild-type p53 protein, inhibits sequence-specific DNA binding, and sequesters p53 in the cytoplasm, thereby preventing its nuclear entry (Elmore *et al*., 1997; Hussain *et al*., 2007). Here we show a new type of cross-talk between HBx and p53, in which HBx-mediated methylation of DNA inhibits specific DNA binding of p53 (Figure 17 and 19), and p53 is then unable to interact with its cognate binding sites if a methylated cytosine is present. The survivin promoter contains a region containing a p53-binding element,

and methylation of the region inhibits the binding of p53 (Nabilsi *et al*., 2009). In an independent study, HBx increased the expression of survivin, suggesting that the survivin promoter may be a target of HBx-mediated methylation (Zhang *et al* 2005). We reported recently that poly(ADP-ribose) polymerase 1 (PARP-1)-mediated poly(ADP-ribose)ylation (PARylation) of p53 is necessary for the transcriptional repression of MTA1 (Lee *et al*., 2012). PARP-1 is an abundant nuclear enzyme that plays important roles in DNA repair, recent studies have demonstrated its function in epigenetic modification (Kraus *et al*., 2008). Inhibition of PARP-1 by 3-aminobenzamide increased and altered the pattern of DNA methylation in the Htf9 promoter and transfected 216 CpG-like sequence containing foreign DNA (Zardo and Caiafa *et al*., 1998; Zardo *et al*., 1999). Our data together with those of other groups may suggest that PARP-1-mediated PARylation on certain cellular proteins plays a role in maintaining the methylation pattern of genomic DNA. Whether the PARylation of p53 is linked to HBx-mediated DNA methylation remains as an interesting question.

Several recent studies have shown that MTA1 can be a molecular target for antimetastatic therapy. Recently, several studies have shown that

MTA1 could be a molecular target for anti-metastatic therapy. Qian *et al*. examined the therapeutic value of MTA1 levels in malignant melanoma cells, results that down-regulation of MTA1 by RNAi successfully suppressed the growth in vitro and experimental metastasis of mouse B16F10 melanoma cells in vivo (Qian *et al*., 2007). In prostate cancer, silencing MTA1 significantly suppressed the invasion and angiogenic activity of the prostate cancer cell lines and delayed tumor formation and development in mouse xenografts (Kai *et al*., 2011). Further, the cell invasion, migration and adhesion capabilities were decreased after inhibition of MTA1 expression mediated by MTA1-siRNA transfection in SiHa cells (Rao *et al*., 2001). These studies demonstrated that MTA1 may serve as an anti-metastatic therapeutic target for cancer therapy. In this study, we found that the expression of MTA1 was significantly greater in both the cancerous regions and the adjacent noncancerous regions than in the normal liver (Figure 20A). This result suggests that MTA1 also plays a role in precancerous changes in HBV-associated HCC. Epigenetic modification has emerged recently as an attractive therapeutic strategy against HBV-associated HCC. DNMT inhibitor demethylation agents, such as 5-aza-2'deoxycytidine,

and histone deacetylase inhibitors, such as SAHA, tributyrin, and valproic acid, have been tested preclinically against HCC and have showed promising results (Herceg and Paliwal, 2011). Interestingly, 5-aza-2'deoxycytidine can increase the sensitivity of hepatoma cell lines to 5-fluorouracil, which represses the transcriptional expression of MTA1, as demonstrated in our recent study (Lee *et al*., 2012) and another study (Kanda *et al*., 2005). In the present study, we showed that MTA1 expression was upregulated by DNA methylation, which may provide a rationale for epigenetic combination therapy to HBV-associated HCC in patients with an increased MTA1 expression level. In summary, the present study highlights the important role of DNA methylation in MTA1 expression in HBx-positive HCC.

V. CONCLUSIONS

In the present study, I investigated the molecular details of the regulation of the expression of MTA1. I found that p53 trans-repressed the expression of the MTA1 gene via the p53 binding elements located in its upstream promoter. Moreover, p53 was PARylated directly by PARP-1 when it formed a p53-associated repressing complex with HDAC1 and HDAC2 on the MTA1 promoter. Hence, here I report that MTA1 is a new target gene of p53 and that PARylation of p53 has an important function in the p53-mediated repression of the expression of the MTA1 gene. These findings may raise issues regarding the spectrum of clinical application of PARP-1 inhibitors, which may be related to our finding of gene-specific regulation mediated by PARylated p53.

In addition, I report on an important epigenetic regulation of the MTA1 promoter that is mediated by HBx during hepatocarcinogenesis. A CpG island and its methylation patterns of the human MTA1 promoter were characterized in HBx-expressing cell lines and in tissues obtained from patients with HBV-associated HCC. I also found that the HBx-induced methylation of the MTA1 promoter was linked to a decrease in the tumor suppressor function of p53, which further supports the oncogenic and

metastatic potential of HBx in the development and progression of HBV-associated HCC. This findings shed light on the basic molecular mechanisms involved in HBx-induced HCC progression and support a role for DNMTs as a potential target for antimetastatic therapy.

