INTRODUCTION

Hepatitis C virus (HCV) is a major cause of non-A and non-B hepatitis, which frequently leads to liver cirrhosis and hepatocellular carcinoma (HCC) (Caselmann & Alt, 1996; Koike, 2007). As a member of the family Flaviviridae, HCV contains a positive-stranded RNA genome of 9.5 kb, encoding a polyprotein processed into four structural proteins and six non-structural proteins (Suzuki et al., 2007). In addition to its function in capsid assembly, the core protein has been strongly implicated in HCC pathogenesis by virtue of its role in the alteration of various signalling pathways, transcriptional activation, modulation of immune responses, apoptosis and lipid metabolism (Koike, 2007; Liang & Heller, 2004). In addition, core has been directly implicated in cellular transformation and immortalization (Ray et al., 1996). Furthermore, direct induction of HCC by core in transgenic mice has been reported (Moriya et al., 1998). Despite steadily increasing knowledge about core in HCV-associated oncogenesis, its mechanism of action is still controversial.

Oxidative stress generating reactive oxygen species (ROS) induces multiple types of cell damage, including DNA breaks, protein modifications, lipid peroxidation and mitochondrial failure (Matés et al., 2008; Ott et al., 2007). If not properly repaired, the oxidative damage can lead to cell death that, according to the extent of oxidative stress, may be necrotic or apoptotic (Ott et al., 2007). \( \text{H}_2\text{O}_2 \), a representative ROS, can induce apoptosis in many different types of cells (Li et al., 2003; Maheshwari et al., 2009; Tamura et al., 2003; Wu et al., 2011). A number of proteins can be activated, but the tumour suppressor p53 appears to play a key role in this process (Achanta & Huang, 2004). The activated p53 triggers expression of the Bcl-2 family proteins Bax and PUMA, which target the mitochondria to induce cytochrome \( \text{c} \) release and subsequent activation of caspase-9, leading to activation of the intrinsic apoptotic pathway (Gao et al., 2001). In addition, p53 can activate the extrinsic apoptotic pathway by upregulating Fas expression (Li et al., 2003).

Mouse double minute 2 (MDM2) maintains p53 at a low level by acting as an E3 ubiquitin (Ub) ligase targeting p53 for Ub-dependent proteasomal degradation (Michael & Oren, 2003). However, p53 becomes rapidly stabilized and activated in response to cellular stresses, such as DNA damage and oncogene activation (Enoch & Norbury, 1995). Activation of p53 in response to DNA damage involves a series of post-translational modifications by DNA-damage-sensing kinases to both itself and MDM2, which facilitate the dissociation of the MDM2–p53 complex and contribute to the stabilization of p53 (Enoch & Norbury, 1995). p53 is also regulated via another route involving p14 and MDM2 (Zhang & Xiong, 2001). Expression of p14 is usually induced in response to aberrant growth signalling from Myc and Ras through the E2F transcription factor. p14 then directly binds
to MDM2 and prevents it from targeting p53 for degradation by inhibiting the E3 ligase activity of MDM2 (Gallagher et al., 2006; Pomerantz et al., 1998; Zhang & Xiong, 2001), promoting the rapid degradation of MDM2 (Zhang et al., 1998), and preventing nuclear export of MDM2 and p53 (Zhang & Xiong, 2001). Although the two pathways both require inactivation of MDM2 for p53 stabilization, it is unclear whether they cross-talk or affect each other in order to stabilize p53 under oxidative stress.

