A Dissertation for the Degree of Doctor of Philosophy

Inhibitory effects of resveratrol on hepatitis B virus X-protein (HBx)-induced hepatocellular carcinoma (HCC)

B형 간염 바이러스 X-단백질로 유도된 간세포암종에서 resveratrol의 억제 효과

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Veterinary Pathobiology and Preventive Medicine
Department of Veterinary Medicine
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ABSTRACT

Inhibitory effects of resveratrol on hepatitis B virus X-protein (HBx)-induced hepatocellular carcinoma (HCC)

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Resveratrol is one of the active compounds isolated from red grapes, berries and peanuts. The purpose of our present study was to investigate inhibitory effects of resveratrol in hepatitis B virus (HBV)-induced hepatocellular carcinoma (HCC) using HBV X-protein-overexpressing Huh7 (Huh7-HBx) human hepatoma cells and the possible mechanisms in *vitro* and *in vivo*.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showed that resveratrol decreased cell viability. Fluorescence-

activated cell sorter analysis showed that it induced G1 cell cycle arrest without increasing the sub-G1 phase cell population. Therefore, we evaluated its effect on regulation of cyclin D1, which is critically involved in G1/S transition. Resveratrol lowered cyclin D1 transcription. Western blot analysis of the effects of resveratrol on upstream cyclin D1 transcriptional signaling, extracellular signal-related kinase (ERK), $p90^{RSK}$, Akt, and $p70^{S6K}$ revealed inhibition of Akt but not ERK signaling pathways. Collectively, these results indicated that resveratrol inhibits Huh7-HBx proliferation by decreasing cyclin D1 expression through blockade of Akt signaling. We investigated the anti-carcinogenic effect and mechanism of resveratrol in xenograft model mice implanted with Huh7-HBx cells. Intraperitoneal injection of resveratrol reduced the tumor size in the mice. Expression of survivin was reduced, whereas cyclin D1 was not affected.

In conclusion, resveratrol inhibits Huh7-HBx cell proliferation by decreasing cyclin D1 expression through blockade of the Akt signaling pathway *in vitro* and reduces the tumor size on xenograft model mice implanted with Huh7-HBx cells by decreasing the expression of survivin in tumor specimen.

Keywords : Hepatocellular carcinoma (HCC), Hepatitis B virus (HBV), Hepatitis B Virus X-protein (HBx), Resveratrol , cyclin D1, Akt, Survivin

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LIST OF ABBREVIATIONS

HCC	**Hepatocellular carcinoma**
HBV	**Hepatitis B virus**
HBx	**Hepatitis B virus X-protein**
HCV	**Hepatitis C virus**
CMV	**Cytomegalovirus**
ERK	**Extracellular signal-related kinase**
Bcl	**B-cell lymphoma line**
MAPK	**Mitogen-activated protein kinase**
PI3K	**Phosphatidylinositol-3-kinase**
TGFβ	**Transforming growth factor**
JAK	**Janus kinase**
STAT	**Signal transducer and activator of transcription**
IGF2R	**Growth factor 2 receptor**
wnt	**Wingless and Int-1**
NF-kB	**Nuclear factor kappa-B**
AP-1	**Activating protein-1**
VEGF	**Vascular endothelial growth factor**
MCL-1	**Myeloid leukemia–1**
HBXIP	**Hepatitis B X-interacting protein**
IAP	**Inhibitor of apoptosis**

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Abstract

Liver cancer occurs very frequently worldwide and hepatocellular carcinoma (HCC) accounts for more than 80% of total primary liver cancer cases. Here, the anticarcinogenic effects of resveratrol against hepatitis B virus (HBV)-induced HCC were investigated using HBV X-protein-overexpressing Huh7 (Huh7-HBx) human hepatoma cells. MTT assay showed that resveratrol decreased cell viability. Fluorescence-activated cell sorter analysis showed that it induced G1 cell cycle arrest without increasing the sub-G1 phase cell population. Therefore, we evaluated its effect on regulation of cyclin D1, which is critically involved in G1/S transition. Resveratrol lowered cyclin D1 transcription. Western blot analysis of the effects of resveratrol on upstream cyclin D1 transcriptional signaling, extracellular signal-related kinase (ERK), $p90^{RSK}$, Akt, and $p70^{S6K}$ revealed inhibition of Akt but not ERK signaling pathways. Collectively, these results indicated that resveratrol inhibits Huh7-HBx proliferation by decreasing cyclin D1 expression through blockade of Akt signaling. We investigated the anti-carcinogenic effect and mechanism of resveratrol in xenograft model mice implanted with Huh7-HBx cells. Intraperitoneal injection of resveratrol reduced the tumor size in the mice. Expression of survivin was reduced, whereas cyclin D1 was not affected. These results demonstrated that treatment with resveratrol might be helpful to manage of HBV-induced HCC

by regulating survivin.

1. Introduction

1.1. HCC

Cancer is one of the leading causes of death worldwide (Jemal et al. 2011; Mathers et al. 2008). The burden of cancer is increasing continuously worldwide because of population aging and lifestyle habits such as smoking and westernized diets (Jemal et al. 2011). More than 50% of cancer cases and 60% of cancer-induced deaths occur in economically developing countries (Jemal et al. 2011). According to the reported cancer statistics, the highest mortality rate was observed for liver cancer in the United States from 1994 to 2003 (El–Serag et al. 2007). According to the GLOBOCAN 2008 estimates of new cancer cases and death incidences, more than 700,000 new cases of liver cancer and more than 600,000 cancer-associated deaths are reported annually worldwide, and about half of these occur in China (Ferlay et al. 2010). The highest incidence rate for liver cancer is reported in East and South-East Asia and in Middle and Western Africa, whereas the lowest rates are detected in the EU and Central Asia (Ferlay et al. 2010).

Hepatocellular carcinoma (HCC) is responsible for more than 80% of all primary liver cancers in the world (El–Serag et al. 2007; Perz et al. 2006). The epidemiologic features of HCC include unique geographical variations, racial groups, gender among others. (El–Serag et al. 2007). Furthermore,

HCC nearly never occurs in healthy liver, but chronic liver damage during cirrhosis contributes to cancer progression (El–Serag et al. 2007). Therefore, understanding the epidemiological features and molecular mechanisms of HCC can improve the prevention and management of HCC.

1.2. Epidemiologic features of HCC

HCC cases prevail in Eastern Asia, as well as in Middle and Western Africa (El–Serag et al. 2007; Ferlay et al. 2010): China accounts for more than a half of all the world's cases, and incidence rates of HCC are markedly high in Senegal, Gambia, and South Korea (>20/100,000). Moreover, HCC cases are detected 2 to 4 times more often in men than in women in these areas (El–Serag et al. 2007; Ferlay et al. 2010). HCC incidence rates are low (<5.0/100,000) in Canada, Colombia, the United Kingdom, and Australia and moderate (5.0~20.0/100,000) in Spain, Italy, and Greece (Parkin et al. 2002). In the period from 1993 to 1997, an increase in the number of liver cancer cases was reported in the Khon Kaen region of Thailand (88.0/100,000 for men and 35.4/100,000 for women) (Parkin et al. 2002). However, this increase in liver cancer occurrence was associated with endemic liver fluke infection; moreover, the major type of liver cancer detected in this outbreak was intrahepatic cholangiocarcinoma rather than HCC (Okuda et al. 2002).

Between 1993 and 1997, a decline in HCC incidence rates was observed in Hong Kong, Shanghai, Singapore, China, and Japan (Mcglynn et al. 2001; Parkin et al. 2002). All newborns were vaccinated against hepatitis B virus (HBV) in many Asian countries, and the effect of vaccination on HCC rates was evident (Chang et al. 1997). At the same time, the number of HCC cases increased in the countries with typically low incidence rates of liver cancer, including the United States, the United Kingdom, and Australia (Mcglynn et al. 2001). The reasons for this are not clear thus far; one of the hypothesis is that this increase in incidence rates might be related to hepatitis C viral (HCV) infection (El–Serag et al. 2007).

HCC incidence rates vary among different ethnic populations, even the ones living in the same region: the rates in Chinese are higher than in ethnic Indians living in Singapore, and the rates in Asians are twice as high as those in African Americans in the United States (Parkin et al. 2002).

The HCC incidence rates are higher among men than among women (usually the ratio of HCC incidence rate between men and women ranges from 2:1 to 4:1) in almost all populations (El–Serag et al. 2007). The underlying causes of this phenomenon are thought to be related to the higher exposure of men to risk factors such as hepatitis B virus (HBV) and HCV infection, alcohol consumption, smoking, and iron Deficiency (El–Serag et al. 2007). Moreover, previously reported studies have shown that androgens had

an effect on HCC progression (Rudolph et al. 2000), and that there is a positive correlation between circulating testosterone level and HCC progression in men (Yu et al. 1993; Yu et al. 2001)

1.3. Main risk factors for HCC

In areas with high HCC incidence rates, the dominant risk factor for HCC is chronic HBV infection (El–Serag et al. 2007). High HCC rates in Asia and Africa are associated with the prevalence of HBV infection in these regions (El–Serag et al. 2007; Fontham 2008). Unlike other parts of Asia, the main hepatitis virus in Japan is HCV, which started spreading after the World War II (Armstrong et al. 2000).

In areas with low HCC incidence rates, increase in HCC occurrence is linked to the increase in the number of patients with cirrhosis (El–Serag et al. 2007). It is believed that liver cirrhosis arises from complications of HCV infection due to administration of a wrong drug or infusion of contaminated blood from the national supplies (Armstrong et al. 2000; Wong et al. 2000).

1.3.1. HBV

HBV infection is considered the most common cause of HCC worldwide (El–Serag et al. 2007). Almost 80% of patients with HBV-related HCC have liver cirrhosis while in the absence of cirrhosis, HBV does not

typically cause HCC (El–Serag et al. 2007). HBV infection is usually acquired by vertical transmission in areas associated with high HBV-related HCC incidence rate such as Asia and Africa, and more than 90% of HBV patients follow a chronic course (El–Serag et al. 2007). In regions with the low incidence rate of HCC, HBV infection is acquired in adulthood by horizontal transmission, and acute infections generally resolve spontaneously (El–Serag et al. 2007).

The risk of developing HCC is lower in persons immune to HBV, and the spontaneous or artificially induced development of antibodies against hepatitis B antigens was reported to result in improved clinical status (Beasley 1988). Hepatitis B vaccination is regarded as the most effective approach in preventing HBV infection. Results from the Taiwan study showed that the number of HCC cases can be reduced through HBV vaccination, and the reduction in mortality from HBV-related HCC was confirmed over several decades (El–Serag et al. 2007; Kane 2003).

The pathogenesis of HBV-related HCC has not been fully understood, but HBx, HBV transactivator protein, is thought to have an oncogenic effect linked to HBV-associated HCC (Birrer et al. 2003; Zhang et al. 2006). Hwang *et al.* reported that Hbx antigen was detected in the sera of 40% of patients with HCC and in the liver tissues of 85% of patients with HCC (Hwang et al. 2003). Moreover, according to another study, Hbx antigen was

highly expressed in 76% of liver cancer tissues and 100% of cirrhosis tissues (Zhang et al. 2005).

