Functional Characterization of Sirtuin 1 (SIRT1) in Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide. In Hong Kong where there is a high prevalence of hepatitis B infection, HCC is the third most common cancer. Despite the cancer can be eradicated by curative surgery, most of the patients are often diagnosed at an advanced stage when it is no longer amenable to curative therapies. The prognosis is very poor for patients who have unresectable tumors, with the median survival of ~ 6 months. Therefore, there is an urgent need in the understanding of HCC carcinogenesis at molecular level and to identify novel molecular targets for the development of more efficacious therapeutics. Silent mating-type information regulation 2 homologue 1 (SIRT1), a class III histone deacetylase, is the mammalian homologue of yeast Sir2 and functions as a NAD^+-dependent histone deacetylase. SIRT1 is involved in multiple cellular functions including metabolism, cellular stress response, and cell differentiation. Although SIRT1 is up-regulated in various human and mouse malignant tumors, the exact role of SIRT1 in tumorigenesis remains elusive. Therefore, the goal of this study is to characterize the functional role of SIRT1 in HCC with the following objectives: 1) To investigate the association between SIRT1 and HCC; 2) To investigate the effect of SIRT1 knockdown on HCC growth using cell models; and 3) To identify SIRT1-regulated genes and pathways in HCC.

Using paired HCC specimens and cell lines I showed that SIRT1 is differentially overexpressed in a subset of HCC. Immunohistochemical analysis of HCC tissue microarray further revealed that high expression of SIRT1 in HCC tumors was significantly associated with tumor grade. Secondly, using lentivirus-mediated RNA interference to knockdown SIRT1 expression resulted in profound suppression of HCC cell growth. Importantly, suppression of SIRT1 in p53 wild type HCC cells resulted in the induction of cellular senescence associated with the accumulation of acetylated-p53 and p21 respectively. On the other hand, increased propensity to apoptotic cell death was evidenced by the enhanced PARP cleavage in p53-null or -mutant cells upon SIRT1 knockdown. The cellular

senescence or cell death upon gene knockdown of SIRT1 is associated with reduced expression of telomerase reverse transcriptase (TERT) and PTOP, which is a member of the shelterin complex, leading to the formation of telomere dysfunction-induced foci (TIF). Accordingly, the restoration of PTOP or TERT expression significantly restored cell growth and reduced senescence imposed by SIRT1 knockdown. In addition, the expression of TERT was associated with the expression of SIRT1 in clinical specimens, suggesting that SIRT1 might similarly regulate TERT expression *in vivo*. Finally, SIRT1-silencing sensitized HCC cells to doxorubicin treatment.

My study revealed a novel role of SIRT1 in the pathogenesis of HCC, provided a better understanding of the molecular pathogenesis of HCC, and might facilitate future development of SIRT1 inhibitors as HCC therapeutics.

摘要

原發性肝癌是世界上最常見的五大惡性腫瘤之一，在香港地區肝癌死亡率位第三。肝癌起病隱匿,絕大部分患者就診時已界中、晚期, 完全失去了手術治愈的機會，並且肝癌的化療效果不佳，因此預後很差，肝癌晚期病人存活期僅有 3-6 個月時間。目前對 HCC 發生、發展和轉移復發的精確分子機制尚不完全清楚。因此,探討其發生發展和轉移復發的精確分子機制,尋找早期診斷肝癌、預測轉移的生物標誌物和幹預治療的靶分子具有重要的研究意義。Sirtuin 1 是一種組蛋白脫乙酰基酶，同時也是與酵母染色質 Sir2 同源性最高的一個,分布於細胞核中。通過對組蛋白及多種非組蛋白進行去乙酰化修飾來調節基因表達,參與細胞衰老、凋亡、分化，應激耐受及能量代謝等生理活動。目前雖然人們在多種腫瘤組織中發現 SIRT1 表達增高，並且認爲它在腫瘤形成過程中發揮著重要作用，但是 SIRT1 發揮作用的潛在分子機制仍然不爲人們所知。因此我們的課題旨在研究 SIRT1 在肝細胞癌發生發展中的重要作用。我們的研究目標包括三個部分：1.分析 SIRT1 在肝細胞癌中的表達及與臨床病理參數之間的相關性；2.鑒定 SIRT1 在肝癌細胞增值中的作用；3.進一步發現 SIRT1 調控的靶蛋白及相關信號通路。

我們的研究發現 SIRT1 在肝癌細胞系和人類肝癌組織中表達增高，而在正常肝組織中表達水平極低。爲了進一步研究和證實 SIRT1 的重要性，我們運用組織芯片技術對 150 例肝癌組織及癌旁組織中 SIRT1 的表達水平進行檢測，結果顯示 SIRT1 在肝癌中的表達水平和腫瘤的病理分級呈正相關性(P=0.006)。在細胞模型研究中，當下調 SIRT1 的表達水平時，肝癌腫瘤細胞系表現出明顯的

生長抑制。同時，SIRT1 表達水平的下調促進肝癌細胞（野生型 p53）的老化並且同時促進 p53 突變及缺失肝癌細胞的凋亡。而肝癌細胞細胞的老化與細胞內 p53 乙酰化水平和 p21 表達水平的增高具有明顯的相關性。我們進一步發現 SIRT1 的沈默能夠導致端粒的功能障礙，這種功能障礙與端粒酶逆轉錄酶 (hTERT), 保護端粒 1 蛋白（POT1）及 POT1 相互作用蛋白 1 （PTOP）表達水平的下調息息相關。 眾所皆知，這些蛋白在端粒維持中發揮著重要作用，因此我們的結果顯示 SIRT1 可能通過調節端粒相關蛋白基因的表達從而維持端粒結構，而在這之前，人們從來沒有發現 SIRT1 在這個過程中發揮著作用。最後我們發現在肝癌組織中 SIRT1 的表達水平和 TERT 的表達具有顯著的相關性。

我們的研究首次揭示了 SIRT1 在端粒維護中的重要作用，並進一步強調了 SIRT1 活性抑制劑將有望成爲抗腫瘤藥物潛在靶點。

PUBLICATIONS

Conference abstract

Juan Chen, Bin Zhang, Nathalie Wong, Anthony W.I. Lo, Ka-Fai To, Anthony W.H., Paul B.S. Lai and Ben C.B. Ko." Sirtuin 1 (SIRT1) is upregulated in a subset of hepatocellular carcinoma and is essential for telomeric maintenance and tumor cell growth" in the proceeding of EMBO meeting, 4 -7 September 2010, Barcelona, Spain.

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LIST OF ABBREVIATIONS

AceCS2	Acetyl-CoA synthetase 2
ADH	Dehydrogenase
AFB1	Aflatoxin B1
APC	Adeomatous polyposis coli
BRAC1	Breast cancer 1
BRAC2	Breast cancer 2
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CIN	Chromosomal instability
CNs	Cirrhotic nodule
CPS1	Ccarbamoyl phosphate synthetase 1
CR	Caloric restriction
CVD	Cardiovascular disease
DDR	DNA damage response
DLC1	Deleted in liver cancer 1
DMEM	Dulbecco's Modified Eagle Medium
DNs	dysplastic nodules
eNOS	Endothelial nitric oxide synthase
FXR	farnesoid X receptor
GDH	Glutamate dehydrogenase
H3K9	Histone H3 lysine 9
HBV	Hepatitis B virus
HCC	Hhepatocellular carcinoma
HCV	Hepatitis C virus
HIC1	Hypermethylated in cancer 1
HOXA10	Homeobox protein A10

hTERT	Human Telomerase Reverse Transcriptase
IDH2	Isocitrate dehydrogenase 2
MEM	Minimum Essential Medium
NAFLD	Nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NCoR	(nuclear receptor co-repressor
PARP	poly ADP ribose polymerase
PC	Prostate cancer
PGC-1α	PPAR gamma coactviator 1 alpha
PML	Promyelocytic leukemia protein
POT1	Protection of telomeres 1
PPAR-γ	Peroxisome proliferator-activated receptor-gamm
PTOP	TINF2-interacting protein
RASSF1A	Ras association domain family 1A gene
RB	Retinoblastoma
rDNA	Recombination of ribosomal DNA
RRs	Relative risks
SA-β-gal	Senescence-associated β-galactosidase
TERF1	Telomeric repeat-binding factor 1
TIF	Telomere dysfunction Induced Foci
TINF2	TERF1-interacting nuclear factor 2
TNF-α	Tumor necrosis factor alpha

Chapter 1 Introduction

1.1 Background of hepatocellular carcinoma (HCC)

Primary liver cancer is the fifth most common malignancy worldwide and the third common cause of cancer mortality (El-Serag and Rudolph 2007). Approximately 560,000 new liver cancer cases are diagnosed and it causes ~550,000 deaths each year. Liver cancer exhibits distinct geographic distribution. More than 70% of all new cases are diagnosed in Asia, a region where there are about 75% of all HBV carriers in the world (Lai, Ratziu et al. 2003). Of particular importance, China alone accounts for 55% of all HCC cases worldwide (Parkin, Bray et al. 2005). In Hong Kong, HCC is the fourth common cancer and the third leading cause of cancer deaths. According to statistics of HK cancer registry 2008, 1,745 new cases were diagnosed and 1,499 deaths due to liver cancer were recorded. The high mortality of liver cancer stems in part from its resistance to existing anticancer agents, a lack of biomarkers that can detect surgically respectable incipient disease, and insufficient knowledge regarding the underlying mechanisms of its pathogenesis that limits the use of chemotherapeutic drugs (Farazi and DePinho 2006). Liver cancer comprises diverse, histologically distinct primary hepatic neoplasms, which includes hepatocellular carcinoma (HCC), intrahepatic bile duct carcinoma (Cholangiocarcinoma, hepatoblastoma, bile duct cystadenocarcinoma, haemangiosarcoma) and epitheliod haemangioendothelioma (MacSween 2002). In most countries, 75-90% of liver cancer cases are HCC (Okuda, Nakanuma et al. 2002).

1.1.1 Epidemiology of HCC

HCC shows significant epidemiologic differences with regard to region, age and genders. The major geographic distribution of HCC is in North America, Northern Europe, sub-Saharan Africa, as well as in Central and Eastern Asia (Figure 1.1). Majority of HCC (>80%) cases occurs in either sub-Saharan African or

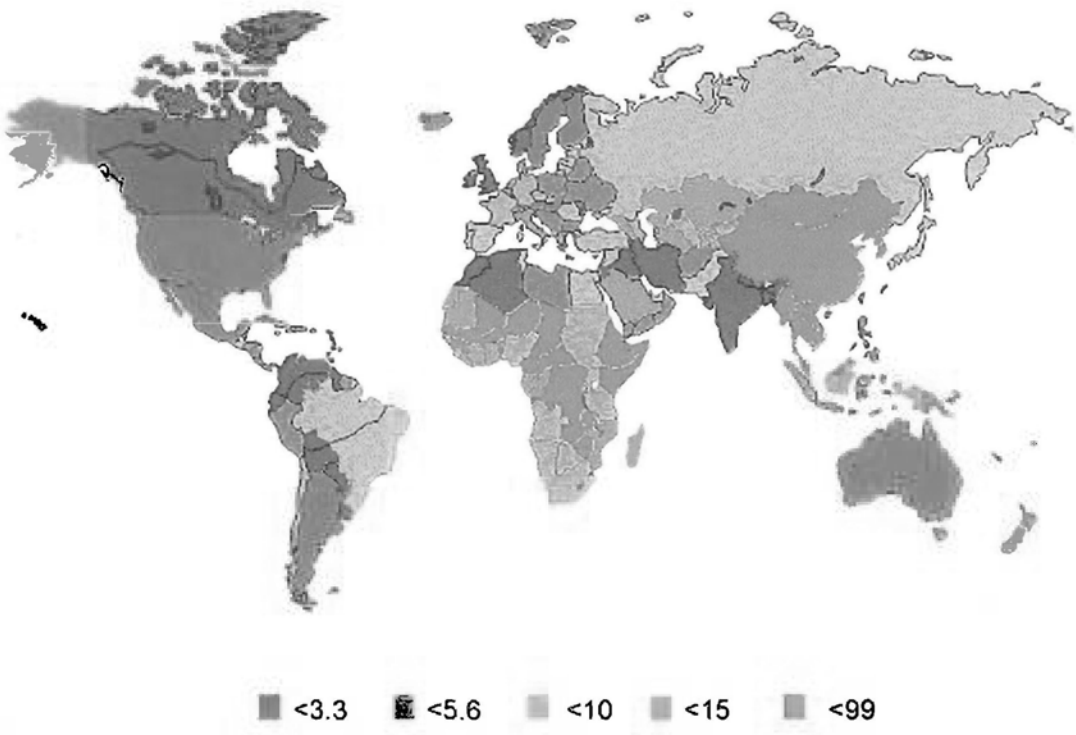


Figure 1.1 Global incidence of liver cancer in males estimated in year 2000. The rates are reported per 100,000 persons.

This figure was adapted from Katherine A et al. 2007

in Eastern Asia. China alone accounts for over 50% of the total cases (age standardized incidence rate: men, 35.2/100,000; women, 13.3/100,000). North and South American, Northern Europe tend to have a lower incidence rate (<5.0/100,000). In most areas, female incidence rate peak in the age group 5 years older than that of male. In high-risk African populations (e.g. The Gambia, Mali), male incidence rate peaks between 60 and 65 years old, while that of female peaks between 65 and 70 (McGlynn and London 2005). However, in low-risk region, the highest rate was found at age 75 or beyond. A similar correlation of risk and age is also noted in high-risk Asian populations (eg, in Hong Kong and Shanghai). On the other hand, HCC is biased towards the male gender, for the ratio between male to female is about 2~4:1. The reason for high rates of HCC in males is not fully understood, but may be partially explained by the gender-related risk factors, such as the higher chance of infection by HBC or HCV, as well as alcohol and cigarettes consumptions in males.

1.1.2 Risk factors of HCC

The major risk factors for HCC are infection with hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol consumption and dietary exposure to food contaminated with aflatoxin B1 (AFB1). The suspected risk factors include diet, obesity, tobacco smoking, diabetes and insulin resistance, and iron overload (McGlynn and London 2005) (Table 1.1). In high-risk areas, HBV and AFB1 are the main factors for HCC development, whereas HCV and alcohol consumption are more important factors in low- to medium-rate areas. However, HBV and HCV infections together account for more than over 80% of HCC cases in the world.

1.1.2.1 Hepatitis B virus (HBV)

More than 350 million people worldwide are chronically infected with HBV. (Maddrey 2001; Zhu 2003). In HCC high-risk regions such as Asia and Africa, HBV infection is associated with majority of liver cirrhosis and over 80% of HCC cases. Chronic HBV infection is associated with the development of cirrhosis, which is the principal predisposing factor for HCC development (el-Serag 2001).Hepatitis B virus

Table 1.1 Relationship between different factors and the risk of hepatocellular carcinoma development

Evidence	Decreased risk	Increased risk
Convincing		Hepatitis B virus, hepatitis C virus, aflatoxin B_1, cirrhosis, alcohol, male gender, hemochromatosis, thorotrast
Probable	Vegetables	Iron levels, vinyl chloride, obesity, diabetes mellitus
Possible	Selenium, green tea	Tobacco, anabolic steroids, androgen levels, parity, schistosomiasis, NASH
Unclear		Arsenic

belongs to the Hepadnavirus family. The HBV virion is 42 nm in diameter and consists of an outer lipid envelope formed by hepatitis B surface antigen and an icosahedral nucleocapsid. The nucleocapsid encloses the viral DNA and a DNA polymerase that has reverse transcriptase activity (Locarnini 2004). The HBV genome is a partially double-stranded relaxed circular DNA with a length of about 3200 nucleotides. The minus strand contained four partially overlapping open-reading frames encoding the envelope (pre-S/S), core (pre-C/C), polymerase (P) and X proteins（X）respectively (Tsai and Chung 2010) (Figure1.2). After binding to a yet unknown cell surface receptor, the HBV genome is transferred to the nucleus by host proteins, and subsequently assumes a highly stable confirmation named covalently closed circular DNA (cccDNA). The cccDNA serves as a template for transcription of viral RNA, which is translated into proteins.

Several lines of evidence support the direct involvement of HBV in the carcinogenesis of liver cancer. Firstly, integration of HBV into DNA has been associated with a wide range of genetic alterations in the host genome, including chromosomal deletions, production of fusion transcripts, translocations and genomic instability (Bonilla Guerrero and Roberts 2005; Feitelson and Lee 2007), which may alter the expression of tumor suppression genes, oncogenes and microRNAs (Feitelson and Lee 2007). Secondly, HBx plays a central role in HBV infection and hepatocarcinogensis. Transgenic mice expressing HBV X (HBx) protein developed HCC, which started with the formation of multi-focal areas of altered hepatocyets followed by progression to malignant carcinoma (Lok 2000). HBx transactivates many cytoplasmic signaling molecules, including JAK/STAT, protein kinase C (PKC), PI3K, c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK), Ras，Raf，MAPK, nuclear factor-κB (NF-κB), Smad and Wnt respectively (Lee, Kang-Park et al. 2001; Majano, Lara-Pezzi et al. 2001; Nijhara, Jana et al. (Lee, Kang-Park et al. 2001; Majano, Lara-Pezzi et al. 2001; Nijhara, Jana et al. 2001; Feitelson, Reis et al. 2009). In addition, HBx decreases proteasomal degradation of β-catenin and increases the downstream targets of β-catenin such as c-myc and cyclin D1 (Ding, Xia et al. 2005). Moreover, HBx causes transcriptional

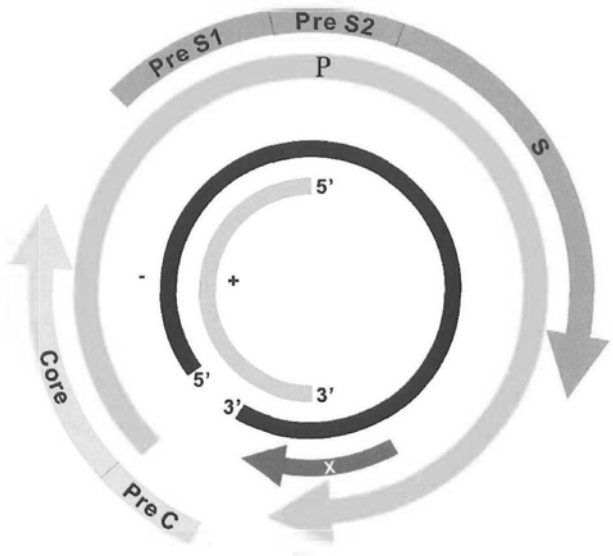


Figure 1.2 The structure of HBV virus. Organization of the hepatitis B virus genome. The inner circles represent the full-length minus (-) strand (with the terminal protein attached to its 5' end) and the incomplete plus (+) strand of the HBV genome. This figure was modified form a figure published in (Beck et al. 2007).

repression of the p53 gene (Lee and Rho 2000). It also interacts with p53 to block several important p53-dependent activities, including apoptosis, DNA repair and cell-cycle arrest (Kremsdorf, Soussan et al. 2006). Finally, pre-S/S protein transactivates several host genes to stimulate HCC development. Two deletion mutants of the pre-S/S proteins were found in the serum and tumor in 60% of HCC patents (Fan, Lu et al. 2001). The mutant pre-S large surface antigens can induce oxidative DNA damage and genomic instability through the induction of endoplasmic reticulum (ER) stress (Hsieh, Su et al. 2004; Wang and Weinman 2006; Gwak, Lee et al. 2008). It also stimulates cell cycle progression and cell proliferation by upregulating COX2 (cyclooxygenase-2) (Wang and Weinman 2006). In addition, a recent study found that pre-S2 increases malignant transformation of human HCC cell lines by upregulating human telomerase reverse transcriptase and inducing telomerase activation (Liu, Luan et al. 2007). Therefore, HBV is an important player in hepatocytes transformation and it is involved in the multi-step tumorigenesis.

1.1.2.2 Hepatitis C virus (HCV)

Chronic Hepatitis C virus (HCV) infection is another major risk factor for HCC development. Approximately 170 million people worldwide are seropositive for anti-HCV antibodies and, of these, an estimated 127 million are chronically infected (Ray Kim 2002; Bosch, Ribes et al. 2005). HCV infection are found in 28-76% of HCC cases in Europe (with an increasing gradient from north to south) and in 80-90% of HCC patients in Japan (el-Serag 2001; Bosch, Ribes et al. 2005). A meta-analysis revealed that HCC risk of was increased by 17-fold among HCV-infected patients compared with HCV-negative controls (Donato, Boffetta et al. 1998).