Aberrant oxidative stress in the liver of hepatitis C patients mostly occurs as a result of chronic inflammation (González-Gallego et al., 2011). HCV infection or overexpression of specific HCV proteins (mainly the core protein) can also enhance the production of ROS, accompanying other mitochondrial dysfunctions (Korenaga et al., 2005; Okuda et al., 2002). Oxidative stress, imposed either by viral proteins or inflammation, has been considered as an important pathological mechanism in hepatitis C and other chronic liver diseases (Farinati et al., 2007; González-Gallego et al., 2011). Free radicals can trigger a cascade of epigenetic, genomic and post-genomic alterations that lead to HCC (Farinati et al., 2007). However, it is still unclear how hepatocyes maintain their growth potential and support virus replication under the oxidative microenvironment that causes cell death. It might be possible if the key regulators of ROS-mediated apoptosis are dysfunctional in these cells. Actually, p53 is the most frequently mutated tumour suppressor gene in human cancer including HCC, but half of all human primary tumours still have WT p53. Another way to inactivate p53 is through the inactivation of p14, because then MDM2 is not inhibited by p14 and is free to induce p53 degradation (Esteller et al., 2001). Indeed, the INK4a/ARF locus is found to be frequently silenced via DNA methylation in p53-positive HCC tumours (Esteller et al., 2001; Robertson & Jones, 1999), especially in HCV-associated tumours (Peng et al., 2002). However, the mechanism by which p14 is epigenetically inactivated and its actual role in p53 inactivation in HCV-associated tumour cells under oxidative stress remain unknown. In the present study, we investigated the mechanism by which H$_2$O$_2$ induced apoptosis in human hepatocytes, focusing on the activation of p53 via the p14–MDM2 pathway. We then investigated whether and how core from either exogenous expression or infection with cell culture-derived HCV overcomes H$_2$O$_2$-induced apoptosis in p53-positive human hepatocytes, enabling their continuous cell proliferation under oxidative stress.

RESULTS

HCV core overcomes H$_2$O$_2$-induced apoptosis

Initially, we examined whether H$_2$O$_2$ inhibited cell growth and induced apoptosis in human hepatocytes. According to the data from the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Fig. 1a), treatment with H$_2$O$_2$ reduced the viability of HepG2 cells in a dose-dependent manner. In addition, H$_2$O$_2$ induced cell death of HepG2 cells as demonstrated by the Trypan blue exclusion assay (Fig. 1b). Furthermore, according to the data from FACS analysis, H$_2$O$_2$ substantially increased the sub-G₁ fraction of HepG2 and Huh-7.5 cells (Fig. 1c). These results suggested that H$_2$O$_2$ induced apoptotic cell death of human hepatocytes to inhibit their growth. However, the potential of H$_2$O$_2$ to inhibit cell growth (Fig. 1a) and to induce cell death (Fig. 1b) was severely impaired in HepG2 cells that stably expressed core. In addition, the potential of H$_2$O$_2$ to induce apoptosis was much weaker both in core-expressing HepG2 cells and Huh-7.5 cells infected with HCV (Fig. 1c). However, neither the potential of H$_2$O$_2$ to induce apoptosis nor the effect of core on it was clearly observed in p53-negative Hep3B cells (Fig. 1c). These results suggested that core overcame H$_2$O$_2$-induced apoptotic cell death in human hepatocytes via a p53-dependent pathway(s).

HCV core blocks activation of the p53-dependent apoptotic pathways by H$_2$O$_2$

Next, we investigated the molecular mechanism by which core overcame H$_2$O$_2$-induced apoptotic cell death. H$_2$O$_2$ usually induces apoptosis via activation of p53 that can trigger both extrinsic and intrinsic apoptotic pathways (Achanta & Huang, 2004; Li et al., 2003). Indeed, treatment of p53-positive human cell lines, including HepG2, Chang liver and Huh-7 cells, with H$_2$O$_2$ resulted in upregulation of p53 and subsequent activation of apoptosis-related molecules, including Bax, caspase-8, -9 and -3, and poly(ADP-ribose) polymerase (PARP) (Fig. 2a, b). In addition, a dramatic increase in caspase-3 and -7 activities (potential apoptotic markers) was also observed in H$_2$O$_2$-treated HepG2 cells (Fig. 2c). However, these effects were undetectable or negligible in p53-negative Hep3B cells (Fig. 2a–c), suggesting a role for p53 in H$_2$O$_2$-induced apoptosis of human hepatocytes.

To confirm that p53 was critical for the activation of apoptotic pathways by H$_2$O$_2$, we attempted to knock down p53 in HepG2 cells using a specific small interfering RNA (siRNA). As a result, the effects of H$_2$O$_2$ on Bax, caspase-3 and PARP almost completely disappeared in HepG2 cells (Fig. 2d). Furthermore, exogenous expression of p53 in Hep3B cells successfully restored the potential of H$_2$O$_2$ to activate Bax, caspase-3 and PARP (Fig. 2e). These results confirmed that H$_2$O$_2$ activated apoptotic pathways in human hepatocytes via upregulation of p53.