Previous studies suggested that HBx can regulate apoptosis in a number of different ways: (1) by blocking activation of caspase 3, which plays a central role in cell apoptosis (Han et al. 2000): (2) by interacting with B-cell lymphoma line (Bcl) -2 in mitochondria and inducing mitochondrial-dependent cell death through the loss of mitochondrial membrane in HBV-infected hepatocytes (Terradillos et al. 2002); (3) by upregulating the expression of survivin, which inhibits apoptosis of hepatic carcinoma cells and is overexpressed in many human cancers (Donghua et al. 2003; Zhang et al. 2005); (4) by activating the RAS-RAF-Mitogen-activated protein kinase (MAPK) signal-ing pathway, which is commonly associated with the transformation process (Mansour et al. 1994); (5) by stimulating the Phosphatidylinositol-3-kinase (PI3K) /Akt pathway, which blocks the transforming growth factor (TGFβ)-induced apoptosis (Lee et al. 2001); (6) by activating Janus kinase (JAK) / Signal transducer and activator of transcription (STAT) pathway, which stimulates hepatocyte proliferation (Cressman et al. 1995), and (7) by controlling cell cycle checkpoints including cyclins, kinases, among others. (Koike et al. 1994). According to a recently reported study, HBx accelerated hepatocarcinogenesis by upregulating survivin in liver cancer tissue of HBx-transgenic mice (Zhang

et al. 2014). Survivin is known to inhibit apoptosis and accelerate cancer cell proliferation (Fields et al. 2004), and it has been suggested to play an important role in the progression and recurrence of HCC (Fields et al. 2004).

1.3.2. HCV

Some studies provided evidence that HCC was associated with HCV infection; HCV antibodies were detected in the blood of a significantly higher number of patients with HCC (up to 70%) than in patients with cirrhosis or control patients in Spain and Italy (Bruix et al. 1989; Colombo et al. 1989). Meta-analysis of data from various case-control studies suggested that the risk of developing HCC was 17 times higher in HCV-infected patient than in HCV-negative control population. (Donato et al. 1998; El–Serag et al. 2007; Shi et al. 2005) These results indicate that chronic HCV infection plays an important role in the development of HCC (Saito et al. 1990).

Previous studies reported that environmental factors, including older age at infection, gender, heavy alcohol consumption, obesity, diabetes, and co-infection with HBV, play a more crucial role than viral factors in the progression of HCV infection to cirrhosis (Cramp 1999; Freeman et al. 2001). Several studies suggested that antiviral therapy may decrease the risk of HCC development in patients with HCV-related cirrhosis (Bruno et al. 2001; Ikeda et al. 1999; Yoshida et al. 1999); however, randomized controlled

studies did not support this finding (Niederau et al. 1998; Okanoue et al. 1999; Valla et al. 1999)

1.4. Molecular mechanisms of HCC

Cirrhosis is the most common condition observed prior to HCC development. The risk of developing HCC increases exponentially at the cirrhosis stage (El–Serag et al. 2007). Typically, proliferation of liver cells increases in chronic hepatitis; however, this proliferation is reduced at the stage of cirrhosis owing to deterioration of hepatic regenerative ability (Delhaye et al. 1996). Many studies have suggested several mechanisms that could explain reduced liver regeneration in patients with cirrhosis. First, telomere shortening limits the hepatocytic regenerative capacity (Levy et al. 1992; Wege et al. 2003). Chronic liver damage accelerates telomere shortening, and consequently, leads to the accumulation of senescent hepatocytes in cirrhotic liver (Wiemann et al. 2002). If the length of telomeres is critical, DNA damage signals cell-cycle arrest or induction of apoptosis (Di Fagagna et al. 2003; Wright et al. 1992). Telomerase dysfunction can result in chromosomal translocations or chromosomal gain or loss in daughter cells (Artandi et al. 2000; Maser et al. 2007; Smogorzewska et al. 2002). Farazi *et al.* (2003) reported that initiation of

liver tumors is accelerated in carcinogen-treated telomerase-deficient mice in comparison with control mice. Furthermore, several studies reported that telomeres are critically short in patients with HCC (Plentz et al. 2004; Plentz et al. 2007).

Second, decrease in hepatocytic proliferation can promote cancer development (El–Serag et al. 2007) since studies have shown that chemical inhibition of hepatocyte proliferation accelerated the development of carcinogen-induced tumor in rats (Van Gijssel et al. 1997).

Alterations in micro- and macro-environment significantly affect cancer development in patients with cirrhosis. In cirrhosis, the changes in liver mass and loss of liver function can alter cytokine secretion and stimulate proliferation accelerating carcinogenesis (El–Serag et al. 2007; Giannelli et al. 2005).

Several alterations in molecular pathways that occur at the stage of cirrhosis can promote carcinogenesis. The alterations in cell cycle and apoptosis pathways are linked to hepatocarcinogenesis. In humans, the most frequently altered pathways in the development of HCC are the p53, p27, Rb, and TGFβ-insulin-like growth factor 2 receptor (IGF2R) pathways, accounting for 60% - 100% of all HCC cases (El–Serag et al. 2007). The p53 pathway is affected on multiple levels in human HCC (Bressac et al. 1991). The impairment of p53 function leads to the proliferation of hepatocytes with

dysfunctional telomerase and chromosomal instability (Artandi et al. 2000; El–Serag et al. 2007). The Rb pathway is suppressed by the inhibition of p16 in more than 80% of patients with HCC (Azechi et al. 2001). In addition, the expression of gankyrin, which inhibits p53 and Rb, increases in these patients (Higashitsuji et al. 2000). The downregulation of p27 also promotes the development of HCC (Ito et al. 1999). Alterations in TGFβ - IGF2R signaling lead to cell cycle arrest and loss of IGF2R commonly observed in HCC patients (Breuhahn et al. 2004). The TGFβ pathway induces apoptosis by activating Smad3, which downregulates Bcl-2 (Yang et al. 2006).

Activation of oncogenic pathways is an important factor in the development of HCC. Activation of the Wingless and Int-1 (Wnt) /β-catenin pathway is commonly observed in human and mouse HCC (De La Coste et al. 1998; Terris et al. 1999), and its stimulation can induce hepatocarcinogenesis (Colnot et al. 2004). Activation of the PI3K/Akt signaling and dysregulation of phosphatase and tensin homolog, which negatively regulates Akt, are observed in more than 40% of HCC patients (Hu et al. 2003).

1.5. Resveratrol

Despite the intensive advances in improving prognosis, cancer is still one of the leading causes of death worldwide (Lee et al. 2011). More than

250 population-based studies reported that the risk of cancer decreases by about 50% for each person who consumes more than five servings of vegetables and fruits a day in comparison to people who consume less than two servings (Surh 2003). Many studies revealed that macro- and micro-nutrients, as well as phytochemicals in plants, can reduce the risk of various cancers by altering cellular signaling pathways that regulate cell proliferation and differentiation (Surh 2003). Various phytochemicals have been reported to modulate molecular signaling pathways (Bode et al. 2001; Byun et al. 2010; Ermakova et al. 2006; He et al. 2008; Jung et al. 2008; Jung et al. 2010; Lee et al. 2008; She et al. 2001; Zykova et al. 2008).

1.5.1. Resveratrol and its pharmacological activities

Regular consumption of red wine is often suggested as the most likely explanation of the French Paradox, the French have a relatively low incidence of cardiovascular diseases despite consumption of diet high in saturated fat (Renaud et al. 1998). The consumption of wine and grape juice has been reported to inhibit platelet aggregation (Seigneur et al. 1990), enhance vasorelaxation (Fitzpatrick et al. 1993), reduce atherosclerosis (Wang et al. 2005), and inhibit lipid peroxidation (Fuhrman et al. 1995).

Resveratrol, a phytoalexin commonly found in grapes and wine, received little attention until it was predicted to explain the cardioprotective

effect of red wine (Siemann et al. 1992). Since then, many studies have confirmed that resveratrol can prevent or inhibit the progression of various diseases, including cardiovascular diseases (Bradamante et al. 2004), disorders of the immune system such as chemically induced edema (Chen et al. 2005), osteoarthritis (Elmali et al. 2005), airway inflammation (Birrell et al. 2005), ischemia-reperfusion injury such as myocardial infarction (Ray et al. 1999; Sato et al. 2000), and stroke (Sinha et al. 2002).

1.5.2. Resveratrol and anticancer effects

The chemopreventive activity of resveratrol in cancer has been well studied. Resveratrol exhibited various pharmacological properties and chemoprevention effects in human cancers, including HCC (Aggarwal et al. 2004; Hayashibara et al. 2002; Liao et al. 2005). Jang *et al.* proposed that resveratrol acts as an antioxidant and antimutagen and inhibits cellular events related to tumor initiation and progression (Jang et al. 1997). In the study by Bode *et al*., topical administration of resveratrol reduced the number of tumors in mice (Bode, et al. 2001). Administration of low doses of resveratrol, equivalent to that obtained from dietary sources, was shown to have a therapeutic effect in a rat colon carcinogenesis model (Tessitore et al. 2000). Administration of higher doses of resveratrol, i.e. pharmacologically achievable concentration, increased the survival rate of mice with

neuroblastoma from 0% to 70% (Chen et al. 2004). In another study, resveratrol was suggested to enhance the effect of the anticancer drug, 5-fluorouracil (FU) in murine liver cancer (Wu et al. 2004).

A number of previous studies suggested that resveratrol has antiproliferative and pro-apoptotic effects in various cancer cell lines including liver, lung, pancreas, prostate, ovary, cervix, among others. (Aggarwal et al. 2004). In addition, resveratrol has been shown to induce apoptosis *in vitro* and *in vivo* via modulation of various signaling pathways (Garvin et al. 2006; Zhou et al. 2005).

Resveratrol is well-known for suppressing cell-cycle proteins (Bhardwaj et al. 2007; Tze-Chenhsieh et al. 1999), growth factors (Serrero et al. 2001; Shih et al. 2002), and angiogenesis and metastasis (Hsieh et al. 1999; Woo et al. 2003), upregulating the Fas pathway (Clément et al. 1998; Delmas et al. 2003), inhibiting mitochondrial pathway (Dörrie et al. 2001; Tinhofer et al. 2001), activating the p53 pathway (Lu et al. 2001; Narayanan et al. 2003; Shih et al. 2002), inhibiting the Rb pathway (Adhami et al. 2001; Kim et al. 2003), MAPK pathway (Shih et al. 2002; Stewart et al. 2004), and Nuclear factor kappa-B (NF-kB) and activating protein (AP) -1 signaling pathways (Surh et al. 1999). Moreover, resveratrol administration resulted in decreased expression of survivin, an anti-apoptotic protein associated with various cancers (Aggarwal et al. 2004; Hayashibara et al. 2002; Zhao et al.

2010).

1.6. Resveratrol on HBx-induced HCC

Hepatocellular carcinoma (HCC), which is the most highly epidemic and malignant cancer globally, occurs widely in Asia as well as central and west Africa (Kew 2010). Currently, HCC has the sixth highest incidence of all cancers, worldwide, and numerous new patients are diagnosed every year. Furthermore, HCC is estimated to have the third highest annual cancer mortality rate (Parkin et al. 2005). Pre-existing HBV infection is the main etiological factor for HCC (Kew 2010). Despite the increasing availability and administration of HBV vaccinations worldwide, HBV remains the main cause of development of HCC (Tian et al. 2013) and outbreaks of HBV-induced HCC are expected to increase consistently (Kew 2010). Recent studies have indicated that the interaction of viral and host genetic factors may be critically involved in both the sensitivity to HBV and the development of disease (Sun et al. 2009). In addition, it has been reported that the relationship between HBV infection and the progression of HCC may mainly involve viral factors. Recent studies have reported that HB viral loads are related to malignant transformation of primary hepatocytes (Wu et al. 2008; Yang et al. 2002); (Viana et al. 2009). In 1975, the close link between chronic HBV infection and the progression of HCC was first

reported (Blumberg et al. 1975), and the anti-apoptotic activity of HBV X protein (HBx) (Chirillo et al. 1997); (Su et al. 1997) has been recognized as the predominant cause of this tumor. The molecular mechanisms underlying the carcinogenicity of HBV involve transactivation by and abnormal accumulation of viral proteins, gene activation, and recombination of the HBV subgenome (Yu et al. 1999). However, the precise mechanisms of HBV-induced carcinogenesis have not been fully elucidated yet.