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국 문 초 록

간암 진행과정에서 전이활성인자 MTA1의 전사조절 기전 규명

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전이활성인자 MTA1은 세포내 유전자발현을 조절하는 인자인 NuRD 복합체 구성 단백질로서 유전자 발현을 억제하는 기능을 가지고 있다. MTA1은 정상조직에서는 약한 발현을 보이지만 간암을 포함한 여러 종류의 암조직에서 과발현 되어 있고, 침윤과 전이와의 높은 상관관계를 보이고 있음이 보고되어 있다. 하지만, 암세포에서 MTA1 유전자 발현 조절기전은 거의 알려져 있지 않다.

첫 번째 단원에서 암세포에서 MTA1 발현 조절 기전을 알아보기 위해 MTA1 프로모터에 결합하는 전사인자를 분석하였으며, MTA1 유전자 발현 조절 전사인자인 p53을 찾아내었다. MTA1 프로모터에는 3부분의 p53 결합부위가 존재하고 있으며, MTA1 프로모터 활성과 단백질 발현은 p53을 활성화 시키는 약물인 5-fluorouracil (5-FU)처리에 의해 감소하였다. 5-FU에 의한 MTA1 발현의 감소는 wild-type p53을 가지고 있

는 세포에서만 관찰되었으며, 이때 p53과 히스톤탈아세틸화효소 1/2가 MTA1 프로모터로 오며, 전사활성표지자인 H3K9Ac이 감소함을 확인하였다. 또한 p53에 의한 MTA1 발현 감소에 관여하는 p53복합체 인자들을 확인하기 위해, DNA 침강법으로 침강된 MTA1 프로모터 p53 결합부위 결합단백질을 LC/MS/MS 분석을 통해 분석하였으며, poly(ADP-ribose) polymerase 1 (PARP-1) 단백질을 찾아 내였다. 흥미롭게도 p53은 PARP-1에 의해 poly(ADP-ribose)ylation되었으며, 이는 p53에 의한 MTA1 유전자의 감소에 필요함을 알 수 있었다. 본 연구를 통해 poly(ADP-ribose)-ylation된 암억제 유전자인 p53이 MTA1 프로모터와의 결합을 통해 MTA1의 발현을 억제한다는 새로운 사실을 알 수 있었다.

MTA1의 발현은 B형간염 바이러스 X 단백질 (HBx)에 의해 증가한다고 알려져 있지만 HBx에 의한 MTA1 단백질의 조절 기전은 거의 알려져 있지 않다. 두 번째 단원에서는 HBx가 MTA1의 발현을 증가시키는 전사조절 기전에 대해 알아보았다. 먼저, MTA1 프로모터 5' 상위서열 분석을 통해 MTA1 프로모터는 두 부위의 CpG island를 가지고 있음을 알 수 있었다. 인간 간세포주 Chang 세포에 HBx 단백질을 과발현시켜, bisulfite-modified direct sequencing을 통해 DNA methylation변화를 확인해 본 결과 CpG island1 부분의 DNA methylaion이 18%에서 49%로 증가하였으며, 또한 크로마틴 면역침강법을 통해 HBx 단백질이 CpG island1부위로 DNMT3a와 DNMT3b를 유도한다는 사실을 알 수 있었다.

In silico 분석 방법을 통한 CpG islannd1 분석결과 CpG islannd1 부위에는 p53 결합부위가 존재하였다. 흥미롭게도 DNA 침강법을 통해 p53은 methylation된 DNA에는 결합하지 않음을 알 수 있었다. 추가로, 마우스 MTA1 프로모터부위에도 p53 결합부위를 가지고 있는 CpG island가 존재하며, HBx 단백질이 과발현될 때 p53단백질의 결합이 감소함을 확인하였다. 또한 HBx 형질전환 마우스 간 조직에서 MTA1과 DNMT3의 발현이 증가해 있음을 관찰할 수 있었다. 마지막으로, 인간 정상 간조직과 간암조직 임상샘플분석결과 MTA1의 발현과 DNMT3a의 발현이 유의성 있는 상관관계를 보였으며 ($r = 0.5686$, $P = 0.0001$), DNMT3b는 미미한 상관관계를 보였다 ($r = 0.3162$, $P = 0.0103$). 본 연구를 통해 HBx 단백질이 MTA1 프로모터 CpG island의 methylation을 증가시키고, 이로 인해 p53의 DNA 결합하는 것을 방해함으로서, MTA1의 발현을 증가시킨다는 새로운 사실을 알 수 있었다.

주요어 : MTA1, p53, PARP-1, poly(ADP-ribose)ylation; HBx; 간암, DNA methylation