We next investigated the effect of core on the activation of apoptotic pathways by H$_2$O$_2$. Both stable and transient expression of core effectively blocked upregulation of p53 in p53-positive human hepatocytes (Fig. 2a, b). In addition, core could downregulate levels of exogenous p53 in Hep3B cells (Fig. 2e). As a consequence, the potential of H$_2$O$_2$ to activate apoptotic molecules such as Bax, caspase-3 and PARP was severely impaired or undetectable in the
In the presence of core (Fig. 2a–c, e), we concluded that p53 was required not only for the activation of apoptotic pathways by H$_2$O$_2$, but also for its abrogation by core.

**HCV core blocks activation of the p14–MDM2–p53 pathway by H$_2$O$_2$**

We next investigated how H$_2$O$_2$ and core regulated levels of p53 in human hepatocytes. According to the data shown in Fig. 2, core downregulated levels of p53 derived from both endogenous and exogenous origin. Therefore, it was possible to hypothesize that core antagonized H$_2$O$_2$ by inhibiting its ability to stabilize p53. To prove this hypothesis, we examined whether H$_2$O$_2$ and core affected the p14–MDM2–p53 pathway, which is closely associated with p53 stabilization (Gallagher et al., 2006; Pomerantz et al., 1998).

Treatment with H$_2$O$_2$ upregulated levels of p14, whilst it downregulated levels of MDM2 in HepG2 cells, resulting in upregulation of p53 (Fig. 3a). In contrast, core downregulated levels of p14, whilst it upregulated levels of MDM2 in HepG2 cells, in the presence or absence of H$_2$O$_2$. The basal level of p53 in the absence of H$_2$O$_2$ was slightly higher in core-expressing cells, as demonstrated previously (Kwun & Jang, 2003). However, it was obvious that core downregulated levels of p53 in the presence of H$_2$O$_2$ (Fig. 3a). These results suggested that H$_2$O$_2$ and core oppositely regulated levels of p14 and MDM2 to modulate p53 stability.

To prove that upregulation of p14 by H$_2$O$_2$ was critical for p53 stabilization, we attempted to knock down p14 in the control cells using a specific siRNA. Knock-down of p14 in H$_2$O$_2$-treated cells to the level in untreated cells almost completely abolished the potential of H$_2$O$_2$ to modulate levels of MDM2 and p53 (Fig. 3b). In addition, complementation of p14 expression in core-expressing cells by transient transfection of p14 expression plasmid almost completely abolished the potential of core to modulate levels of p53.
of MDM2 (Fig. 3c). Under these conditions, H2O2 could upregulate p53 in core-expressing cells as in the control cells. These results suggested that H2O2 downregulated levels of MDM2 and subsequently stabilized p53 by upregulating levels of p14, whilst core abolished the effect of H2O2 by downregulating levels of p14.

**Fig. 2.** HCV core suppresses activation of the p53-dependent apoptotic pathway by H2O2. (a) Four human cell lines, as indicated, were transiently transfected with core expression plasmid and then either mock-treated or treated with 400 μM H2O2 as described in Fig. 1. Levels of p53, core, γ-tubulin, and cleaved active forms of caspase-8 (41 kDa), caspase-9 (35 kDa) and PARP (84 kDa) were determined by Western blotting. (b) HepG2 and Hep3B cell lines with or without core expression were treated with 400 μM H2O2 as described in Fig. 1. Levels of p53, Bax, core, γ-tubulin, and cleaved forms of caspase-3 (20 kDa) and PARP (84 kDa) were determined by Western blotting. (c) Caspase-3/7 activities in cells prepared as above were measured as described in Methods. (d) HepG2 and HepG2-core cells were transiently transfected with the indicated amounts of p53 shRNA plasmid and treated with 400 μM H2O2 as described in Fig. 1. Levels of p53, Bax, core, γ-tubulin, and cleaved forms of caspase-3 (20 kDa) and PARP (84 kDa) were determined by Western blotting. (e) Hep3B and Hep3B-core cells were transiently transfected with the indicated amounts of p53 expression plasmid and treated with 400 μM H2O2 as described in Fig. 1, followed by Western blotting.
HCV core represses p14 expression via DNA methylation