HBx, a multi-functional viral factor of HCC, has been reported to participate in the life cycle of the virus and the development of HCC. These activities are mediated by signaling cascades that participate in the modulation of cell proliferation and survival including the STAT3/survivin signaling pathway. HBx promotes apoptosis of p53/p73-expressing hepatoma cells (Knoll et al. 2011). The complex, cell-context-dependent interaction between HBx and p53 tumor suppressor family members in the modulation of apoptosis is crucial for induction of HBV-associated HCC and in anticancer therapy (Knoll et al. 2011).

Resveratrol (3,5,4′-trihydroxy stilbene) is a polyphenolic antioxidant compound produced by plants like berries, grapes, and peanuts (Vastano et al. 2000), and has also been found in Japanese knotweed (Wicklow et al. 2015). Resveratrol has been reported to inhibit the STAT3/survivin signaling pathway, which blocks apoptosis of tumor cells (Quoc Trung et al. 2013).

STAT proteins are involved in cell proliferation and apoptosis and act as cytokine signaling molecules and downstream effectors of growth factor receptors (Espinoza et al. 2012). A recent study showed that the promoter of tumor suppressor genes is methylated by acetylated STAT3, inhibiting gene transcription. Additionally, it has been shown that resveratrol induces promoter demethylation and inhibits STAT3 acetylation, restoring the expression of tumor suppressor genes (Lee et al. 2012). The STAT3 family and survivin, its downstream effector, are involved in the regulation of cell survival and proliferation, and survivin normally plays a role as anti-apoptotic protein (Quoc Trung et al. 2013). The associated Akt signaling pathway is normally decreased in apoptosis and cyclin is involved in the regulation of cell cycle progression (Parekh et al. 2011). However, the effect of resveratrol on HBx-associated HCC development and its molecular mechanisms have not been elucidated yet.

Therefore, in this present study, we investigated the effect of resveratrol on HBx-associated HCC proliferation and the underlying molecular mechanisms using HBx-overexpressing Huh7 (Huh7-HBx) human hepatoma cells. Moreover, we investigated its effects on expression of survivin, downstream effectors of the STAT3 pathway, and cyclin D1 in BALB/c nude mice implanted with Huh7-HBx cells.

2. Materials and methods

2.1. Reagents

Resveratrol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RNase was purchased from Amresco LLC (Solon, OH, USA). The following antibodies against target proteins were purchased from the specified manufacturers. HBx, survivin, phosphorylated (p)-$p70^{S6K}$, $p70^{S6K}$ (Cell Signaling Biotechnology, Beverly, MA, USA), p-ERK, ERK, cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-$p90^{RSK}$, $p90^{RSK}$ (Signal Way Antibody, College Park, MD, USA), p-Akt, Akt, p-p53, p-53 (Pierce Chemical, Rockford, IL, USA), and β-actin (Sigma-Aldrich, St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco (Grand Island, NY, USA).

2.2. Cell culture

Cytomegalovirus (CMV)-expressing Huh7 and Huh7-HBx human hepatoma cells were provided by Dr. Jung, Guhung (College of Natural

Science, Seoul National University, Seoul). The cells were cultured in DMEM containing penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FBS at 37 °C under an atmosphere of 5% CO_2.

2.3. Western blot analysis

Huh7 human hepatoma cells were seeded into 6-cm dishes at a density of 1.5×10^5 cells per dish and cultured in RPMI 1640 medium supplemented with 10% FBS before resveratrol treatment. Then, the cells were treated with resveratrol at varying concentrations, after which the cellular proteins were harvested and centrifuged at 14,000 rpm at 4 °C for 10 min. The supernatants were collected and the proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto nitrocellulose membranes (Pall Corporation, Port Washington, NY). The membranes were blocked with TBS containing 0.05% Tween-20 and 5% fat-free dry milk for 2 h at 20 ± 5 °C. Then, the membranes were incubated overnight with specific primary antibodies at 4 °C. Subsequently, they were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature and the protein bands were visualized using BeyoECL Plus reagent (GE Healthcare Life Sciences, Little Chalfont, UK) while β-actin was used as the loading control.

2.4. MTT assay

The cytotoxicity of resveratrol was estimated using the MTT assay. Briefly, Huh7 cells were seeded in 96-well plates at a density of 5×10^3 cells per well, cultured in RPMI 1640 medium supplemented with 10% FBS, and then treated with resveratrol at varying concentrations for 48 h. Then, the cells were treated with MTT solution at 0.2 mg/mL for 3 h, and incubated for 30 min at 37 °C in an atmosphere of 5% CO_2. Next, the supernatants were removed and the formazan crystals that had formed were dissolved with dimethyl sulfoxide (DMSO). Finally, the absorbance of the solution at 540 nm was determined using a microplate reader (Tristar LB 941, Berthold Technologies GmbH and Co. KG, Germany).

2.5. FACS analysis

Huh7 cells were seeded into 6-cm dishes at a density of 1.5×10^5 cells per dish and cultured in RPMI 1640 medium containing 10% FBS. The cells were treated with resveratrol at varying concentrations before trypsinization, and centrifuged at 2,000 rpm at 4 °C for 2 min. The supernatants were removed, the pellets were washed twice with phosphate-buffered saline (PBS), and the cells were fixed with cold 70% ethanol (v/v) overnight at -20 °C. Then, the cells were stained with PI solution containing RNase (0.2 mg/mL) and analyzed using the Guava EasyCyte TM Flow

Cytometer (Millipore, Billerica, MA, USA). Per group, 10,000 cells were analyzed.

2.6. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Huh7 cells were seeded into 6-cm dishes at a density of 1.5×10^5 cells per dish, cultured in RPMI 1640 medium containing 10% FBS, and treated with resveratrol at varying concentrations. Total RNA was isolated using RNA-Bee reagent (Tel-Test, Inc., Friendswood, TX, USA) following the manufacturer's instructions and cDNA was synthesized using a reverse transcription system purchased from Promega Corporation (Fitchburg, WI, USA). The cDNA and specific primers were mixed with the Maxima SYBR Green qPCR master mix (Fermentas, Vilnius, Lithuania). The primer sequences were as follows: cyclin D1 (NM 007631.2), forward, 5′-agcagaagtgcgaagaggagg-3′ and reverse, 5′-ggcagtcaagggaatggtctc-3′ and β-actin (X03672) forward, 5′-tgtccaccttccagcagatgt-3′ and reverse, 5′-agctcagtaacagtccgcctaga-3′. The primers were obtained from Bioneer Corporation (Daejeon, Republic of Korea).

2.7. Xenograft assay *in vivo*

The animal protocol used in this study was reviewed and approved

by the ethics committee of the Seoul National University (SNU-IACUC). A mice xenograft model was established using 6-week-old female BALB/c (nu/nu) mice (Orient Inc, Seoul, Korea).

Tumor cells (5×10^6 Huh7-HBx cells) were suspended in 0.2 ml of serum-free DMEM and injected subcutaneously into the right flank of each nude mouse. When the tumor size reached approximately 100 mm^3 (after 21 days), the mice were randomly divided into a vehicle group, and 50 mg/kg and 100 mg/kg resveratrol-treated groups (n = 3/group). For the two resveratrol-treated groups, the nude mice were injected intraperitoneally with resveratrol starting at 21 days after inoculation. Intraperitoneal injections were given daily for 3 weeks. The vehicle group was injected with normal saline. The body weight and the tumor size were measured every 3 days for 21 days during treatment. The tumor volume (V) was calculated using the following formula: $V\ (mm^3) = 1/2\ ab^2$, where *a* and *b* represent the long diameter and perpendicular short diameter (mm) of the tumor, respectively. At 21 days after treatments, the experiments were ended and the mice were sacrificed by using CO_2. The transplanted tumors were sampled and weighed. The tumor growth inhibition rate was calculated using the following formula:

Inhibition ratio (%) = $[(W_{vehicle} - W_{treated}) / W_{vehicle}] \times 100\%$

2.8. *In vivo* sample preparation and western blot analysis

Tumors were removed from mice and sliced into pieces of 0.05 g each and homogenized with RIPA lysis buffer (10 mM Tris-Cl (pH 7.1), 100 mM NaCl, 1 mM EGTA, 10% glycerol, 0.5% Triton X-100, protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktail (Sigma-Aldrich). Samples were centrifuged at 13,000 rpm and supernatants were collected and separated by SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes (Pall Corporation, Port Washington, NY). Western blot analysis was conducted as described above.

2.9. Pathologic examination

Tumors were removed from mice, fixed in 10% buffered formalin, and embedded in paraffin. For pathologic examination, 4-μm-thick tissue sections were stained with hematoxylin and eosin(H&E).

2.10. Immunohistochemical analysis

Formalin-fixed paraffin sections were hydrated and heat-mediated antigen retrieval was carried out when necessary. The sections were incubated with primary antibody overnight at 4 °C.

2.11. Statistical analysis

Results are expressed as the mean ± standard deviation (SD) of at

least 3 independent experiments. Statistical analysis was performed with SPSS Statistics Software. Student's *t*-test was used to compare differences between two groups, and one-way ANOVA was used for analysis and post-hoc comparisons were performed by using the tukey HSD for comparing differences between multiple groups. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Survivin expression is induced in Huh7-HBx cells

First, we examined HBx expression in Huh7-CMV, Huh7-HBx cells and primary hepatocytes isolated from HBx transgenic mice. As expected, Huh7-HBx but not the Huh7-CMV cells expressed HBx and HBx transgenic mice were verified by PCR using specific primer sets. (Fig. 1A). In addition, the expression of survivin, a representative hepatocarcinoma biomarker, was strongly induced in Huh7-HBx cells and primary hepatocytes isolated from HBx transgenic mice. Therefore, HBx is coupled with the expression of survivin and they were confirmed to be hepatocellular carcinoma (Fig. 1B).

3.2. Resveratrol inhibits cell proliferation and decreases survivin expression in Huh7-HBx cells

We evaluated the anti-proliferating effect of resveratrol using the Huh7-HBx HCC cell line. Compared to day 0, the cell number of the Huh7-HBx cells was increased by 2.5-fold on day 2. Resveratrol at 100 μM decreased the Huh7-HBx cell number to 33% on day 2. (Fig. 2A). To investigate whether the effect of resveratrol is specific to Huh7-HBx cells, we conducted the MTT assay in Huh7-CMV cells as well. The results showed that resveratrol decreased the viability of both Huh7-CMV and Huh7-HBx cells (Fig. 2A and B), indicating that the effect was not confined to HBx-overexpressing cells.

Next, we determined whether the expression of survivin protein is affected by resveratrol in Huh7-HBx cells. Consistent with the MTT assay results, western blot analysis showed that 100 μM of resveratrol decreased survivin protein expression (Fig. 2C). Collectively, these results indicated that resveratrol decreases cell viability and survivin expression in Huh7-HBx HCC cells.

