trace amounts of endogenous AID protein were detectable in HepG2 cells without any stimulation. AID protein expression was markedly increased after TNF-α treatment. In contrast, AID protein was not detected in cells treated with AID-targeting siRNA, irrespective of stimulation with TNF-α (Figure 2f). A time course study revealed that AID protein expression was detected after stimulation with TNF-α (Figure 2g). We further confirmed the proinflammatory cytokine-mediated expression of endogenous AID protein in a non-neoplastic primary hepatocyte cell line that retained primary hepatocyte characteristics (Figure 2h) (Aly *et al.*, 2007). Taken together, these data suggest that proinflammatory cytokine signaling induces *AID* gene expression in human hepatocytes.

*AID expression in hepatocytes is achieved through NF-κB activation*

Our findings that proinflammatory cytokines induced *AID* transcripts in hepatocytes led us to test whether AID expression is mediated by an NF-κB-dependent mechanism, because both TNF-α and IL-1β activate the transcription factor NF-κB and contribute to the expression and regulation of various genes. Activation of the classical NF-κB pathway converges on IκB kinase complex (IKK), a protein complex composed of two kinase subunits (IKK-α and IKK-β) and a non-catalytic subunit (IKK-γ/NF-κB expansion modulator (NEMO)). To clarify whether IKK is involved in TNF-α-mediated AID expression, we examined the effects of wild-type IKK-α and IKK-β on AID expression. The AID expression levels were significantly upregulated in the cells expressing IKK-α, IKK-β or NF-κB RelA protein itself (Figure 3a).

Next, we examined the TNF-α-mediated *AID* expression with NF-κB signaling inhibitors. We found that the NF-κB inhibitory reagents SN50 and MG132 significantly reduced TNF-α-induced NF-κB activation, and pretreatment with SN50 or MG132 almost completely suppressed the TNF-α-induced increase in *AID* transcripts (Figure 3b). Furthermore, TNF-α-induced AID protein expression was almost completely abolished by co-production of the super-repressor form of IκBα, a specific NF-κB inhibitor (Figure 3c). We then used siRNA to reduce the expression of endogenous IKK-γ/NEMO. Transfection of IKK-γ-specific siRNA, but not control siRNA, reduced endogenous IKK-γ protein levels in HepG2 cells and the lower levels were sustained for at least 3 days. Under these conditions, TNF-α stimulated AID expression in control cells. In contrast, TNF-α failed to elicit an increase in AID protein in cells in which endogenous IKK-γ was reduced by siRNA (Figure 3d). Taken together, these findings indicate that the induction of AID expression in human hepatocytes by TNF-α is achieved through the activation of NF-κB.

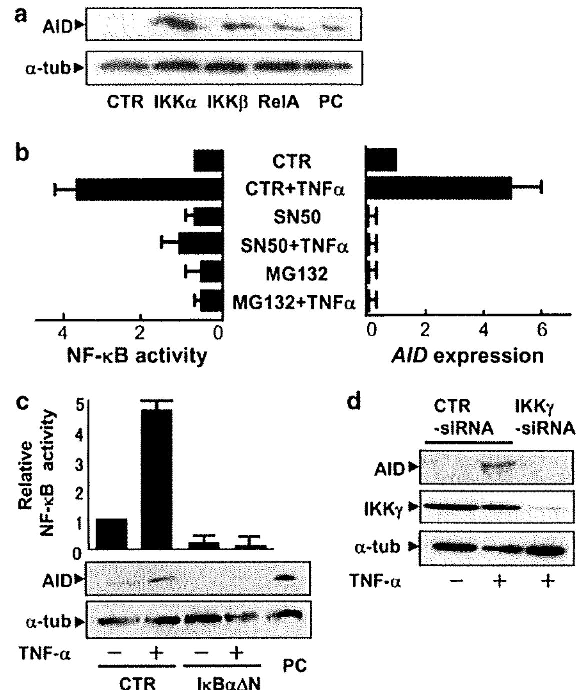



**Figure 3** AID expression in human hepatocytes is regulated by NF-κB activity. (**a**) HepG2 cells were transfected with plasmid for the expression of IKK-α, IKK-β or RelA, or with a control vector (CTR). After 48 h, lysates of the transfected cells were immunoblotted with anti-AID antibody (upper panel) or anti-α-tubulin antibody (α-tub, lower panel). The lysate from the BL2-lymphoma cell line, which contains a high expression level of endogenous AID, was used as a positive control for AID expression (PC). (**b**) Effects of the NF-κB inhibitors on TNF-α-induced NF-κB activity and endogenous AID expression. HepG2 cells were transfected with pNF-κB-Luc, followed by the treatment with MG132 (2 μg/ml) or SN50 (50 μg/ml) for 2 h, and further subjected to TNF-α (100 ng/ml) stimulation for 12 h. Luciferase activities were monitored in each extract and normalized by the activity of *Renilla* luciferase (left graphs). Total RNA was also isolated from each cell and the expression levels of *AID* mRNA were measured by quantitative real-time RT–PCR (right graphs). The data present the means of the relative luciferase activity and *AID* expression in three independent experiments. (**c**) pcDNA3-IκBαΔN was transfected into Hep3B cells and then treated with TNF-α (100 ng/ml) for 24 h. Empty vector was used as a control (CTR). Each sample was harvested and luciferase activities were monitored to quantify the endogenous NF-κB activities. Cell lysates were also probed with anti-AID (upper panel) or anti-α-tubulin (α-tub, lower panel). Lysate from the BL2-lymphoma cell line was used as a positive control (PC). (**d**) HepG2 cells were transfected with siRNA targeting IKK-γ/NEMO for 48 h and then treated with TNF-α (100 ng/ml) for 24 h. Whole-cell lysates were probed by anti-AID antibody (upper panel), anti-IKK-γ/NEMO antibody (middle panel) or anti-α-tubulin antibody (α-tub, lower panel).

*HCV core protein triggers AID expression via NF-κB*

Previously, we demonstrated that the HCV core protein has the potential to induce NF-κB activation in human hepatocytes (Marusawa *et al.*, 1999). Thus, the expression of AID protein was analysed by immunoblotting in the presence or absence of various HCV proteins. There was a significant upregulation of AID protein expression in HepG2 cells transfected with expression plasmid encoding the whole HCV genome (Figure 4a). Moreover, marked induction of AID was observed in core-producing cells. In contrast, the production of nonstructural viral protein resulted in little change in the expression

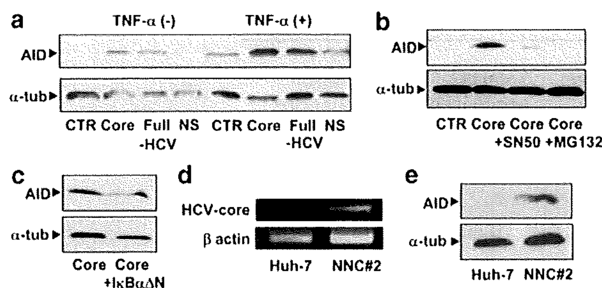



**Figure 4** AID expression is enhanced by production of the HCV core protein. (**a**) HepG2 cells were transfected with plasmids encoding the whole HCV genome (Full-HCV), core protein (Core), non-structural protein (NS) or control vector (CTR). After 48 h, lysates were extracted from the cells with or without treatment with TNF-α (100 ng/ml) for 24 h. AID (upper panel) or α-tubulin (α-tub, lower panel) expression levels were analysed by immunoblotting. (**b**) HepG2 cells were transfected with plasmid encoding the core protein. After 24 h, the cells were treated with MG132 (2 μg/ml) or SN50 (50 μg/ml) for 2 h and stimulated with TNF-α (100 ng/ml) for 24 h. Lysates from these cells were collected and AID or α-tubulin (α-tub) expression levels were analysed by immunoblotting. (**c**) HepG2 cells were transfected with plasmids encoding the core protein with or without the super-repressor form of IκBα (IκBαΔN), a specific NF-κB inhibitor. After 48 h, lysates were extracted and AID (upper panel) or α-tubulin (α-tub, lower panel) expression levels were analysed by immunoblotting. (**d**) Total RNA was extracted from HCV full-genome replicon cells and control parental cells lacking HCV replication. Semiquantitative RT–PCR analyses were performed using specific primers for *HCV-core* (upper panel) or *β-actin* as an internal control (lower panel). (**e**) Whole-cell lysates were collected from the cells of HCV full genome replicon cells or control cells and subjected to immunoblotting analyses using anti-AID antibody (upper panel) or anti-α-tubulin antibody (α-tub, lower panel).

levels of endogenous AID protein. Consistent with the findings of the synergy of proinflammatory cytokine stimulation and viral protein on NF-κB activation (Marusawa *et al.*, 1999), the treatment of whole HCV protein- or core-producing cells with TNF-α further enhanced the expression of AID expression.

To determine whether HCV core-induced AID expression was achieved in an NF-κB-dependent manner, the effects of NF-κB inhibitors and the super-repressor form of IκBα on AID expression were examined in the viral core-expressing cells. We found that incubation of the cells with NF-κB inhibitors, SN50 and MG132, reduced the expression of AID in the cells expressing the viral protein (Figure 4b). Moreover, co-production of the super-repressor form of IκBα with HCV core protein resulted in the reduced expression of endogenous AID protein in HepG2 cells (Figure 4c).

To further confirm the aberrant upregulation of AID by HCV infection, we analysed the expression levels of endogenous AID in the full-genome HCV replicon cells producing whole HCV RNA and proteins (NNC#2 cells) (Ishii *et al.*, 2006). Consistent with a previous report, the expression of the *HCV core* in the replicon cells was confirmed by RT–PCR (Figure 4d). Immunoblotting analyses revealed that AID protein expression was dramatically enhanced in the full-genome replicon cells compared with that in the parental cells lacking HCV replication (Figure 4e). These results demonstrate that HCV core protein induces the expression of AID via NF-κB activation in human hepatocytes.