HCV is a RNA virus of the Flaviviridae family. The HCV positive-stranded RNA genome is translated into a single polyprotein of 3010 or 3011 amino acids, which is processed into structural (core, envelope glycoproteins E1 and E2) and nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins by host and viral proteases (Chisari 2005; Lindenbach and Rice 2005). Viral particles interact with

specific surface receptors and are probably internalized. Once inside the cytoplasm, the single-stranded, positive-sense RNA genome is released. The genomic RNA functions directly as a messenger RNA for the translation of viral proteins, and serves as template for RNA replication (Lindenbach and Rice 2005; Moradpour, Penin et al. 2007). Viral assembly is thought to occur in the endoplasmic reticulum of the infected cells. HCV is distinctive from HBV in several aspects. First, HCV is an RNA virus that will is not subjected to reverse transcription and thus cannot be integrated into host genomes. Second, HCV shows high propensity to yield chronic infection, with a infection rate of 60-80% versus 10% in HBV (Rehermann and Nascimbeni 2005). This might due to the immune invasion of HCV quasi-species generated from high rates of replication errors (Weiner, Erickson et al. 1995; Rehermann and Nascimbeni 2005). Third, HCV shows high propensity to promote liver cirrhosis compared with HBV. About 5-10% of HCV-infected patients develop liver cirrhosis after 10 years of infection, a frequency that is 10-20 folds higher than that induced by HBV (Rehermann and Nascimbeni 2005). The underlying molecular mechanisms of HCV-related HCC are still unclear. Since there is no integration of viral nucleic acid, viral pathology may contribute to oncogenesis through cirrhosis and the regeneration of liver cells. Alternatively, different HCV proteins have been implicated in the process of hepatocarcinogenesis. HCV core protein is a structural viral protein that packages the viral genomic RNA. Transgenic mice expressing the core gene developed HCC (Moriya, Fujie et al. 1998). Interestingly, these mice developed steatosis without inflammation after 3 month, and progressed into HCC in 26-31% of male transgenic mice after 16-19 months (Moriya, Fujie et al. 1998). HCV core protein appears to have a diverse range of functions and interacts with many cellular proteins. It can modulate the expression of the cyclin-dependent inhibitor p21, which is a major target of p53. It also regulates the activities of cyclin /cyclin-depent kinase complex involved in cell-cycle control and tumor formation (Yamanaka, Kodama et al. 2002; Kwun and Jang 2003). HCV core protein also interacts with p73 and this interaction results in the nuclear translocation of core protein and prevents p73α-dependent cell growth arrest in p53-dependent manner (Alisi, Giambartolomei

et al. 2003; Benard, Douc-Rasy et al. 2003). In addition, HCV core protein up-regulates the expression of transforming growth factor (TGF)-beta 1 and connective tissue growth factor (CTGF), suggesting that HCV core protein may contribute to the hepatic fibrogenesis (Taniguchi, Kato et al. 2004; Shin, Hur et al. 2005). Moreover, HCV core protein can active the Raf/MAPK signal pathway (Hayashi, Aoki et al. 2000; Erhardt, Hassan et al. 2002), and inhibit T-cell responsiveness through its interaction with complement receptor C1qR on T cells (Kittlesen, Chianese-Bullock et al. 2000). On the other hand, the NS5A protein was found to be involved in a large number of cellular functions by interacting with members of the cellular signaling apparatus, transcription activation machinery and cell cycle-regulatory kinases (e.g. p53, p21, growth factor receptor-bound protein 2). HCV NS5A binds directly to p53, inhibits its transactivation, leading to the inhibition of p21 expression (Majumder, Ghosh et al. 2001; Lan, Sheu et al. 2002). In addition, it also interacts with and sequesters hTAF(II)32 and hTAF(II)28, component of TFIID and essential coactivator of p53 (Lan, Sheu et al. 2002). Furthermore, NS5A interacts with growth factor receptor-bound protein 2 to inhibit mitogenic signaling while simultaneously promoting the PI3K-AKT cell survival pathway by interaction with p85 PI3K, which may represent a crucial step in HCV persistence and pathogenesis (He, Nakao et al. 2002). In summary, HCV proteins can inhibit p53 tumor suppressor functions and interfere with other signaling pathways to accelerate malignant transformation.

1.1.2.3 Aflatoxins

Aflatoxins are strong carcinogens for HCC development. Aflatoxins are naturally occurring mycotoxins that are produced by many species of *Aspergillus*, a fungus, most notably by *Aspergillus flavus* and *Aspergillus parasiticus*. These fungi grow readily on foodstuff, such as corn and peanuts that are stored in warm and damp conditions. Although there are at least 13 different types of aflatoxins produced in nature, aflatoxins B1 (AFB1) is the most potent carcinogen in animal studies. After entering the body, aflatoxins is metabolized by the liver to a reactive epoxide

intermediate, AFB1-exo-8,9-epoxide, which is later detoxified through a series of metabolic processes. The AFB1-exo-8,9-epoxide is able to react with guanine nucleotides in the hepatocyte DNA to form a number of adducts (Wild and Turner 2002). These DNA adducts, or secondary DNA damage arising from them, consequently lead to genetic changes that push the hepatocytes toward transformation (Smela, Currier et al. 2001). The characteristic genetic change is the G:C to T:A transversion on the third base in condon 249 of p53 (resulting in an Arg→ Ser alternation in the p53 protein). Of significance, high frequency of p53 249^{ser} mutation has been observed in HCC patient from China and Southern Africa, where high level of aflatoxin was found in daily diet (Wang and Groopman 1999). Another study revealed that polymorphism of the X-ray repair cross-complement 3 (XRCC3) gene Thr241Met may be associated with the risk of AFB1-related HCC among the Guangxi population (Long, Ma et al. 2008). In regions where exposure to aflatoxin is high and HBV infection is prevalent, these two risk factors may act synergistically to push HCC carcinogenesis (Albano, French et al. 1999; el-Serag 2001) .

1.1.2.4 Alcohol

Heavy alcohol intake, defined as daily ingestion of more than 50-70 g/day for prolong period of time, is an important HCC risk factor. A meta-analysis has shown a dose response relationship between alcohol consumption and HCC with relative risks (RRs) of 1.19, 1.40 and 1.81 for 25, 50 and 100 g of alcohol intake per day, respectively (Corrao, Bagnardi et al. 2004).

Despite the increasing understanding of the effects of alcohol on liver functions, the evidence of a direct carcinogenic effect of alcohol on hepatocytes is scarce. However, it has been suggested that it is the hepatic metabolism of alcohol rather than alcohol *per se that* underlies the mechanism by which alcohol cause hepatocyte damage. Ethanol is metabolized through three main catabolic / metabolic pathways including the alcohol dehydrogenase (ADH), catalase and microsomal ethanol oxidizing system (comprising predominantly of inducible cytochrome P450 (CYP2E1). ADH-dependent ethanol metabolism involves the conversion of ethanol to

acetaldehyde to acetate. These reactions result in the production of acetaldehyde and free radicals, which can bind to and rapidly damage cell constituents including proteins and DNA (Ekstrom and Ingelman-Sundberg 1989; Albano, French et al. 1999; Wu and Cederbaum 2003). CYP2E1 was induced after chronic excessive alcohol intake (Morimoto, Hagbjork et al. 1993; Dupont, Lucas et al. 1998; Lieber and Leo 1998). Similar to ADH-dependent metabolism, induction of CYP2E1 also leads to the conversion of ethanol to acetaldehyde and production of free radicals which lead to cellular damage through lipid peroxidation (Morimoto, Hagbjork et al. 1993; Dupont, Lucas et al. 1998; Lieber and Leo 1998). In addition, CYP2E1 induction is associated with changes in cell cycle progression (Kwon, Jovanovic et al. 2003; Hoffler, Dixon et al. 2005) and nutritional aberrations of folate, zinc, vitamin A (Barch, Kuemmerle et al. 1984; Djordjevic, Nikolic et al. 1998; Esfandiari, Villanueva et al. 2005). Importantly, CYP2E1 activity can alter immune system responses leading to increased susceptibility to HBV and HCV infection (Djordjevic, Nikolic et al. 1998; Albano, French et al. 1999; McKillop and Schrum 2005).

1.1.2.5 Nonalcoholic fatty liver disease

Nonalcoholic fatty liver disease (NAFLD) is the most common etiology of chronic liver disease in the United States and other developed countries. It encompasses a clinicopathologic spectrum of disease ranging from isolated hepatic steatosis to non- alcoholic steatohepatitis (NASH). NASH is the more aggressive form of fatty liver disease (Ong and Younossi 2007). It may account for a large proportion of idiopathic or cryptogenic cirrhosis (CC), which predisposes these patients to the development of HCC (Bugianesi 2007). The overall incidence of HCC arising in cryptogenic cirrhotic patients is reported to be 6.9 % (Bugianesi, Leone et al. 2002).

1.1.3 Genetic and epigenetic changes in HCC

The development of HCC proceeds through a multistep process that is

comparatively less well characterized than that of other type of cancers. As Figure 1.3 shown, various HCC etiological agents cause constant cycle of damage followed by repair, resulting in chronic liver injury. Continuous cycles of destructive-regenerative process fostered a chronic liver disease condition that culminates in liver cirrhosis. Subsequently, hyperplastic nodules will be formed, followed by the formation of dysplastic nodules, which may finally evolve to HCC. Molecular analysis of human HCC revealed many genetic and epigenetic alternations including chromosomal aberrations, DNA mutations, HBV integration associated DNA rearrangements, CpG island methylation, DNA hypomethylation, deletions and amplifications and microsatellite instability.

1.1.3.1 Chromosomal and genetic alternations in hepatocarcinogenesis

Many efforts have been directed towards charting the genomic events in HCC, with the main goals of understanding the genetic basis of this disease and identifying new therapeutic targets. Recent studies implicated a relationship between chromosomal instability (CIN), which occurs at an early stage and accumulates during hepatocarcinogenesis, and the development of malignant phenotype (Wong and Ng 2008). The most frequently deleted chromosome regions are found to be on 1p, 4q, 8p, 16q, 16p, 9p, 13q, 6q and 17p, whereas the most frequent chromosomal gains are on 1q, 8q, 20q and 17q (Fujimoto, (Fujimoto, Hampton et al. 1994; Boige and Laurent-Puig 1997; Marchio, Meddeb et al. 1997; Nagai, Pineau et al. 1997; Piao, Park et al. 1998; Guan, Fang et al. 2000; Wong, Lai et al. 2000; Balsara, Pei et al. 2001; Laurent-Puig, Legoix et al. 2001; Nishimura, Nishida et al. 2002). Moreover, high levels of allelic losses seem to be associated with more advanced tumor stages and more aggressive tumor behavior, suggesting that cumulative loss of heterozygosity during tumor progression may lead to inactivation of genes that regulate cell growth and adhesion, therefore conferring selection advantage for increased aggressiveness of the tumor (Tamura, Nakamori et al. 1997). Several oncogenes and tumor-suppressor genes residing at the 'gain' and 'loss' of chromosome regions have been found. These include c-*myc* (8q24); *EMS1* (11q13), *RB* (13q13-14), liver related putative tumour

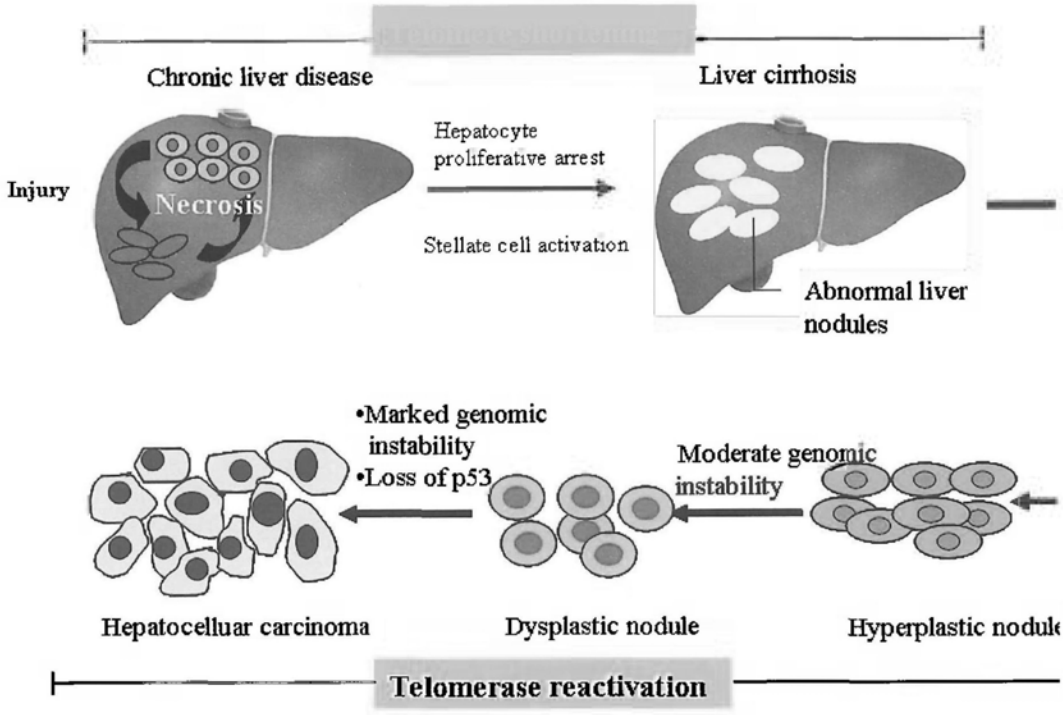


Figure 1.3 Histopathological progression of HCC. After hepatic injury (Hepatitis B virus infection, Hepatitis C virus infection, alcohol and aflatoxin B1), hepatocytes necrosis followed by hepatocytes proliferation occurs. Continuous cycles of necrosis-regenerative proliferation foster a chronic liver disease condition that culminates in liver cirrihosis. Subsequently, hyperplastic nodules are observed followed by dysplastic nodules, which can finally evolve to HCC. This figure was modified from a figure published in (Paraskevi et al., 2006)

suppressor (*LTPS*) (8p23.3); *TP53* (17p13.1); *cyclin A* (4q32-q-ter); and down-regulated in liver malignancy (*DRLM*) (4q21-22) (Fujimoto 1994; Fujimoto, Hampton et al. 1994; Hsu, Peng et al. 1994; Kuroki, Fujiwara et al. 1995; Yumoto, Hanafusa et al. 1995; Kawate, Fukusato et al. 1999; Liao, Zhao et al. 2000; Harada, Nagai et al. 2002). In addition, HBV-related HCC seems to show more frequent chromosomal aberration than HCV-related HCC (Zondervan, Wink et al. 2000). Moreover, losses at chromosome 4q, 16q, and 17p (including the TP53 region) appeared significantly more prevalent in HBV-associated liver cancers than non-viral HCC, suggesting these chromosomal abnormalities may be associated with HBV infection (Zondervan, Wink et al. 2000). Several other chromosomal loci have been specifically identified in HBV-, HCV-, or non-viral related HCC: a gain of 10q was detected exclusively in cases with HCV infection; whereas an amplification of 11q13 was more frequently found in HBV-positive HCC. Although these genomic alternation–aetiology correlations are intriguing, it has to be noted that several other studies have failed to uncover significant difference in chromosomal aberration between HBV- and HCV-associated HCC (Guan, Fang et al. 2000; Koo, Ihm et al. 2001; Thorgeirsson and Grisham 2002).

Telomere shortening limits the number of cell division and may affect the regenerative ability of organ system during aging and chronic diseases. Telomere shorting has been recognized as a key feature of chronic hyperproliferative liver disease, and the telomere length tends to decrease with the progression of chronic liver disease (Kitada, Seki et al. 1995; Urabe, Nouso et al. 1996; Miura, Horikawa et al. 1997). These observations have suggested that telomere shortening is associated with chronic liver disease, and hepatocyte turnover contributes to the induction of genomic instability that drives HCC development (Farazi and DePinho 2006). In this regard, analysis of telomere length of hepatocytes in HCC established a correlation between telomere shortening and increased chromosomal instability (Plentz, Caselitz et al. 2004; Plentz, Schlegelberger et al. 2005). Another aspect of telomere biology in HCC is the telomerase activation. The human telomerase reverse transcriptase (hTERT) mRNA was detected in nearly 90% (17/19) of livers with HCC and

telomerase activity was detected in nearly 89% (17/19) of liver tumor tissues (Nagao, Tomimatsu et al. 1999; Lee, Hsu et al. 2004; Shimojima, Komine et al. 2004). Moreover, telomerase re-activation has been reported to increase microvessel density and HCC recurrence after resection (Kobayashi, Kubota et al. 2001; Piao, He et al. 2004). Taken together, these data suggest a model wherein telomere shortening promote chromosomal instability during early stages of hepatocarcinogenesis, and telomerase reactivation restores chromosomal stability to a level compatible with cancer cell viability which driving malignant progression (Farazi and DePinho 2006).

1.1.3.2 Mutational analysis in hepatocellular carcinoma

In human HCC, *p53* and β-catenin probably are the most frequently mutated genes (Honda, Sbisa et al. 1998; Jayshree and Sridhar 2000; Jeng, Sheen et al. 2000; Ishizaki, Ikeda et al. 2004), but mutation have been found in many other genes, including breast cancer 1 (*BRAC1*), breast cancer 2 (*BRAC2*), Retinoblastoma (*Rb)*, adeomatous polyposis coli (*APC*), etc (Farshid, Hsia et al. 1994; Katagiri, Nakamura et al. 1996; Lin, Shi et al. 1996; Su, Abdalla et al. 2001). Recent study suggested that β-catenin mutations was associated with epigenetic alternations whereas p53 mutations correlated with high level of chromosomal instability (Nishida, Nishimura et al. 2007).

p53 gene is the most common tumor suppressor gene known to be mutated at very high frequency in different tumors. However, the frequency of p53 mutation in HCC only ranges from 13 to 33% in Asian countries (Ng, Chung et al. 1994; Ng, Srivastava et al. 1994; Ming 1998). In general, mutation of p53 was found in exons 5-9 in a random fashion. However, the hotspot of p53 mutation for HCC is the G:C to T:A transversion in codon 249 of exon 7 (Bressac, Kew et al. 1991). This point mutation is strongly associated with dietary AFB1 exposure or HBV infection, as evidenced in China and Africa (Hussain, Schwank et al. 2007). Mutant p53 has a much longer half-life than the wild-type p53, which results in the overexpression of this protein. Clinicopathological analysis of mutant p53 in HCC showed that overexpression of p53 is associated with poor cellular differentiation (Ng, Lai et al.

1995) and it may serve as a prognostic marker for poor clinical outcomes when combined with other biomarkers (Ivanov, Kolev et al. 2009).

β-catenin is a crucial downstream component of the Wnt signaling pathway, which plays a critical role in the control of cellular proliferation, motility, morphology and embryonic development (Moon, Bowerman et al. 2002). Mutation of β-catenin has been frequently reported. Most β-catenin mutation has found within exon of 3 at codon 32-37. As a consequence of the mutation, β-catenin is protected from degradation and results in its accumulation in the nuclei, leading to the activation of Wnt target genes (Terris, Pineau et al. 1999; Wong, Fan et al. 2001; Taniguchi, Roberts et al. 2002). β-catenin mutations and increased nuclear expression had been reported in human HCC (Edamoto, Hara et al. 2003; Ishizaki, Ikeda et al. 2004). Other reports also showed that β-catenin mutations and overexpression are related to early-stage HCC (Thorgeirsson and Grisham 2002; Peng, Chen et al. 2004). In addition, HCV-related HCC showed high frequency of mutation and nuclear accumulation of β-catenin than HBV-related HCC (Huang, Fujii et al. 1999; Hsu, Jeng et al. 2000). These findings indicated that both the function of mutant β-catenin and localization may be crucial in hepatocarcinogenesis.

1.1.3.3 Epigenetic changes in HCC

The term 'epigenetic' generally refers to heritable changes in DNA methylation and histone modification that modify gene transcription but do not involve changes of the DNA sequence (Bird 2007). In mammalian genome, DNA methylation is mainly found in the cytosine residues of CpG dinucleotides within the repetitive elements or promoter-related CpG islands. The hypermethylation of the CpG islands in the promoter regions has become the well-categorized epigenetic change to occur in tumors. CpG island methylation has been found in virtually every type of human neoplasm and is associated with the inappropriate transcriptional silencing of genes (Baylin and Herman 2000; Jones and Baylin 2002). Epigenetic alterations also play a very important role in human HCC. Aberrant DNA methylation has been detected in the earliest stages of hepatocarcinogensis, and to a greater extent during tumor

progression (Lee, Lee et al. 2003). Specific hypermethylation of events have been observed at the regulatory region of *p16(INK4a)*, Retinoblastoma (*Rb)*, E-cadherin, Ras association domain family 1A gene (*RASSF1A)*, *COX2*, Glutathione S-transferase pi-1 (*GSTSP1*), phosphate and tensin homolog (*PTEN*), suppressor of cytokine signaling (*SOSC-1*), deleted in liver cancer 1 (*DLC1*) and apoptosis-associated speck-like protein (*ASC*), etc (Liew, Li et al. 1999; Matsuda, Ichida et al. 1999; Wong, Lo et al. 1999; Yoshikawa, Matsubara et al. 2001; Zhong, Tang et al. 2002; Wong, Lee et al. 2003; Kubo, Yamamoto et al. 2004; Murata, Tsuji et al. 2004; Wang, Wang et al. 2007).

The well-known tumor suppressor *p16(INK4A),* also known as cyclin-dependent kinase inhibitor 2A, is frequently inactivated in HCC by promoter methylation (Liew, Li et al. 1999; Pang, Ng et al. 2003). *p16* is an important component of the cell cycle. It regulates the G_1-S cell cycle transition by inhibiting the cyclin D-cyclin-dependent kinase CDK4/CDK6-mediated phosphorylation of retinoblastoma protein (pRb). Analysis of methylation status of p16 gene in HCCs and its surrounding dysplastic nodules (DNs) and cirrhotic nodule (CNs) showed that the frequency of p16 hypermethylation increased progressively from CNs (15/24, 62.5%) to DNs (26/37, 70.3%) to HCC (15/18,83.3%) (Shim, Yoon et al. 2003). Loss of p16 function is also associated with the phosphorylation of tumor suppressor pRb, thereby limits its function, resulting in unregulated cellular proliferation (Serrano, Hannon et al. 1993). On the other hand, hepatoma cells treated with demethylating agent increases *p16(INK4A)* expression that is associated with cell growth inhibition (Maeta, Shiota et al. 2005).