3.3. Resveratrol induces G1 phase cell cycle arrest in Huh7-HBx cells

We considered two hypotheses for inhibitory effect of resveratrol against Huh7-HBx cells. Firstly, resveratrol likely inhibits cell proliferation, and secondly, it may induce apoptosis. To determine whether resveratrol inhibits cell proliferation and/or induces apoptosis of Huh7-HBx cells, we conducted FACS analysis. We synchronized the cell cycle at the G1 phase using serum starvation and induced cell cycle progression by serum stimulation with or without 100 μM resveratrol. The FACS analysis showed similar cell cycle profiles for both the control and resveratrol-treated groups at 0 h (Fig. 3A and B). However, the G0/G1 phase ratio of the control group decreased over 24 h while the population of cells in the S and G2/M phases increased. This result indicated that the serum stimulation successfully induced cell cycle progression (Fig. 3A and B). In contrast to the control group, the overall cell cycle profiles of the resveratrol-treated groups did not significantly change during the 24-h treatment, indicating that resveratrol induced cell cycle arrest at the G1 phase (Fig. 3A and B). Furthermore, the population of cells in the sub-G1 phase, an indicator of apoptosis, was not altered in both the control and resveratrol-treated groups. Taken together, these results led us to conclude that resveratrol decreases the viability of Huh7-HBx cells by inhibiting cell proliferation, not by inducing apoptosis.

3.4. Resveratrol decreases cyclin D1 protein transcription in Huh7-HBx cells

After determining that resveratrol induced G1 phase arrest, we evaluated its effects on the expression of cell cycle progression-related proteins in Huh7-HBx cells. Cyclin D1 is a well-known protein that is responsible for G1/S transition (Shapiro 2006) and, therefore, we evaluated its expression in resveratrol-treated Huh7-HBx cells. Western blot analysis showed that resveratrol decreased the protein expression of both cyclin D1 and survivin (Fig. 4A). Next, to determine whether resveratrol inhibits cyclin D1 protein transcription, we examined its effects on mRNA expression of cyclin D1. qRT-PCR analysis showed that resveratrol decreased mRNA expression of cyclin D1, indicating that it inhibits cyclin D1 protein translation in Huh7-HBx cells (Fig. 4B).

3.5. Resveratrol blocks Akt but not ERK signaling in Huh7-HBx cells

Transcription of cyclin D1 gene is regulated by the ERK or Akt signaling pathways (Dubey et al. 2015) therefore, we evaluated the effect of resveratrol on the expression of these proteins. Western blot analysis revealed that resveratrol decreased the phosphorylation of Akt and $p70^{S6K}$, which is a downstream protein of the Akt pathway (Fig. 5A). However, the phosphorylation of ERK and its downstream protein $p90^{RSK}$ were not altered (Fig. 5B), indicating that resveratrol decreased cyclin D1 protein transcription through blockade of the Akt signaling pathway. Taken together, these results led us to conclude that resveratrol inhibits the proliferation of Huh7-HBx cells by decreasing cyclin D1 protein transcription through blockade of the Akt signaling pathway.

3.6. Treatment with resveratrol reduces tumor volume and development in xenograft model mice implanted with Huh7-HBx cells

To investigate the effect of resveratrol on xenograft model mice implanted with Huh7-HBx cells, we observed the effect of intraperitoneal injection of resveratrol on the clinical status and tumor volume after growing the tumors to approximately 100 mm^3 for 21 days.

The tumor volume in the vehicle group was increased at day 21 of the experiment. Resveratrol, at doses of 50 mg/kg and 100 mg/kg, markedly reduced the tumor volumes (1,626.2 ± 152.26 mm^3, $p < 0.01$ and 1,321.96 ± 1024.80 mm^3, $p < 0.01$) as compared to vehicle group (4,604.96 ± 198.05 mm^3) (Fig. 7A). The tumor weight was increased in the vehicle group at day 21. Resveratrol significantly reduced the tumor weight in the groups treated with 50 mg/kg and 100 mg/kg (5.35 ± 0.73 g and 3.61 ± 2.63 g, $p < 0.05$) (Fig. 7B). At day 21, mice implanted with tumor cells showed severe tumor development (Fig. 7C). Compared to the vehicle group, resveratrol markedly inhibited tumor development. The mean inhibition ratios on day 21 were 32.2 ± 9.69% and 53.3 ± 34.74% for 50 mg/kg and 100 mg/kg resveratrol, respectively (Fig. 7D). No significant differences in body weight between the resveratrol-treated groups and the vehicle group were observed (Fig. 7E), indicating that resveratrol is not toxic to the mice. Together, these results indicated that resveratrol has antitumor potential at doses of 50 mg/kg and

100 mg/kg in xenograft model mice implanted with Huh7-HBx cells.

3.7. Intraperitoneal injection of resveratrol reduces tumor development and expression of survivin in tumor specimens

We evaluated inhibitory effects of resveratrol in tumor specimens by pathological examination. Tumor specimens were collected after treatment for 21 days, at the end of the experiment. The specimens were evaluated using H&E staining and immunohistochemical staining for survivin. Resveratrol decreased tumor development and survivin expression in the tumor specimens as compared to that in the vehicle group (Fig. 8A). The level of protein expression was checked using western blotting. The survivin/actin expression ratio was significantly reduced in the groups treated with 50 mg/kg and 100 mg/kg resveratrol (0.77 ± 0.06, $P < 0.05$ and 0.60 ± 0.03, $P < 0.01$, respectively) as compared to that in the vehicle gteroup (1.0 ± 0.14) (Fig. 8B).

3.8. Effect of resveratrol on protein expression in tumor specimens

The tumor suppressor protein p53 and cell proliferation-related protein cyclin D1 were evaluated using western blot analysis. The p53/actin expression ratio (1.81 ± 0.34) was significantly increased in the group treated with 100 mg/kg resveratrol as compared to the vehicle group (1.0 ± 0.0, $P < 0.05$) (Fig. 9A). However, cyclin D1 did not show any significant changes in the resveratrol-treated groups as compared to the vehicle group (Fig. 9B).

4. Discussion

In the present study, we demonstrated that resveratrol inhibits proliferation of Huh7-HBx cells by blocking the Akt signaling pathway. However, the detailed molecular mechanisms underlying the resveratrol-induced blockade of the Akt signaling pathway were not investigated in this study. Previous studies have reported these molecular mechanisms. Choi *et al* (2010) reported that resveratrol decreases the activity of phosphatidylinositide 3-kinase (PI3K), an upstream kinase of Akt, *in vitro*. Furthermore, it has been reported that inhibition of PI3K activity decreases Akt signaling (Bruix et al. 2005), and numerous other studies have reported similar findings in various cell lines (El–Serag et al. 2007; Huh et al. 2015; Seo et al. 2013; Siegel et al. 2013). Based on these previous reports, we propose that resveratrol might target PI3K activity to attenuate Akt signaling pathway in Huh7-HBx cells.

Most previous reports have demonstrated that either inhibition of cell proliferation or induction of apoptosis decreases the viability of cancer cells (Jemal et al. 2011; Kolodziej et al. 2015; Lee et al. 2011; Parkin 2001). Based on these findings, we conducted FACS analysis to determine which of these actions mediated the effects of resveratrol. The results clearly showed that it induced cell cycle arrest at the G1 phase but did not alter the apoptotic cell population of Huh7-HBx cells. This result is inconsistent with previous

studies, as resveratrol previously has been reported to induce apoptosis and decrease cell proliferation in different cancer cells (Dai et al. 2015; Mathers et al. 2008; Perz et al. 2006). This discrepancy might be due to differences in cell culture conditions and context-dependent cellular antiproliferative and apoptotic effects of resveratrol. In addition, we opined that resveratrol might be more effective in preventing carcinogenesis than in reducing already grown cancer cells.

Cyclin D1, as well as other cell cycle-related proteins, have been reported to be unstable. These cell cycle-related proteins can be temporarily regulated depending on the cell cycle status. For instance, the expression of cyclin D1 can be induced during G1/S transition and subsequently downregulated, suggesting that the protein expression of cyclin D1 is tightly regulated by the balance between cyclin D1 degradation and synthesis (Bashir et al. 2003). Therefore, our determination that resveratrol decreased cyclin D1 protein expression led us to consider two possible explanations for its effects. Resveratrol decreased cyclin D1 protein expression either by inhibiting mRNA transcription or by increasing proteasome-mediated degradation. Evaluation of the mRNA level revealed that treatment with resveratrol significantly decreased cyclin D1 mRNA expression. This result indicated that resveratrol inhibits cyclin D1 protein transcription. However, it is possible that resveratrol regulates the degradation rate of cyclin D1 as well;

further studies are required to verify this.

Previous studies *in vivo* have reported that resveratrol inhibited tumor growth in nude mice bearing HepG2 cells and downregulated NF-κB and VEGF cells (Yu et al. 2010), and that it prevented the development of spontaneous HCC in HBx transgenic mice (Lin et al. 2012). However, the mechanisms of resveratrol in HBV-induced HCC *in vivo* are largely unknown. Resveratrol activated the expression of p53 (Bobrowska-Hagerstrand et al. 2006) and downregulated MCL-1 (Tsutsui et al. 2010), anti-apoptotic protein and survivin (Quoc Trung et al. 2013), and downstream of STAT3 pathway (Quoc Trung et al. 2013). Recently, acetylated STAT3 has received attention as a tumor-progressing factor (Lee et al. 2012). Survivin (encoded by *BIRC5* (Altieri 2006)), an inhibitor of apoptosis protein (Eckelman et al. 2006), acts as a regulator of cell division, a modulator of cell death, and a stress response factor in cell proliferation and survival (Altieri 2006); (Eckelman et al. 2006). Survivin regulates gene expression, protein–protein interaction by inhibition of apoptosis through multiple pathways (Altieri 2008). Meanwhile, a recent study clarified that HBx induces hepatocarcinogenesis together with survivin (Zhang et al. 2009; Zhang et al. 2005) through modulation of oncoprotein HBx-interacting protein (HBXIP), a cofactor of survivin in apoptosis suppression (Zhang et al. 2014). Moreover, HBV-induced HCC shows p53 inactivation by

mutations (O'dell et al. 2012) and activation of the Akt signaling pathway (Guerrieri et al. 2013). Thus, we hypothesized that the mechanisms of resveratrol might involve survivin, p53, and the Akt signaling pathway in nude mice implanted with Huh7-HBx cells. Resveratrol obviously decreased the expression of survivin and increased that of p53 in tumor specimens. In contrast, it had no effect on the expression of cyclin D1. These results demonstrated that resveratrol showed anti-tumor activities in nude mice implanted with Huh7-HBx cells by downregulating survivin and upregulating p53.

In conclusion, the present study suggested the anti-carcinogenesis activity of resveratrol in Huh7-HBx cells and its molecular mechanisms. Resveratrol was found to inhibit proliferation of Huh7-HBx cells by inducing cell cycle arrest at the G1 phase. It decreased cyclin D1 expression through attenuating Akt signaling pathway; however, it did not block the ERK signaling pathway. Thus, this study showed that resveratrol effectively decreases cell proliferation by suppressing cyclin D1 protein expression through downregulation of Akt signaling pathway. Although several further studies are needed to elucidate exact molecular mechanisms of these inhibitory effects, this study suggests resveratrol as a potent anti-carcinogenic agent *in vitro*. Our *in vivo* data showed that resveratrol

exhibited anti-tumor activities on HBV-induced HCC and reduced tumor development in mice implanted with Huh7-HBx cells by decreasing the expression of survivin, downstream of the STAT3 pathway. The expression of the tumor suppressor protein p53 was normally increased, whereas the cell proliferation-related protein cyclin D1 was not affected by resveratrol treatment. Thus, survivin may be a therapeutic target and resveratrol could play an important role in the treatment of HBV-induced HCC.