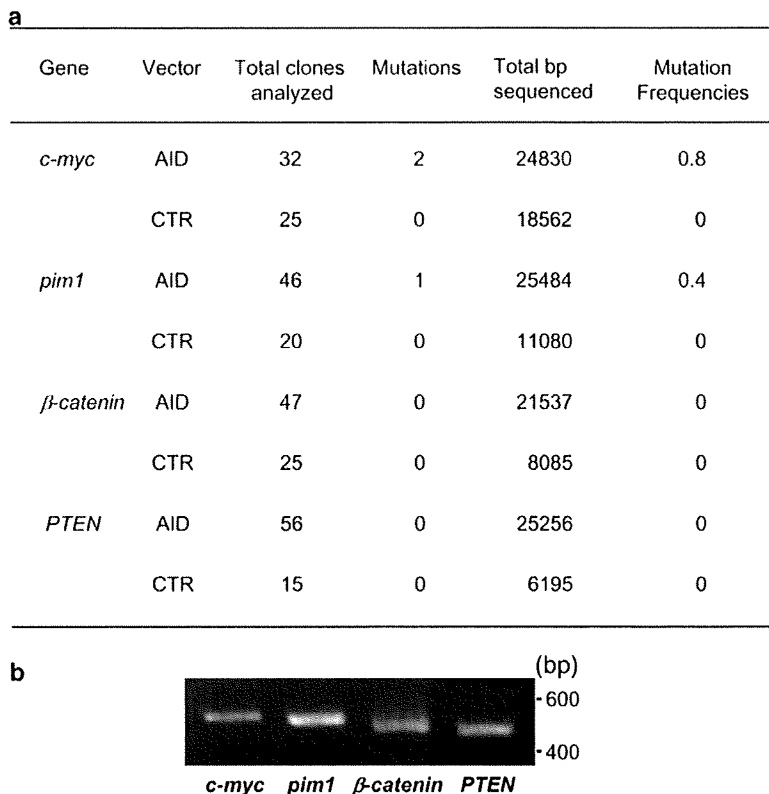
*Expression of AID is sufficient to induce nucleotide alterations in the tumor-related genes in human hepatocytes*

To determine whether aberrant AID expression could contribute to the accumulation of genomic mutations in hepatocytes, we investigated the mutation occurrence in the *c-myc* and *pim1*, both of which are thought to be common targets for abnormal editing in the lymphoma cells of AID Tg mice (Kotani *et al.*, 2005). For this purpose, we established a conditional system that allows for AID activation in the cells in response to an estrogen analog, 4-hydroxytamoxifen (OHT) (Doi *et al.*, 2003). The OHT treatment triggers a post-translational conformational change and thus prompt activation of AID in AID-estrogen receptor (ER)-expressing cells. When the AID ER-expressing HepG2 cells were treated with OHT for 8 days, we found the emergence of nucleotide alterations in the *c-myc* and *pim1* genes in the cells with AID activation, whereas the mutation frequencies in AID-activating cells did not show a statistically significant difference compared to the frequencies in the cells without AID activation (Figure 5a). In contrast, none of the nucleotide substitutions was detected in the control cells with OHT treatment. As the *c-myc* and *pim1* genes were found to be the possible targets for AID in HepG2 cells, we asked whether AID could induce genomic mutations in other tumor-related genes, *β-catenin* and *PTEN*, both of which are thought to be involved in human hepatocarcinogenesis (Thorgeirsson and Grisham, 2002). None of the nucleotide alterations, however, were observed in the *β-catenin* and *PTEN* genes after the OHT treatment. As it has been shown that the AID-mediated somatic mutation is induced in a transcription-dependent manner, we examined the expression levels of these four tumor-related genes in HepG2 cells. RT–PCR analyses revealed evidence showing that the transcription levels of *β-catenin* and *PTEN* genes were comparable with those of *c-myc* and *pim1* genes (Figure 5b).

Consistent with the findings observed in cultured human hepatoma-derived cells with AID activation, sequence analyses of HCC tissues of the AID Tg mice revealed that the *c-myc* gene, which was reported to accumulate high mutation frequencies in T-cell lymphoma, contained nucleotide alteration accompanied by upregulation of *c-myc* transcripts (Supplementary Figure). Taken together, these data suggest that the specificity for mutation of target genes is present in hepatocytes, as was observed in T-cell lymphoma cells (Kotani *et al.*, 2005).

## Discussion

Deregulated expression of the *AID* gene is often observed in human lymphoid malignancies, including non-Hodgkin lymphoma and chronic lymphocytic leukemia (Greeve *et al.*, 2003; Heintel *et al.*, 2004).



**a**

| Gene | Vector | Total clones analyzed | Mutations | Total bp sequenced | Mutation Frequencies |
|---|---|---|---|---|---|
| *c-myc* | AID | 32 | 2 | 24830 | 0.8 |
| | CTR | 25 | 0 | 18562 | 0 |
| *pim1* | AID | 46 | 1 | 25484 | 0.4 |
| | CTR | 20 | 0 | 11080 | 0 |
| *β-catenin* | AID | 47 | 0 | 21537 | 0 |
| | CTR | 25 | 0 | 8085 | 0 |
| *PTEN* | AID | 56 | 0 | 25256 | 0 |
| | CTR | 15 | 0 | 6195 | 0 |



**Figure 5** Mutation frequencies in various tumor-related genes in HepG2 cells with AID activation. (**a**) AID-expressing HepG2 cells and empty vector-expressing cells (CTR) were treated with OHT for 8 days. After the amplification of each tumor related gene, the PCR products were subcloned and sequence analyses were performed. Mutation frequencies were calculated per the total bases analysed $\times 10^{-4}$. (**b**) Total RNA was extracted from HepG2 cells and semiquantitative RT–PCR analyses were performed using specific primers for *c-myc*, *pim1*, *β-catenin* and *PTEN*.

Moreover, animal models demonstrate that aberrant expression of AID causes an accumulation of DNA mutations in various genes leading to tumorigenesis (Okazaki *et al.*, 2003; Kotani *et al.*, 2005). These findings suggest that inappropriate or deregulated AID expression increases the mutation rate of genes that are not normally attacked by AID and are associated with tumor development (Kinoshita and Nonaka, 2006). Under physiologic conditions, however, basal expression levels of AID are not high and the expression of AID is restricted to activated B cells located at the germinal centers. In this study, we clearly demonstrated for the first time that AID expression in hepatocytes is strongly induced in response to proinflammatory cytokine stimulation via NF-κB activation.

At present, little is known about how AID expression is regulated, except that it is under the control of B-cell activating stimulation. It was shown that CD40 ligation induces NF-κB binding to the two potential NF-κB sites located in the 5′ upstream region of the *AID* gene, suggesting that NF-κB participates in the regulatory process of the *AID* gene in B cells (Dedeoglu *et al.*, 2004). In this study, we examined the mechanisms of AID regulation by proinflammatory cytokines and demonstrated that NF-κB activation is involved in TNF-α- as well as IL-1β-induced *AID* gene transcription in hepatocytes. The induction of *AID* transcripts is regulated in an NF-κB-dependent manner in both human lymphoid and human epithelial cells, which suggests the presence of a common mechanism underlying the regulation of *AID* gene transcription.

Several proinflammatory cytokines, including TNF-α and IL-1β, play important roles in the pathophysiology of chronic inflammatory disease, including chronic hepatitis (Tilg *et al.*, 1992; Gonzalez-Amaro *et al.*, 1994). IκB kinase-dependent NF-κB signaling pathways are reported to link inflammation to tumorigenesis (Greten *et al.*, 2004; Pikarsky *et al.*, 2004; Karin and Greten, 2005). Activation of this pathway leads to increases in a variety of genes that encode mediators of inflammatory cytokines, chemokines, cell adhesion molecules, and apoptosis inhibitors (Pahl, 1999; Balkwill and Mantovani, 2001). In this study, we identified AID as a target gene of the IKK-β-dependent NF-κB activation pathway in human hepatocytes. A connection between inflammatory stimulation and AID expression led to the speculation that one possible mechanism for increased susceptibility to HCC in the liver during chronic inflammation is inappropriate expression of AID in hepatocytes via NF-κB activation.

An important finding in this study is that HCV infection, one of the major causes of HCC, might trigger

aberrant AID expression in hepatocytes. We previously demonstrated that among the HCV proteins, the core protein functioned in the activation of NF-κB and that NF-κB-dependent transcriptional activities induced by TNF-α were synergistically enhanced by the presence of a viral core protein (Marusawa *et al.*, 1999). Consistent with our previous findings, the TNF-α-induced AID expression was strongly enhanced by the presence of HCV core protein. Interestingly, HCV-associated HCC shows various mutations in proto-oncogenes, but non-viral HCC does not (Machida *et al.*, 2004). Taken together, these findings suggest that the upregulation of AID induced by HCV infection in chronically damaged liver might contribute to enhanced susceptibility to mutagenesis, resulting in the accumulation of somatic mutations leading to the production of sufficient genomic alterations and eventually to cancer development.

In this study, we showed that constitutive and ubiquitous expression of AID in Tg mice caused the development of HCC as well as T-cell lymphoma. Moreover, aberrant AID expression was capable of triggering accumulation of nucleotide alterations in the *c-myc* gene in cultured hepatoma-derived cells *in vitro*. It has been demonstrated that deregulation of c-myc has been implicated in the etiology of a wide variety of human cancers (Pelengaris *et al.*, 2002). The deregulating mutations impact either on the *c-myc* gene itself or on upstream regulatory sequences, and point mutations or gene amplification of *c-myc* was reported in human HCC (Feitelson, 2006). Moreover, several studies revealed that the mouse models with overexpression of c-myc developed liver cancers (Murakami *et al.*, 1993; Coulouarn *et al.*, 2006). Thus, it is tempting to speculate that increased AID expression might be responsible for the enhanced susceptibility of the hepatocytes to somatic gene alterations in tumor-related genes including *c-myc*, which might facilitate HCC development. In the current study, however, the *c-myc* mutation frequency in AID-activating HepG2 cells did not show a statistically significant difference compared with that in the cells without AID activation. It has been supposed that the AID-mediated somatic mutation is induced in a transcription-dependent manner and the preference of target genes is variable between the cells with AID expression (Kotani *et al.*, 2005). Thus, there is room for further investigation to identify the frequent target genes of AID in human hepatocytes.

In conclusion, our findings provide the first evidence that AID is induced in response to proinflammatory cytokines via the NF-κB/IKK-β signaling pathway and might be responsible for the development of HCC in the underlying chronic liver disease. Understanding the mechanisms of AID upregulation in human hepatocytes might provide a new strategy for preventing the development and progression of HCC in patients with chronic liver disease.