Next, we investigated the mechanism by which core repressed p14 expression. According to our previous reports, core induced promoter hypermethylation of the p16 gene (Park et al., 2011), which is closely associated with p14 in the INK4a/ARF locus (Gil & Peters, 2006). Therefore, we examined whether core induced promoter hypermethylation of p14 to inhibit its expression in human hepatocytes. Indeed, core derived from either stable transfection or HCV infection induced promoter hypermethylation of p14 in the presence or absence of H$_2$O$_2$ (Fig. 4a, b), resulting in downregulation of its expression (Fig. 4c, d). In contrast, the DNA methylation pattern of p14 was little affected by treatment with H$_2$O$_2$ (Fig. 4a, b), although its expression was upregulated by H$_2$O$_2$ in both HepG2 and Huh-7.5 cells (Fig. 4c, d), indicating that H$_2$O$_2$ regulated p14 expression via a different mechanism(s). Treatment with a universal DNA methyltransferase (DNMT) inhibitor, 5-Aza-2’dC, almost completely abolished the potential of core to induce DNA methylation of p14, restoring its protein levels in core-expressing cells to those in control cells (Fig. 3a, b). As a consequence, the potential of core to upregulate levels of MDM2 and subsequently downregulate those of p53 in the presence of H$_2$O$_2$ was no longer effective (Fig. 4c, d). Under these conditions, treatment with H$_2$O$_2$ almost equally activated Bax and caspase-3 in control and core-expressing cells (Fig. 4c, d). In addition, caspase-3 and -7 activities were similarly activated by H$_2$O$_2$ in these cells (Fig. 4e, f). Taken together, we concluded that core repressed p14 expression via DNA methylation to upregulate levels of MDM2, to downregulate levels of p53 and, finally, to inhibit activation of apoptotic pathways in the presence of H$_2$O$_2$.

HCV core induces Ub-mediated proteasomal degradation of p53 in the presence of H$_2$O$_2$

Finally, we investigated whether core actually induced p53 degradation in the presence of H$_2$O$_2$ by repressing p14 expression via DNA methylation. Treatment with a proteasomal inhibitor, MG132, elevated levels of p14 in both control and core-expressing cells (Fig. 5a). However, the effect of core on p14 expression was little affected under these conditions, probably because core did not act on p14 by affecting its proteasomal degradation. Levels of MDM2 and p53 in control and core-expressing cells were elevated to similar levels under the same conditions. These results suggested that H$_2$O$_2$ induced proteasomal degradation of MDM2 to stabilize p53 in control cells, whilst core stabilized MDM2 to enhance proteasomal degradation of p53.

To prove that H$_2$O$_2$ induced ubiquitination of MDM2 for its proteasomal degradation in control cells but not effectively in the presence of core, we introduced HA-tagged Ub along with MDM2 into these cells and immunoprecipitated Ub-complexed products. As shown in Fig. 5(b), H$_2$O$_2$ increased the amount of Ub-complexed MDM2 in control cells, whilst the effect was much weaker in the presence of core. Treatment with 5-Aza-2’dC that recovered levels of p14 in core-expressing cells (Fig. 4c) almost completely abolished the potential of core to inhibit ubiquitination of MDM2 by H$_2$O$_2$. These results suggested that H$_2$O$_2$ induced degradation of MDM2 via the Ub–proteasome system in control cells by upregulating levels of p14, whilst core inhibited its degradation by downregulating levels of p14 via DNA methylation.