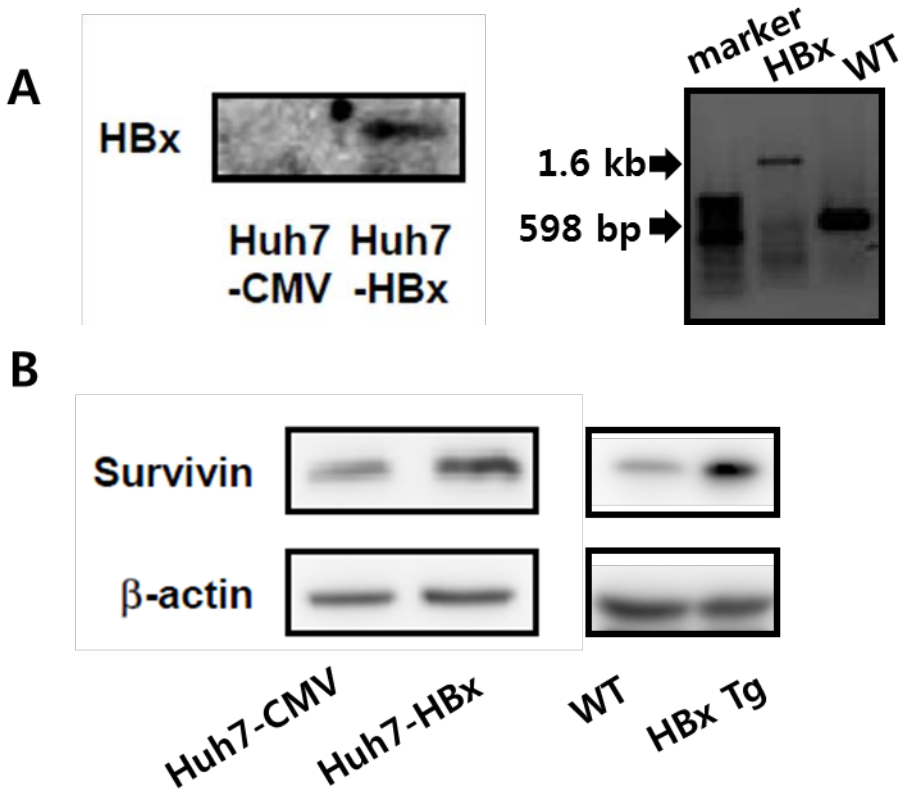
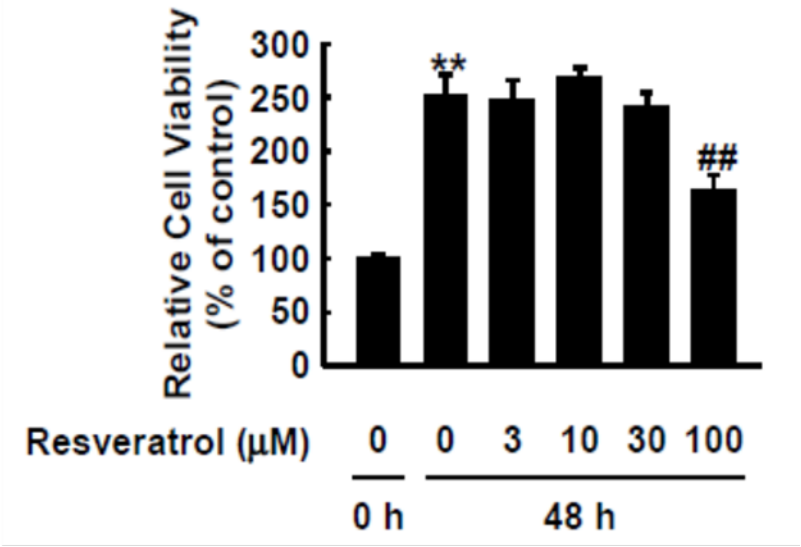


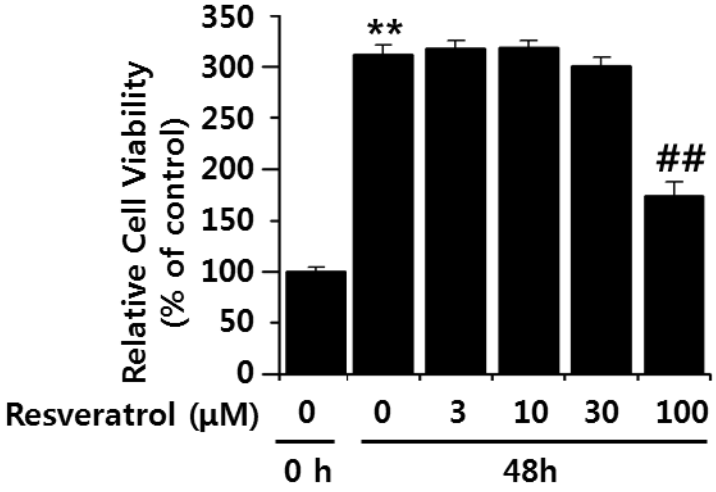
Figure 1. **Protein expression of HBx and survivin in Huh7-HBx cells.**

(A) HBx protein expression was upregulated in Huh7-HBx cells compared to Huh7-CMV cells and HBx transgenic mice were verified by PCR using specific primer sets. (B) Survivin protein expressions were upregulated in Huh7-HBx cells compared to Huh7-CMV cells and survivin protein expressions was upregulated in HBx transgenic mice compared to wild-type mice. Western blot analysis was conducted with β-actin as loading control. Results represent at least 3 independent experiments producing similar results.

A


Relative Cell Viability
(% of control)
300
250
200
150
100
50
0
**
##
Resveratrol (μM)
0
0
3
10
30
100
0 h
48 h

B


Relative Cell Viability
(% of control)
350
300
250
200
150
100
50
0
**
##
Resveratrol (μM)
0
0
3
10
30
100
0 h
48h

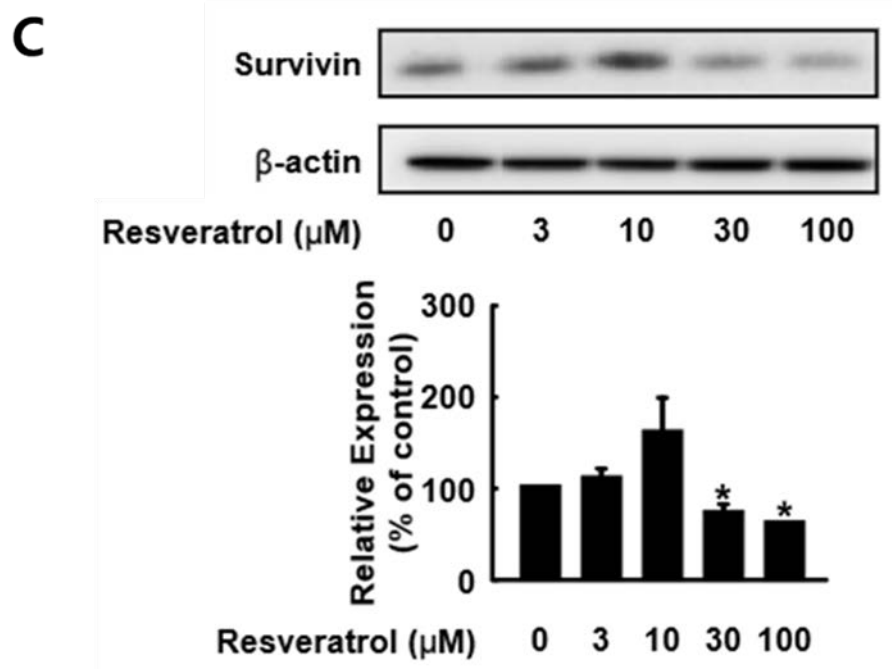


Figure 2. Effects of resveratrol on cell proliferation and survivin expression in Huh7-HBx cells.

(A and B) Resveratrol inhibited cell proliferation of Huh7-HBx and Huh7-CMV cells incubated with varying concentrations for 48 h. Results are mean ± standard deviation (SD) of at least 3 independent experiments; **, $p < 0.01$ comparing control group at 0 and control group at 48 h. $^{\#\#}$, $p < 0.01$ comparing control and resveratrol-treated groups at 48 h. (C) Resveratrol decreased survivin protein expression in Huh7-HBx cells treated with varying concentrations for 24 h. Western blot analysis was conducted with β-actin was used as loading control. Graphs are quantification of result of at least 3 independent experiments; *, $p < 0.05$ comparing resveratrol-treated and control groups.

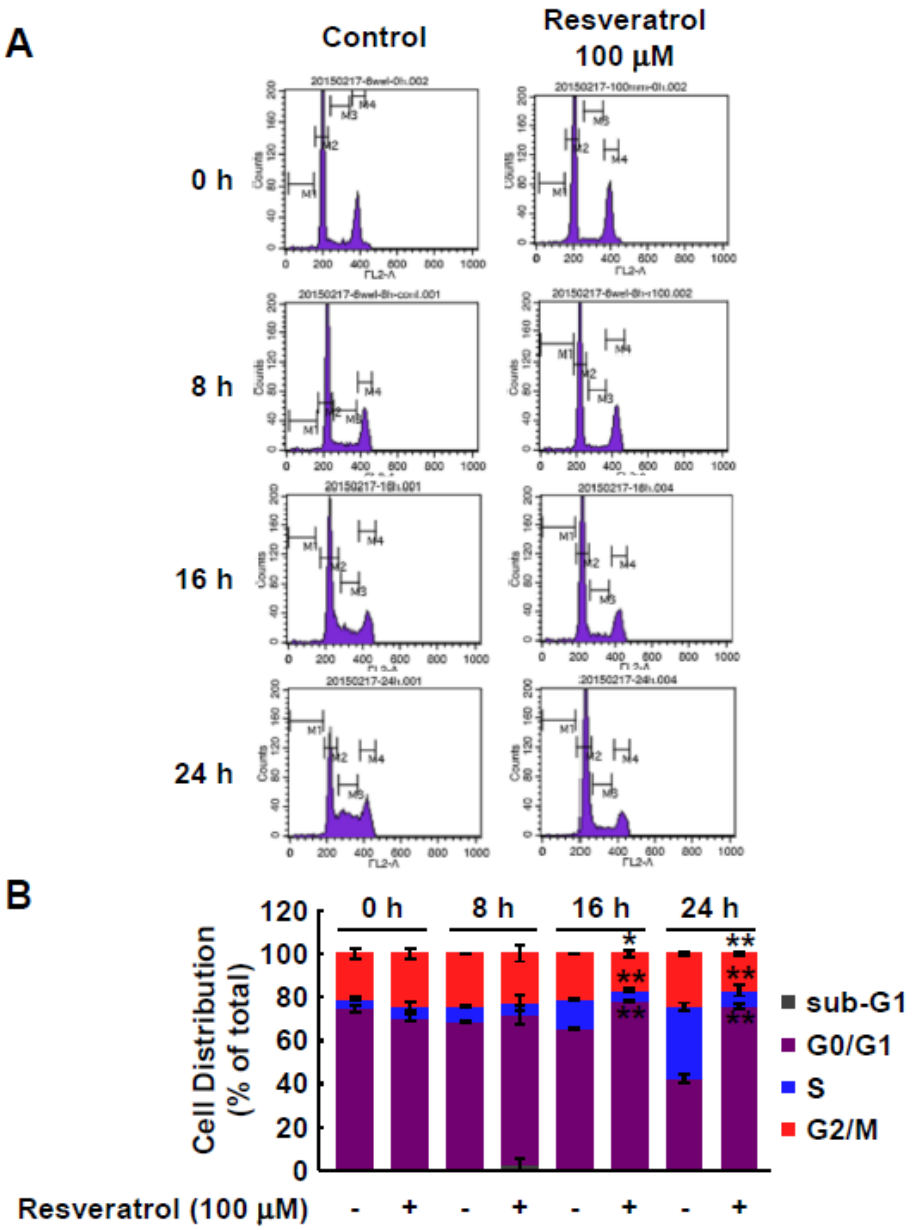


Figure 3. Effect of resveratrol on cell cycle progression of Huh7-HBx cells.