## Materials and methods

### *AID Tg mice*

Generation of AID Tg mice was described previously (Okazaki *et al.*, 2003). Tissue samples from the mice were removed and fixed in 4% (w/v) formaldehyde, embedded in paraffin, stained with hematoxylin and eosin (H&E), and examined for histological abnormalities.

### *Cell culture and transfection*

Human hepatoma-derived cell lines, Hep3B, Huh-6 and HepG2, were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Tokyo, Japan) containing 10% fetal bovine serum. For plasmid transfection, we used Trans-IT LT1 transfection reagent (Mirus Bio Corporation, Madison, WI, USA).

siRNA duplexes composed of 21-nucleotide sense and antisense strands used for targeting IKK-γ/NEMO and AID were obtained from Dharmacon Research (Lafayette, CO, USA). Transfection of siRNA was described previously (Matsumoto *et al.*, 2006).

Primary human hepatocytes were isolated from surgical specimens of patients with metastatic liver tumors undergoing partial hepatectomy (Tateno *et al.*, 2000; Tanaka *et al.*, 2006), after informed consent for the use of resected tissues was obtained in accordance with the Declaration of Helsinki Principle. An established non-neoplastic human primary hepatocyte cell line that retained primary hepatocyte characteristics was also used (Aly *et al.*, 2007).

A full genome HCV replicon system was established by transfecting to the cells with the NN strain (genotype 1b) (Ishii *et al.*, 2006).

A system that allows conditional expression of the active form of AID was established by stable transfection of the HepG2 cells with pAID-ER-BOSbsr vector encoding the active form of AID fused with the hormone-binding domain of the human ER designated as AID-ER (Doi *et al.*, 2003).

### *Reagents and antibodies*

Recombinant human TNF-α and IL-1β were obtained from Peprotech EC (London, UK) and R&D Systems Inc. (Minneapolis, MN, USA), respectively. NF-κB inhibitors MG132 and SN50 were purchased from Biomol International LP (Plymouth Meeting, PA, USA). Polyclonal antibody against human AID was generated using purified recombinant AID protein as an immunogen (Ta *et al.*, 2003). Mouse monoclonal antibodies against human IKK-γ/NEMO and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Calbiochem (San Diego, CA, USA), respectively.

### *Plasmids*

pcDNA3-IκBαΔN, for expression of the super-repressor form of the IκB-α protein, was made by inserting the cDNA fragment of human IκB-α into the *Bam*HI-*Eco*RI sites of pcDNA3 (Invitrogen, Carlsbad, CA, USA). The cDNA fragment for pcDNA3-IκBαΔN (amino acids 37–317) was synthesized by RT–PCR with the oligonucleotide primers 5′-CGCGGATCCATGAAAGACGAGGAGTACGA-3′ (forward) and 5′-CCGGAATTCTCATAACGTCAGACGCTGGC-3′ (reverse). The expression plasmids pcDNA3-IKK-α and IKK-β were described previously (Marusawa *et al.*, 2001). The plasmids for the expression of various HCV proteins, including pCMV-3010 for the expression of full viral proteins, pCMV-core for the expression of the core, and pCMV-NS for the expression of the non-structural protein, were described previously (Marusawa *et al.*, 1999).

### *Semiquantitative and quantitative real-time RT-PCR*

Total RNA was extracted from the cells using Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan) according to the

manufacturer's protocol. For the RT reaction, 1 μg of total RNA was reverse transcribed into 20 μl of cDNA using the Superscript III first-strand synthesis system and oligo(dT)$_{12-18}$ primers (Invitrogen). PCR amplification was performed using Takara Ex Taq DNA polymerase (Takara, Tokyo, Japan). The oligonucleotide primers for the amplification of *AID*, *cIAP1* and *HCV-core* were as follows: *AID*, 5′-AAATGTCC GCTGGGCTAAGG-3′ (forward) and 5′-GGAGGAAGAGC AATTCCACGT-3′ (reverse); *cIAP1*, 5′-CTCTGAGGTTTAG CATTTCA-3′ (forward) and 5′-CTCCAGGTCCAAAATGA ATA-3′ (reverse); *HCV-core*, 5′-TCGTTGGTGGAGTTTAC CTG-3′ (forward) and 5′-GCGGAATGTACCCCATGA-3′ (reverse).

Quantification of gene expression was performed by quantitative real-time RT–PCR using the 7300 Real-Time PCR System (PE Applied Biosystems, Foster City, CA, USA) (Matsumoto *et al.*, 2006). The 6-carboxyfluorescein-labeled probe specific for human *AID* was 5′-TCGGCGTGAGACC TACCTGTGCTAC-3′. Standard curves for *AID* were generated for every target using a 10-fold serial dilution series of five independent transcripts derived from BL2-lymphoma cells, which contain a high expression level of endogenous *AID*. To assess the quantity of isolated RNA as well as the efficiency of cDNA synthesis, target cDNAs were normalized to the endogenous mRNA levels of the housekeeping reference gene *18S rRNA* (Matsumoto *et al.*, 2006). For simplicity, the ratios were represented as relative values compared with expression levels in a lysate from control cells.

*Subcloning and sequencing of the tumor-related genes*
Genomic DNA from the cultured cells and mouse liver tissues was prepared using the DNeasy Tissue Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol.

The oligonucleotide primers for *c-myc* were designed to amplify the sequences between exon 1 and intron 1 of the human *c-myc* gene using 5′-GCCGAATTCTTGCAGCTGC TTAGACGCTG-3′ (forward) and 5′-ATCCTCGAGCCAA CACTCCTTTTGCCAGC-3′ (reverse). The primers for human exon 1 to exon 4 of the human *pim1* gene were as follows: 5′-GCCGAATTCGTCCAAAATCAACTCGCTT-3′ (forward) and 5′-ATCTCTAGACGAAGTCGATGAGCTT GA-3′ (reverse). The primers for the amplification of the human *β-catenin* and *PTEN* genes were as follows: 5′-G CCGAATTCCTGATTTGATGGAGTTGGAC-3′ (*β-catenin*-forward), 5′-ATCCTCGAGAACGCTGGACATTAGTGG GA-3′ (*β-catenin*-reverse), 5′-GCCGAATTCTCATCAAAG AGATCGTTAGCAG-3′ (*PTEN*-forward), and 5′-ATCCTC GAGTGTCTCTGGTCCTTACTTCC-3′ (*PTEN*-reverse). The primers for the amplification of the mouse *c-myc* gene were as follows: 5′-GTTAAGCTTGCGTTTTTTTCTGACT CGCTG-3′ (forward) and 5′-GTTGAATTCCCTACCCC AACTACTCTTGA-3′ (reverse). After the amplification of each gene using high-fidelity Phusion Taq polymerase (FINNZYMES), the PCR products were subcloned by insertion into the pcDNA3 vector and further subjected to sequence analyses as described previously (Marusawa *et al.*, 2000).

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Medicine in focus

# Aberrant AID expression and human cancer development

Hiroyuki Marusawa *

*Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, 54 Kawara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan*

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**Abstract**

Cancer develops via a multistep process that occurs through the accumulation of somatic mutations of tumor-related genes that govern cell proliferation, regeneration, and apoptosis. The question how normal cells acquire the genetic changes that lead to malignant transformation is, however, unknown at present. Activation-induced cytidine deaminase (AID) produces immune-diversity by inducing somatic hypermutations and class-switch recombinations in human immunoglobulin genes. Unfortunately, this function of AID as a genome mutator could aim at the generation of somatic mutations in various host genes of non-lymphoid tissues and contribute to tumorgenesis. Notably, aberrant AID expression can be triggered by several pathogenic factors, including *Helicobacter pylori* infection and proinflammatory cytokine stimulation, in human epithelial cells, whereas AID expression is absent in those cells under physiologic conditions. Thus, aberrant AID activity in epithelial tissues may provide the critical link between inflammation, somatic mutations, and cancer development.
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*Keywords:* Activation-induced cytidine deaminase; Mutation; Cancer

## 1. Introduction

In contrast to normal human cells that replicate their DNA with exceptional accuracy, most cancer cells arise from a stepwise accumulation of genetic changes. The genomes of incipient cancer cells acquire alterations in the nucleotide sequences of proto-oncogenes, tumor-suppressor genes, and other genes that control cell proliferation, regeneration, and apoptosis (Hahn & Weinberg, 2002). Because normal mutation rates cannot account for the accumulation of multiple mutations in tumor cells (Loeb, Loeb, & Anderson, 2003), certain molecular mechanisms must be present to account for the nucleotide alterations observed in most human cancer cells. One mechanism that may account for the enhanced susceptibility to mutagenesis is a genetic defect in the DNA repair pathways. For example, impairments of the mismatch repair system result in a familial colorectal cancer syndrome, defects in nucleotide excision repair are associated with skin cancer, and defects in homologous recombination and double-strand break repair are associated with breast cancer and lymphoma (Hoeijmakers, 2001). The frequency of such defects in the DNA repair system, however, is generally low among human cancers. Thus, how a large number of genetic mutations arise during the course of cancer development remains a fundamental question. We provide an overview of the novel molecular mechanism

* Tel.: +81 75 751 4319; fax: +81 75 751 4303.
*E-mail address:* maru@kuhp.kyoto-u.ac.jp.

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by which normal epithelial cells acquire DNA mutations under pathologic conditions, including chronic inflammation, and pathogenic viral or bacterial infections.