Human RASSF1 gene generates two transcriptional variants, namely RASSF1A and RASSF1C, from distinct CpG island promoters. Analysis of the methylation status of the *RASSF1A* in primary liver tissues demonstrated that 93% (14/15) of HCC were methylated at the CpG island of *RASSF1A* and hypermethylation was independent of hepatitis virus infection (Schagdarsurengin, Wilkens et al. 2003). Furthermore, hypermethylation of RASSF1A in HCC correlates with AFB1-induced DNA damage, suggesting that exposure to environmental carcinogens may be

involved in altered methylation of genes involved in cancer development (Zhang, Ahsan et al. 2002). Suppression of RASSF1A expression by methylation causes mitotic defects, leading to HCC development (Macheiner, Gauglhofer et al. 2009).

Other tumor suppressor genes that regulate various cellular pathways have been also characterized. Deleted in liver cancer 1 (DLC1), a negative regulator of Rho family GTPases, was frequently inactivated via hypermethylation in human HCC (Wong, Lee et al. 2003). E-cadherin is one of the calcium-dependent cell-cell adhesion glycoprotein expressed in the epithelial cells. CpG methylation around the promoter region of the E-cadherin gene was detected in 67% of HCCs examined compared to 46% of liver tissues showing chronic hepatitis or cirrhosis (Kanai, Ushijima et al. 1997).

1.1.4 Signaling pathways related with HCC

As the consequence of the genetic and epigenetic changes discussed above, deregulation of various signaling pathways, such as Wnt/β-catenin, Ras, transforming growth factor-β (TGF-β), p14ARF/p53, p16INK4A/Rb and PTEN/Akt pathways, has been associated with hepatocarcinogesis,. In the following section, I will briefly summarize the role of two of the most common cellular pathways, namely the Wnt/β-catenin and Ras pathway, in the pathogenesis of human HCC.

1.1.4.1 Wnt/β-catenin signaling pathway

Wnt/β-catenin signaling pathway plays a critical role in embryogenesis, cell proliferation, motility and morphology (Moon, Bowerman et al. 2002). β-catenin is the central player in the Wnt signaling pathway. In the absence of Wnt signaling, β-catenin is bound to the degradation complex consisting of Axin, APC and glycogen synthase kinase (GSK)-3β and is phosphorylated by GSK-3β at the serine and threonine residues. The phosphorylated β-catenin is then degraded via the ubiquitin-proteasome system that maintains the cytoplasmic β-catenin at a low level. Upon binding of Wnt ligands, Fizzled and LRP5/6 (low density lipoprotein receptor-related protein), to the transmembrane co-receptors, the Wnt/β-catenin

signaling transduction is triggered. This is followed by the phosphorylation of the signaling transducer Dvl (disheveled), which then binds to and suppresses the activity of GSK-3β, thereby stabilizing β-catenin. Accumulation of the phosphorylated β-catenin in the cytoplasm facilitates its nuclear translocalization, where it functions as co-activator of TCF/LEF (T-cell factor/lymphoid enhancing factor) to upregulate proto-oncogenes expression, such as *Cyclin D1* and *c-myc* (He, Sparks et al. 1998; Brabletz, Jung et al. 1999; Shtutman, Zhurinsky et al. 1999).

Activation of Wnt/β-catenin signaling pathway by APC gene mutations has been reported in colon cancer (Cottrell, Bicknell et al. 1992). Unlike colon cancer, APC mutations are infrequent in HCC, but β-catenin and Axin 1 mutations are frequently identified (de La Coste, Romagnolo et al. 1998; Huang, Fujii et al. 1999; Hsu, Jeng et al. 2000; Satoh, Daigo et al. 2000; Taniguchi, Roberts et al. 2002). In human HCC, β-catenin mutations and nuclear localization ranged from 13-34% and 11-43% respectively (de La Coste, Romagnolo et al. 1998; Huang, Fujii et al. 1999; Hsu, Jeng et al. 2000; Taniguchi, Roberts et al. 2002). HCV-associated HCC was reported to show higher frequencies of both β-catenin mutations and nuclear accumulation than HBV-associated HCC. β-catenin mutations are also mutated in some hepatoblastomas and hepatocelluar adenomas (Koch, Denkhaus et al. 1999; Wei, Fabre et al. 2000). Moreover, adenomas with beta-catenin activation was reported to have a higher risk for malignant transformation (Zucman-Rossi, Jeannot et al. 2006). The AXIN1 gene is located on the chromosome 16p13 which is deleted in about 30% of HCC cases (Laurent-Puig, Legoix et al. 2001). In addition, biallelic inactivations of AXIN1 via mutations associated with LOH or homozygous deletion are found in 5-8% of HCC (Satoh, Daigo et al. 2000; Laurent-Puig, Legoix et al. 2001). In these tumors, inactivation of Axin 1 prevents phosphrylation of β-catenin which results in the accumulation of β-catenin and subsequent activation of Wnt target genes. As for the positive regulators, Wnt ligands and frizzled receptors were found to be overexpressed in HCC and is associated with β-catenin accumulation and activation respectively (Merle, Kim et al. 2005). Likewise, overexpression of Dvl-1 and Dvl-3 are found in human HCC (Chan, Chan et al. 2006). Apart from the above-mentioned

genes, other members of Wnt/β-catenin signaling pathway, such as LPR5/6 and their antagonists, Dickkopf 1 (DKK1), are also subjects of investigation (Niehrs 2006).

1.1.4.2 Ras-Raf-MEK-ERK signaling pathway

Ras-Raf-MEK-ERK pathway is one of the most significant cellular signaling sequences in the development and maintenance of HCC. The Ras signaling is initiated when a ligand binds to its tyrosine kinase receptors, such as the epidermal growth factor receptor (EGFR), the insulin-like growth factor receptor (IGFR) or the vascular endothelial growth factor receptor (VEGFR), which induces phosphorylation of tyrosine residues on the cytoplasmic domain of the receptor. Subsequently, Ras protein is recruited to the activated receptor and converted to its active GTP-bound form by Son of Sevenless Protein (SOS). Activation of Ras in turn activates the serine threonine kinases of the Raf-family. Once activated, Raf phosphorylates MEK1/2 kinases which leads to the activation of ERK1/2. Activated ERK1/2 translocates to the nucleus and regulates the expression of target genes including *c-Fos, CREB* (cAMP responsive element binding protein), *Elk1, c-Jun* and *c-myc*. The dysregulation of this pathway is mainly due to the oncogenic transformation of Ras and Raf isforms, or overexpression of the Ras and Raf genes (Hopfner, Schuppan et al. 2008). Mutation of Ras genes in HCC is rare. However, overexpression of Ras protein has been reported in human HCC and cirrhotic liver (Nonomura, Ohta et al. 1987). In spite of the low incidence of Ras gene mutations, downregulation of the physical inhibitor of Ras-Raf-MEK-ERK pathway has been frequently found human HCC. Inactivation of the inhibitors might result in persistent activation of the downstream pathway during hepatocarcinogenesis. For example, Raf-1 kinase inhibitory protein (RKIP) was downregualted in human HCC which leads to increased ERK1/2 activity (Schuierer, Bataille et al. 2006). In contrast, ectopic expression of RKIP suppressed the Raf activity and in turn altered HCC cell proliferation and migration (Lee, Tian et al. 2006). Similarly, Spred-1, another inhibitor of Ras-Raf-MEK-ERK pathway, was frequently underexpressed in human HCC. The expression level of it was correlated with the incidence of tumor invasion

and metastasis (Yoshida, Hisamoto et al. 2006). Despite its important function in cellular transformation, it is worth noting that Ras signaling may induce cell senescence or apoptosis, particularly in untransformed cells (Serrano, Lin et al. 1997; Downward 1998). The RAS-association domain family (RASSF1) can directly interact with Ras to induce Ras-mediated apoptosis (Khokhlatchev, Rabizadeh et al. 2002). These data suggested that RASSF1 may function as a gatekeeper against the oncogenic activity of Ras. In human HCC, RASSF1 is frequently epigenetic silenced via hypermethylation of the CpG island (Schagdarsurengin, Wilkens et al. 2003; Zhong, Yeo et al. 2003). Loss of RASSF1 expression in HCC may shift the balance of RAS activities towards grow promotion.

1.2 The Silent Information Regulator 2 (SIR2) family

The silent information regulator 2 (SIR2) family, or sirtuins, is a group of nicotinamide (NAD^+)-dependent deacetylase/ADP-ribosyltransferases initially discovered in yeast. SIR2 proteins are conserved in diverse organisms from yeast to flies and to worms. In the past decade, substantial evidence has suggested that SIR2 family is involved in the regulation of aging and caloric restriction (CR)-induced longevity in lower organisms such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans* (Tissenbaum and Guarente 2001) and *Drosophila melanogaste*r (Rogina and Helfand 2004). Recently, evidences suggested that sirtuins are involved in the regulations of various aspects of CR response including fat metabolism (Picard, Kurtev et al. 2004), stress resistance (Brunet, Sweeney et al. 2004; Cohen, Miller et al. 2004), glucose homeostasis (Rodgers, Lerin et al. 2005), insulin secretion (Moynihan, Grimm et al. 2005; Bordone, Motta et al. 2006), and physical activity (Chen, Steele et al. 2005).

1.2.1 *SIR2* and aging in non-mammalian species

SIR2 was first identified as one of the four genes (*SIR1-4*) that mediates the silencing of the extra copy of mating-typing information loci in yeast (Klar, Fogel et

al. 1979). Ten years after this discovery, Gottlied and Esosito (Gottlieb and Esposito 1989)1989) demonstrated that *SIR2* is the only *SIR* gene required to suppress recombination of ribosomal DNA (rDNA), which contains 100–200 tandem repeats on chromosome 12. Subsequently, it has been shown that *SIR2* is involved in gene silencing near telomere (Aparicio, Billington et al. 1991). Later, Sinclair and Guarente found that the pace of aging in yeast is set by the generation of the extrachromosomal ribosomal DNA circles (ERCs) through homologous recombination within the rDNA repeats (Sinclair and Guarente 1997). The accumulation of ERCs leads to enlarged nucleoli, cell-cycle arrest and cellular senescence. Consequently, Kaeberlein et al showed that increased the dosage of *SIR2* extends the replicative lifespan, whereas the deletion of *SIR2* accelerates the generation of ERCs and thus, shorten lifespan of yeast (Kaeberlein, McVey et al. 1999).

Sir-2.1, the ortholog of SIR2 in Caenorhabditis elegans, extends the lifespan of the worm by a distinct mechanism (Tissenbaum and Guarente 2001). In *C*.elegans, downregulation of the insulin-IGF pathway results in dauer formation during development and lifespan extension in adults (Kenyon 2001). *Sir-2.1* requires the forkhead transcription factor Dauer Formation Protein 16 (DAF-16) for lifespan extension (Tissenbaum and Guarente 2001). In response to stress, *sir-2.1* binds to DAP-16 in a way that is dependent on a class of scaffolding proteins termed 14-3-3 (Berdichevsky, Viswanathan et al. 2006). The formation of this complex results in the transcription activation of the DAF-16 target genes. In *Drosophila melanogaster*, an extra copy of *Sir2* gene extends lifespan, whereas a decrease in *Sir2* blocks the life-span-extending effect of calorie reduction (Rogina and Helfand 2004).

It is intriguing that *SIR2* defines lifespan in various organisms, despite its diverse regulatory mechanisms in different species. The relationship between *SIR2* and lifespan may be explained by its unique enzymatic activity as nicotinamide adenine dinucleotide (NAD)-dependent deacetylase, which connects *SIR2* activity to cellular energy state (Imai, Armstrong et al. 2000). The close relationship between *SIR2* and NAD places *SIR2* at a central position as a mediator of caloric restriction (CR) and

lifespan. CR is a dietary regimen that extends the lifespan from yeast to mammals. SIR2 is required for lifespan extension by moderate CR in yeast, worms and flies (Lin, Defossez et al. 2000; Rogina and Helfand 2004; Wang and Tissenbaum 2006). CR produces more oxidative metabolic state reflected by high levels of NAD^+ which is sensor of the redox state. NAD^+ in turn activates SIR2 members and facilitates survival mechanisms such as inhibition of apoptosis and senescence, activation of stress-response pathways (Vaquero, Sternglanz et al. 2007). However, the requirement for the *SIR2* in CR-induced longevity is not without controversy. CR-induced lifespan extension was independent of *SIR2* in the yeast strain BY4742 (Kaeberlein, Kirkland et al. 2004). One studies indicated that the severe CR condition (0.05% glucose) extends yeast lifespan by a different mechanism that is independent of *SIR2* (Kaeberlein, Kirkland et al. 2004; Lin and Guarente 2006). Finally, *SIR2* inactivation along with calorie restriction caused a dramatic chronological life-span extension (the length of yeast cell viability in a quiescent stationary phase) in certain long-lived mutant strains, which suggested that effects of *SIR2* on chronological life span are opposite to replicative life span (Fabrizio, Gattazzo et al. 2005). Further studies on this topic are necessary to reach a consensus.

1.2.2 The mammalian Sirtuins: SIRT1-7

The mammalian sirtuins family consists of seven members, namely SIRT1-7. These proteins are characterized by a highly conserved 275 amino acid NAD-dependent core domain, first identified in the founding yeast SIR2 protein (Frye 2000). SIRT1-7 are a very heterogeneous group of proteins that are involved in different functions and exhibit different cellular localization and enzymatic activities (Figure 1.4). In general, three mammalian sirtuins (SIRT1, SIRT6, and SIRT7) are predominantly localized to the nucleus (although SIRT1 also demonstrates some important cytoplasimc functions). In the nucleus, a majority of SIRT1 is associated with euchromatin, whereas SIRT6 is associated with heterochromatin, and SIRT7 is localized to the nucleolus (Michishita, Park et al. 2005). SIRT2 is the only sirtuins member with predominant cytoplasmic localization (North, Marshall et al. 2003). On

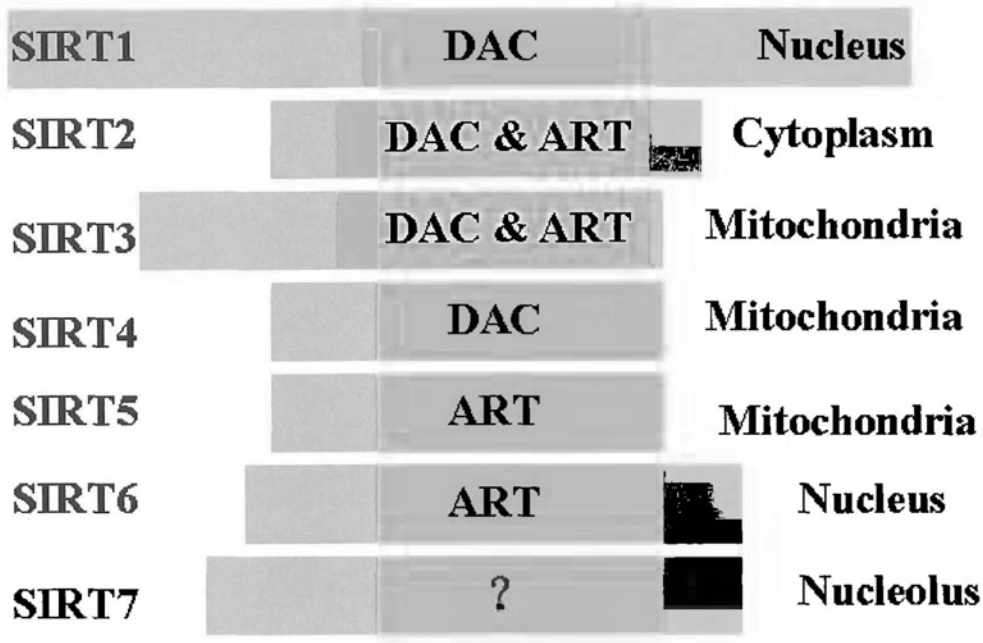


Figure 1.4 Mammalian sirtuins. SIRT1-7 has a NAD^+-dependent catalytic core domain that act as deacetylase (DAC) or mono-ADP-ribosyl transferase (ART) (yellow region). Additional N-terminal and/or C-terminal sequences of variable length may flank this core domain (blue region).These seven members exhibited different celluar localization.

This figure was modified from a figure published in (Shaday Michan et al. 2007).

the other hand, SIRT3, SIRT4, and SIRT5 are found to be localized to mitochondria. In terms of activity, SIRT1 and SIRT5 exhibit deacetylase activity (Vaziri, Dessain et al. 2001), whereas SIRT4 and SIRT6 are mono-ADP-ribosyl transferase (Liszt, Ford et al. 2005; Haigis, Mostoslavsky et al. 2006). Both deacetylase and mono-ADP-ribosyl transferase activities have been found in SIRT2 and SIRT3 (North, Marshall et al. 2003; Shi, Wang et al. 2005). No robust deactylase or mono-ADP-ribosyl transferase activities could be identified in SIRT7. Sirtuin enzymatic activity requires the metabolic cofactor β-nicotinamide adenine dinucleotide (NAD^+) (Imai, Armstrong et al. 2000; Landry, Sutton et al. 2000). The sirtuin-mediated deacetylation reaction involves hydrolysis of one NAD^+ and transfer of the acetyl group to ADP-ribose, leading to the formation of deacetylated substrate, nicotonimde and *O*-acetyl-ADP ribose as final products. As for the mono-ADP-ribosyl transferase, sirtuin can transfer ADP-ribose from NAD^+ to targeted proteins.

1.2.3 Function of SIRT1

Being a prototypical member of the sirtuin family, the biological role of SIRT1 has been subjected to intense investigation. Evidence suggested that mammalian SIRT1 plays a major role in metabolism, cell growth and differentiation, cellular stress response and tumor development. SIRT1 carries out these activities in part via regulating chromatin function through histone deaceylation. On the other hand, SIRT1 also regulates cellular activities via deacetylation of non-histone proteins. These SIRT1 substrates can be divided into three groups. The first are transcription related factors such as p53 (Luo, Nikolaev et al. 2001; Vaziri, Dessain et al. 2001), members of the FOXO family (Brunet, Sweeney et al. 2004; Daitoku, Hatta et al. 2004; Kobayashi, Furukawa-Hibi et al. 2005), PGC1-α (Rodgers, Lerin et al. 2005), PPARγ (Picard, Kurtev et al. 2004), NF-κB (Yeung, Hoberg et al. 2004; Chen, Zhou et al. 2005), E2F1 (Wang, Chen et al. 2006), etc. The second group includes chromatin-related enzymes such as histone acetyl-transferases (HATs) p300 (Bouras, Fu et al. 2005), the p300-associated factor (PCAF) (Fulco, Schiltz et al. 2003), the

DNA-dependent protein kinase subunit Ku80 (Cohen, Miller et al. 2004), and the transcriptional corepressors such as NcoR/SMRT (Picard, Kurtev et al. 2004). The third group includes signaling factors like Smad7 (Kume, Haneda et al. 2007).

1.2.3.1 SIRT 1 regulates chromatin structure and transcription

Similar to yeast Sir2, mammalian SIRT1 facilitates the formation of heterochromatin, which is a highly condensed region of the genome characterized by histone hypoacetylation and gene repression. SIRT1 is recruited to chromatin through interactions with a variety of transcription factors and coregulators, such as chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting proteins 1 and 2 (CTIP1 and CTIP2) (Senawong, Peterson et al. 2003; Senawong, Peterson et al. 2005). CTIP1 and CTIP2 enhance transcriptional repression mediated by COUP-TF and have been implicated in hematopoietic cell development and malignancies. Upon recruitment to chromatin, SIRT1 can deacetylate histone H3 lysine 9 ("H3K9"), H4 lysine 16 ("H4K16") and H1 lysine 9 and 26 ("H1K9, H1K26")(Imai, Armstrong et al. 2000; Vaquero, Scher et al. 2004). Besides direct deacetylation of histones, SIRT1 also interacts with histone-modifying enzymes and regulates their activities. For example, SIRT1 negatively regulates the activity of the histone acetyltransferase p300 by deactylating the lysine residues at positions 1020 and 1024 to promote hypoacetylation of nucleosome and affect gene expression outcomes (Bouras, Fu et al. 2005).

Histone acetylation and histone methylation are often coordinately regulated (Wang, Schones et al. 2009). In this regard, SIRT1 induces the formation of H3 tri-methyl K9 (H3-tri-MeK9) and H4 mono-methyl K20 (H4-mono-MeK20), two chromatin marks that are associated with repressed chromatin. It also induces the formation of H3 di-methyl K9 (H3-di-MeK9), a "boundary" separating active and inactive chromatin domains (Murayama, Ohmori et al. 2008). Although in most cases the underlying mechanisms for SIRT1-induced histone methylation remain elusive, several recent reports showed that SIRT1 can recruit histone methyltransferase (suppressor of variegation 3-9 homolog 1 or SUV39H1) to target site, thereby

regulating both acetylation and methylation (Vaquero, Scher et al. 2007). SIRT1 not only recruits and deacetylates SUV39H1 which resulting in increased levels of trimethylation of H3K9 at SIRT1 target site (Vaquero, Scher et al. 2007), it also form a complex with SUV39H1 and nucleomethylin, a nuclear protein that binds to H3K9me at the rDNA locus (Murayama, Ohmori et al. 2008). This complex senses cellular energy status and facilitates the formation of the silent chromatin at the rDNA locus through H3K9 deacetylation and methylation. These observations suggested that deacetylase activity of SIRT1 provides a critical link between energy status and rRNA transcription (Murayama, Ohmori et al. 2008). Interestingly, DBC1 (deleted in breast cancer) disrupts the SIRT1-SUV39H1 complex. Furthermore, DBC1 directly interacts with both SIRT1 and SUV39H1 catalytic domain and inhibits their activity (Kim, Chen et al. 2008; Zhao, Kruse et al. 2008; Li, Chen et al. 2009). These results suggested that DBC1 is an important regulator of heterochromatin formation and genomic stability by disrupting the SUV39H1-SIRT1 complex via inactivating both enzymes.