(A and B) Resveratrol blocked G1-S transition of Huh7-HBx cells treated with 100 μM for indicated times as shown by FACS analysis. Results represent at least 3 independent experiments that produced similar results. Graphs are quantification of result of at least 3 independent experiments; *, $p< 0.05$ and **, $p < 0.01$ comparing control and resveratrol-treated groups.

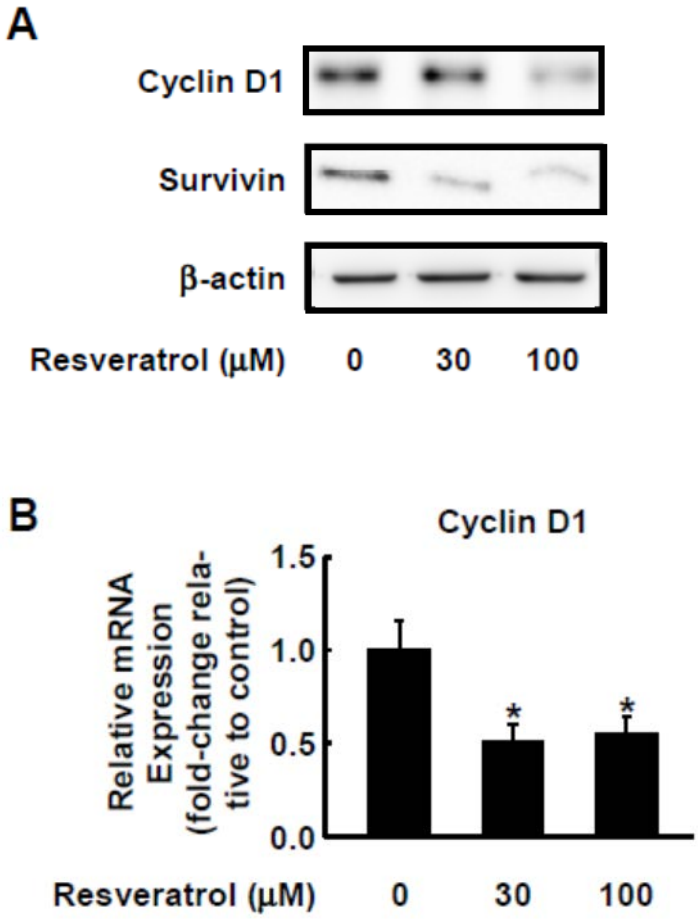


Figure 4. **Effects of resveratrol on cyclin D1 and survivin expression in Huh7-HBx cells.**

(A) Resveratrol decreased cyclin D1 and survivin protein expression in Huh7-HBx cells treated with varying concentrations for 8 h. Western blot analysis was conducted with β-actin as loading control. Results represent at least 3 experiments that produced similar results. (B) Resveratrol decreased cyclin D1 mRNA expression in Huh7-HBx cells treated with varying concentrations for 4 h. qRT-PCR analysis was conducted with β-actin as

loading control. Graphs are quantification of results of at least 3 independent experiments; *, $p < 0.05$ comparing control and resveratrol-treated groups.

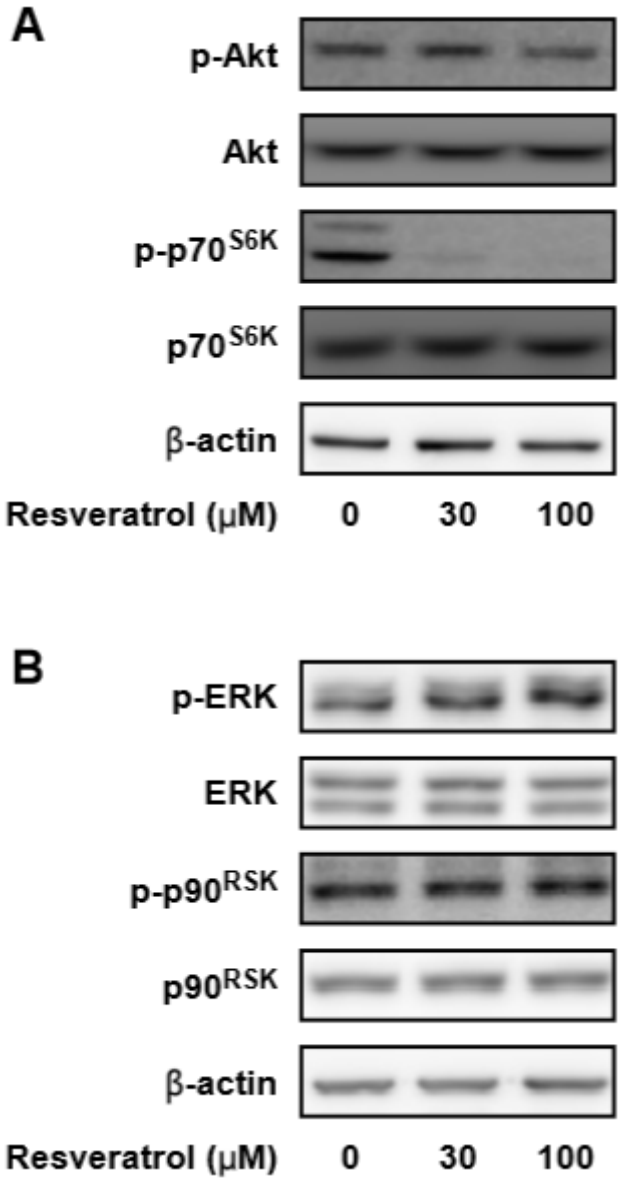


Figure 5. Effects of resveratrol on ERK and Akt signaling pathway in Huh7-HBx cells.

(A and B) Resveratrol blocked Akt, but not ERK signaling pathway in Huh7-HBx cells treated with varying concentrations for 4 h. Western blot analysis

was conducted with β-actin as loading control. Data represent at least 3 independent experiments that produced similar results; p-ERK, phosphorylated extracellular signal-regulated kinase.

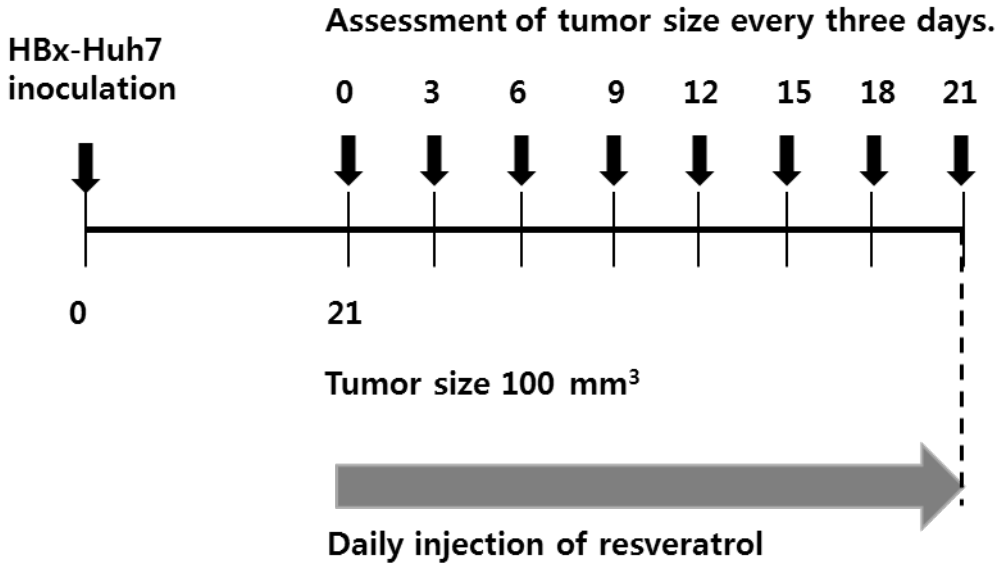
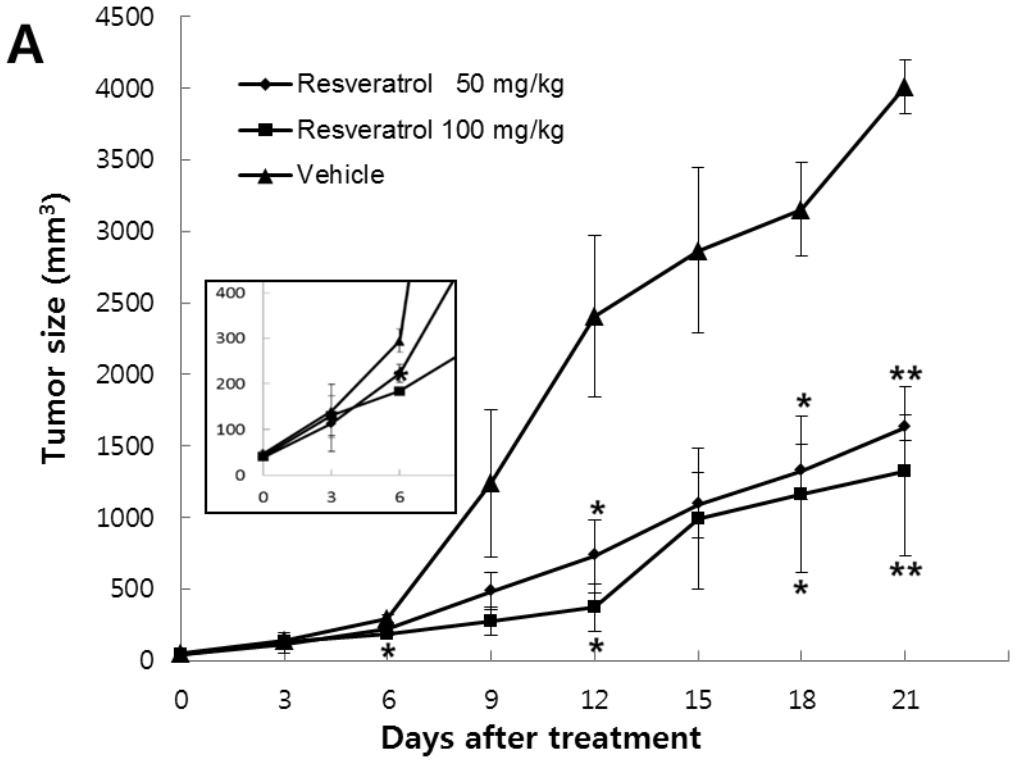
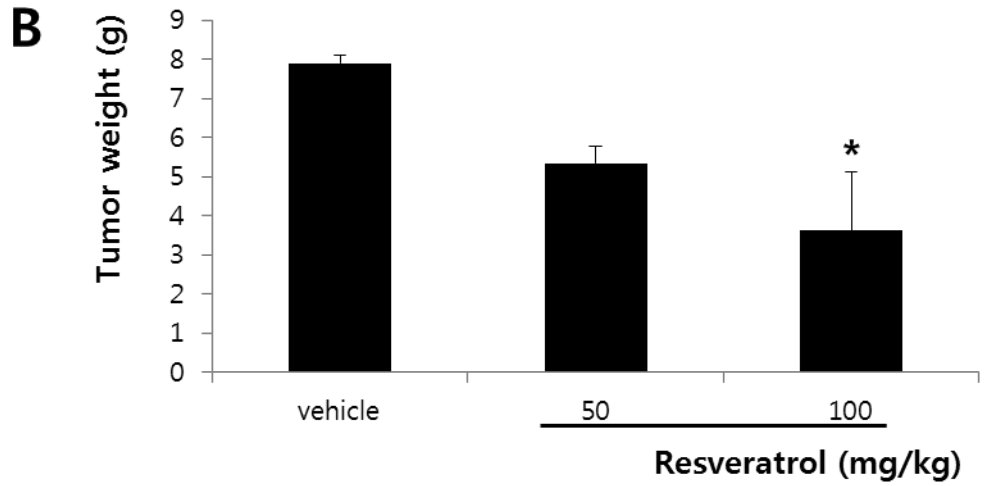