It is relatively well established that MDM2 directly binds to the N-terminal domain (aa 1–43) of p53 and mediates its degradation via the Ub–proteasome system (Haupt et al., 1997). Therefore, we investigated whether H$_2$O$_2$ and core affected the interaction between MDM2 and p53 to modulate degradation of p53. For this purpose, we employed a mammalian two-hybrid assay in which reporter gene activity was determined by the level of interaction between G4-MDM2 and p53-VP16 (Kwun & Jang, 2004). Treatment with H$_2$O$_2$ decreased the luciferase...
activity in control cells (Fig. 5c), probably because it interfered with the interaction between MDM2 and p53 by downregulating levels of MDM2. As a consequence, H2O2 downregulated levels of Ub-complexed p53, resulting in upregulation of its protein levels in control cells (Fig. 5d). In contrast, core elevated luciferase activity both in the presence and absence of H2O2, indicating that it increased the interaction between MDM2 and p53 (Fig. 5c). This was probably because core upregulated levels of MDM2 under these conditions. As a consequence, levels of Ub-complexed p53 were much higher in core-expressing cells, resulting in downregulation of its protein levels in core-expressing cells (Fig. 5d). Treatment with 5-Aza-2′dC strongly interfered with the interactions between MDM2 and p53 both in control and core-expressing cells (Fig. 5c), probably because levels of MDM2 under these conditions were almost undetectable in these cells (Fig. 5b). As a result, levels of Ub–p53 complexes were very low both in control and core-expressing cells, resulting in accumulation of p53 in these cells (Fig. 5d). Taken together, we concluded that H2O2 upregulated levels of p14 and induced proteasomal degradation of MDM2 to stabilize p53, whilst core counteracted this effect by repressing p14 expression via DNA methylation.

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Fig. 4. HCV core suppresses activation of the p53-dependent apoptotic pathways by downregulating p14 expression via DNA methylation. (a) HepG2 and HepG2-core cells were either mock-treated or treated with 400 μM H2O2 in the presence or absence of 5 μM 5-Aza-2′dC as described in Fig. 1. Total genomic DNA prepared from these cells was subjected to methylation-specific PCR (MSP) to measure the relative amount of unmethylated (U) and methylated (M) forms of the p14 promoter. (b) Huh-7.5 cells were either mock-infected or infected with HCV at m.o.i. 0.1 for 2 h, and treated with H2O2 and 5-Aza-2′dC for an additional 60 h, followed by p14 MSP as described in (a). (c) HepG2 and HepG2-core cells were prepared as described in (a), and subjected to Western blotting to determine levels of p14, MDM2, p53, Bax, cleaved caspase-3, core and γ-tubulin. (d) Huh-7.5 cells prepared as described in (b) were subjected to Western blotting. (e) Caspase-3/7 activities were measured from HepG2 and HepG2-core cells prepared as in (a). (f) Caspase-3/7 activities were measured from Huh-7.5 cells prepared as in (b).
DISCUSSION

HCV infection is characterized by a systemic oxidative stress that is most likely caused by a combination of chronic inflammation and proteins encoded by HCV (Farinati et al., 2007; Gonza´lez-Gallego et al., 2011; Korenaga et al., 2005; Okuda et al., 2002). Several oxidative markers have been detected in serum, peripheral blood mononuclear cells and liver specimens from hepatitis C patients (Choi & Ou, 2006; Mate´s et al., 2008; Muriel, 2009; Ott et al., 2007). ROS generated under oxidative stress are well recognized for playing dual roles in the development and progression of HCC (Gonza´lez-Gallego et al., 2011). ROS, such as free radicals and peroxides, can induce oxidative DNA damage, rendering cells more susceptible to spontaneous or mutagen-induced alterations associated with cell transformation (Farinati et al., 2007). In addition, ROS can activate several cellular signalling pathways including the mitogen-activated protein kinase (MAPK), NFkB, phosphatidylinositide 3-kinase (PI3K), β-catenin/Wnt and angiogenesis signalling pathways (Farinati et al., 2007; Koike, 2007). Oxidative stress, however, can cause cell death via either apoptosis or necrosis depending on the extent of oxidative stress (Li et al., 2003; Maheshwari et al., 2009; Tamura et al., 2003; Wu et al., 2011). According to the present study, ≤400 μM H2O2 induced apoptotic cell death of human hepatocytes. Induction of apoptosis by ROS may serve as an anticancer defence mechanism, preventing damaged cells from undergoing aberrant cell proliferation (Campisi, 2005). In this respect, hepatocytes in the liver of hepatitis C patients should evolve a mechanism(s) evading...
Inhibition of H$_2$O$_2$-induced apoptosis by HCV core