SIRT1 can also modulate chromatin function by promoting alterations in the methylation of DNA, leading to the repression of transcription. SIRT1 localized specially to the promoters of abberantly silenced tumor suppressor genes, such as E-cadherin, where the CpG islands are hypermethylated in breast cancer and colon cancer cells (Pruitt, Zinn et al. 2006). Inhibition of SIRT1 levels or activity increased H4-K16 and H3-K9 acetylation at such promoter and sufficed to induce reexpression of corresponding genes (Pruitt, Zinn et al. 2006). On the other hand, it has been shownt that SIRT1 can be recruited to the DNA near a double strand break (O'Hagan, Mohammad et al. 2008), which is required for the recruitment of DNA methyltransferae 3B (DNMTB) and the subsequent heritable methylation of CpG islands at the reporter gene promoter, leading to silencing of the associated gene (O'Hagan, Mohammad et al. 2008). These results implicated a critical role for SIRT1 in DNA methylation and epigenetic silencing, although details of the mechanism remain to be elucidated.

1.2.3.2 SIRT1 regulates cell survival and apoptosis

Besides histone modification, posttranslation modification of transcription factors, including acetylation, constitutes an additional level of transcriptional control. SIRT1 deactylates several transcription factors involved in the regulation of cell cycle progression and apoptosis, which may play a role in the pathogenesis of cancer (Figure 1.5).

1.2.3.2.1 SIRT1 regulates p53-dependent apoptosis

Besides histone, SIRT1 also plays a role in the regulation of p53. The activity of p53 can be regulated via phosphorylation, acetylation and ADP-ribosylation respectively. Acetylation of human p53 lysine residues (K320, K373 and K382) favours its stabilization and transactivation activation (Ito, Kawaguchi et al. 2002). SIRT1 functions as an NAD-dependent p53 deacetylase (Vaziri, Dessain et al. 2001). SIRT1 physically interacts with and deacetylates p53 at multiple lysine including K320, K373 and K382. Furthermore, SIRT1-dependent deacetylation of p53 inhibits its transactivation activity and represses apoptosis in response to DNA damage and oxidative stress (Luo, Nikolaev et al. 2001). The effect of SIRT1 on p53 is further supported by the observation that SIRT1 is recruited to the promyelocytic leukemia protein (PML) nuclear bodies where it binds to and deacetylates p53. SIRT1 antagonizes PML-induced acetylation of p53 and rescues PML-mediated premature cellular senescence (Langley, Pearson et al. 2002). On the other hand, p53 also plays a role in the regulation of SIRT1 activity. Two p53 binding sites that were identified in the SIRT1 gene promoter repress SIRT1 gene transcription (Nemoto, Fergusson et al. 2004) al. 2004). In addition, the tumor suppressor hypermethylated in cancer 1 (HIC1) forms a transcriptional repression complex with SIRT1, and this complex directly binds to the SIRT1 promoter and represses its transcription, and thereby attenuates p53-dependent apoptotic response (Chen, Wang et al. 2005). Since p53 is able to transactivates HIC1 transcription, it has been proposed that SIRT1-HIC1-p53 act in a complex feedback loop (Michan and Sinclair 2007). Since aging increases promoter hypermethylation and epigenetic silencing of HIC1, it was also speculated

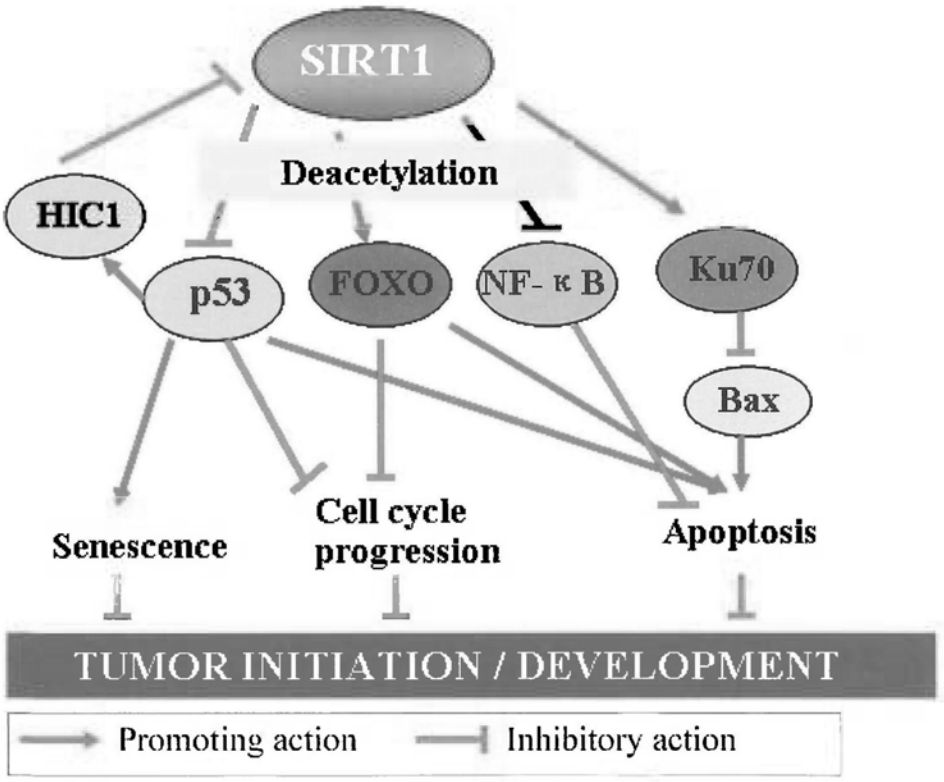


Figure 1.5 Molecular interconnection between SIRT1 and pathways involved in tumor initiation/ progression.

This figure was modified from a figure published in (Dimitrios Anastasiou et al. 2006).

that the resultant upregulation of SIRT1 may deacetylate p53 and increase the risk for neoplastic transformation and tumorigenesis (Chen, Wang et al. 2005). On the other hand, SIRT1-deficient cells exhibited p53 hyperacetylation after DNA damage, and SIRT1 mutant mice showed increased ionizing radiation-induced thymocyte apoptosis (Cheng, Mostoslavsky et al. 2003). However, other reports showed that inhibition of SIRT1 catalytic activity increased p53 acetylation but did not alter cell survival following DNA damage in certain cell lines (Solomon, Pasupuleti et al. 2006). Accordingly, the elimination of p53 in SIRT1-null mice does not relieve the phenotypes observed from mice deficient for SIRT1 alone. While SIRT1 interacted with p53, SIRT1 had little effect on p53-dependent transcription and did not affect the sensitivity of thymocytes and splenocytes to radiation-induced apoptosis (Kamel, Abrol et al. 2006). These discrepancies suggested that the relationship between SIRT1 and p53 is complex and the regulation of p53 by SIRT1 may involve the input of other mechanisms in addition to deacetylation.

1.2.3.2.2 The role of SIRT1 in E2F1- , Bax- and p73-mediated apoptosis

In addition to p53-dependent apoptosis, other mechanisms by which SIRT1 regulates cell survival have been reported. E2F1 has important role in regulating cell proliferation and apoptosis in neoplasia mainly through stimulating the transcription of several genes in the apoptotic pathway (Nahle, Polakoff et al. 2002). Once DNA damage occurs, E2F1 is overexpressed to induce premature S-phase entry and results in apoptosis (Johnson, Schwarz et al. 1993). E2F1 directly binds to the SIRT1 promoter and induces its expression at transcriptional level. Furthermore, SIRT1 binds to E2F1 and inhibits E2F1 activities, forming a negative feedback loop (Wang, Chen et al. 2006). This mutual regulation between E2F1 and SIRT1 protects against DNA damage in U20S and H1299 cancer cell lines (Wang, Chen et al. 2006).

Though DNA repair factor Ku70 can promote genomic stability and prevent the transformation of normal cells to cancer cells, Ku70 can also inhibit Bax-mediated apoptosis. DNA damage promotes Ku70 acetylation on multiple lysine residues, disrupting the association with Bax, which on release transits to the mitochondira and

initiates apoptosis (Cohen, Lavu et al. 2004). SIRT1 deacetylates Ku70, causing it to sequester the proapoptotic factor Bax away from mitochondria, thereby inhibiting stress-induced apoptotic cell death (Cohen, Miller et al. 2004). However, the site of SIRT1-mediated deacetylation of Ku70 is not yet clear.

The p53-related tumor suppressor p73 shares significant amino-acid sequence identity with p53. SIRT1 also binds to and deacetylates p73 to suppresses its transcriptional activity and inhibits apoptosis (Dai, Wang et al. 2007).

1.2.3.2.3 SIRT1 regulates stress resistance and cell survival mediated by Forehead Box Class O (FOXO) transcription factors

An alternative pathway by which SIRT1 promotes cell survival is through the regulation of FOXO transcription factors. FOXO transcription factors, including FOXO-1, FOXO-3a, FOXO-4 and FOXO-6, respond to DNA damage and oxidative stress and regulate expression of cell-cycle, DNA repair and apoptosis related genes (Furukawa-Hibi, Kobayashi et al. 2005). FOXOs can regulate cell fate by modulating the expression of genes involved in apoptosis (Fas ligand, proapoptotic BH3-only protein, Bim and TRAIL), in cell cycle progression (cyclin D1 and D2, p27/Kip1, Gadd45), and stress response (Gadd45, Mn-SOD). Similar to p53, the FOXOs are regulated by post-transcriptional modifications including phosphorylation and acetylation. In response to oxidative stress, FOXO proteins are phosphorylated by protein kinase B (Akt) and translocated into the nucleus. However, HATs may acetylate FOXO proteins and negatively regulates their transactivation activity (Fukuoka, Daitoku et al. 2003; Daitoku, Hatta et al. 2004; Motta, Divecha et al. 2004; van der Horst, Tertoolen et al. 2004). SIRT1 has been shown to deacetylate FOXO-1, FOXO-3a and FOXO-4. SIRT1 binds and deacetylates FOXO1 at residues acetylated by cAMP-response element-binding protein-binding protein (CBP). Moreover, deacetylation of SIRT1 modulates the transcriptional ability of FOXO1 and increases the expression of FOXO-targeted genes, such as manganese superoxide dismutase (MnSOD) and $p27^{kip1}$ (Daitoku, Hatta et al. 2004). This observation indicated that SIRT1 may promote cell survival by promoting cellular repair in response to

oxidative damage. The LXXLL motif of FOXO1 was found to be indispensable for its transcriptional activity and for SIRT1 binding (Nakae, Cao et al. 2006). In response to oxidative stress, SIRT1 forms a complex with FOXO3 and deacetylates it. SIRT1 had a dual effect on FOXO3 function by enhancing FOXO3's ability to induce cell cycle arrest and resistance to oxidative stress but also inhibits FOXO3's ability to induce cell death after cellular stress (Brunet, Sweeney et al. 2004). Peroxide stress induces FOXO4 acetylation and inhibits its transcriptional activity. Interestingly, SIRT1 enhances the transcriptional activity of FOXO4 through deactylation, thereby promotes mammalian cellular defense against oxidative stress (van der Horst, Tertoolen et al. 2004). In addition, the deacetylation of FOXO4 proteins by SIRT1 promotes expression of $p27^{kip}$ and growth arrest and DNA–damage-inducible α (GADD4α) to induce cell cycle arrest (Brunet, Sweeney et al. 2004; Kobayashi, Furukawa-Hibi et al. 2005). Thus, SIRT1 appears to promote cell-cycle arrest and DNA repair downstream of FOXO proteins, shifting FOXO-induce response away from apoptosis towards cell survival by deacetylation.

1.2.3.2.4 The role of SIRT1 in NF-κB-dependent transcription and cell survival

Apart from its anti-apoptotic function, SIRT1 may promote apoptosis under certain conditions via modulating nuclear factor NF-κB activity. NF-κB is dimeric transcription factor that regulates the expression of numerous genes controlling immune and inflammatory response, cell proliferation, differentiation and apoptosis. Phosphorylation of NF-κB on Ser276 recruits HAT-containing complex to target promoters, while acetylation of NF-κB affects both its DNA binding and transactivation activity (Chen, Mu et al. 2002; Zhong, May et al. 2002; Kiernan, Bres et al. 2003). SIRT1 physically interacts with the RelA/p65, the most prevalent form of NF-κB, and inhibits its transcriptional activity by deacetylating RelA/p65 at lysine 310. Deacetylation inhibits the transactivation potential of RelA/p65, which sensitizes cells to apoptosis in response to tumor necrosis factor alpha (TNF-α) (Yeung, Hoberg et al. 2004). Some studies have shown that inhibition of NF-κB in hepatocytes may promote hepatocarcinogensis (Maeda, Kamata et al. 2005; Luedde,

Beraza et al. 2007). Interestingly, SIRT1 can suppress NF-κB-mediated transcription through interacting with transducin-like enhancer of split-1 (TLE1) (Ghosh, Spencer et al. 2007). On the other hand, resveratrol, a small-molecule sirtuins activator, inhibits NF-κB-mediated transcription, but whether this effect is directly or solely through activation of SIRT1 is still unclear (Holmes-McNary and Baldwin 2000; Manna, Mukhopadhyay et al. 2000). As chronic inflammation and activation of NF-κB may contribute to several type of cancer, treatment with resveratrol may be a potential chemopreventative or chemotherapeutic agent.

1.2.3.2.5 The role of SIRT1 in cellular senescence

Primary cells undergo a limited number of cell divisions. Cell replications lead to progressive telomere shortening which ultimately induces replicative senesecence. Oncogene or other stress stimuli also induce the similar replicative senescence in cells (Hanahan and Weinberg 2000). Cellular senescence has been proposed as a major contributor to mammalian aging and also a tumor suppressor mechanism. Whether SIRT1 inhibits or contributes to cellular senescence is not clear. However, SIRT1 level decreases during replicative senescence in primary human fibroblasts, (Michishita, Park et al. 2005; Abdelmohsen, Pullmann et al. 2007).

Several tumor suppressor genes (such as p53, $p19^{ARF}$ and Rb) regulate senescence by inducing a permanent withdrawal from the cell cycle. p53 regulates the expression of several genes , including p21, PUMA and Bax, to initiate cell-cycle arrest, senescence or apoptosis (Vousden and Lu 2002). Increased p53 acetylation is associated with senescence induced by oncogenic Ras and promyelocytic leukemia protein (PML) is required for p53 acetylation and senescence upon oncogene expression. (Pearson, Carbone et al. 2000). It is expected that SIRT1 can block oncogene-induced senescence via inducing p53 deacetylation. In support of this notion, Langley et al has reported that SIRT1 antagonizes PML-induced acetylation of p53 and rescues PML-mediated premature cellular senescence in primary mouse embryonic fibroblasts (MEFs) (Langley, Pearson et al. 2002). In contrast, sirtinol, a SIRT1 inhibitor, induces senescence-like growth arrest in human breast cancer

MCF-7 and lung cancer H1299 cells. However, SIRT1 has also been shown to promote cellular senescence. Primary SIRT1$^{-/-}$ mouse embryonic fibroblasts exhibited greater proliferative capacity and were resistant to replicative senescence in response to prolonged cell culture (Chua, Mostoslavsky et al. 2005). However, the induction of senescence is not impaired in response to oncogene activation and DNA damage in MEFs lacking SIRT1 (Chua, Mostoslavsky et al. 2005). In addition, SIRT1 protein decreased significantly with serial cell passages in both human and murine cells or in dividing tissues of aged mice (Sasaki, Maier et al. 2006). A recent study showed that resveratrol，a sirtuin activator，induces senescence-like growth inhibition in osteocarcinoma U2OS cells. Part of this effect may due to the activation of SIRT1 (Rusin, Zajkowicz et al. 2009). The regulatory role of SIRT1 in senescence, whether positive or negative, could have influence on tumorigenesis in the elderly.

1.2.3.2.6 The role of SIRT1 in DNA repair and genome integrity

Two recent reports suggested that SIRT1 plays an important role in the repair of damaged DNA and maintaining genome stability. Wang et al found that SIRT1-deficient embryo cannot survive beyond middle gestation stages (Wang, Sengupta et al. 2008). Analyzing the *SIRT1*$^{-/-}$ mice during embryonic development showed that SIRT1 deficiency results in incomplete chromosome condensation and chromosome instability, impaired heterochromatin formation and abnormal mitosis (Wang, Sengupta et al. 2008). In addition, SIRT1 deficiency also decreases the ability to repair DNA-double strand breaks (DSBs) induced by γ–irradiation, characterized by reduced DNA foci formation that are composed of γ-H2AX, RAD51, BRCA1, and NBS1 respectively. This is associated with reduced expression of γ-H2AX, but not that of RAD51, BRCA1, and NBS1, in *SIRT1*$^{-/-}$cells. Consistently, γ-H2AX expression was restored when SIRT1 was reconstituted into *SIRT1*$^{-/-}$ cells (Wang, Sengupta et al. 2008). Moreover, the *SIRT1*$^{+/-}$*p53*$^{+/-}$ mice developed spontaneous tumor in multiple tissues. The tumors contained extensive aneuploidy and chromosomal aberrations including chromosome breaks and translocations,, chromosomal deletions and dicentric chromosomes (Wang,

Sengupta et al. 2008). These results suggested that SIRT1 deficiency severely impairs genome integrity and stability.

Studies also suggested that under basal state SIRT1 binds to and represses repetitive DNA as well as a functionally diverse set of genes across the mouse genome (Oberdoerffer, Michan et al. 2008). In response to DNA damage, SIRT1 dissociates from these loci and relocalizes to DNA breaks to promote repair and maintain genome integrity. Efficient recruitment of SIRT1 to damaged DNA requires DNA damage signaling through ATM and H2AX. Importantly, recruitment of both RAD51 and NBS1 are impaired in the absence of SIRT1. In addition, overexpression of SIRT1 in $p53^{-/-}$ mice promotes genomic stability and suppresses tumorigenesis in vivo (Oberdoerffer, Michan et al. 2008).

1.2.3.3 The role of SIRT1 in cancer

An area of considerable debate is whether SIRT1 plays a role in tumorigenesis and tumor progression. Reduced expression of SIRT1 has also been reported in some cancers (Wang, Zheng et al. 2008) , consistent with its putative role as tumor suppressor involved in DNA repair and cell growth suppression. However, increased expression of SIRT1 has also been associated with the tumorigenesis of prostate cancer (Huffman, Grizzle et al. 2007), colorectal cancer (Nosho, Shima et al. 2009), leukemia (Bradbury, Khanim et al. 2005) and skin cancer respectively (Hida, Kubo et al. 2007). SIRT1 is also the only histone deacetylase that is significantly overexpressed in leukemia lymphoblast as compared with normal lymphoblasts (Liu, Liu et al. 2009). Analysis of gene expression in CD133 cancer stem cells in glioblastoma revealed that SIRT1 mRNA expression is increased average 4.9 times in poorly differentiated, CD133 positive cells compared with more differentiated CD133 negative cells (Liu, Yuan et al. 2006). SIRT1 is also significantly increased in mice with poorly differentiated adenocarcinomas , as well as in human prostate cancer cells (Huffman, Grizzle et al. 2007). In human prostate cancer, HIC-1 may contribute to the increased expression of SIRT1 (Huffman, Grizzle et al. 2007). Concordantly, Jung et al also found that SIRT1 is overexpressed in human prostate

cancer (PCa) tissues as well as PCa cells, and inhibition of SIRT1 leads to FOXO1 acetylation and transcriptional activation in PCa cells (Jung-Hynes, Nihal et al. 2009). In human colorectal cancer tissues, SIRT1 is overexpressed in 37% (180/450) tumor (Nosho, Shima et al. 2009). SIRT1 expression is associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer, suggesting involvement of SIRT1 in gene silencing in this tumor subtype (Nosho, Shima et al. 2009). Moreover, overexpression of SIRT1 was frequently observed in all kinds of non-melanoma skin cancers including squamous cell carcinoma, basal cell carcinoma, Bowen's disease and actinic keratosis (Hida, Kubo et al. 2007). In tumor cells, SIRT1 promotes cell survival by deactylating cell cycle regulators, such as p53, p73, Rb, and FOXOs, and represses the transcription of tumor suppressors such as E-cadherin and MLH1(a mismatch repair protein) (Pruitt, Zinn et al. 2006). In addition, SIRT1 is overexpressed in chemo-resistant cancer cell lines derived from neuroblastoma, osteosarcoma, mammary and ovarian carcinomas respectively when compared to their drug-sensitive counterparts (Chu, Chou et al. 2005). Importantly, SIRT1 inhibition significantly reversed the resistance phenotype and reduced expression of the multidrug resistance molecule P-glycoprotein. Consistently, ectopic overexpression of SIRT1 induces P-glycoprotein expression and renders cancer resistant to the chemotherapy drug doxorubicin (Chu, Chou et al. 2005).