## 2. Nucleotide-editing enzymes that can induce mutations in DNA and/or RNA

To maintain homeostasis and conserve genetic information, cells have several systems to prevent mutations, and repair any changes in nucleotide sequences, thus avoiding harmful sporadic nucleotide alterations, so-called "somatic mutations". In contrast to normal cells, however, cancer cells usually acquire a variety of somatic mutations during the transformation process. Recently, a novel enzyme family was highlighted in association with the mechanism of mutagenesis. Cytidine deaminases are enzymes involved in DNA and/or RNA editing by converting cytosine to uracil, resulting in nucleotide alterations in target sequences. Among them, the apolipoprotein B-editing catalytic polypeptide (APOBEC) represents a clustered family characterized by a zinc-binding catalytic domain with the consensus amino acid sequences (Cascalho, 2004). The human APOBEC family comprises a series of molecules, including APOBEC1, -2, -3A, -3B, -3C, -3DE, -3F, -3G, -3H -4 and activation-induced cytidine deaminase (AID) (Conticello, Thomas, Petersen-Mahrt, & Neuberger, 2005). APOBEC family member-induced cytidine deamination has a critical role in mediating subtle changes in the DNA or RNA sequences that can produce diverse physiologic functions of target genes (Pham, Bransteitter, & Goodman, 2005). For example, APOBEC1 induces a mutation in apoB mRNA at a specific site that results in the generation of a premature stop codon, thereby producing a truncated form of apoB (Chen et al., 1987; Powell et al., 1987). A full-length apoB mRNA product, apoB-100, is a component of very-low-density and low-density lipoprotein, whereas the truncated apoB is secreted in the triglyceride-rich chylomicrons that carry dietary fat (Chan, 1992). In contrast to APOBEC1, APOBEC-3G is involved in cellular defense against retroviruses by inducing mutations into the viral genome. It has been shown that deamination activity of APOBEC-3G contributes to its antiviral activity against human immunodeficiency virus (HIV)-1 and restricts viral growth through a massive deamination of cytosines in the viral-minus DNA strands (Goff, 2003; KewalRamani & Coffin, 2003). Interestingly, more recent study reported that a deamination-independent mechanism might also be involved in APOBEC-3G antiviral activity (Iwatani et al., 2007).

## 3. AID is capable of inducing mutations in DNA sequences

Among the APOBEC family, AID has a unique ability with favorable function. Antigen stimulation of activated B lymphocytes triggers somatic hypermutations, which diversifies the variable region of the immunoglobulin genes, and AID expression is essential for this process. The finding that AID induces the production of somatic hypermutations in the immunoglobulin gene indicates that AID can induce nucleotide alterations in human DNA sequences. The activity of AID as a genome mutator leads to the question of whether AID induces inappropriate mutations in non-immunoglobulin genes.

The link between AID expression and unfavorable consequences in various organs was first revealed by phenotypic analyses of a transgenic mouse model with AID expression. Constitutive and ubiquitous AID expression in transgenic mice induced the development of lymphomas (Okazaki et al., 2003). Moreover, point mutations are massively introduced in various non-immunoglobulin genes, including the proto-oncogene *c-myc* in lymphoma cells. Interestingly, those mice also develop epithelial tumors including micro-adenomas and dysgenetic lesions of the respiratory bronchioles in the lung. Further phenotypic analyses revealed that AID-transgenic mice develop neoplasia in other epithelial tissues, including liver and stomach (Endo et al., 2007; Matsumoto et al., 2007). These findings indicate that aberrant AID expression might cause tumorigenesis in both lymphoid and non-lymphoid organs, via the accumulation of somatic mutations in tumor-related genes (Fig. 1). Fortunately, AID transcription is restricted to activated B lymphocytes and thus almost no AID expression is observed in most human tissues under physiologic conditions.

## 4. Aberrant AID expression in gastric epithelial cells in association with *Helicobacter pylori* infection

The majority of human gastric cancers arise in the stomach with clinical features of chronic gastritis (Aoi, Marusawa, Sato, Chiba, & Maruyama, 2006). The most important causative pathogen for chronic gastric inflammation and a class one carcinogen for human gastric cancer is *H. pylori* infection (Chiba, Seno, Marusawa, Wakatsuki, & Okazaki, 2006). The mechanisms that link *H. pylori*-induced chronic gastric inflammation and cancer development remain unclear, but it is thought to involve a multistep process of genetic alterations. Indeed, several studies have reported various nucleotide alter-

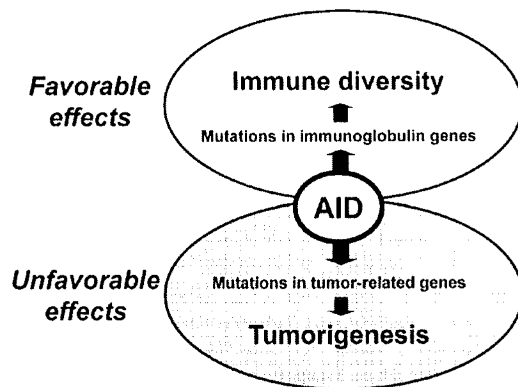


Fig. 1. Dual effects induced by AID activation. A schematic depicting the favorable and unfavorable effects of AID. AID acts as a cytidine deaminase that is capable of inducing nucleotide alterations in human DNA sequences. Under the physiological condition, AID is a protein indispensable for the diversification of immunoglobulin genes by somatic hypermutation and class-switch recombination. On the other hand, AID is able to induce genome-wide mutations in a variety of mammalian non-lymphoid cells, and thus can contribute to the production of unfavorable genetic changes in the tumor-related genes leading to carcinogenesis.

ations in tumor-related genes, including *TP53* in gastric cancer cells (Fenoglio-Preiser, Wang, Stemmermann, & Noffsinger, 2003). These somatic mutations are also observed in non-cancerous stomach tissues with *H. pylori* infection, suggesting that nucleotide alterations accumulate in gastric epithelial cells during the course of chronic inflammation. The *in vivo* findings that AID-transgenic mice develop gastric neoplasms suggest that aberrant AID expression in gastric epithelial cells contributes to cancer development via the accumulation of somatic mutations (Matsumoto et al., 2007). Surprising findings were obtained by analyses of AID expression in human stomach tissue specimens with *H. pylori*-related chronic gastritis and gastric cancers. In contrast to normal gastric mucosa, aberrant AID expression is present in gastric epithelial cells of the stomach tissues with *H. pylori*-positive chronic gastritis (Matsumoto et al., 2007). In addition, AID protein is expressed in neoplastic cells in approximately 80% of *H. pylori*-infected gastric cancer tissues. Because AID expression is specifically upregulated in human gastric epithelial cells and neoplastic cells with *H. pylori*-induced chronic inflammation, the relationship between *H. pylori* infection, proinflammatory cytokine stimulation, and AID expression was further investigated *in vitro*. Although AID expression is low in cultured human gastric epithelial cells, marked upregulation of AID is induced in response to either *H. pylori* infection or tumor necrosis factor (TNF)-α stimulation. AID expression in human gastric epithelial cells by *H. pylori* infection or TNF-α is induced by the activation of the transcription factor NF-κB, indicating that AID expression is regulated through an NF-κB activation pathway in human gastric epithelial cells. Notably, aberrant AID expression triggers the accumulation of nucleotide alterations in the *TP53* gene in human gastric epithelial cells. Taken together, these findings provide evidence that AID is induced in response to *H. pylori* infection or proinflammatory cytokine stimulation via the NF-κB signaling pathway and is capable of contributing to the generation of somatic mutations in tumor-related genes in gastric epithelial cells. Thus, inflammation-mediated AID expression might underlie the development of human gastric cancer via *H. pylori*-associated chronic gastritis (Fig. 2).

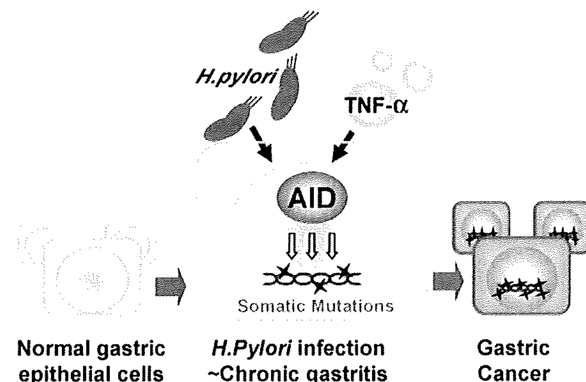


Fig. 2. AID links chronic inflammation to gastric cancer development via its mutagenic activity. This figure presents a model that depicts how AID plays a role in the development of human gastric cancer. Human gastric epithelium lacks endogenous AID expression under the physiological condition. *Helicobacter pylori* infection and the resultant inflammatory stimulation, however, trigger the aberrant AID expression in gastric epithelial cells. Constitutive AID activation in gastric epithelium results in the accumulation of somatic mutations in various target genes. If critical nucleotide changes in the tumor-related genes may be induced by AID activity, the resultant gastric epithelial cell can acquire the transformation, leading to the development of gastric cancer cells.

## 5. Conclusion and future aspects

Proinflammatory cytokine induction of AID expression via the NF-κB activation pathway is not limited to gastric epithelial cells. Indeed, AID expression is also mediated by TNF-α or interleukin-1β in human hepatocytes (Endo et al., 2007). More importantly, hepatitis C virus (HCV) strongly triggers AID expression in hepatocytes in collaboration with proinflammatory cytokines (Endo et al., 2007), and ectopic AID expression is observed in human liver specimens with chronic hepatic inflammation caused by HCV infection (Kou et

al., 2007). Thus, AID possibly has a role in enhancing genetic susceptibility to mutagenesis, leading to the development of hepatocellular carcinoma in the setting of HCV-related chronic liver disease.

In conclusion, these recent findings support the idea that aberrant expression of the endogenous DNA mutator AID in epithelial cells provides a novel link between inflammation, mutagenesis, and cancer development. There might be more examples of human cancers that arise due to chronic inflammation that causes mutational accumulation mediated by AID activity.

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REVIEW

# A novel mechanism for inflammation-associated carcinogenesis; an important role of activation-induced cytidine deaminase (AID) in mutation induction

**Tsutomu Chiba · Hiroyuki Marusawa**

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**Abstract** Inflammation is a risk for cancer development; however, its mechanism is unknown. Recent studies have revealed that activation-induced cytidine deaminase (AID), which plays essential roles in both class-switch recombination and somatic hypermutation of immunoglobulin gene in B lymphocytes, is aberrantly expressed in non-lymphoid cells not only by *H.pylori* and HCV infection but also by various proinflammatory cytokines, leading to the generation of gene mutations. These findings not only suggested a new mechanism of inflammation-associated carcinogenesis but has also opened up a new field of tumor biology.