cell death caused by oxidative stress to support virus replication and tumour progression. Indeed, core can overcome ROS-mediated apoptosis via induction of antioxidant gene expression (Okuda et al., 2002) and activation of antiapoptotic factors such as NFkB (Kato et al., 2000). The present study also showed that core overcame apoptosis provoked by H$_2$O$_2$ by inactivating p53 via inhibition of p14 expression. Therefore, it is likely that core contributes to HCC formation not only by facilitating cell transformation via induction of ROS, but also by providing survival benefits to cells against oxidative stresses (Fig. 6).

Several previous reports have suggested that p53 plays a key role during ROS-induced apoptosis (Achanta & Huang, 2004). Consistently, according to the present study, H$_2$O$_2$ could activate apoptotic pathways in p53-positive human hepatocytes, but not in p53-negative human hepatocytes. Both the intrinsic and extrinsic apoptosis pathways were activated by H$_2$O$_2$, which is consistent with previous reports (Achanta & Huang, 2004; Li et al., 2003). Interestingly, the potential of H$_2$O$_2$ to induce apoptosis was almost completely abolished by overexpression of core alone or infection with cell culture-derived HCV. Several lines of evidence argue that this effect is closely associated with the potential of core to downregulate levels of p53. First, core exhibited this effect in p53-positive HepG2 cells, but not in p53-negative Hep3B cells. Second, it was possible to abolish the effect almost completely by silencing p53 expression in HepG2 cells. Third, exogenous expression of p53 made Hep3B cells susceptible to the H$_2$O$_2$-mediated activation of apoptotic pathways and its inhibition by core.

Several previous studies have demonstrated that core binds to p53 (Kao et al., 2004; Otsuka et al., 2000), resulting in either inhibition or activation of p53 followed by anti- or pro-apoptotic effects (Kao et al., 2004; Lu et al., 1999; Otsuka et al., 2000). In addition, core enhances the transactivational activity of p53 by affecting its post-translational modifications (Kao et al., 2004). Core also counteracts p53-mediated growth suppression through activation of the MAPK and PI3K/Akt pathways, which enhances cell growth and proliferation. Therefore, it has so far been established that core targets the p53 pathway via at least three different means: physical interaction, post-translational modifications and modulation of p53 regulatory pathways (Kao et al., 2004). In the present study, we provide another mechanism by which core inactivates p53: proteasomal degradation of p53.

In general, post-translational modifications of p53 and MDM2 by ATM (ataxia telangiectasia mutated) activated in response to DNA double-strand breaks destabilize their interactions, leading to stabilization and activation of the p53 pathway (Khosravi et al., 1999). In the present study, we found that H$_2$O$_2$ can stabilize p53 via activation of the p14–MDM2 pathway, which is usually activated in response to oncogenic stimuli such as c-Myc and Ras. For this effect, H$_2$O$_2$ upregulated levels of p14 via an unknown mechanism(s) and induced Ub-dependent proteasomal degradation of MDM2, resulting in stabilization of p53. In contrast, core destabilized p53 via the same pathway simply by downregulating levels of p14. Therefore, H$_2$O$_2$ and core appear to oppositely regulate the p14–MDM2 pathway to modulate levels of p53. Interestingly, knock-down of p14 in the H$_2$O$_2$-treated cells using a specific siRNA almost completely abolished the potential of H$_2$O$_2$ to upregulate levels of p53. In addition, complementation of p14 in the core-expressing cells by either transient transfection of the p14 expression plasmid or treatment with 5-Aza-2’dC almost completely abolished the potential of core to downregulate levels of p53. Therefore, it is possible to assume that the two pathways initiated from p14 and DNA damage cross-talk to regulate p53 in response to oxidative stress. Otherwise, repression of p14 expression by core may simply provide excess amounts of active MDM2, which can override the signal from the DNA damage that prevents p53 degradation. More extensive studies are required to provide a final conclusion on this observation.