In contrast, several recent studies supported that SIRT1 serves as a tumor suppressor. Recent study found that SIRT1 expression is much lower in breast cancer type I susceptibility protein (BRCA1)-associated breast cancer than BRCA-wildtype breast cancer both in human and transgenic mouse (Wang, Zheng et al. 2008). The author further demonstrated that BRCA1 binds to the SIRT1 promoter and increases SIRT1 expression, and BRAC1 deficiency reduces SIRT1 expression, which may be responsible for the malignant transformation of BRCA1-mutant cells. Concordantly, overexpression of SIRT1inhibits BRCA1 mutant cell growth and tumor formation in vivo (Wang, Zheng et al. 2008). SIRT1 also negatively regulates the expression of anti-apoptotic genes in a BRCA-1 dependent manner. A tumor suppressive function of SIRT1 is also evident in other mouse models. The ectopic

induction of SIRT1 in a β -cantenin-driving mouse model of colon cancer significantly reduces tumor formation, proliferation and animal morbidity (Firestein, Blander et al. 2008). Furthermore, SIRT1 deacetylates β -catenin and suppresses its ability to activate transcription and drive cell proliferation (Firestein, Blander et al. 2008). Subsequent studies showed SIRT1 knockdown increase the growth of colon cancer xenografts (Kabra, Li et al. 2009). In particular, increased SIRT1 expression results in sarcoma and lymphoma development in p53-heterozygous mice (Wang, Sengupta et al. 2008). The author further found that *SIRT1*$^{-/-}$ mouse embryonic fibroblasts displayed chromosome aneuploidy and DNA damage repair defects (Wang, Sengupta et al. 2008).

Whether SIRT1 acts as an oncogene or as tumor suppressor remains to be determined. Nevertheless, these findings provide evidence that SIRT1 plays a critical regulator in cancer development.

1.2.3.4 SIRT1 functions in metabolic regulation

Organismal energy homeostasis is the balanced regulation of energy intake, storage, and expenditure. Disturbance of energy homeostasis can lead to many diseases such as diabetes, obesity, and heart failure. There has been intense scrutiny of the role of sirtuins in metabolic pathways. Although the detailed function of SIRT1 are not yet fully elucidated, growing evidence suggested that SIRT1 senses nutritional availability and relays this information to effectors that govern energy utilization and adaptation (Haigis and Sinclair 2010).

1.2.3.4.1 SIRT1 in homeostatic functions: regulating glucose metabolism

Blood glucose concentration is controlled within a narrow range under different physiology conditions. Under starvation, maintenance of serum glucose is achieved in part by implementing a program of hepatic gluconeogenesis (Finkel, Deng et al. 2009). During fasting, hepatocytes induce gluconeogenesis to supply other tissues with glucose. Several studies have shown that this nutrient response is under control of SIRT1 activity. Regulation of hepatic glucose production is an important

physiologic function of FOXO1. In hepatocytes, SIRT1 binds to and deacetylates FOXO1, leading to the induction of FOXO1-dependent transcription of glucogenetic genes, including glucose-6-phosphatase (*G6pc*) and phosphoenolpyruvate carboxykinase (*Pck1*), as well as insulin-like growth factor-binding protein 1 (*Igfbp1*) (Frescas, Valenti et al. 2005). In the liver, the transcription factor PPAR gamma coactviator 1 alpha (PGC-1α) is another key regulator of glucose production through activation of the entire gluconeogenic pathway. SIRT1 interacts with PGC-1α in a ternary complex with hepatocyte nuclear factor (HNF-4), an essential transcription factor in PGC-1α's gluconeogenic function. In this protein complex SIRT1 is likely to be the sensor for nutrient fluctuations via NAD^+ and induces PGC-1α-dependent gluconeogenic gene expression and hepatic glucose output following fasting (Rodgers, Lerin et al. 2005).

In addition to the regulation of hepatic gluconeogenesis, SIRT1 also regulates serum glucose levels by regulating insulin secretion. In response to glucose, insulin is secreted by the β-cells in the pancreatic islets of Langerhans to promotes glucose intake and catabolism in peripheral tissues (Anastasiou and Krek 2006). Two studies using transgenic mice demonstrated that SIRT1 positively regulates glucose-induced insulin secretion in pancreatic β-cells (Moynihan, Grimm et al. 2005; Bordone, Motta et al. 2006). Moynihan et al showed that increased dosage of SIRT1 in pancreatic beta cells improves glucose tolerance and enhances insulin secretion in response to glucose in beta cell-specific SIRT1-overexpressing transgenic mice (BESTO) (Moynihan, Grimm et al. 2005) . Microarray analyses of beta cells revealed that SIRT1 regulates genes involved in insulin secretion, including mitochondrial uncoupling protein 2 (UCP2). Consistently, Bordone found that SIRT1 positively regulates insulin secretion in pancreatic beta cells and SIRT1 mice show blunted insulin secretion. Moreover, they further demonstrated that SIRT1 represses the gene UCP2 by binding directly to the UCP2 promoter to affect insulin secretion (Bordone, Motta et al. 2006). UCP2 is a mitochondrial inner membrane protein with ubiquitous tissue distribution, mediating proton leak across the inner membrane by uncoupling substrates oxidation from ATP synthesis. Thus, SIRT1 can promote more efficient

mitochondrial ATP production by suppressing UCP2 expression, which subsequently promotes insulin secretion. Indeed, Islets from BESTO mice show enhanced insulin secretion with decreased amounts of UCP2 and increased ATP production (Moynihan, Grimm et al. 2005). Taken together, these data implicates that SIRT1 acts as a positive regulator of insulin secretion in wild-type cells by repressing UCP2, thereby allowing coupling of glucose metabolism to ATP synthesis.

Furthermore, SIRT1 has a protective role in promoting the survival of pancreatic β-cells during oxidative stress. Under normal conditions, FOXO1 is rapidly excluded from the nucleus in β cells. In response to hyperglycemia (or H_2O_2), acetylated FOXO1 is retained in the nucleus, where it engages Pml and SIRT1. Deacetylation of FOXO1 by SIRT1 promotes FOXO1-dependent transcription of *NeuroD* and *MafA*, two transcription factors required for the expression of the *Insulin2* (*Ins2*) gene to prevent apoptosis (Kitamura, Kitamura et al. 2005). Based on this finding, SIRT1 seems to play a protective role against glucose-induced cytotoxicity in pancreatic β-cells, an underlying cause of β-cells degeneration seen in diabetic patients with chronically high serum glucose levels (Anastasiou and Krek 2006).

1.2.3.4.2 SIRT1 function in lipid metabolism

White adipose tissue (WAT) is another major site of metabolic regulation. WAT functions to store triglyceride, an important energy source when glucose availability is limited. During starvation or fasting, adipose triglyceride stores are mobilized to free fatty acid, which can be utilized by other tissue for energy production (Flier 2004). In addition to energy store, WAT also serves as an endocrine organ by secreting hormones, such as adiponection, leptin and inflammatory cyokines. The accumulation of WAT during aging could lead to insulin resistance, type 2 diabetes and atherosclerosis (Gabriely and Barzilai 2001).

In cultured adipocytes, SIRT1 promotes fat mobilization by repressing PPAR-γ (peroxisome proliferator-activated receptor-gamma) activity by docking with its cofactors NCoR (nuclear receptor co-repressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptors) (Picard, Kurtev et al. 2004). Consistently,

SIRT1$^{-/-}$ mice showed lower levels of blood fatty acid released from WAT upon fasting compared with wild type mice (Picard, Kurtev et al. 2004). These results suggested that starvation of animals causes SIRT1 to interact with PPAR-γ DNA-binding sites and thereby represses target genes that drive fat storage. Acetyl-CoA is a small molecule which is central to the synthesis of fatty acids, amino acid, the tricarboxylic acid cycle and ketone bodies. Acetyl-CoA synthetase 1 (AceCS) catalyzes the formation of acetyl-CoA from acetate, CoA and ATP. SIRT1 deacetylates and thereby actives AceCS1(Hallows, Lee et al. 2006). SIRT1 expression also led to a pronounced increase in AceCS1-dependent fatty-acid synthesis from acetate. Considering SIRT1 is induced by calorie restriction, it is plausible that SIRT1 alters the rate of fatty acid synthesis and regulates how much is shuttled into the tricarboxylic acid cycle for ATP production upon food limitation. Adiponectin is an adipocyte-derived hormone that enhances insulin sensitivity, improves fatty acid oxidation in skeletal muscle, and suppresses hepatic gluconeogenesis. SIRT1 also increases adiponectin transcription in adipocytes by activating FOXO1 and enhancing FOXO1 and C/EBP alpha interaction (Qiao and Shao 2006). Importantly, low level of SIRT1 and FOXO1 expression was found in the adipose tissues from obese and diabetic mice (Qiao and Shao 2006). Adiponectin gene expression is diminished in both obesity and type2 diabetes, however the underlying mechanism is poor understand. This observation suggested that decreased SIRT1 and FOXO1 expression and impaired FOXO1-C/EBPα transcription complex formation may contribute to the diminished adiponectin gene expression in obesity and type 2 diabetes.

1.2.3.4.3 Cardioprotective roles of SIRT1

Cardiovascular disease (CVD) is the most prevalent disease worldwide. Growing experimental evidence suggests that SIRT1 has both direct and indirect effects on the cardio-vascular system by improving cardiac function and vascular function and repair. SIRT1 plays an important role in cardiac development and the growth of myocardial cells to maintain cardiac function. SIRT1-null embryos show developmental defects in the heart (Cheng, Mostoslavsky et al. 2003; McBurney,

Yang et al. 2003), while the expression of SIRT1 is high in the heart in the wild-type embryos during embryogenesis (Sakamoto, Miura et al. 2004). In isolated neonatal cardiomyocytes, inhibitors of SIRT1 activity cause a moderate increase in basal cell death and an upregulation in the expression of the hypertrophy-associated genes such as atrial naturietic factor (ANF). In addition, overexpression of SIRT1 protects cardiac myocytes from apoptosis in response to serum starvation and significantly increased the size of cardiac myocytes (Alcendor, Kirshenbaum et al. 2004). This result suggests endogenous SIRT1 plays an essential role in mediating cell survival and maintaining modest hypertrophy. Importantly, SIRT1 expression was increased significantly in hearts from dogs with heart failure induced by rapid pacing superimposed on stable, severe hypertrophy, which suggesting SIRT1 has cardioprotective effect in pathologic hearts in vivo during heart failure (Alcendor, Kirshenbaum et al. 2004). Pillai et al. also provides evidence supporting the protective role of SIRT1 against heart failure. Activation of poly (ADP-ribose) polymerase-1 (PARP) by oxidative stress has been regards as a major cause of caspase-independent myocyte cell death contributing to heart failure. Increased activity of PARP correlates with reduced SIRT1 deacetylase activity both in failing hearts tissue and cultured cardiac myocytes. Moreover, myocyte cell death induced by PARP activation is prevented only when SIRT1 is intact (Pillai, Isbatan et al. 2005). Some recent studies have demonstrated that SIRT1 protects heart from oxidative stress through activation of FOXO-1 dependent antioxidant enzyme expression and deactivation of proapoptotic p53 and FOXO3a (Alcendor, Gao et al. 2007; Vahtola, Louhelainen et al. 2008) .

Another major function of SIRT1 relevant to CVD is its ability to regulate endothelial cell physiology. Endothelial cells are involved in many aspects of vascular biology, including atherosclerosis, barrier function, blood clotting, inflammation, formation of new blood vessels and vasoconstriction and vasodilation. Mattagajasingh et al. have identified an important and direct role of SIRT1 in controlling endothelium-dependent vascular relaxation (Mattagajasingh, Kim et al. 2007). Endothelial nitric oxide synthase (eNOS), an enzyme that generates nitric

oxide, is atheroprotective and promotes vessel relaxation. SIRT1 promotes endothelium-dependent vasodilation by targeting endothelial nitric oxide synthase (eNOS) for deacetylation, which in turn stimulating eNOS activity and increasing endothelial nitric oxide (NO). The corresponding study in mice upon CR has confirmed deacetylation of eNOS in response to SIRT1 activation (Mattagajasingh, Kim et al. 2007). This study has implicated the important role for SIRT1 in endothelium-dependent eNOS-mediated vascular homeostasis: a role that may become more prominent during times of low calorie intake. Oxidative stress can induce the premature senescence in vascular endothelial cell, which is likely determinant of cardiovascular disease (Chen and Goligorsky 2006). SIRT1 inhibition induces premature senescence-like phenotype in human umbilical vein endothelial cells (HUVEC) in parallel with increased PAI-1 and decreased eNOS expression (Ota, Akishita et al. 2007). Conversely, overexpression of SIRT1 (Ota, Akishita et al. 2007) or activation of SIRT1 with resveratrol (Csiszar, Labinskyy et al. 2008), or induction of SIRT1 by cilostasol (Ota, Eto et al. 2008) prevents oxidative stress-induced premature senescence in endothelia cell endothelia cells. Moreover, cilostazol increases phosphorylation of eNOS with a dose-dependent increase in SIRT1 expression. These data raise the possibility of a vasodilatory feed-forward loop between SIRT1 and eNOS. Like cell endothelia cells, vascular smooth muscle cells (SMC) are critical for the control of blood pressure and the remodeling of aging and diseased arteries. In vascular smooth muscle cells (VSMCs), SIRT1 has been shown to inhibit the expression angiotensin II type I receptor (AT1R), which mediating Ang II –dependent vasoconstriction and sodium retention (Miyazaki, Ichiki et al. 2008). This observation has identified a important role of SIRT1 in the control of blood pressure via SMC and the renin-angiotension system. Taken together, these data suggests that SIRT1 plays an important role in controlling hypertension at the level of the vessel wall.

1.2.3.4.4 SIRT1 and neurodegeneration

Neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease

and amyotrophic lateral sclerosis are increasingly prevalent in aging society. Many studies demonstrate that activation of SIRT1 could attenuate neuronal degeneration and death in animal models of neurodegenerative disease and exert a neuroprotective effect.

Immunohistochemistry analysis has shown that SIRT1 is highly expressed incentral nervous system during embryogenesis and in adult mice (Sakamoto, Miura et al. 2004). Abnormalities in the central nervous system have been found in SIRT1 knockout mice. In these mice, multiple retinal cell layers are significantly thinner and the inner and outer nuclear layers were disorganized (Cheng, Mostoslavsky et al. 2003). In the brain, nicotinamide mononucleotide adenylyl-transferase1 (Nmnat1), an enzyme in the NAD biosynthetic pathway that generates NAD within the nucleus, is responsible for the delayed axonal degeneration observed in the Wallerian degeneration slow (*wld*^{s}) mice (Araki, Sasaki et al. 2004). Importantly, SIRT1 has been demonstrated to be the downstream effector of increased Nmnat activity that leads to axonal protection (Araki, Sasaki et al. 2004). SIRT1 has since been shown to be upregulated in mouse models of Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and in primary neurons challenged with neurotoxic insults (Kim, Nguyen et al. 2007). Overexpression of SIRT1 and resveratrol both promote neuronal survival. Furthermore, neuronal SIRT1 activation is responsible for the neurons protection by calorie restriction against Alzheimer disease amyloid deposition (Qin, Yang et al. 2006). The mechanism of the neuroprotection by SIRT1 is associated with its downregulation of proapoptotic factors, such as p53 (Vaziri, Dessain et al. 2001; Kim, Nguyen et al. 2007), FOXO (Brunet, Sweeney et al. 2004; Motta, Divecha et al. 2004) and NF-kappa B (Chen, Zhou et al. 2005). However, suppression of apoptosis is probably not the only mechanism by which SIRT1 protects neurons. It is known that the maintenance of mitochondrial number and function is critical for normal neuronal function, and mitochondrial dysfunction may contribute to pathogenesis of many neurodegenerative disease such as Alzheimer's disease, Parkinson's disease (Beal 2005). PGC-1α, a master regulator of mitochondrial number and function, is highly neuroprotective. Activation of PGC-1α protects neurons from oxidative

stress-induced death (St-Pierre, Drori et al. 2006). As mention above, PGC-1α is one of downstream target of SIRT1. Therefore it is likely that neuroprotective ability of SIRT1, at least in part, from its regulation of PGC-1α. However, the modulation of SIRT1/ PGC-1α pathway in the central nervous system is not well documented, and requires further study.

1.2.4 Function of SIRT2

Mammianlian SIRT2 is predominantly localized in cytoplasm, although it does transiently reside in the nucleus during the phases of mitosis (North and Verdin 2007). In the cytosol, SIR2 colocalizes with microtubules and deacetylates α-tubulin on lysine 40 (North, Marshall et al. 2003), thereby participating in the regulation of microtubule dynamic. However, the physiological consequences of α-tubulin deacetylation by SIRT2 are not yet clear. Importantly, SIRT2 is a critical regulator of mammalian cell cycle. SIRT2 protein level increases dramatically during mitosis and its overexpression delays mitotic phase of cell cycle (Dryden, Nahhas et al. 2003). During mitosis, SIRT2 is multiply phosphorylated at the G2/M transition of the cell cycle and shuttles to the nucleus and colocalizes with chromatin (Dryden, Nahhas et al. 2003; Inoue, Hiratsuka et al. 2007). In nucleus, SIRT2 deacetylates histone H4 at lysine 16 (H4K16) leading to global decrease of H4K16 acetylation, which may aid in chromatin condensation (Vaquero, Scher et al. 2006). As overexpression of SIRT2 delays mitotic exit, SIRT2 might act as a mitotic checkpoint protein by blocking the entry to chromosome condensation during mitosis (Inoue, Hiratsuka et al. 2007).

In addition, SIRT2 also interacts with several transcription factors. In adipocyte, SIRT2 deaectylates FOXO1 to promote FOXO1's binding to PPAR-γ, which lead to repression on PPAR-γ transcriptional activity and subsequent inhibition of adipocyte differentiation (Wang and Tong 2009). The deacetylation of FOXO1 by SIRT2 aslo influences FOXO1-induced autophagic process leading to cell death (Zhao, Yang et al. 2010). SIRT2 similarly interacts with and deacetylates FOXO3 to increase its transactivation activity (Wang, Nguyen et al. 2007). Furthermore, SIRT2 deacetylates p53 and down-regulates its transcriptional activity. The regulation of p53 by SIRT2 is

augmented by its interaction with 14-3-3 β/γ protein (Jin, Kim et al. 2008). SIRT2 also interacts with homeobox protein A10 (HOXA10), a sequence-specific DNA-binding transcription factor important for development regulation (Bae, Swanson et al. 2004).

A role of SIRT2 in cancers has been suggested. SIRT2 is located at a genomic region frequently deleted in human gliomas. The RNA level of SIRT2 was diminished in gliomas tissue and glioma cell lines while overexpression of SIRT2 markedly reduced the capacity of glioma cell to form colonies (Hiratsuka, Inoue et al. 2003). A recent report suggested that a 4-gene expression signature, including SIRT2, predicts survival of patients with escphageal adenocarcinoma (Peters, Rees et al. 2010).

1.2.5 Functions of mitochondrial sirtuins (SIRT3-5)

Recent proteomic analysis demonstrated that more than 20% of mitochondrial proteins, including many longevity regulators and metabolism enzymes, are subjected to lysine acetylation (Kim, Sprung et al. 2006). SIRT3 is the most extensively studied mitochondrial sirtuin that exhibits the most robust deacetylase activity when compared to SIRT4 and SIRT5 (Lombard, Alt et al. 2007). So far, several mitochondrial proteins have been identified as SIRT3 targets which link SIRT3 to various metabolic pathways. Acetyl-CoA synthetase 2 (AceCS2) was the first mitochondrial protein identified as the substrate of SIRT3 (Hallows, Lee et al. 2006; Schwer, Bunkenborg et al. 2006). AceCS2 converts free acetate, which is generated from endogenous cellular reactions or absorbed from the gut, into an active metabolite acetyl-CoA for energy production through TCA cycle. Acetylation of AceCS2 inactivates the enzyme, whereas deacetylation by SIRT3 activates it. On the other hand, SIRT1 deacetylates and activates the cytoplasmic acety-CoA synthetase, AceCS1 (Hallows, Lee et al. 2006). Considering the AceCS1 and AceCS2 are the only known mammalian AceCSs for acetate conversion under ketogenic conditions such as diabetes and prolonged fasting (Fujino, Kondo et al. 2001; Yamashita, Kaneyuki et al. 2001), it is speculated that SIRT1 and SIRT3 serve as

important mediators for acetate metabolism under stress. In addition, two other mitochondrial matrix proteins, glutamate dehydrogenase (GDH), an enzyme important for amino acid metabolism, and isocitrate dehydrogenase 2 (IDH2), a key enzyme for TCA cycle, are also deacetylated and activated by SIRT3 (Lombard, Alt et al. 2007; Schlicker, Gertz et al. 2008). Moreover, SIRT3 physically interacts with FOXO3a in mitochondria and increases FOXO3a-dependent gene expression (Jacobs, Pennington et al. 2008). Interestingly, several components of the electron transport chain (ETC) are regulated by SIRT3, including NADH dehydrogenase alpha subcomplex 9 (NDUFA 9) which is a subunit of ETC complex I (Ahn, Kim et al. 2008), and succinate dehydrogenase flavoprotein (SdhA) which is subunit of ETC complex II (Cimen, Han et al. 2010). Their interaction with SIRT3 increases both complex I and II activities, suggesting that SIRT3 is central player in energy metabolism. In addition, one study indicates that SIRT3 is an important regulator in adaptive thermogenesis in brown adipose tissue (BAT). Caloric restriction activates SIRT3 expression in both white and brown adipose tissue, and cold exposure increases SIRT3 levels in BAT (Shi, Wang et al. 2005). Overexpression of SIRT3 in brown adipocyte promotes the expression of PGC-1α, uncoupling protein 1 (UCP1) and other mitochondria-related genes, which finally lead to enhancement of mitochondrial electron transport activity (Shi, Wang et al. 2005).