**Keywords** Activation-induced cytidine deaminase (AID) · Inflammation · Carcinogenesis · Helicobacter pylori · Gene mutation

## Introduction

Since Virchow's era, a causal relationship between inflammation and cancer development has been proposed in a variety of chronic inflammatory diseases. In particular, many cancers of digestive organs, some of which are caused by infectious agents, are known to arise on a backgroud of chronic inflammation [1, 2]. These include *Helicobacter pylori* (*H. pylori*)-induced gastric cancer, hepatitis C virus (HCV)- and hepatitis B virus (HBV)-related hepatocellular carcinoma, Barrett's esophageal adenocarcinoma, colitis-associated colon cancer and cholangiocarcinoma accompanied by primary sclerosing cholangitis (PSC) [2]. There are many pathways that can lead to cancer development through inflammation. First, microorganisms such as *H.pylori*, HBV and HCV may directly modulate cellular function, giving the cells growth advantages and resistance to apoptosis [3, 4]. Inflammation also induces many mediators and cellular effectors that appear to be involved in carcinogenesis. These include various cytokines, chemokines and growth factors [2, 5, 6]. Moreover, cyclooxygenase 2 (COX2) produced in inflammatory condition is known to enhance tumorigenesis through various mechanisms [7]. In addition, reactive oxygen species (ROS) induced during inflammation have been shown to have mutagenic activity [8].

On the other hand, mutation is recognized as a hallmark of cancer. Indeed, cancers are derived from a clonal proliferation of the transformed cells caused by accumulation of various genetic alterations in proto-oncogenes, tumor-suppressor genes, and other genes that control cell proliferation, regeneration, and apoptosis. Supporting this idea, recent studies analyzing a large number of genomes in human cancers have revealed that a single cancer cell generally possesses approximately 70–90 mutations, 10–15 of which are so-called "driver genes" that contribute to cancer development [9]. Because normal mutation rates cannot account for such multiple mutations in cancer cells, certain molecular mechanisms must be present to explain a large number of nucleotide alterations. One mechanism for the enhanced susceptibility to mutagenesis may be a defect in DNA repair systems. Indeed, dysfunction of the mismatch repair system results in familial colorectal cancer syndrome, and defects in the nucleotide excision repair system are associated with colon and skin cancer [10, 11]. However, the frequency of such defects in the DNA repair system is

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T. Chiba (✉) · H. Marusawa
Department of Gastroenterology and Hepatology,
Graduate School of Medicine, Kyoto University,
Kawahara-cho 54, Shogoin, Sakyo,
Kyoto 606-8507, Japan
e-mail: chiba@kuhp.kyoto-u.ac.jp

generally low in human cancers. Considering these facts, it is tempting to hypothesize that inflammation may trigger gene alterations during carcinogenesis.

Previous studies have identified many exogenous mutagenic agents, and several possible intrinsic mutagens have also been proposed. Among these, ROS produced during inflammation are known to elicit mutations, particularly G to T transversions [11]. However, a recent study has shown that G to T transversion accounts for only a minor proportion of the total mutations in human cancer cells, and instead C/G to T/A transition is the most prevalent mutation especially in gastrointestinal malignancies [12]. Thus, alternative mechanisms for mutagenesis during inflammation-associated carcinogenesis should be considered.

## Activation-induced cytidine deaminase (AID) in B lymphocytes is a physiologic genome mutator essential for somatic hypermutation of immunoglobulin genes

Generally, cells have several systems to prevent mutations so as to avoid harmful nucleotide alterations, so-called "somatic mutations". However, there is one type of cell in which somatic mutations frequently occur under physiological condition. That cell type is B lymphocytes in which immunoglobulin genes undergo somatic hypermutations to generate molecular diversity against many antigens. Prof. Honjo's group at Kyoto University first cloned the gene responsible for immunoglobulin class switch recombination in 1999 [13]. They found that this gene was closely related to apolipoprotein B RNA-editing cytidine deaminase 1, which converts cytosine nucleotides to uracils in RNA, and named it activation-induced cytidine deaminase (AID). Interestingly, they subsequently found that this molecule is also responsible for somatic hypermutation of immunoglobulin genes [14]. Notably, AID is the only human enzyme known to induce DNA mutation in human genomes, although under normal conditions it is expressed only in B cells. AID theoretically induces C/G to T/A transitions by its cytidine deaminase activity. In this regard, it should be emphasized that a recent report on systemic sequencing of cancer genomes clearly demonstrated that the most prevalent mutation pattern in human cancers is C/G to T/A transition, a pattern similar to that induced by AID [12].

## AID transgenic mice develop not only lymphomas but also various types of cancers

After cloning of the AID gene, many investigators have found overexpression of AID in human lymphoid malignancies, suggesting involvement of AID in human lymphomagenesis [15, 16]. Prof. Honjo's group established AID transgenic mice and, as expected, nearly 100% of these developed lymphomas, again suggesting roles of AID in lymphomagenesis, probably by inducing a range of mutations [17]. We then wanted to see whether these mice also developed cancers, because they expressed AID not only in lymphocytes but also in other cells including epithelial cells. Interestingly, it was found that in addition to lymphomas these mice developed many types of cancers, including lung, liver, and gastric cancers, and cholangiocarcinomas [17–19]. These observations prompted us to speculate that AID may be involved in human carcinogenesis, though expression of AID had been believed to be strictly restricted to B cells under normal conditions.

## AID is strongly expressed in HCV-infected liver and liver cancers and in *H.pylori*-infected gastritis mucosa and gastric cancers

We, therefore, first examined whether AID is expressed in clinical specimens of liver tissues of patients with HCV infection, and surprisingly we were able to show by immunohistochemistry strong expression of AID not only in liver cancers but also in chronic hepatitis tissues [20, 21]. AID expression was also found in both chronic gastritis mucosa and gastric cancer tissues of *H.pylori*-infected patients, and eradication of *H.pylori* could reduce its expression [19, 22]. Later, aberrant AID expression was also revealed in cholangioepithelium of patients with PSC [23], and in colonic mucosa and cancer tissues of patients with inflammatory bowel disease, but not in normal colonic mucosa [24]. All these data strongly suggested the involvement of AID in inflammation-associated carcinogenesis in human.

## AID is induced via NFκB activation by *H.pylori* and HCV core protein, and causes gene mutations

We next examined AID expression in human hepatocytes and gastric cells *in vitro*, and tried to elucidate the mechanisms for AID expression in non-lymphoid cells. We first observed that AID is induced by expression of HCV core protein or by *H.pylori* infection. Then, because AID expression in B cells was known to be induced by NFκB activation through CD40 ligation by T cells, and because both *H.pylori* and the core protein of HCV enhance NFκB activation [4, 25], the roles of NFκB in AID expression in epithelial cells were examined. It was found that introduction of the gene for the core protein of HCV into human hepatocytes induced AID expression via NFκB activation [20]. Induction of AID expression in human gastric cells by *H.pylori* infection was also found to be dependent on NFκB [19]. Because *H.pylori* deficient for Cag pathogenicity island (PAI) completely lost

its ability to induce both NFκB activation and AID expression [19], it was considered that certain *H.pylori* factors that are introduced into epithelial cells through the *H. pylori* type IV secretion machinery could cause AID expression via NFκB activation. In this regard, Viala et al. [26] reported that *H.pylori*-derived peptidoglycan introduced via the type IV secretion apparatus is responsible for NFκB activation. We further observed *in vitro* that *H.pylori* infection resulted in mutations in various genes including *p53*, which could be inhibited by knockdown of endogenous AID using AID siRNA [19]. Taken together, the following scenario may be illustrated: both *H.pylori* and HCV infection generate gene mutations by inducing AID expression through NFκB activation (Fig. 1).

In addition to *H.pylori* and HCV core protein themselves, AID expression is also induced by IL1β and TNFα through NFκB activation, by IL4 and IL13 through STAT6, and by TGFβ[20, 24]. These cytokines are known to enhance AID expression in B lymphocytes. Thus, these cytokines appear to be involved in inflammation-associated cancer development by accelerating gene mutations (Fig. 2).

## Lessons for cancer research in the study of AID

### Roles of NFκB in carcinogenesis

Recent cancer studies have focused on the important roles of NFκB in inflammation-associated carcinogenesis. Indeed, NFκB is activated not only by microorganisms but also by many cytokines. However, most investigators are interested in its growth-promoting and anti-apoptotic activity, and in its role in enhancing inflammation during cancer development.

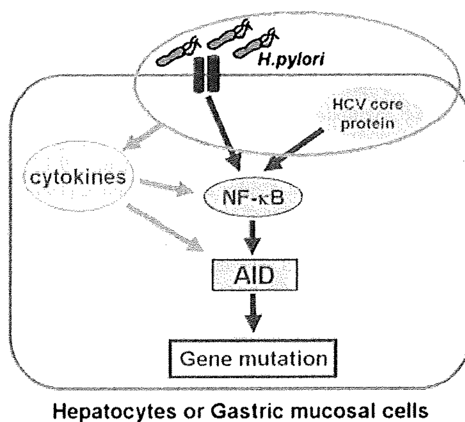


**Fig. 1** Induction of AID and gene mutations by *H.pylori* and HCV infection. Certain *H.pylori* factors that are introduced into epithelial cells through the *H.pylori* type IV secretion machinery and HCV core protein could cause AID expression via NFκB activation. *H.pylori* and HCV can also induce AID expression indirectly by enhancing various cytokines production

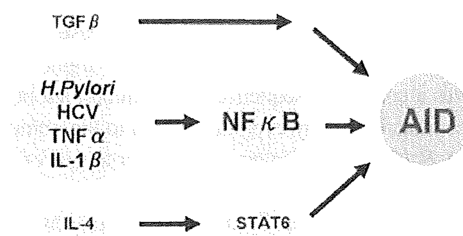


**Fig. 2** Induction of AID by various cytokines. In addition to *H.pylori* and HCV core protein themselves, various cytokines induced by inflammation can also elicit AID expression

In contrast, our studies demonstrated that NFκB activation is involved in generating gene mutation by inducing AID expression, which may suggest a new role for NFκB in inflammation-associated carcinogenesis (Fig. 3).