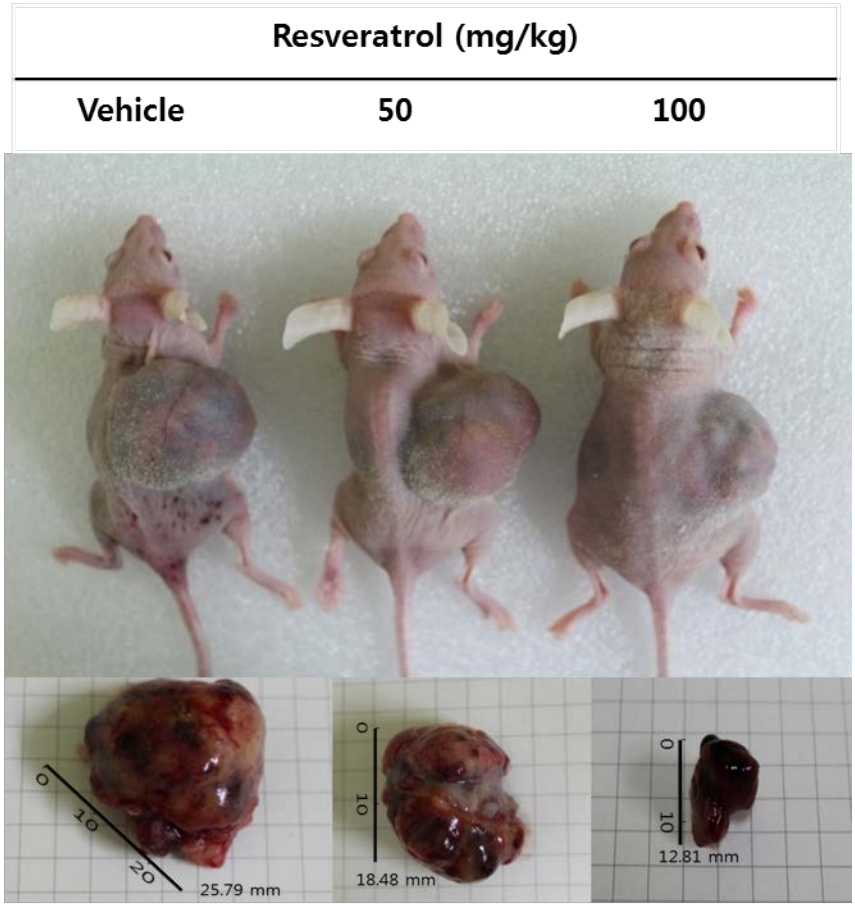
Figure 6. The entire schedule of *in vivo* study.

Tumor cells were injected subcutaneously into the right flank of each nude mouse. When the tumors' size progress to approximate 100 mm^3 (at 21 days), resveratrol was injected intraperitoneally for 3 weeks every day. At 21 days after treatment, the experiments were ended and the mice were sacrificed. The transplanted tumors were sampled and weighed.

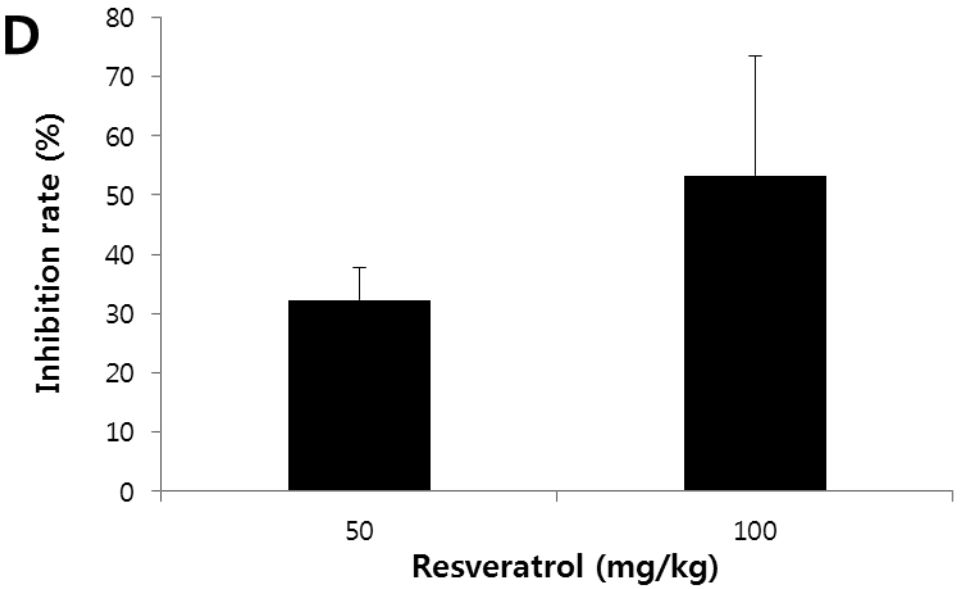

A
Resveratrol 50 mg/kg
Resveratrol 100 mg/kg
Vehicle
Tumor size (mm3)
Days after treatment
0
500
1000
1500
2000
2500
3000
3500
4000
4500
0
3
6
9
12
15
18
21
*
**


B
Tumor weight (g)
0
1
2
3
4
5
6
7
8
9
vehicle
50
100
Resveratrol (mg/kg)
*

C


Resveratrol (mg/kg)
Vehicle
50
100
0
10
20
25.79 mm
0
10
18.48 mm
0
10
12.81 mm

D


Inhibition rate (%)
80
70
60
50
40
30
20
10
0
50
100
Resveratrol (mg/kg)

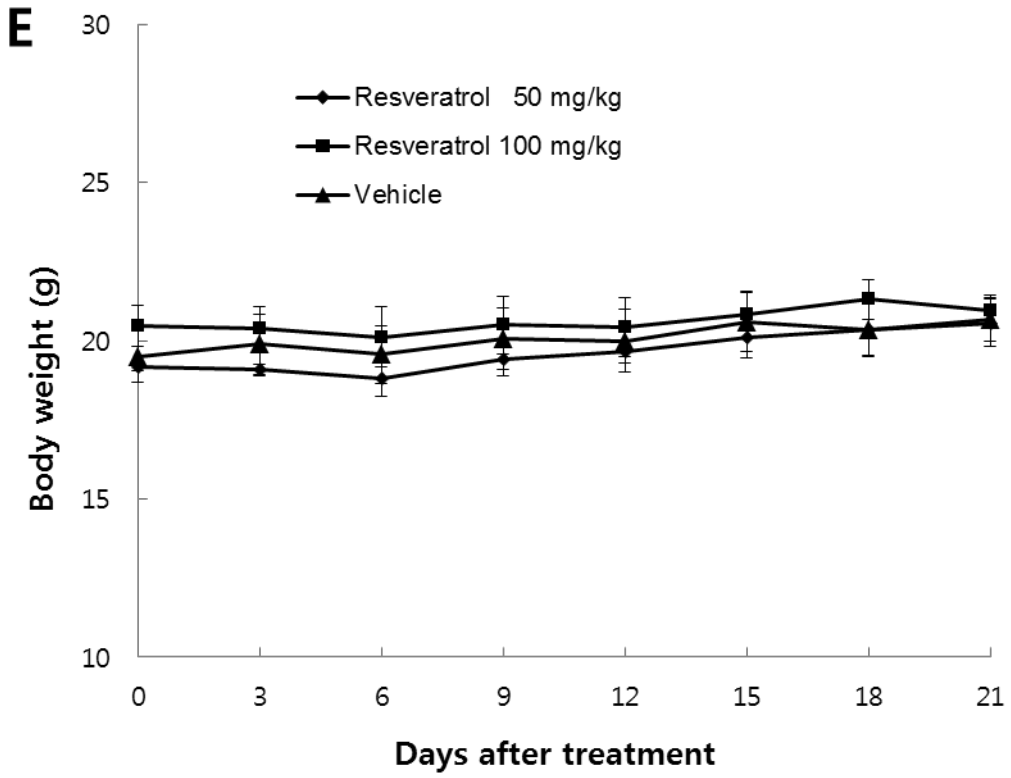


Figure 7. Inhibitory effects of resveratrol in xenograft model mice implanted with Huh7-HBx cell *in vivo*.

5×10^{6}/0.2 mL Huh7-HBx cells were inoculated into the right upper flank of BALB/c (nu/nu) mice subcutaneously. Then, the mice were randomly allocated to vehicle group and two treatment groups at 21 days after inoculation. (A) Tumor size curve. The size of tumors were measured from day 1 to day 21 each 3-day. (B) The tumor weight on 21 days at the end of study. (C) Representative photographs of nude mice bearing Huh7-HBx cell

at the end of study. (D) The Inhibition rate of tumor growth was calculated using the formula:

$$Inhibition\ rate\ (\%) = \frac{(tumor\ weight\ of\ vehicle\ group - tumor\ weight\ of\ treated\ group)}{tumor\ weight\ of\ vehicle\ group}\ x\ 100\%$$

(E) Body weight curve. Results were expressed as the mean ± S.D. (n=3) *, significance at $p < 0.05$ and **, $p < 0.01$, between resveratrol treated group and vehicle group and one-way ANOVA was used for analysis and post-hoc comparisons were performed by using the tukey HSD.