Besides functioning as a metabolic regulator, SIRT3 may be involved in longevity. Ang II type 1 receptors (AT1A) knockout mice display marked prolongation of life span and the longevity phenotype was associated with upregulation of SIRT3 expression in the kidney (Benigni, Corna et al. 2009). Furthermore, polymorphisms in SIRT3 gene are associated with survival in the elderly (Rose, Dato et al. 2003; Bellizzi, Rose et al. 2005). However, at the molecular level, the regulation of cell survival by SIRT3 is controversial. SIRT3 is required for cell survival under genotoxic stress (Yang, Yang et al. 2007) but in contrast, SIRT3 participates in Bcl-2 and JNK2-regulated apoptosis (Allison and Milner 2007).

SIRT4 functions as an efficient mitochondrial ADP-ribosyl transferase with no detectable deacetylation activity. SIRT4 uses NAD+ to ADP-ribosylate and

downregulate glutamate dehydrogenase (GDH) activity by at least 50% (Haigis, Mostoslavsky et al. 2006). GDH is known to promote the metabolism of glutamate and glutamine to generate ATP, which promotes insulin secretion (Haigis, Mostoslavsky et al. 2006). Therefore, SIRT4-mediated inactivation of GDH leads to the inhibition of insulin secretion in pancreatic β-cells (Haigis, Mostoslavsky et al. 2006). Moreover, SIRT4 also interacts with the insulin-degrading enzyme (IDE) (Ahuja, Schwer et al. 2007), thus providing an alternative possible mechanism for how SIRT4 affects insulin secretion.

In contrast to SIRT3, SIRT5 has weak mitochondrial deacetylase activity (Michishita, Park et al. 2005). A recent study found that SIRT5 deacetylates and upreguates the activity of carbamoyl phosphate synthetase 1 (CPS1), an enzyme regulating urea cycle for excess amminia detoxification and disposal (Nakagawa, Lomb et al. 2009). SIRT5-mediated regulation of CPS1 is important for the adaptation to conditions linked to increases amino acid catabolism such as CR and prolonged fasting. Moreover, SIRT5 is found to interact with cytochrome c in mitochondrial intermembrane space (Nakamura, Ogura et al. 2008). However, the biochemical activity and biological functional changes following deacetylation of cytochrome c have not been delineated.

1.2.6 Function of SIRT6

SIRT6 is a nuclear protein that functions originally as a nuclear ADP-ribosyl transferase (Liszt, Ford et al. 2005). Recently, SIRT6 was found to have deacetylase activity as well, specifically targeting histone H3 on lysine 9 (H3K9) (Michishita, McCord et al. 2008). The acetylation of H3K9 by SIRT6 plays an important role in maintaining telomere integrity. Depletion of SIRT6 leads to telomere dysfunction with end-to-end chromosomal fusions and premature cellular senescence (Michishita, McCord et al. 2008). Furthermore, deletion of SIRT6 results in cellular hypersensitivity to DNA damage as well as increased genomic instability, manifested as chromosomal breaks and fusions (Mostoslavsky, Chua et al. 2006). Consistently, SIRT6 knockout mice displayed signs of premature aging, such as decreased size, loss

of subcutaneous fat, lymphocyte apoptosis, abnormal spine curvature and metabolic defects leading to premature death after only 4 weeks of age (Mostoslavsky, Chua et al. 2006). These results suggested that mammalian SIRT6 play a key role in DNA repair and maintenance of genomic stability in cells and is also necessary to maintain organismal health and to prevent the development of several progeroid pathologies.

In addition, SIRT6 functions at chromatin to attenuate NF-κB signaling pathway (Kawahara, Michishita et al. 2009). SIRT6 interacts with the NF-κB RELA subunit and deacetylates histone H3 lysine 9 (H3K9) at NF-κB target gene promoters, causing them to become less active. Consistently, in SIRT6-deficient cells, hyperacetylation of H3K9 at these target promoters is associated with increased RELA promoter occupancy and enhanced NF-κB-dependent modulation of gene expression, apoptosis, and cellular senescence.

1.2.7 Function of SIRT7

SIRT7 localizes to nucleoli, associate with condensed chromosomes during mitosis and is widely expressed in proliferating mouse tissue, such as liver, spleen and testes (Michishita, Park et al. 2005; Ford, Voit et al. 2006). So far, no deacetylase or ADP-ribosyltransferase activity has been demonstrated for SIRT7, and no specific interacting partners or targets have been identified. One study implicated a role of SIRT7 in the regulation of rRNA genes (rDNA) transcription via interaction with RNA polymerase I (Pol I) (Ford, Voit et al. 2006). SIRT7 is also impicated in the regulation of cell survival and proliferation, although these effects may be cell-specific. Depletion of SIRT7 in primary cardiomyocytes triggers apoptosis and also diminishes resistance to oxidative and genotoxic stress (Vakhrusheva, Smolka et al. 2008). In contrast, overexpression of SIRT7 inhibits cell growth in mouse embryonic fibroblasts (MEFs) (Vakhrusheva, Braeuer et al. 2008). Moreover, increased SIRT7 expression was observed in breast cancer as compared to nonmalignant breast tissue (Ashraf, Zino et al. 2006).

Chapter 2 Materials and Methods

2.1 Plasmids and antibodies

Lentivirus plasmid vectors pLKO.1-puro, and pLKO.1-puro vectors containing MISSION shRNA targeting SIRT1 (clone SH2421) or non-targeting shRNAs (clone SHC001) were from Sigma-Aldrich. Vectors expressing TERT (pLV102-TERT), PTOP (pLV102-TPP1) and GFP (pLV102-GFP) were from GeneCopoeia.

SIRT1 (1104-1) and TERT (1531-1) antibodies were from Epitomics; POT1 (ab21382) and PTOP (ab57595) antibodies were from Abcam; p53 (2524), acetyl-p53 (K382) (2525), phospho-H2AX (Ser139) (2577) and PARP (9542) antibodies were from Cell Signaling Technology; p16 (C-20) (sc-468) antibody was from Santa Cruz Biotechnology; p27 (554228) antibody was from BD Biosciences. SIRT1 antibodies (HPA006295), β-ACTIN antibodies (A5316) and α-tubulin antibodies (T5168) were from Sigma-Aldrich.Anti-mouse, -rabbit HRP-linked secondary antibodies were purchased from GE Healthcare Ltd (Pittsburgh, PA, USA). Secondary antibody polymer-HRP anti-rabbit (K4003) was from Dako North America, Inc.

2.2 Patients and specimens

Tumorous liver tissues and the corresponding adjacent non-tumoral liver tissues were collected from 40 patients who underwent curative surgery for HCC at The Prince of Wales Hospital, Hong Kong. Total RNAs and proteins were extracted from these specimens. Informed consent was obtained from each patient recruited, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong.

2.3 Cell culture

Materials

- Dulbecco's Modified Eagle Medium (DMEM) (Carlsbad, CA, USA)
- Minimum Essential Medium (MEM) (GIBCO)
- Penicillin (10,000 units/ml)-Streptomycine(10.000 μg/ml) (GIBCO)
- Fetal bovine serum (GIBCO)

HepG2, SK-Hep-1, PLC5, Hep3B, SNU-449, SNU-423, and Huh-7 cells were obtained from American Type Culture Collection recommendations (ATCC, Rockville, MD). Huh7 cell line was acquired from the Health Science Research Resources Bank (Osaka, Japan). HKCI-4 and HKCI-2 cells were established in Prof. Nathalie Wong's laboratory (Wong, Yeo et al. 2009). Huh-7, SNU423, Hep3B and PLC5 were cultured according to recommendations in DMEM medium containing 10% fetal bovine serum and , 100 Unit/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Grand Island, NY). HepG2 was maintained in MEM containing 10% fetal bovine serum and, 100 Unit/ml penicillin and 100 μg/ml streptomycin. SNU-449 were maintained in complete RPMI medium (Gibco BRL, Grand Island, NY). HKCI-4 and HKCI-2 cell lines were maintained in RPMI 1640 glutamax with HEPES buffer supplemented with 10% fetal bovine serum, 10 μg/ml selenium, 10 μg/ml transferrin and 10 μg/ml insulin. All cells were maintained at standard cell culture conditions (37℃, 5% CO_2 in a humidified incubator) as recommended by the vendors.

2.4 Tissue microarrays and immunohistochemistry

Materials

- Xylene
- Ethanol (100%,95%,70%)
- 3% Hydrogen peroxide
- Phosphate buffered saline, PBS (GIBCO)
- TBS : 0.05 M Tris, 0.15 M NaCl (pH7.6)

- DAB chromogen / substrate kit (Diagnostic BioSystems)
- Citrate buffer: 10mM citric acid (pH 6.0)
- Blocking buffer: 10% goat serum (Dako) in PBS

The HCC tissue microarrays were generated from formalin-fixed, paraffin-embedded archive tissue of 180 paired HCC tumors arranged in tissue array blocks, among which 153 cases possessed complete follow-up data. The samples were collected at The Prince of Wales Hospital from 1995 to 2002.

The slides were deparaffinized through xylenes and graded ethyl alcohols and then rinsed in water, followed by quenching of endogenous peroxidase activity by a 3% solution of hydrogen peroxide for 10 minutes. Antigen retrieval was performed by boiling the slides in 0.01mol/l sodium citrate buffer, PH 6.0, in a microwave oven at 800w power for 10 minutes. The sections were then cooled down and rinsed in washing buffer. The slides were incubated overnight with the anti-SIRT1 antibody (Sigma-Aldrich, 1:400 dilution) after being blocked with 10% goat normal serum for 30 minutes. After washed by TBS, the sections were reacted with the secondary goat anti-rabbit antibody (Labelled Polymer-HRP) for 1 hour and then detected by using DAB chromogen /substrate kit according to manufacturer's protocol. The stained slides were counterstained with hematoxylin and mounted. To evaluate IHC staining of SIRT1, the scoring of SIRT1 was carried out by two independent pathologists according to the proportion of tumor cells with positive nuclear staining (negative, none; weak, <=10%; moderate, 10 to <=50%; strong, >50%).

2.5 Western blot analysis

Materials

- Cold PBS
- RIPA Lysis buffer: 20 mM Tris-Cl (pH7.6), 150 mM NaCl, 1 mM EDTA, 1 mM $NaVO_3$, 1 mM β-glycerol phosphate, 1 % Triton X-100, 0.1% SDS, 1 mM dithiothreitol, 1 mM phenylmethylsufonyl fluoride and protease inhibitor

- Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA)
- Separating gel: acrylamide mix (Bio-Rad), 0.4 M Tris-Cl (pH 8.8), 1 % SDS, 1 % ammonium persulphate and 0.04 % N, N, N', N'-Tetramethylethylenediamine (TEMED)
- Stacking gel: acrylamide mix (Bio-Rad), 0.13 M Tris-Cl (pH 6.8), 1 % SDS, 1 % ammonium persulphate and 0.001 % N, N, N', N'-Tetramethylethylenediamine (TEMED)
- Laemmli loading buffer: 2% SDS, 10 % glycerol, 60 mM Tris (pH 6.8), 0.005 % bromophenol blue and 100 mM β-ME
- 1 X SDS running buffer: 25 mM Tris base, 192 mM glycine and 0.1% SDS
- 1 X Transfer buffer: 39 mM glycine, 48 mM Tris base and 20 % methanol
- Whatman filter paper
- Trans-blot cell (Bio-Rad Laboratories)
- Hybond Enhanced chemiluminescence nitrocellulose (ECL) membrane (Amersham Biosciences, Piscataway, NJ)
- TBS-T: 20 mM Tris-Cl (pH 7.5), 500 mM NaCl, 0.05% Tween-20
- Blocking solution: 5 % Non-fat dry milk in TBS-T
- Western blotting substrate ECL™ (Pierce) (Rockford, IL, USA)
- BenchMark™Pre-Stained Protein Ladder (Invitrogen)

After treatments, cells were harvested and lysed with radio-immunoprecipitation assay (RIPA) lysis buffer. After being rotated at 4℃ for 20 minutes, the cell lysates were cleared by high speed centrifugation at 4℃ for 15 minutes. The supernatants were collected as total cell extracts. Protein concentration was determined using the Bio-Rad protein assay and indicated amount of protein samples with loading buffer were boiled at 95°C for 10 min. Equal amount of protein from each sample was loaded into each well of SDS-polyacrylamide gel（SDS-PAGE）followed by electrophoresis at the voltage 120 V. For each set of SDS-PAGE electrophoresis, 10μl of protein ladder was also loaded onto the gel to indicate the size of the proteins.

After electrophoresis, the proteins from the gel were transferred electrophoretically to a nitrocellulose membrane using a trans-blot cell. In brief, the gel and membrane were sandwiched by 3 sheets of filtered paper, and then were held tightly by a gel holder cassette inside the buffer tank. By placing the gel near the cathode with the membrane near anode, the SDS-PAGE was subjected to a constant voltage in 1 X Transfer buffer. The protein transfer was carried out at 90 V for 3 hours in cold room. Then, the membrane was blocked with blocking buffer at room temperature for 1 hour with gentle shaking. After rinsing with TBS-T twice, the membrane was probed by primary antibodies diluted by blocking buffer at 4°C for overnight with agitation. After washing with TBS-T three times, the membrane was then incubated with secondary antibodies diluted by blocking buffer for 1 hour at room temperature. Lastly, the membrane was finally washed three times with TBS-T before detecting its immunoblotted proteins using the ECL™ Western blot detection system. The membrane was then wrapped in plastic wrap and exposed to X-ray film for a proper exposure time. Band intensities were quantified using ImageJ software.

2.6 Real-time quantitative polymerase chain reaction (qPCR)

Materials

- Trizol Reagent RT (Molecular Research Center, Inc.)
- High Capacity RNA-to-cDNA Master Mix (Applied Biosystems)
- Power SYBR®Green PCR Master Mix (Applied Biosystems)

Primers, the sequence as following (Sigma):

TERT:

Forward: 5'GCGTTTGGTGGATGATTTCT3',

Reverse: 5'CAGGGCCTCGTCTTCTACAG3',

PTOP:

Forward: 5'CCTTGAGGAGCACCTTTCAG3',

Reverse: 5'CAGTGTCAGGCAGCTTTCAG3',

POT1:

Forward: 5’TGGGTATTGTACCCCTCCAA3’,

Reverse: 5’TTGATGAAGCATTCCAACCA3’,

TRF1

Forward: 5’GGCAGCGGCAAAAGTAGTAG3’,

Reverse: 5’GTCTTGTTGCTGGGTTCCAT3’,

TRF2

Forward: 5’GTACCCAAAGGCAAGTGGAA3’,

Reverse: 5’TGACCCACTCGCTTTCTTCT 3’

RAP1

Forward: 5’ATGATGATCCACCCACACCT3’,

Reverse: 5’AAGTAGCCTCCAGCTCACCA3’,

TINF2

Forward: 5’ACTAGGGGAGGCCATAAGGA3’,

Reverse: 5’GGGTCTGGCATGGACTCTTA3’,

PINX1:

Forward: 5’GAAAGGGTTTAGGGGCTCAG3’,

Reverse: 5’TCCGAGGAATCTGTGGTTTC3’,

TANK1

Forward: 5’CGCATGTTGTTTCATGGTTC3’,

Reverse: 5’CTGTTCCTCCTCCAATTCCA3’;

KU70

Forward: 5’AAAAGACTGGGCTCCTTGGT3’,

Reverse: 5’TGTGGGTCTTCAGCTCCTCT3’,

AURKA:

Forward: 5’AACCTCTGCTTCCTGGGTTT 3’

Reverse: 5’ ACGTTTTGGACCTCCAACTG 3’

AURKB:

Forward: 5’ GGAGAGTAGCAGTGCCTTGG 3’

Reverse: 5’ GCTCATGAGGACAAGTGCAG 3’

CENPA:

Forward: 5' TCCGAAAGCTTCAGAAGAGC 3'

Reverse: 5' AGGCGTCCTCAAAGAGATGA 3'

BUB1:

Forward: 5' ATGACCCTCTTGGTGAATGG 3'

Reverse: 5' TGAATCTTGGGTCATTGTGG 3'

β-ACTIN

Forward: 5' CTCTTCCAGCCTTCCTTCCT 3'

Reverse: 5' AGCACTGTGTTGGCGTACAG 3'

2.6.1 RNA extraction

Total RNA was extracted with TRI Reagent. For RNA extraction from frozen tissue samples, 50mg of frozen tissue was homogenized with 2ml TRI Reagent, followed by 10 minutes incubation at room temperature for the complete dissociation of nucleoprotein complexes. The homogenate was supplemented with Chloroform (0.4 ml) and shaken vigorously for 15 seconds. The mixture was centrifuged for 15 minutes at 12,000 × g at 4℃ after incubation at room temperature for 10 minutes. The upper RNA-containing aqueous phase was transferred to a fresh tube and mixed with 1.0ml isopropanol. Then the sample was stored at room temperature for 5 minutes and centrifuge at 12,000g for 8 minutes at 4℃. RNA precipitate formed a white pellet on the bottom of the tube. The pellet was then washed with 75% ethanol (1ml) and spun down at 6,000 × g for 5 minutes at 4℃. The RNA pellet was dried at room temperature and dissolved in 50μl DEPC-treated water. The quantity and quality of the RNA were measured by spectrometry.

For cells grown in monolayer, cells were directly lysed by TRI Reagent after wash with PBS. For cells confluent in 35 mm culture dish, 1 ml of TRIZOL Reagent was added and cell lysates were passed through a pipette for several times. Chloroform (0.2 ml) was added, incubated, and centrifuged. RNA remained in the upper aqueous layer was transferred to a new tube and 0.5 ml isopropanol was added to precipitate the RNA. The RNA pellet was then washed with 75% ethanol (1.0 ml),

spun down, dried and re-dissolved in DEPC-treated water as described above.

2.6.2 cDNA synthesis

The genomic DNA was digested by DNaseI. Then, Complementary DNA (cDNA) was synthesized from total RNA using High Capacity RNA-to-cDNA Master Mix .In brief, the reaction mixtures were prepared according to manufacturer's instruction. The cDNA was amplified by sequential incubation at 25 °C for 2 minutes and 42 °C for 30 minutes which was then followed by 85 °C for 5 minutes. The synthesized cDNA was stored at -20 °C for future use.

2.6.3 Real-time quantitative PCR

Quantitative PCR experiments were performed using the SYBR Green PCR core reagent kit (Applied Biosystems) and the reactions were carried out using an ABI 7900 real-time PCR system (Applied Biosystems). The mRNA level of target genes were normalized to the mRNA level of beta-actin and the $\Delta\Delta$Ct method of relative quantification was used to compare the level of expression in different treatment. The reaction was carried out in a total volume of 10μl containing 1 ×SYBR®Green PCR Master Mix ,2 μM of each forward and reverse primers, and 1 μ l of cDNA (synthesized from DNase treated total RNA as described in 2.6.2). The reaction was initiated by carryover decontamination at 50 °C for 2 minutes, followed by a hot-start step 95 °C for 2 minutes. Amplification was done in 40 cycles of 15 seconds 95 °C and 30 seconds 60 °C.

2.7 Transformation and plasmid preparation

Materials

- One Shot® Stbl3™ Chemically Competent E. coli (Invitrogen)
- LB plates (10 g/L typtone, 10 g/L NaCl, 5 g/L yeast extract and 15 g/L agar)
- Antibiotics (100 μg/ml ampicillin)
- PureLink™HiPure Plasmid Midiprep Kit (Invitrogen)

Plasmid was mixed with 100 μl of competent cells and incubated on ice for 30 minutes. Then the bacterial cells were put in 42 °C heat block for 1 mintues and placed on ice for 2 minutes. The entire bacterial cells were plated on a LB plate containing 100 μg/ml ampicillin and incubated overnight at 37 °C.

A single clone was picked and grown for 16 hours in 100ml LB medium containing ampicillin. The plasmid DNA was isolated by using PureLink™HiPure Plasmid Midiprep Kit according to manufacture's protocols. In brief, the bacterial culture was pelleted by centrifugation at 5000 rpm for 10 min at 4°C. After removing the supernatant, 4 ml R3 buffer was added to resuspend the cells and then 4 ml L7 buffer added to lyse the cells. The lysate was then neutralized by 4 ml N3 buffer. The cell debris was removed by centrifugation at 10 000 rpm for 20 min at 4°C. The supernatant was loaded onto the equilibrated column and drained by gravity flow. The column was washed by washing buffer and DNA was was eluted by adding Elution buffer. The DNA plasmid was precipitated by addition of absolute isopropanol. After centrifugation at 10,000 rpm for 15 min at 4°C, the pellet was washed carefully by 70% ethanol and then air dried. Finally, the DNA was dissolved in 200 μl TE buffer.

2.8 Transient transfection

- FuGENE® HD Transfection Reagent
- Opti-MEM®I Reduced-serum Medium (GIBCO)

HCC cells were seeded in 6-well flat bottomed plates 24 hours prior to transfection. FuGENE® HD Transfection Reagent was used for transfection according to manufacture's protocol. In brief, plasmid DNA was diluted in Opti-MEM and then the FuGENE HD reagent was mixed with DNA/Opti-MEM complex. The entire FuGENE HD/DNA mixture was added drop-wise to the cells after incubation at room temperature for 15 minutes. Cells were then incubated for 24 hours before subjecting to further treatment.