### Different spectra of gene mutations in different cancers

With the exception of *p53*, the cancer genes that are frequently mutated differ for different types of cancers. For instance, *k-ras* mutations are found in the majority of the pancreatic cancers whereas only a few gastric cancers have *k-ras* mutations [27]. Moreover, although *apc* mutations are rarely found in hepatocellular carcinomas, many colon cancers possess this mutation. The reason for such distinct mutation patterns in different cancers is not clear. Because the importance of each gene as a tumor suppressor or as a tumor enhancer may be different in different cell types such as pancreatic cells, gastric cells and colonocytes, we may be able to find distinct spectra of mutated genes in cancer cells that have arisen from different tissues. In this context, it is interesting to note that the genes targeted by AID are different in different cells [18]. Indeed, induction of AID causes mutations at *p53* and *βcatenin* but not at *c-myc* in gastric cells, whereas it induces *c-myc* mutations in hep-

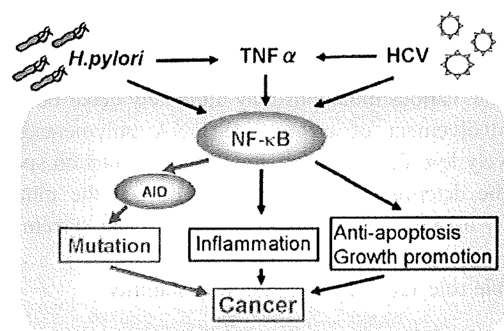


**Fig. 3** Important roles for NFκB in inflammation-associated carcinogenesis. NFκB is known to play roles in inflammation-associated carcinogenesis by further enhancing inflammation and also through its anti-apoptotic or growth promoting action. Moreover, NFκB contributes to inflammation-associated carcinogenesis by enhancing gene mutations through AID induction

atocytes and lymphocytes [19–21]. In the case of immunoglobulin gene somatic hypermutation in B lymphocytes, AID recognizes a consensus sequence in the immunoglobulin gene to induce mutations. However, in addition to the immunoglobulin gene, *Bcl6* is known to be an excellent target for AID, for reasons that are not clear at present. Unlike the immunoglobulin genes, we have so far been unable to find any consensus sequence in various cancer-related genes that is recognized by AID. One mechanism may be that AID targets genes that are undergoing active transcription [28]; however, it is clear that other mechanisms by which AID recognizes specific target genes are also present. In any case, it is interesting to speculate that AID is involved in the development of distinct mutation patterns in different cancers.

### Relationship between mutation induction and repair systems

As already mentioned, AID deaminates cytotsine to produce uracil and, after DNA replication, the paired guanine is mutated to adenine. Then, after further DNA replication, the original cytosine is mutated to thymidine, and thus eventually a C/G to T/A transition develops. The importance of the induction of C/G to T/A mutation by AID may be supported by the fact that C/G to T/A transitions are the most prevalent mutation found in human GI cancers [12]. However, although C/G to T/A transitions are the most prevalent after AID induction, other mutations also develop [29]. In agreement with these data, a range of mutations for which AID is entirely responsible do occur even in immunoglobulin gene somatic hypermutation. The reason why AID induces different mutations is not completely clear at present. However, it is possible that several repair systems including mismatch repair and excision repair become involved after cytosine deamination by AID, and it is important to note that these repair systems do not always provide high-fidelity repair [30, 31]. For instance, when excision repair is initiated by uracil endoglycosidase before DNA replication, abasic site is produced. When DNA replication starts in such a situation, various mutations may ultimately occur because of the involvement of error-prone DNA polymerases with relatively low fidelity. Thus, the ultimate mutation spectrum may be determined by a balance between the mutagenic activity of AID and the involvement of repair systems [32].

### Possible role for AID in genomic instability

Recent studies have shown that, in addition to mutation induction, AID is also responsible for chromosomal translocations through production of double-strand DNA breaks in the development of B cell malignancies. Indeed, Nussenzweig et al.[33, 34] reported that AID is required for chromosomal breaks in not only *IgH* but also *c-myc* that lead to *c-myc/IgH* translocations. In this connection, cancer cells possess a considerable number of gene duplications and deletions. Because gene deletion requires DNA breaks and AID can induce DNA breaks, it may be reasonable to consider that ectopically-expressed AID is involved in not only gene mutations but also gene deletions in cancer cells. Thus, whether AID is able to explain accumulation of both mutations and deletions of genes during carcinogenesis is an interesting question to be clarified in future studies.

## Summary

The finding that AID expression is induced in non-lymphoid cells in various inflammatory conditions has not only proposed a new mechanism of inflammation-associated carcinogenesis but has also opened up a new field of tumor biology. Recent studies have suggested the importance of both genetic and epigenetic changes in cancer development, and the relative importance of each is always a matter of discussion. Under such circumstances, we believe that the study of AID is able to "reinforce" cancer genetics. Moreover, a very recent study has demonstrated that AID is involved in programmed DNA demethylation of the zdebrafish genome during embryonic development. Thus, it is tempting to speculate that AID plays important roles in linking between genetics and epigenetics [35].

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# Adipose Tissue-Derived Mesenchymal Stem Cells as a Source of Human Hepatocytes

Agnieszka Banas,[1] Takumi Teratani,[1] Yusuke Yamamoto,[1,2] Makoto Tokuhara,[3] Fumitaka Takeshita,[1] Gary Quinn,[1,4] Hitoshi Okochi,[3] and Takahiro Ochiya[1]

**Recent observations indicate that several stem cells can differentiate into hepatocytes; thus, cell-based therapy is a potential alternative to liver transplantation. The goal of the present study was to examine the *in vitro* hepatic differentiation potential of adipose tissue-derived mesenchymal stem cells (AT-MSCs). We used AT-MSCs from different age patients and found that, after incubation with specific growth factors (hepatocyte growth factor [HGF], fibroblast growth factor [FGF1], FGF4) the CD105$^+$ fraction of AT-MSCs exhibited high hepatic differentiation ability in an adherent monoculture condition. CD105$^+$ AT-MSC-derived hepatocyte-like cells revealed several liver-specific markers and functions, such as albumin production, low-density lipoprotein uptake, and ammonia detoxification. More importantly, CD105$^+$ AT-MSC-derived hepatocyte-like cells, after transplantation into mice incorporated into the parenchyma of the liver. *Conclusion:* Adipose tissue is a source of multipotent stem cells that can be easily isolated, selected, and induced into mature, transplantable hepatocytes. The fact that they are easy to procure *ex vivo* in large numbers makes them an attractive tool for clinical studies in the context of establishing an alternative therapy for liver dysfunction.** (HEPATOLOGY 2007;46:219-228.)

Regenerative medicine holds promise for the development of stem-cell-based therapy of the liver and may allow transplanting hepatocytes or liver devices generated *in vitro* from stem cells. The establishment of stem cell therapy requires multipotential, immunocompatible stem cells and a direct differentiation strategy, that is not followed by post-transplantation complications or unwanted differentiation such as tumor formation. Many types of stem cells from different sources have being investigated for hepatic differentiation ability; mostly mouse, but also monkey and human embryonic stem (ES) cells have been used.[1] We have established the direct differentiation of mouse ES cells into functional hepatocytes in an adherent monoculture condition by the use of the HIFC method.[2,3] In our method, growth factors directing hepatic fate specification have been identified on the basis of an *in vivo* transplantation of ES cells into liver-injured animals.[4]

ES cells have enormous potential; however, many limitations, such as teratoma formation followed by tumor genesis, immunogenicity, and ethical issues, are arresting their clinical usage. Adult human stem cells are promising candidates for liver regeneration,[5-10] and their usage might sidestep obstacles, such as ethical concerns and risks of rejection. Currently, the focus is on mesenchymal stem cells (MSCs), found in human bone marrow (BM),[11] adipose tissue (AT),[12,13] scalp tissue,[14] placenta,[15] and umbilical cord blood (UCB)[16] as well as in various fetal tissues.[17] These stem cells can differentiate *in vitro* into multiple types of lineages such as: chondrogenic, osteogenic, adipogenic,[11] myogenic,[18]

*Abbreviations: ES, embryonic stem; HIFC, hepatic induction factor cocktail; HGF, hepatocyte growth factor; FGF, fibroblast growth factor; MSC, mesenchymal stem cell; BM, bone marrow; AT, adipose tissue; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic acid; TGF, transforming growth factor; MACS, magnetically activated cell-sorting; HCM, hepatocyte culture medium; OsM, oncostatin M; TTR, transthyretin; ALB, albumin; TDO2, tryptophan 2,3-dioxygenase; AFP, alpha-fetoprotein; CK, cytokeratin; HNF, hepatocyte nuclear factor; LDL, low-density lipoprotein; PAS, periodic acid-Schiff; GPT, glutamic-pyruvic transaminase; FITC, fluorescein isothiocianate; CD, cluster of differentiation antigen.*

*From the [1]Section for Studies on Metastasis, National Cancer Center Research Institute 1-1, Tokyo, Japan; [2]Department of Biology, School of Education, Waseda University, Nishi-Waseda, Tokyo, Japan; [3]Department of Surgery, International Medical Center of Japan, Tokyo, Japan; and [4]Effector Cell Institute, Inc., Tokyo, Japan.*

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*Address reprint requests to: Takahiro Ochiya, Ph.D., Head of Section for Studies on Metastasis, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan. E-mail: tochiya@ncc.go.jp; fax: (81) 3-3541-2685.*

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**Table 1. Adipose Tissue Donor Information**

| Donor (n) | Age (Years) | Gender | Height (cm) | Weight (kg) | BMI (kg/m²) | Comments |
|---|---|---|---|---|---|---|
| 1 | 54 | male | 158 | 61.7 | 24.7 | gastric cancer |
| 2 | 45 | male | 180 | 86.2 | 26.6 | gastric cancer |
| 3 | 43 | female | 165 | 62.0 | 22.8 | gastric cancer |
| 4 | 55 | male | 164 | 67.3 | 25.0 | gastric cancer |
| 5 | 45 | female | 149 | 47.2 | 21.5 | gastric cancer |
| 6 | 36 | female | 160 | 42.0 | 16.4 | gastric cancer |

NOTE. Adipose tissue was harvested from 6 cancer patients (three males, three females), undergoing gastrectomy. Patients' parameters such as: BMI, body mass, height, disease are of similar category.