Recently, we and others demonstrated that core downregulates the levels of several tumour suppressors including p16 via promoter hypermethylation (Arora et al., 2008; Lim et al., 2012; Park et al., 2011). For this effect, core upregulates levels of DNMT1 and DNMT3b (Park et al., 2011). Consistently, according to the present study, core induced promoter hypermethylation of p14 in the presence or absence of H$_2$O$_2$, resulting in downregulation of its expression. In addition, the potential of core to downregulate p14 expression was almost completely abolished by treatment with 5-Aza-2’dC. In contrast, H$_2$O$_2$ did not affect DNA methylation of p14. It is not known how H$_2$O$_2$ upregulates levels of p14. H$_2$O$_2$, like other ROS, may activate cellular signalling pathways leading to activation of

![Diagram](http://vir.sgmjournals.org)
E2F1 (Farinati et al., 2007; Koike, 2007), which in turn activates p14 expression. Although core is also known to activate E2F1 under oxidative stress (Lim et al., 2012), it could not lead to activation of p14 expression probably because its promoter was hypermethylated in the presence of core. In conclusion, the present study showed for the first time, to our knowledge, that oxidative stress can stabilize p53 via activation of the p14–MDM2 pathway, which leads to apoptosis. To counteract this effect, core destabilizes p53 by inhibiting p14 expression via promoter hypermethylation and thus overcomes apoptosis provoked by H$_2$O$_2$. Considering the roles of p14 and p53 as tumour suppressors, their inactivation may also contribute to other aspects of HCV-mediated carcinogenesis.

**METHODS**

**Plasmids.** Plasmid pCMV-3 × HA1-Core encodes the full-length core downstream of three copies of the influenza virus haemagglutinin (HA) epitope as described previously (Arora et al., 2008). Plasmids pCMV-myc3-MDM2 and pEGFP-N1-p14 were gifts from B.-J. Park (Pusan National University, Republic of Korea). Plasmids pHA-Ub and pCMV-p53-WT were gifts from Y. Xiong (University of North Carolina at Chapel Hill, NC, USA) and C.-W. Lee (Sungkyunkwan National University, Republic of Korea). Plasmids pHA-Ub and pCMV-p53-WT were gifts from Y. Xiong (University of North Carolina at Chapel Hill, NC, USA) and C.-W. Lee (Sungkyunkwan National University, Republic of Korea), respectively. Plasmids p14 shRNA and p53 shRNA were purchased from Santa Cruz Biotechnology.

**Cell lines and transfection.** HepG2 (KCLB 88065), Hep3B (KCLB 88064) and Huh-7 (KCLB 60104) were obtained from the Korean Cell Line Bank (KCLB). Chang liver (ATCC No. CCL-13) was purchased from the American Type Culture Collection (ATCC). For transient expression, 2 × 10$^5$ cells per 60 mm dish were transfected with 1 μg appropriate plasmid(s) with the use of the WelFect-EX PLUS (WelGENE) following the manufacturer’s instructions. Stable cell lines were established by transfection with either pCMV-3 × HA1 or pCMV-3 × HA1-Core, followed by selection with 500 μg G418 ml$^{-1}$ (Gibco) (Park et al., 2011).

**HCV infection system.** The plasmid pFH-1 containing HCV cDNA from a Japanese patient with fulminant hepatitis behind a T7 promoter (Kato et al., 2003) was linearized at the 3’ end of the HCV cDNA by XbaI digestion. The linearized DNA was then used as a template for in vitro transcription (MEGAscript; Ambion). JFH-1 RNA (10 μg) was delivered to Huh-7.5 cells by electroporation and virus stocks were prepared as described by Zhong et al. (2005). For the determination of virus titres, real-time reverse transcription-PCR analysis was performed as described previously (Takeuchi et al., 1999). Cells were either mock-infected or infected with HCV at m.o.i. 0.1 under the indicated conditions.