2.9 Lentivirus packaging and transduction

Materials

- $CaCl_2$: 2.5 M in bi-distilled water. Sterilize by passage through a 0.2 μm filter.
- 2X HEPES-buffered saline (HBS): 0.05M HEPES, 0.28M NaCl, and 1.5mM Na_2HPO_4 (pH 7.12. Sterilize by passage through a 0.2 μm filter.
- TE: 10mM Tris-HCL, 1mM EDTA (pH 7.9). Sterilize by passage through a 0.2 μm filter.
- Plasmid DNA: pLKO.1-purovector containing MISSION shRNA targeting SIRT1 or non-targeting shRNAs (Sigma). Packaging plasmids pLP1, pLP2, and pLP/VSVG (Invitrogen).
- PEG-*it*™ Virus Precipitation Solution (System Bioscience)

HEK293FT cells in complete culture medium were maintained in a 37 °C incubator with 5% CO_2. Twenty-four hours before transfection, plate HEK293FT cells in 100-mm tissue culture dish at a density of 3 $X10^6$ cells/plate. The transfection mix was prepared in a total volume of 500 μl containing four plasmids mix (10 μg pLK0.1-puro vector, 9μg pLP/VSVG, 10.5 μg pLP1 and 10.5 μg pLP2), 294 μl TE , 156 μl water and 50μl $CaCl_2$. After briefly mixing, 500 μl HBS was added to transfection mix dropwise under agitation by vortexing. The calcium phosphate-DNA suspension (1ml per dish) was added to a 100-mm dish containing cells after incubation at room temperature for 20 minutes. Harvest virus-containing supernatants 48-72 hours post-transfection by removing medium to a 15 ml sterile, capped, conical tube. Afterwards, viruses were concentrated using PEG-it™ virus precipitation solution according to manufacture's protocol. Briefly, the cell debris was removed by centrifugation at 3000g for 15 minutes at 4°C. Then every 4 volumes of lentivirus-containing supernatant were mixed with 1 volume of cold PEG-*it* Virus Precipitation Solution and incubated at 4 °C overnight. After centrifugation at 1500 × *g* for 30 minutes at 4°C, the lentiviral particles may appear as a beige or white pellet

at the bottom of the vessel. Lastly, lentiviral pellet was resuspended in 1/10 to 1/100 of original volume using cold PBS and the concentrated viruses were aliquoted and stored at -80 °C for future use.

HCC cells were seeded in 6-well flat bottomed plates 24 hours prior to infection. The medium containing diluted viruses and 6μg/ml polybrene was added drop-wise to the culture cells with gentle rocking of the plate to ensure even distribution. Cells were then incubated for 48 hours before subjecting to further treatment.

2.10 Immunofluorescence / TIF assay

Materials

- SK-Hep-1 cells
- Cold PBS
- 4 % paraformaldehyde in PBS
- 100 % methanol
- Trition extraction buffer: 300mM sucrose, 20mM HEPES(pH7.9), 50mM NaCl, 3mM $MgCl_2$ and 0.5% Trition X-100
- Primary antibodies: antibodies against TRF2 and γ -H2AX
- Secondary antibodies: FITC/TRITC-conjugated anti-mouse IgG or FITC/TRITC-conjugated anti-rabbit IgG (Calbiochem)
- Blocking buffer: 3 % bovine serum albumin (BSA) in PBS
- TBS: 50 mmol/L Tris-Cl (pH 7.4), 150 mmol/L NaCl
- 25mmol/L glycine
- FluorSave™ reagent (Calbiochem)

SK-Hep-1 cells grown on coverslips in six-well plate were fixed in 4 % cold paraformaldehyde in PBS for 15 min. After fixation, cells were washed with PBS and re-permeabilized with Triton extraction buffer for 10 minutes. Cells were then washed twice for 5 minutes with PBS, and blocking was carried out for 1 hour in

blocking buffer. For double-staining, cells were first incubated with anti-γ-H2AX antibodies in 1:100 dilution in blocking buffer for 2 hours at room temperature, washed extensively with PBS, incubated with FITC-labeled secondary antibodies for 1 hour, and subsequently fixed for 15 minutes with 2% paraformaldehyde in PBS. The formaldehyde was inactivated for 15 minutes using 25 mmol/L of glycine in PBS. Coverslips were then incubated with anti-TRF1 (1:100) antibodies at 4 °C overnight and subsequently by the TRITC-labelled secondary antibodies. Coversilps were washed three times in PBS, stained with DAPI, and mounted on slides with FluorSave™ reagent. Digital images were captured with a Zeiss Axiovert 200 M fluorescence microscope with a CoolsnapTMES2 (Photometrics®) camera.

2.11 Trypan blue exclusive assay

Material

- Trypan blue solution (Sigma)

Cell proliferation in response to SIRT1-silencing was determined by trypan blue exclusion assay. Following treatments, cells were trypsinized and collected in a 1.5-ml Eppendorf tube. The cells were pelleted by centrifugation and resuspended in PBS (500μl). Trypan blue solution (500μl) was added to cell suspension and the number of cells (viable unstained and nonviable blue) was counted after incubation for 5 minutes at room temperature.

2.12 Colony formation assay

Materials

- 0.5% crystal violet
- Methanol
- 10 mg/mL Puromycin (Sigma)

Colony formation assay was used to investigate the effects of SIRT1 silencing on the plating effect of each individual cell transfectant. Cells were seeded on

six-well plates at a density of 1 x 10^3 cells/well 48 hours after infection. Colonies were allowed to grow for 10-14 days in medium containing puromycin (0.25μg/ml). The medium was discarded and colonies were washed twice with PBS carefully. The colonies were fixed in methanol for 15 minutes, and then stained with 0.5% crystal violet for 10 minutes. Uninfected cells were treated similarly, and all died within 1 weeks of culture in the selection medium. Finally, positive colony formation was counted.

2.13 Soft agar assay

Materials

- 0.05% crystal violet
- 0.7% Agarose in PBS
- 1% Agarose in PBS
- 2×DMED+20% FBS

Soft agar assay was conducted to investigate the ability of cells to carry out anchorage-independent growth in soft agar. The base layer of each well was consisted of 1.5 ml solidified media containing DEME with 10% FBS and 0.5% low melting point agarose. It was placed in six-well plates, and allowed to solidify in 4 °C for 10 minutes. The top agar layer was consisted of 5,000 SK-Hep-1 cells suspended in DEME with 0.35% low melting point agarose. The cells were incubated at 37°C in a 5% CO_2 incubator for 3 weeks and then stained for stained with 0.05% crystal violet for 15 minutes The sizes and number of colonies were measured under a microscope (Leica DMIL). The experiment was done in triplicate in different groups.

2.14 BrdU incorporation

Materials

- Cell Proliferation ELISA, BrdU (colorimetric) (Roche Diagnostics Corporation)

DNA synthesis was determined by the bromodeoxyuridine (BrdU) assay

according to manufacturer's instruction. In brief, 1.8×10^4 SK-Hep-1 cells were plated in triplicate in 96-well plates. BrdU (10m M) was added to the culture medium for 6 hours. The BrdU-labeled cells were fixed, and the DNA was denatured in FixDenat solution for 30 min at room temperature. Cells were then incubated with anti-BrdU antibody diluted in staining buffer (100µl total) for 90 minutes at room temperature, followed by washing three times with washing solution. The immune complex was then detected using a 3,3',5,5'-tetramethylbenzidine substrate reaction and measuring the absorbance at 450 nm.

2.15 SA-β-gal assay

Materials

- Potassium Ferrocyanide (Sigma)
- Potassium Ferricyanide (Sigma)
- Magnesium Chloride (Sigma)
- Dimethylformamide
- Glutaraldehyde
- 1X PBS
- Fixative Solution: 0.2% glutaraldehyde in 1X PBS
- X-gal stock: 40 mg/ml in dimethylformamide, store at –20°C protected from light
- SA-b-gal Staining Solution: 5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide, 2mM $MgCl_2$, Once in solution adjust pH to 6.0.
- Complete staining solution: Add 25 µl of 40 mg/ml X-gal per ml of staining solution

The cells were transduced with lentivirus expressing shRNA target SIRT1 or scramle shRNA control,respectively. At 6 days post-transduction, cells were washed twice PBS and then fixed with fixative solution for 15 minutes. The cells were then

incubated overnight with complete staining solution at 37°C. SA-β-gal-positive cells were enumerated by counting over 200 cells in three independent fields.

2.16 Cell viability assay (MTT assay)

Materials

- MTT reagent: Methylthiaolydiphenyl-tetrazolium bromide, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
- MTT solubilization buffer: 10% SDS, 10 mM HCL
- 96 well plate reader (Mutilabel counter Victor^{3} ^{TM}, Perkinelmer) (Waltham Massachusetts, USA)

HCC cells were seeded in 96-well plates (6,000/well) and incubated overnight at 37°C. For the cytotoxicity assay of doxorubicin, HCC cells were exposed to compound for 48 hours. After treatment, 10 μl of MTT reagent was added to each well and incubated at 37°C for 4 hours. Afterwards, solubilization buffer (100 μl per well) was added for incubation overnight at 37°C. Absorbance of each well of the plates was measured at 570 nm. The percentage of cell viability was obtained by the absorbance of treated cells versus untreated cells × 100%. The viability of untreated cells was considered to equal 100%. Mean and standard deviation for each concentration were obtained by three independent experiments.

2.17 Flow cytometry (cell cycle analysis)

Materials:

- Propidium iodide (sigma)
- Rnase (USB) (Cleveland, Ohio, USA)
- PBS (GIBCO)

Cell cycle distribution was determined by fluorescence-activated cell sorting (FACS) analysis. Cells were trypsinized, centrifuged and washed by PBS. Cells were

then fixed with 70 % ethanol at -20°C overnight and stained with propridium iodide (10mg/ml) for 15 minutes in the dark at room temperature. Afterwards, cells were then washed and resuspended in PBS before analyzing for their DNA content using the FACSCalibur flow cytometer (BD Biosciencs) (San Jose, CA, USA). Data acquisition and analysis were done with CellQuest (BD Bioscience)

2.18 Statistical Analysis

SIRT1 expression in HCC and non-tumoral liver were compared by the paired Student's *t*-test. Comprarisons of control cells and SIRT1-depleted cells in functional investigations were conducted by the two-sample *t*-tests. Correlations between SIRT1 and individual clinicopathological parameters were evaluated by the non-parametric Chi-square test and Spearmans rho rank test. All statistical analyses were carried out by statistical program SPSS version 16.0. The difference was considered as significant statistically when the *p*-value was less than 0.05.

Chapter 3 Results

Given the diverse roles of SIRT1 in cell growth and tumorigenesis, the goal of my study is to evaluate the role SIRT1 in the pathogenesis of human HCC. To this end, I will first determine if SIRT1 is differentially expressed in HCC specimen. Next, I will elucidate the functional role and the underlying molecular mechanisms of SIRT1 in HCC pathogenesis.

3.1 SIRT1 is upregulated in HCC

3.1.1 Overexpression of SIRT1 in different HCC cell lines

The expression of SIRT1 in nine HCC cell lines (SK-Hep-1, HepG2, Hep3B, PLC5, SNU449, SNU423, Huh-7, HKCI-2 and HKCI-4) and three normal liver biopsies was determined by western blotting analysis. While SIRT1 expression level was expressed at very low levels in the normal livers, it was significantly overexpressed in all the HCC cell lines (9/9) examined (Figure 3.1).

3.1.2 Over-expression of SIRT1 in clinical specimens

To establish the relevance of SIRT1 expression in HCC, the expression level of SIRT1 protein in tumor was determined by using western blotting analysis. Total protein was extracted from 40 pairs of primary HCC tissues and adjacent non-tumoral liver, which is often considered the premalignant lesion of HCC. A significant portion of patients showed elevated SIRT1 level in HCC (Figure 3.2). Of a total 40 cases, 28 cases (70%) showed elevated SIRT1 expression in tumor (more than 2 fold) compared to adjacent non-tumoral liver (Table 3.1). Furthermore, the average level of SIRT1 was found to be significantly higher in tumor (median 0.25, quartiles 0.95-0.09) relative to non-tumoral liver (median 0.05, quartiles 0.07 – 0.03) (paired t-test $p < 0.001$) (Figure 3.3).

Western blotting analysis revealed that there is an over-expression of SIRT1 in HCC tissue samples, but it is not known whether the over-expression was due to an increase in transcription. Hence, SIRT1 mRNA level in HCC tumor and non-

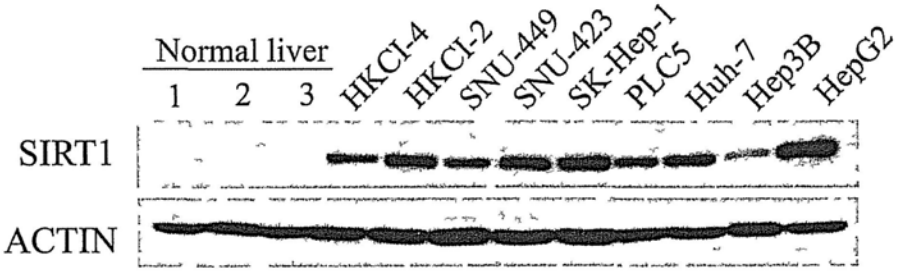


Figure 3.1 Expression of SIRT1 in HCC cell lines and normal liver biopsy. Cell lysates from the indicated cell lines were used for western blotting analysis using SIRT1 antibodies. β-ACTIN was used as loading control.

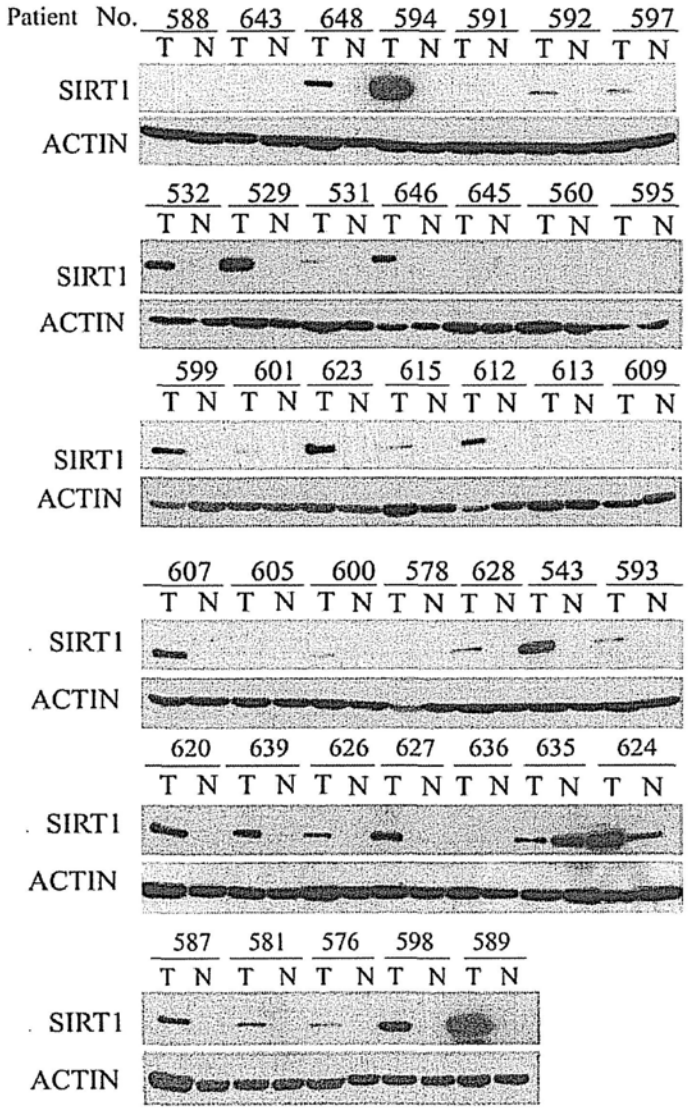


Figure 3.2 Expression of SIRT1 in liver tissues from patients with HCC.

Proteins were extracted from 40 paired HCC tissues and subjected to western blotting analysis using SIRT1 antibodies. β-ACTIN was used as loading control.

Table 3.1 Relative expression of SIRT1 in HCC samples

Fold change (Tumor/Non-tumor)	≤2	>2
Case No. (%)	12 (30%)	28 (70%)

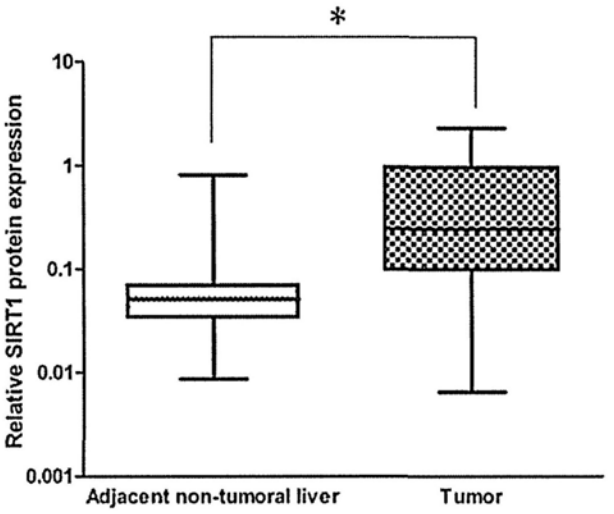


Figure 3.3 Quantification of SIRT1 protein expression in HCC samples.

Expression level of SIRT1 protein in 40 paired HCC samples and their adjacent non-tumoral livers deduced by Western blot was quantified using ImageJ software. SIRT1 expression were normalized to β-ACTIN expression. * $P < 0.001$, paired Student's *t*-test.

-tumorous tissues were determined by real-time quantitative PCR. Real-time quantitative PCR results showed that the average SIRT1 mRNA level was not differed significantly between tumor and non-tumoral liver (Figure 3.4), suggesting that tumor-specific over-expression of SIRT1 was regulated in a transcription-independent manner.

Next, the expression level and localization of SIRT1 in the liver tissue of HCC patients was determined using immunohistochemical analysis. For this purpose, tissue microarrays that contained 150 resectable HCC tumors and their paired adjacent non-tumoral livers were assayed. The samples were collected at The Prince of Wales Hospital from 1995 to 2002. The clinicopathologic parameters of these HCC cases were summarized in Table 3.2. Concordant with the findings from Western blot analysis, distinct SIRT1 staining was found in certain tumors (46/150 cases), whereas the staining was negative in all adjacent non-tumoral livers. In SIRT1 positive HCC, immunostaining could be identified in the tumor hepatocytes, and the staining was predominantly localized to the nucleus (Figure 3.5). SIRT1 positive tumors were further classified into negative, weak, moderate and strong categories according to the score obtained by determining the percentage of positively stained cells on the section (Figure 3.5). Among the 46 positive cases, 21 cases showed strong staining of SIRT1, whereas 8 and 17 cases exhibited moderate and weak staining respectively (Table 3.3).

3.1.3 Association of SIRT1 expression with tumor grade

To further understand how SIRT1 expression is related to the pathogenesis and prognosis of HCC, the correlation between SIRT1 expression and clinicopathological features was analyzed. The data revealed that SIRT1 expression was positively correlated with tumor grade (Spearman rho test, $p< 0.001$), in which 67% of the poorly-differentiated (grade 3) tumors showed SIRT1 over-expression (Table 3.4). Apart from this correlation, no significant association between SIRT1 over-expression and other clinicopathological parameters was found, which included patients' sex, age, tumor stage, tumor numbers, vascular invasion, HBV and HCV

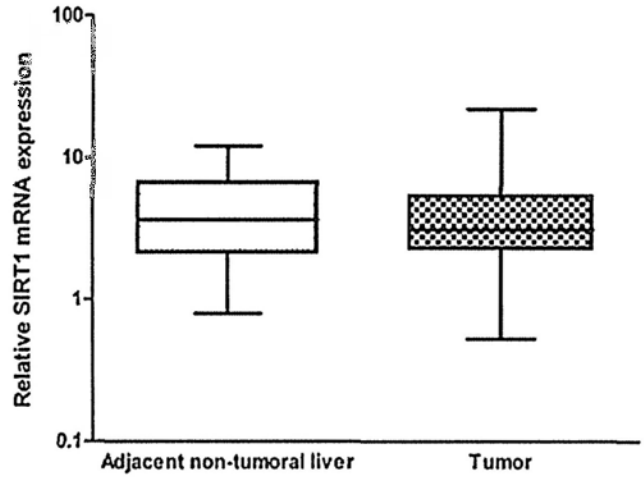


Figure 3.4 SIRT1 mRNA expression in HCC samples. The expression of SIRT1 mRNA in paired tumor and adjacent-nontumoral liver was determined by RT-PCR. SIRT1 mRNA level was normalized to beta-ACTIN mRNA level. $P > 0.05$, paired Student's *t*-test.

Clinicopathological Parameters		No. of the patients
Sex	Male	124
	Female	26
Age (year) (median+/-SD)		54.5+/-13.7
Grade/Differentiation	1/Well	35
	2/Moderate	103
	3/Poorly	12
Stage (T)	1	90
	2	38
	3	22
Multiple Tumor	No	121
	Yes	29
Macroscopic Vascular Invasion	No	137
	Yes	13
Microscopic Vascular Invasion	No	101
	Yes	49
Non-cirrhotic	HBV	31
	HCV	0
	Non-HBV/HCV	14
Cirrhotic	HBV	83
	HCV	1
	Non-HBV/HCV	21
Follow-up (month) (median+/-SD)		23.9+/-39.8

Table 3.2 Clinicopatholgoical information of 150 patients with hepatocellular cacrinoma.