neurogenic[19] and hepatogenic,[7-10] depending on the microenvironment in which they reside. MSCs from BM and UCB have been induced into a hepatic lineage[7-10]; however, the question of whether these are the best sources of stem cells for hepatic replacement and/or regeneration remains. Adipose tissue is an attractive source of multipotent human MSCs. AT-MSCs, so-called processed lipoaspirate (PLA) cells,[12,13,20] adipose-derived stromal cells (ADSCs),[21] adipose-derived adherent stromal cells/adipose-derived adult stem cells (ADASs),[22-24] and adipose tissue-derived stromal cells (ATSCs)[25] are considered to be the multipotent fraction of adherent cells, which, after isolation of the adipose stromal vascular fraction (SVF), attach to plastic culture dishes and remain there as a heterogeneous population of fibroblast-like cells. AT-MSCs are very similar to BM-MSCs.[20,26] Besides the fact that they are more heterogeneous[25], they reveal a surface antigen marker profile[22,26-28] and differentiation potential similar to BM-MSCs.[21,29-33] AT-MSCs are characterized as $CD45^-$ $CD34^+$ $CD105^+$ $CD31^-$[34]; however, there is confusion regarding the CD34 marker. One of the markers defining MSC provenance is CD105 (endoglin).[11,35,36] CD105 is a component of the receptor complex of transforming growth factor (TGF)-beta, a pleiotropic cytokine involved in cellular proliferation, differentiation, and migration. $CD105^+$ cells from bone marrow displayed more colony-forming unit-fibroblasts (CFU-Fs) and revealed a capacity to form bone *in vivo*[37] and differentiate into a chondrogenic lineage.[38] The adipogenic and myogenic differentiation ratio of $CD105^+$ BM-MSCs was not influenced by the age of the donor; however, the ratio usually decreased in older patients.[39] The ratio of the number of $CD105^+$ stem cells in adipose tissue to the age of the donor is not clear, and little is known about the relationship between disease (e.g., cancer) and stem cell potential.

The aim of our present study was to evaluate the hepatogenic potential of AT-MSCs. Because therapy concerns patients, not healthy donors, we have used AT-MSCs from non-obese cancer patients undergoing gastrectomy. We performed magnetically activated cell-sorting (MACS) of the CD105 fraction in order to achieve a multipotent and homogeneous subpopulation of cells. $CD105^+$ AT-MSCs were highly inducible into the hepatic lineage, and derived hepatocyte-like cells expressed the liver markers, proteins, enzymes, and functions of human primary hepatocytes. The differentiation potential of AT-MSCs that we have advanced by *in vivo* transplantation into immunodeficient mice resulted in incorporation of AT-MSC-derived hepatocytes into the $CCl_4$-injured liver. Thus, human AT-MSCs, which might be obtained in a large number of cells, represent a very attractive tool for future stem cell therapy of the liver diseases.

## Materials and Methods

***Isolation and Culturing of AT-MSCs.*** Abdominal subcutaneous adipose tissue was obtained from six gastric cancer patients undergoing gastrectomy at the International Medical Center of Japan in Tokyo. All patients (Table 1) (age: 36-55 years old; mean: 46) exhibited comparable features (body weight, height, BMI, gastric cancer). The hospital's committee of ethics approved this study, and informed consent was obtained from all patients. Adipose tissue was minced with scissors and scalpels into less than 3-mm pieces and isolation of AT-MSCs proceeded as previously described.[12,13] Briefly, after gentle shaking with equal volume of PBS(−), the mixture have separated into two phases. The upper phase (containing stem cells, adipocytes and blood) after washing with phosphate-buffered saline (PBS) (−) was enzymatically dissociated with 0.075% collagenase (type I)/PBS(−) for 1 hour at 37°C with gentle shaking. The dissociated tissue was then mixed with an equal volume of DMEM (GIBCO-BRL, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) and incubated 10 minutes at room temperature. The solution then was separated into two phases. The lower phase was centrifuged at 1,500rpm for 5 minutes at 20°C. The cellular pellet was resuspended in 160mM $NH_4Cl$ to eliminate erythrocytes

and passed through a 40$\mu$m mesh filter into a new tube. The cells were resuspended in an equal volume of DMEM/10% FBS and then centrifuged. Isolation resulted in obtaining $\approx 7.7 \times 10^6$ of adherent cells for a primary culture from 5g of adipose tissue (approximately; $1.0 \times 10^5$ to $4.6 \times 10^6$/1g) (Fig. 1A, step I) after 7-10 days of culture. The cells were suspended in a DMEM/10% FBS plated in concentration 1-5 $\times$ $10^6$cells/75cm$^2$. The cells with 70%-80% confluence were harvested with 0.25% trypsin-EDTA and then either re-pleated at 1.0 $\times 10^5$cells/60-mm dish and used for analysis or sorted using MACS system (Miltenyi Biotec., Bergisch Gladbach, Germany) (Fig. 1A, step II). Representative samples of each cell population were evaluated for hepatic and adipogenic differentiation capacity.

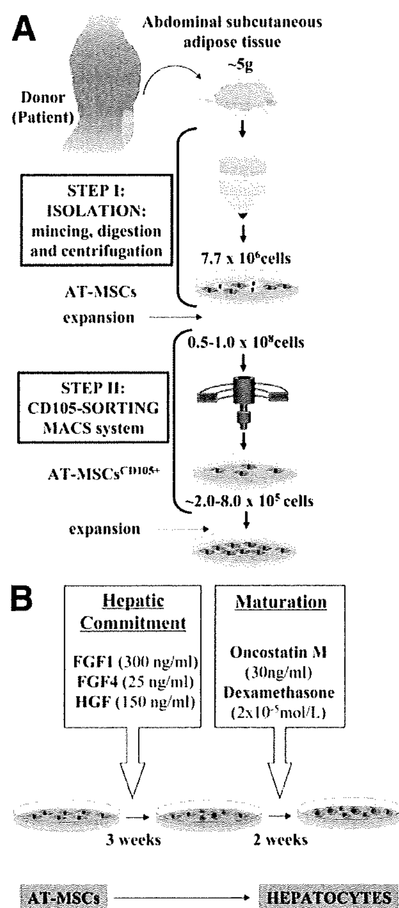


Fig. 1. Experimental strategy. A schematic representation of isolation (Step I), sorting (Step II) (A) and hepatic differentiation (B) of AT-MSCs. AT-MSCs after mincing, digestion with collagenase and centrifugation have been sorted by the MACS system. After expansion, CD105$^+$ AT-MSCs were replated into type I collagen-coated dishes and differentiated into hepatocytes (B). Cells were treated for 3 weeks with a hepatic induction growth factor cocktail (HIFC), followed by 2 weeks of culturing with OsM/dexamethasone, and finally maintained in HCM for 2-5 weeks.

***Isolation of the CD105$^+$ Fraction from AT-MSCs.*** The CD105$^+$ fraction was isolated from AT-MSCs of three donors using CD105-coupled magnetic microbeads (Miltenyi Biotec) (Fig. 1A, Step II). Briefly, trypsinized AT-MSCs (passage 3-8; 0.5-1.0 $\times$ $10^8$cells) were suspended in a MACS buffer (PBS/0.5 bovine serum albumin/2mM EDTA) and incubated with antibodies (8°C, 15 minutes). After rinsing with the MACS buffer and centrifugation (200$\times$g, 10 minutes), the cells were separated on a magnetic column. After the separation, approximately 2.0-8.0 $\times 10^5$ CD105$^+$ cells were achieved, plated in 60mm dishes, expanded, and used for experimental analysis.

***Flow Cytometry.*** The phenotype of AT-MSCs was evaluated by flow cytometry analysis (FACS, Epic XL, Software Expo 32 (Beckman coulter)), by using CD29 (BD Bioscience Pharmingen, Tokyo, Japan), CD31, CD45 (eBioscience, Tokyo, Japan), CD34 (DacoCytomation, Carpinteria, USA) and CD105 (Ancell, Bayport, MN, USA) antibodies, coupled to either phycoerythrin (PE) or fluorescein isothiocyanate (FITC).

**In vitro *Cultivation and Expansion of BM-MSC, BM-HSC, and HepG2 Cells.*** Human BM-MSCs and hematopoietic CD34$^+$ stem cells (BM-HSCs) (Cambrex Corp., Walkersville, MD) were cultured subsequently in a mesenchymal stem cell-growth medium (MSC-GM) and a serum-free Hematopoietic Progenitor Growth Medium (HPGM) (Cambrex). HepG2 cells were cultured in type I collagen-coated dishes under the same conditions as AT-MSCs. Human cryopreserved primary hepatocytes were obtained from TaKaRa (Kyoto, Japan) and cultured in Hepatic Culture Medium (HCM) (Cambrex).

***Hepatic Differentiation by HIFC Method.*** At passage 5-9, 2-3 $\times$ $10^5$ CD105$^+$ AT-MSCs were plated onto 60mm collagen (type I)-coated dishes (Asahi Techno Glass, Tokyo, Japan), and then hepatic induction was performed over a period of three weeks by culturing in a HCM-modified William's E medium containing: transferrin (5$\mu$g/ml), hydrocortisone-21-hemisuccinate ($10^{-6}$M), bovine serum albumin (0.5mg/ml), ascorbic acid (2mM), epidermal growth factor (20ng/ml), insulin (5$\mu$g/ml) gentamicin (50$\mu$g/ml) (Cambrex) and dexamethasone ($10^{-8}$M) and supplemented with HIFC that contained HGF (150ng/ml), FGF1 (300ng/ml), and FGF4 (25ng/ml) (PeproTech EC, London, UK) (Fig. 1B). For the next two weeks, the cells were treated with OsM (30ng/ml) and dexamethasone (2 $\times$ $10^{-5}$mol/l) and then cultured in HCM alone for 2-5 weeks. Hepatic differentiation was identified by cell morphology, immunohistochemistry, RT-PCR analysis, Western blot analysis, and biochemical functions. All were performed

together with undifferentiated AT-MSCs. Human primary hepatocytes or HepG2 cells were used as positive controls.

***RT-PCR.*** Total RNA (0.5$\mu$g) was reverse-transcribed using the SuperScript III Reverse Transcriptase (Invitrogen, Tokyo, Japan) according to the manufacturer's guidelines. PCR analyses were performed using the Ampli Taq Gold kit (Applied Biosystems, Tokyo, Japan). The PCR primer sequences and conditions are listed in Supplementary Table 1.