**Cell viability analysis.** For the determination of cell viability, a MTT assay was performed as described previously (Lee et al., 2012). To induce oxidative stress, 5 × 10$^5$ cells per well in 96-well plates were treated with increasing concentrations of H$_2$O$_2$ (Sigma) for 2 h. After changing to standard medium, the cells were incubated for an additional 36 h. The cells were then treated with 10 μM MTT (Sigma) for 4 h at 37 °C. The formazan compounds derived from MTT by mitochondrial dehydrogenases of living cells were dissolved in DMSO and quantified by measuring A$$_{570}$. In addition, cell death was determined by Trypan blue exclusion assay (Lee et al., 2012). Briefly, 1 × 10$^5$ cells in six-well plates were treated with H$_2$O$_2$ as described above. The cells were collected by trypsinization and 10 μl cell suspension was eluted from them were subjected to Western blotting.

**Cell cycle analysis.** The cell cycle profile was analysed using flow cytometry. Briefly, 5 × 10$^5$ cells were seeded in a 60 mm cell culture dish. After treatment with 400 μM H$_2$O$_2$ for 2 h, the cells were further incubated for 60 h in new culture media. The cells were trypsinized, fixed in 80 % ethanol and resuspended in 50 μg propidium iodide ml$^{-1}$ (Sigma) containing 125 U RNase A ml$^{-1}$ (Sigma). DNA content analysis was performed by flow cytometry using Cell-FIT software (Becton Dickinson Instruments).

**Western blot analysis.** Cells were lysed in buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1% SDS and 1% NP-40) supplemented with protease inhibitors. Cells extracts were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Hybond PVDF, Amersham). Membranes were incubated with antibodies to p14, PARP, caspase-3, caspase-8, caspase-9, Bax, Ub, Bcl2, p53, MDM2 (Santa Cruz Biotechnology), HCV core (Virogen) and γ-tubulin (Sigma). Primary antibodies were detected with the appropriate HRP-conjugated secondary antibodies: anti-mouse IgG (H + L)-HRP (Bio-Rad), anti-goat IgG (H + L)-HRP (Bio-Rad) and anti-rabbit IgG (H + L)-HRP (Bio-Rad). An ECL kit (Amersham) was used to visualize protein bands via a ChemiDoc XRS imaging system (Bio-Rad).

**Caspase-3/7 activity assay.** Caspase-3- and -7 activities were measured using the Caspase-Glo 3/7 Assay (Promega). Briefly, 3 × 10$^5$ cells seeded in 96-well plates were treated with 400 μM H$_2$O$_2$ as above. After adding an equal volume of Caspase-Glo 3 reagent (Promega) to each well, the mixtures were incubated for additional 1 h at room temperature followed by measurement of luminescence.

**Methylation-specific PCR (MSP).** Genomic DNA (1 μg) denatured in 50 μl 0.2 N NaOH was modified by treatment with 30 μl 10 mM hydroquinone (Sigma) and 520 μl 3 M sodium bisulphite (pH 5.0; Sigma) at 50 °C for 16 h. For MSP, the modified DNA (100 ng) was amplified with Tag polymerase using both methylated and unmethylated primer pairs of p14 under conditions described previously (Herman et al., 1996).

**Immunoprecipitation assay.** Immunoprecipitation assay was performed using a Classic Magnetic IP/Co-IP assay kit (Pierce) according to the manufacturer’s specifications. The whole-cell lysates were incubated overnight at 4 °C with an appropriate immunoprecipitation antibody. Protein A/G magnetic beads (Pierce) were then added and incubated for an additional 1 h. The beads were then collected using a magnetic stand (Pierce) and the antigen/antibody complexes eluted from them were subjected to Western blotting.

**Mammalian two-hybrid assay.** To measure the strength of interaction between MDM2 and p53, mammalian two-hybrid assay was performed. Briefly, pG4-DM2, pCMV-p53/VP16 and the Ga4 reporter G5E1b-luc were transfected with either pCMV-3 × HA1 or pCMV-3 × HA1-Core into HepG2 cells. Cells were then treated with 400 μM H$_2$O$_2$ in the presence or absence of 5 μM 5-Aza-2′-dC. At 48 h after transfection, the level of expression from the luciferase activity was analysed as described previously (Kwun & Jang, 2004).

**Statistical analysis.** Each experiment was repeated at least three times in triplicate. The values represent mean ± SD. The difference between the means of the treatment group and the control was assessed with the paired two-tailed t-test; the difference was considered to be significant if $P<0.05$.

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