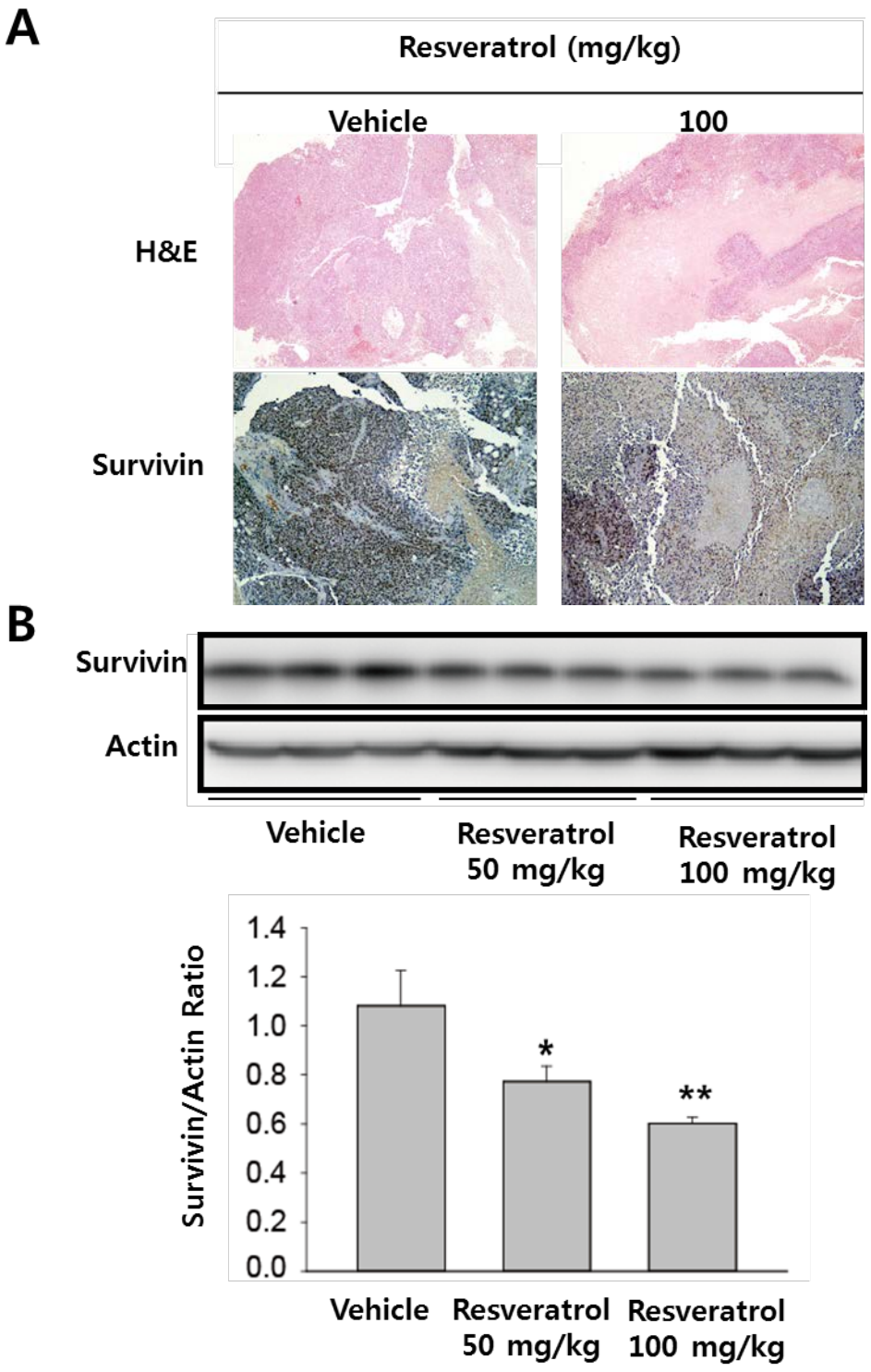
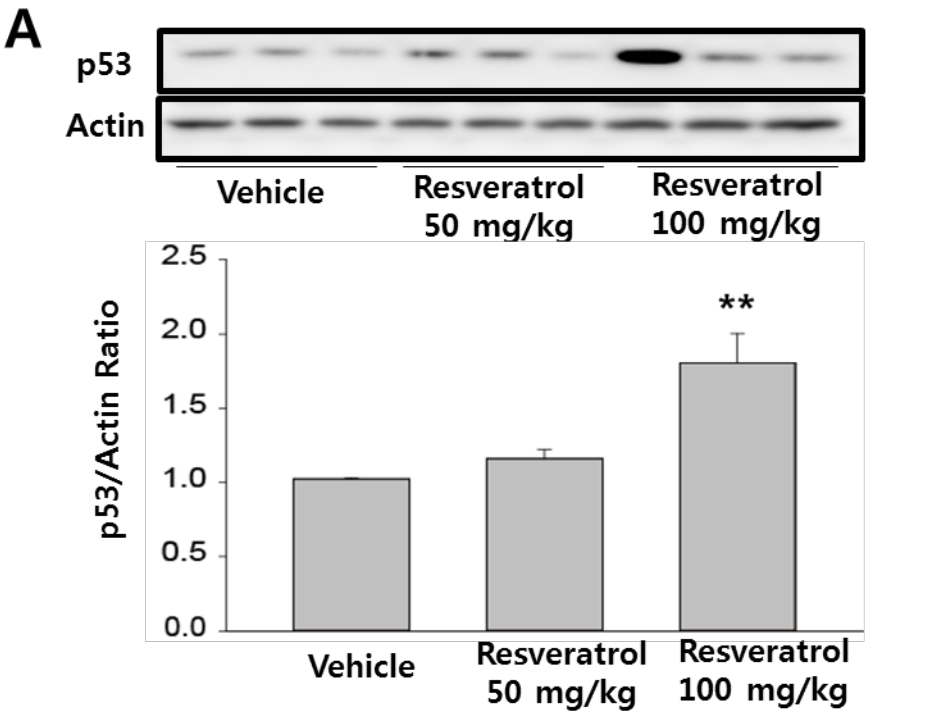

A
Resveratrol (mg/kg)
Vehicle
100
H&E
Survivin
B
Survivin
Actin
Vehicle
Resveratrol 50 mg/kg
Resveratrol 100 mg/kg
Survivin/Actin Ratio
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
**
Vehicle
Resveratrol 50 mg/kg
Resveratrol 100 mg/kg

Figure 8. Effect of resveratrol in tumor specimens.

(A) Pathologic representative status of tumor specimens. Tumor specimens were taken from the xenograft model mice, embedded in paraffin and stained with hematoxylin & eosin (Magnification x 400). Expression of survivin was examined by IHC staining in the tumor tissues of the vehicle and 100 mg/kg resveratrol treated groups. Resveratrol (100 mg/kg treated group) reduced the tumor development and intensity of staining of survivin. (B) The protein expression of survivin. Xenograft model mice implanted with Huh7-HBx cells were treated with 0, 50, 100 mg/kg resveratrol, respectively. Survivin / actin ratio was decreased in both resveratrol treated groups. Survivin / actin expression ratio was significantly reduced at the 50 mg/kg and 100 mg/kg treated groups (0.77 ± 0.06, $p < 0.05$ and 0.60 ± 0.03, $p < 0.01$), compared to vehicle (1.0 ± 0.14). Results were expressed as the mean ± S.D. (n=3). *****, significance at $p < 0.05$ and ******, $p < 0.01$, between resveratrol treated group and vehicle group (Student's *t*-test).


A
p53
Actin
Vehicle
Resveratrol 50 mg/kg
Resveratrol 100 mg/kg
p53/Actin Ratio
2.5
2.0
1.5
1.0
0.5
0.0
**
Vehicle
Resveratrol 50 mg/kg
Resveratrol 100 mg/kg

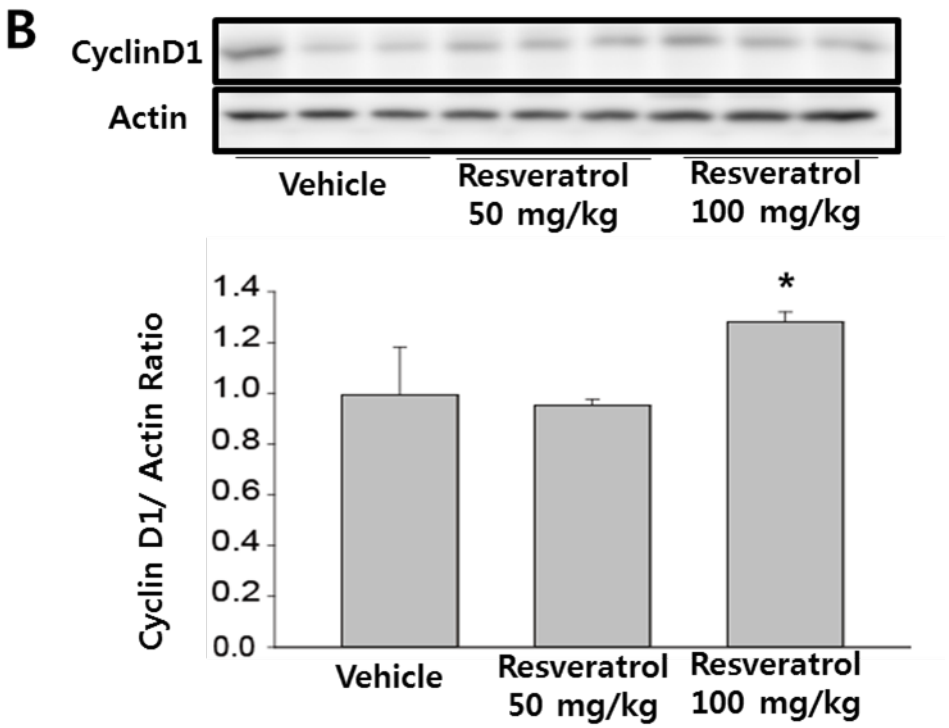

B
CyclinD1
Actin
Vehicle
Resveratrol 50 mg/kg
Resveratrol 100 mg/kg
Cyclin D1/ Actin Ratio
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Vehicle
Resveratrol 50 mg/kg
Resveratrol 100 mg/kg

Figure 9. Effects of resveratrol in expression of various proteins.

(A and B) The protein expression of p53 and cyclin D1 were determined by western blot analysis. The xenograft model mice implanted with Huh7-HBx cells were treated with 0, 50, 100 mg/kg resveratrol, respectively. The proteins were detected by western blot analysis using each antibody. p53/ actin ratio was increased in both resveratrol treated groups and cyclin D1/ actin ratio was not shown any significant changes. p53 / actin expression ratio (1.81 ± 0.34, $p < 0.05$) was increased in 100 mg/kg of resveratrol treated group compared to vehicle group (1.0 ± 0.0). Results were expressed as the mean ± S.D. (n=3). *, significance at $p < 0.05$ and **, $p < 0.01$, between resveratrol treated group and vehicle group (Student's *t*-test).

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국 문 초 록

B형 간염 바이러스 X-단백질로 유도된 간세포암종에서 resveratrol의 억제 효과

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Resveratrol은 적포도, 장과류, 견과류에 함유되어 있는 폴리페놀계 항산화 물질로 항암기능이 알려져 있다.

본 연구에서 B형 간염바이러스 X-단백질이 과발현된 Huh-7 human hepatoma cells (Huh7-HBx)를 이용하여 B형 간염바이러스로 기인된 간세포암종에 대한 resveratrol의 항암 효과 및 기전을 확인하였다. MTT 분석 결과 resveratrol은

Huh7-HBx cells의 활성을 감소시켰다. resveratrol이 Huh7-HBx cells의 증식을 억제하는지 또는 세포자살을 일으키는 지를 확인하기 위하여 FACS로 분석한 결과 resveratrol은 G1 단계에서 세포주기 억류를 유도하였고 sub-G1 단계의 밀도를 증가시키지 않았다.

Resveratrol이 G1 단계에서 세포주기 억류을 유도하는 것이 확인됨에 따라 G1/S 단계의 이행에 깊이 관여하는 것으로 알려진 사이클린 D1의 발현을 확인하였고 그 결과 전사 수준에서 사이클린 D1의 발현을 감소시키는 것으로 확인되었다. 사이클린 D1 전사의 상위 신호 페스웨이인 extracellular signal-related kinase (ERK)와 Akt 신호 페스웨이를 확인한 결과 resveratrol은 Huh7-HBx cells에서 Akt 신호 페스웨이를 억제하고 ERK 신호 페스웨이에는 영향을 주지 않았다. 결과적으로 resveratrol은 Akt 신호 페스웨이를 억제함으로써 사이클린 D1의 발현을 감소시켜 Huh7-HBx cells의 증식을 억제하는 것으로 입증되었다.

In vivo 연구에서, Huh7-HBx cells를 누드마우스에 접종시킨 xenograft 모델 마우스를 이용하여 resveratrol의 항암

효과를 확인하였고 survivin을 억제시켜 암 성장을 감소시켰다. 또한 사이클린 D1의 발현에는 영향이 없는 것으로 입증되었다.

종합적으로 resveratrol은 Akt 신호 페스웨이를 억제함으로써 사이클린 D1의 발현을 감소시켜 Huh7-HBx cells의 증식을 억제하는 것으로 입증되었고 Huh7-HBx cells로 유도한 xenograft 모델 마우스에서 survivin을 억제함으로써 항암작용을 나타내었다.

주요어: 간세포암종, B형 간염 바이러스, B형 간염 바이러스 X-단백질, resveratrol, 사이클린 D1, Akt, survivin

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