***Immunofluorescence.*** Cells were fixed in 4% formaldehyde for 10 minutes, followed by incubation with Protein Block (DakoCytomation) for 30 minutes. Undifferentiated $CD105^+$ AT-MSCs and $CD105^+$ AT-MSC-derived hepatocytes were analyzed by immunohistochemistry with transthyretin (TTR) (1:300), albumin (ALB) (Sigma, Tokyo, Japan) (1:250), cytokeratin-18 (CK-18) (1:200) (Cosmo Bio, Tokyo, Japan), and CYP3A4 (1:2000) (Cosmo Bio) antibodies overnight at 4°C. The rhodamine (1:1000) - or FITC (1:1000)-conjugated secondary antibodies were applied for 30 minutes. Undifferentiated AT-MSCs were stained according to the same protocol used for mouse anti-human vimentin (1:50) (Sigma) and mouse anti-human CD105 (1:40) (Cosmo Bio) antibodies. Nuclei staining have been performed using DAPI (4,6-diamidino-2-phenylindole).

***Western Blot Analysis.*** Western blot was used to detect the presence of CYP3A4, CYP1A1, CYP2C9 as well as NADPH P450 Reductase in AT-MSC-derived hepatocytes. The cells were dissolved in the Mammalian Protein Extraction Reagent (Pierce, Rockford, IL). Proteins were separated on an SDS-polyacrylamide gel and transferred to PVDF (polyvinylidine difluoride) membranes (BIORAD, Tokyo, Japan). Blots were saturated with 5% skim milk in TBS-T for 1 hour at room temperature and afterwards incubated overnight with anti-human rabbit polyclonal CYP3A4 (Cosmo Bio), goat NADPH-cytochrom P-450 reductase (CPR) (Daiichi Pure Chemicals, Tokyo, Japan), mouse CYP1A1 (Santa Cruz Biotechnology, Inc., CA, USA), or rabbit CYP2C9 (Fitzgerald, Concord, USA) antibodies, respectively. Following washing in TBS-T, the membranes were incubated for 30 minutes with sheep anti-rabbit or anti-mouse IgG-HRP-linked whole antibodies (GE Healthcare Bio-Sciences KK, Tokyo, Japan) or rabbit anti-goat IgG (H+L) HRP-linked antibodies (Southern Biotechnology Associates, Inc., Birmingham). Monoclonal antibodies against human GAPDH were used as a control of protein loading (Santa Cruz).

***LDL Uptake.*** LDL uptake was assessed by incubating cells for 4 hours at 37°C with 10$\mu$g/ml acetylated LDL labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Dil-Ac-LDL) purchased from Biomedical Technologies, Inc., Stoughton.

***Periodic Acid-Schiff (PAS) Staining.*** Cells were fixed with 10% formaldehyde oxidized in 1% periodic acid for 10 minutes and rinsed twice with water. Afterwards, cells were treated with Schiff's reagent for 10 minutes, rinsed with water and stained with hematoxylin.

***Ammonia Clearance.*** Cells were cultured in the presence of 2.5mM $NH_4Cl$ and incubated for 30 hours. At 9th, 19th, and 30th hour of incubation, the medium was collected and tested for the concentration of $NH_4Cl$ using Ammonia-Test Wako (Wako Pure Chemicals, Tokyo, Japan).

***Albumin Production.*** The albumin level in the culture medium was evaluated using the Bromocresol Green (BCG) method performed by SRI Communication for the Health Company, Tokyo, Japan.

***$CD105^+$ AT-MSC-Derived Hepatocyte Transplantation into Mice with $CCl_4$-Induced Injury.*** Twenty-four hours after an intraperitoneal injection of 100$\mu$L/20g body weight of olive oil containing 10$\mu$L of $CCl_4$, three BALB/c nude mice ( SLC, Tokyo, Japan) underwent transplantation of *in vitro* generated $CD105^+$ AT-MSC (Donor#6)-derived hepatocytes at $5 \times 10^5$ cells per mouse (0.2 ml of the cell suspension was injected through the tail vein). As a control, $CCl_4$-treated mice (n = 3) and untreated (olive oil) mice (n = 3) were used. $CD105^+$ AT-MSC-derived hepatocytes were collagenase-dispase-treated, while AT-MSCs were trypsinized according to the standard procedure. After centrifugation, the cell pellet was rinsed in PBS (−). Histological analysis of liver tissues was conducted by serial tissue section one day after cell transplantation and stained with hematoxylin and eosin or immunohistochemically examined for human specific albumin expression. Animal studies were carried out in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute.

***Statistical Analysis.*** Results are given as the mean ± SD. The Student *t* test was performed for statistical evaluation, with *P* less than 0.05 considered significant. All *in vitro* results were derived from at least three independent experiments.

## Results

***Characterization of AT-MSCs.*** AT-MSCs were characterized by flow cytometry, immunofluorescence and RT-PCR analysis. Flow cytometry revealed that the cells from all patients (n = 6) were: CD31 (12.9-30%),

**Table 2. Expression of Stem Cell Markers in AT-MSCs, BM-MSCs, and BM CD34$^{+}$ Stem Cells, by RT-PCR Analysis**

| | Bone Marrow-derived Mesenchymal Stem Cells | Adipose Tissue-derived Mesenchymal Stem Cells | Bone Marrow-derived CD34$^{+}$ Stem Cells |
|---|---|---|---|
| CD10 | + | + | − |
| CD13 | + | + | + |
| CD59 | + | + | + |
| CD105 | + | + | + |
| CD166 | + | + | − |
| CD49d | + | + | + |
| SH3 | + | + | − |
| CD29 | + | + | + |
| CD44 | + | + | + |
| CD71 | + | + | + |
| CD90 | + | + | − |
| CD106 | + | +/− | − |
| CD120a | + | + | + |
| CD124 | + | + | + |
| CD11b | − | − | + |
| CD14 | − | +/− | + |
| CD31 | − | +/− | + |
| CD34 | − | + | + |
| CD45 | − | − | + |
| CD48 | − | − | + |
| CD135 | − | − | + |
| CD117 | + | +/− | + |

NOTE. +/− means that mRNA expression vary according to each patient.

CD34 (5.3%-9.9%), CD45 (0.06%-8.4%), CD29 (96%-99%) and CD105 (63.9-98.5) (Supplementary Fig. 1). The number of CD105$^{+}$ cells varies between donors. AT-MSCs were evaluated by the expression of stem cell markers (mesenchymal: CD10, CD13, CD59, CD105, CD166, CD49d, SH3, CD29, CD44, CD71, CD90, and CD106; and hematopoietic: CD120a, CD124, CD11b, CD14, CD31, CD34, CD45, CD48, CD135, and CD117) using RT-PCR analysis and compared with mRNA from BM-MSC and BM-HSC (Table 2). AT-MSCs share similarities in morphology (Fig. 2a,b) and marker profile with BM-MSCs (Table 2); however, AT-MSCs were found to express the mRNA of CD34, CD31 and CD14. The expression of CD106, CD14, CD31 and CD117 varies according to the donor. In addition, we compared the expression of CD105 (Fig. 2c,d) and vimentin (Fig. 2e,f) by immunostaining. The expression of both mesenchymal stem cell markers was lower in unfractionated AT-MSCs than in BM-MSCs. After MACS sorting, the CD105$^{+}$ fraction of AT-MSCs revealed a homogeneous morphology (Fig. 2g) and high proliferation ability (at early passages doubling time= 34-36 hours, whereas the unfractionated population = 36-40 hours). The sorting efficiency was evaluated by CD105 staining (Fig. 2h) and flow cytometry analysis before and after MACS sorting (Fig. 2j). CD105$^{+}$ fraction of AT-MSCs revealed expression of vimentin as well (Fig. 2i). The adipogenic differentiation potential of unfractionated and CD105$^{+}$ AT-MSCs was verified by performing adipogenic differentiation. This resulted in the accumulation of intracellular lipid droplets that could be stained with Oil Red O. CD105$^{+}$ AT-MSCs revealed a higher adipogenic induction ratio (≈60%-82%) than unfractionated AT-MSCs (≈21%-67%) (Fig. 2k). Non-induced AT-MCSs showed no presence of lipid droplets. Similar results were obtained from a different donor age. Chondrogenic induction of CD105$^{+}$AT-MSCs resulted in higher induction ratio than unfractionated AT-MSCs (data not shown) as well. In summary, our data suggest that CD105$^{+}$ fraction exhibits higher homogeneity, proliferation ability and potentiality than unfractionated AT-MSCs.

***Hepatic Differentiation of AT-MSCs.*** We examined the hepatic-differentiation potential of CD105$^{+}$ AT-MSCs. During the initiation step of hepatic differentiation, the cells showed a remarkable transition from a bipolar fibroblast-like morphology (Fig. 3a-c) to a round epithelial-like shape (Fig. 3d-f). The contraction of the cytoplasm progressed further during maturation, and most of the treated cells became quite dense and round with clear or double nuclei in the late stage of differentiation. The representative morphology of differentiated CD105$^{+}$ AT-MSCs from 3 different donors (#1, 2, and 6 as shown in Table 1) is shown in Fig. 3d-f, and contrasted with that of primary human hepatocytes (Fig. 3g,h). We can notice clearly small round or oval-shaped cells with polyhedral structure, with tight cell to cell interactions, and visible bile canaliculi structures (Fig. 3i), similar to primary human hepatocytes (Fig. 3h). CD105$^{+}$ AT-MSCs derived from all three independed donors during hepatic induction underwent morphological changes. CD105$^{+}$ AT-MSC-derived hepatocyte-like cells from donor #2 (Age 45, male) were used for further analysis of liver functions.

***RT-PCR Analysis of Hepatocyte-Specific Markers.*** The temporal expression pattern for a number of hepatocyte-specific genes, such as ALB, AFP, TTR, TDO2, and CYP7A1, and the hepatocyte nuclear factor (HNF)-4-alpha were analyzed by RT-PCR (Fig. 4). The expression of early endoderm differentiation marker-AFP decreased when the cells underwent maturation, whereas the expression of mature hepatocyte markers, such as ALB, TDO2, and CYP7A1 appeared at around day 6 of HIFC treatment and was maintained until day 70 (data not shown). HNF-4-alpha, essential for morphological and functional hepatocyte differentiation had been detected at day 41. Therefore, HIFC induced CD105$^{+}$ AT-MSCs into hepatocyte-like cells revealed a gene expression pattern of