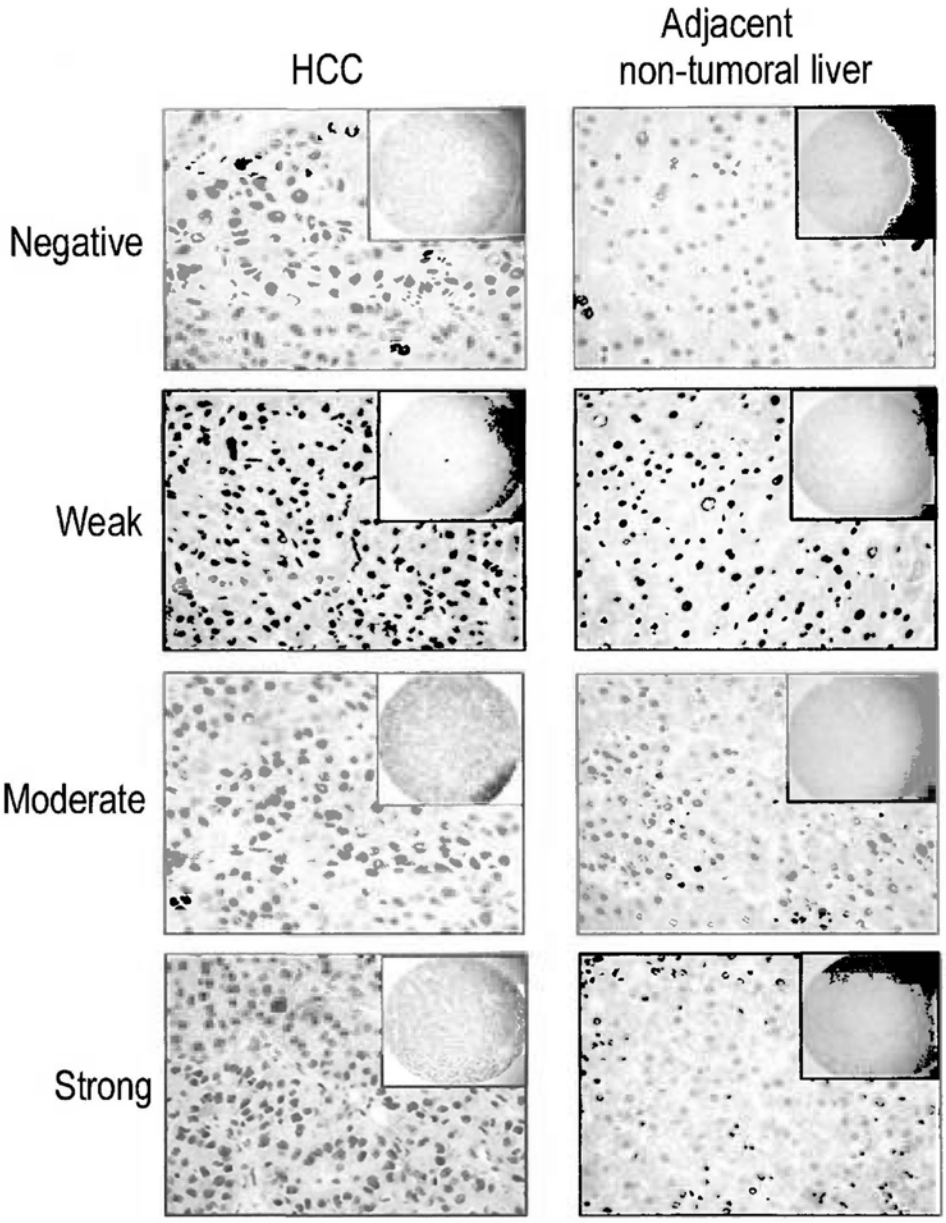


Figure 3.5 Immunohistochemistry analysis of SIRT1 protein expression in HCC and paired adjacent non-tumoral liver. SIRT1 expression was scored according to the proportion of tumor cells with positive nuclear staining (negative, none; weak, <=10%; moderate, 10 to <=50%; strong, >50%). Magnification, X 400; Insert, magnification X 100.

	HCC N=147 N(%)	Non-tumoual liver N=141 N(%)
SIRT1 expression		
Negative	99(70.2%)	141(100%)
Weak	15(10.6%)*	0(0)
Moderate	7(4.9%)*	0(0)
Strong	20(14.2%)*	0(0)

Table 3.3 Quantification of SIRT1 immunostaining in tissue microarrays. Immunostaining of 150-patient tissue microarray using SIRT1 antibodies was semiquantitatively graded as negative, weak, moderate or strong. Statistical analysis was determined using Fisher's exact test. *,$P< 0.001$ for comparison of HCC samples to adjacent non-tumoral livers.

Correlation of clinicopathological parameters with SIRT1 expression

Clinicopathological Parameters		SIRT1 Expression				P-value
		Negative	Weak	Moderate	Strong	
Sex	Male	90	12	5	17	0.162
	Female	14	5	3	4	
Age (median+/-SD)		57+/-13.4	46+/-16.3	56+/-13.8	53+/-11.5	0.544
Grade	1	30	4	1	0	<0.001*
	2	70	12	6	15	
	3	4	1	1	6	
Stage (T)	1	61	13	6	10	0.349
	2	27	2	2	7	
	3	16	2	0	4	
Multiple Tumor	No	83	14	8	16	0.519
	Yes	21	3	0	5	
Macroscopic Vascular Invasion	No	95	16	8	18	0.623
	Yes	9	1	0	3	
Microscopic Vascular Invasion	No	71	13	6	11	0.386
	Yes	33	4	2	10	
HBV	No	8	1	0	3	0.640
	Yes	82	12	5	15	
HCV	No	29	3	1	4	0.965
	Yes	1	0	0	0	
Steatosis	0%	73	14	6	14	0.099
	>0-33%	20	0	2	4	
	>33-66%	9	1	0	1	
	>66%	1	0	0	2	
Cirrhosis	No	33	7	1	4	0.317
	Yes	71	10	7	17	

Table 3.4 Correlation of SIRT1 expressions with clinicopathologic features. *, $P<0.001$

status, and the presence of cirrhosis and steatosis in the nontumorous livers. Besides,when the cumulative actuarial survival (AS) and disease free survival (DFS) was compared between groups by log-rank test, no prognostic significance of SIRT1 expression was demonstrated (p=0.196).

3.2 Effect of lentivirus-mediated knockdown of SIRT1 in HCC cell lines

3.2.1 Knockdown of SIRT1 using shRNAs

Because SIRT1 is over-expressed in HCC, the role of SIRT1 in HCC pathogenesis was explored. Five lentiviral plasmids (pLKO.1-puro) expressing short hairpin RNAs against SIRT1 (shRNA1-5) (Sigma), or non-targeting short hairpin RNA were tested for their efficacy in inducing SIRT1 silencing. SK-Hep-1 cells, a HCC cell line, were transfected with these constructs respectively, and cell lysates were collected 72 hours post-transfection for western blotting analysis. Figure 3.6 showed that all five shRNAs was able to knockdown SIRT1 expression significantly (30~50% reduction). Subsequently, lentiviral vectors encoding shRNA1, shRNA2, or non-targeting short hairpin RNA was further packaged into lentivirus (shSIRT1-1, shSIRT1-2, shCont) respectively. Purified lentiviruses were then transduced into SK-Hep-1 (p53 wild-type), HepG2 (p53 wild-type), Hep3B (p53 deleted), and PLC5 (p53 inactivated) cell lines respectively. Lentiviruses expessing SIRT1 shRNA (shSIRT1-1, shSIRT1-2) efficiently knockdown SIRT1 knockdown in these cell lines (>90%) compared to untreated or scramble shRNA (shCont) transduced cells (Figure 3.7).

3.2.2 Effect of SIRT1 suppression on HCC cell proliferation

To investigate the effect of SIRT1 knockdown on cell proliferation, cells number was determined at regular time intervals. As shown in Figure 3.8A, down-regulation of SIRT1 resulted in a marked decrease in cell number of SK-Hep-1 cells over a course of six days. Similar cell growth inhibition upon SIRT1 depletion

Figure 3.6 SIRT1-shRNA efficiently knockdown SIRT1 expression. SK-Hep-1 cells was transfected with different SIRT1-shRNA. Cell lysates were collected 72 hours post-transfection and probed with rabbit anti-SIRT1 antibodies. β-ACTIN was used as loading control.

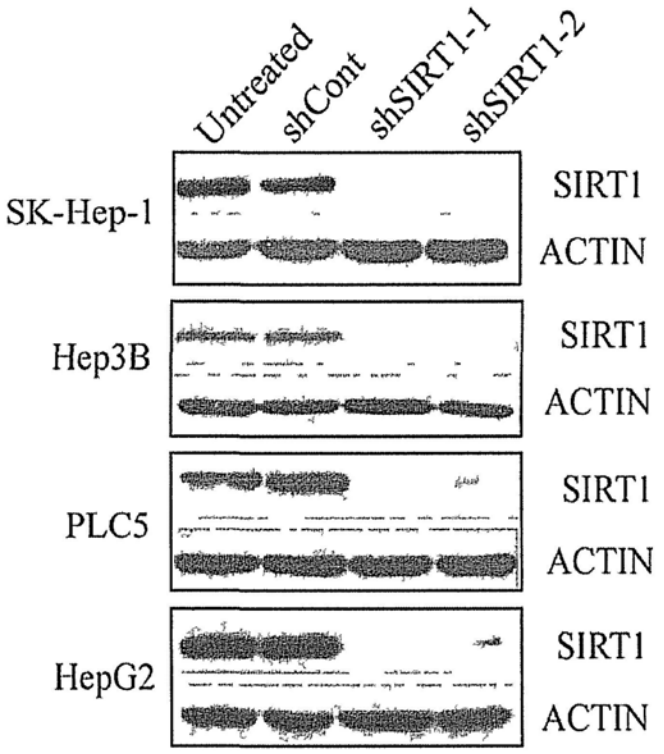
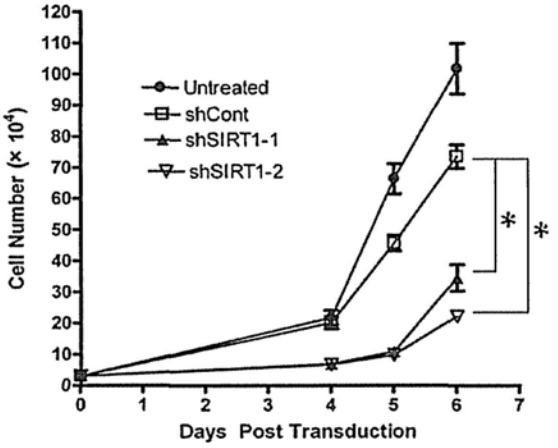


Figure 3.7 Suppression of SIRT1 in four HCC cell lines. Western blot analysis of SIRT1 protein expression was performed in HCC cells transduced with lentiviruses expressing scramble (shCont), or SIRT1-targeting (shSIRT1-1, and shSIRT1-2) shRNA respectively. Cells were harvested at 4 days after viral transduction for western blotting analysis. β-ACTIN was used as loading control.

A



B

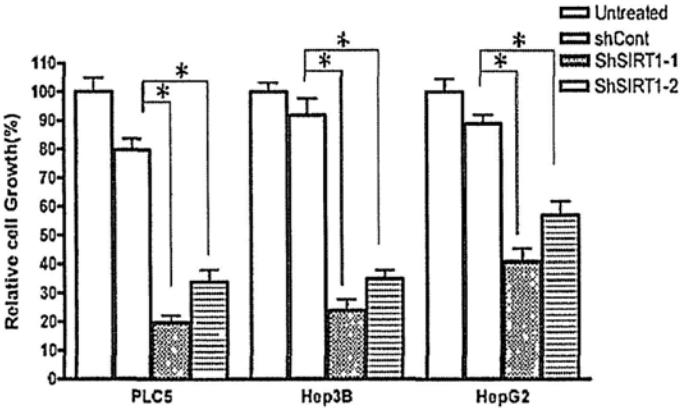


Figure 3.8 Proliferation of HCC cells was suppressed by SIRT1 konckdown. A, SK-Hep-1 cells were infected with the respective lentivirus and cell number was determined by trypan blue exclusion assay at the indicated time. Plots are cumulative cell numbers versus days in culture. *, P< 0.001. **B,** PLC5, Hep3B and HepG2 cells were infected with the respective lentivirus and cell number was determined by trypan blue exclusion assay at day 6 post-tranduction. Values were expressed relative to the cell count in the untreated group, which was set to 100%. *, P< 0.001.

was also observed in several cell lines (HepG2, Hep3B and PLC5) independent of their p53 status (Figure 3.8B). To further test the potential of SIRT1 silencing in suppressing cell proliferation, colony formation assay was carried out using SK-Hep-1 cells. Knockdown of SIRT1 markedly reduced the number and size of puromycin-resistant colonies (Figure 3.9), whereas cell colonies formation in cells tranduced with lentiviruses expressing shCont was not altered. Furthermore, soft-agar assay revealed that downregulation of SIRT1 also inhibited anchorage-independent growth of cells compared to untreated or shCont-expressing cells (Figure 3.10). Taken together, these data suggested that SIRT1 plays an important role in the proliferation and growth of HCC cells.

3.2.3 Effect of SIRT1 suppression on cell cycle distribution

To determine whether the observed cell growth inhibition in SIRT1 knockdown cells was due to reduced cell replication, cellular DNA synthesis was determined by 5-bromo-2'-deoxyuridine (BrdU) incorporation assay. SIRT1 downregulation resulted in a 70% reduction in DNA synthesis compared to untreated or shCont transduced cells (<0.001) (Figure 3.11). To determine if reduced DNA synthesis is associated with a slower rate of cell cycle progression, cell cycle distribution was determined by fluorescence activated cell sorting (FACS) analysis. FACS analysis revealed that SIRT1 silencing induced G1 arrest in SK-Hep-1 and HepG2 cells. Interestingly, SIRT1 silencing in Hep3B cells and PLC5 cells increased the proportion of cells in the G2 phase, suggesting that these cells were arrested at the G2/M transition (Figure 3.12). Taken together, these data suggested that although reduced SIRT1 repressed cell proliferation in general, it has disparate effects on cell cycle distribution in different HCC cells.

3.2.4 Effect of SIRT1 suppression on cellular senescence or apoptosis

Replicative senescence, a state of irreversible growth arrest, can be triggered by multiple mechanisms including telomere shortening, activation of the *INK4a/ARF* locus, and DNA damage. Cellular senescence might defense against tumorigenesis.

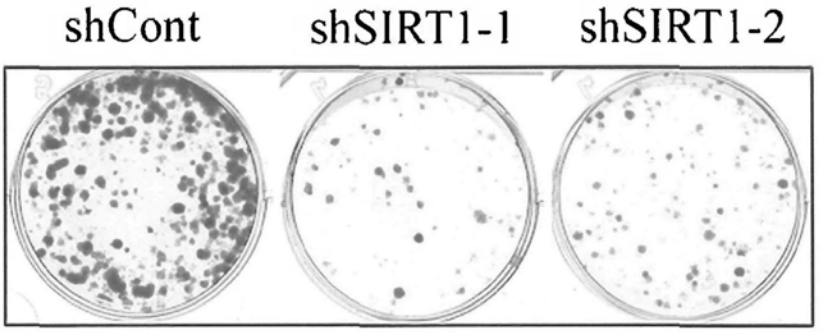


Figure 3.9 Reduced colong formation in SK-Hep-1 cells transduced with lentiviruses expressing SIRT1 shRNAs. SK-Hep-1 cells was transduced with lentivirus expressing shCont, shSIRT1-1 and shSIRT1-2 respectively. Cells were grown for 14 days under the selection of puromycin (0.25 μg/ml) and stained with crystal violet.

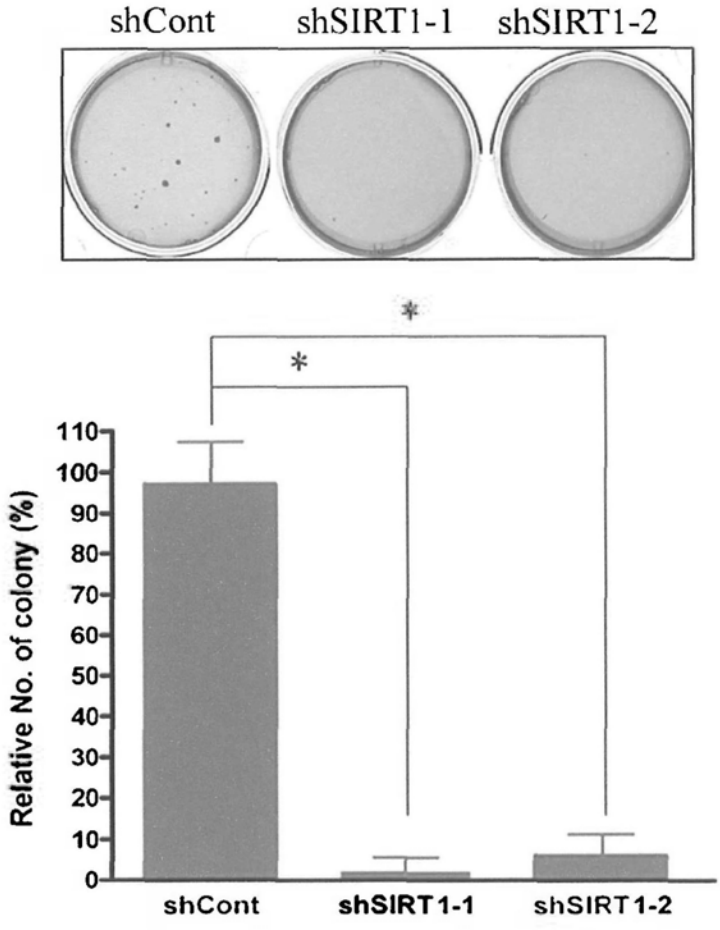


Figure 3.10 Soft agar assay of SK-Hep-1 cells transduced with lentiviruses expressing SIRT1 shRNA. SK-Hep-1 cells was transduced with lentivirus expressing shCont, shSIRT1-1 and shSIRT1-2 respectively. Cells were growth in soft agar for 3 weeks. Quantification of colonies were done by counting macroscopically visible colonies in each well, and expressed as percentage relative to the group expressing shCont. *, $P<0.001$.

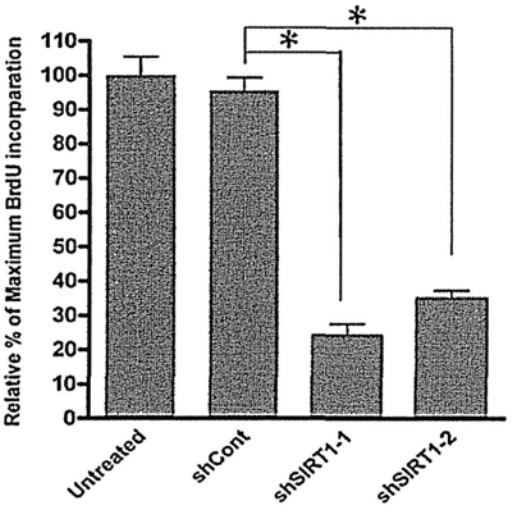


Figure 3.11 BrdU incorporation of SK-Hep-1 cells transduced with lentiviruses expressing the indicated shRNA. SK-Hep-1 was transduced with lentivirus expressing indicated shRNAs. At 4 days after lentiviral transduction, cells were pulsed with BrdU (10 μM) for 4 h. Subsequently cells were stained with anti-BrdU antibody and O.D. was taken at 450 nm. The O.D. values were expressed as percentage relative to the group expressing shCont. *, $P < 0.001$.

		G1	S	G2
SK-Hep-1	shCont	44.29 ±0.36	35.07±1 58	20.64 ±1.58
	shSIRT1-1	69.20±1.24 *	13.40±3.25 *	17.40 ±2.90
	shSIRT1-2	65.86 ±1.75 *	14.57 ±0.72 *	19.57 ±1.90
HepG2	shCont	49.71 ±1.50	27.63 ±0.81	22.66 ±0.35
	shSIRT1-1	68.52 ±0.90 *	20.29±2.50	11.19 ±1.56 *
	shSIRT1-2	65.92 ±2.32 *	20.84±2.56	13.24 ±1.76 *
Hep3B	shCont	57.65 ±0.91	27.84 ±1.21	14.51 ±0.94
	shSIRT1-1	26.14±1.21 *	26.61 ±1.43	47.25 ±1.95 *
	shSIRT1-2	34.21 ±2.41 *	27.73 ±2.76	38.06±0.75 *
PLC5	shCont	66.58 ±1.70	24.40 ±1.31	9.02 ±0.55
	shSIRT1-1	53.59 ±1.35 *	28.62±1.42	17.85 ±0.78 *
	shSIRT1-2	51.84 ±1.38 *	29.07 ±1.22	19.09 ±0.90 *

Figure 3.12 Effect of SIRT1 knockdown on cell cycle distribution. Cell cycle analysis of SK-Hep-1, HepG2, Hep3B and PLC5 cells transduced with lentiviruses expressing the indicated SIRT1 shRNAs for 5 days. Results shown are the mean ± SD of three independent experiments. *, $P<0.001$.

However, tumor cells bypass replicative senescence and undergo immortalization, whereas induction of senescence in tumor cells can suppress tumor growth. Cellular senescence is characterized by a series of features including growth arrest and the induction of senescence-associated β-galactosidase (SA-β-gal). These cellular changes are associated with the induction of the tumor suppressor p53,dephosphorylation of Rb and increased expression of cyclin-dependent kinase (CDK) inhibitors p16, p21 and p27 respectively (Goldstein, Moerman et al. 1994; Dimri, Lee et al. 1995; Serrano, Lin et al. 1997; Collado, Medema et al. 2000; Alexander and Hinds 2001; Beausejour, Krtolica et al. 2003; Jirawatnotai, Moons et al. 2003).

Because SIRT1 has been implicated a role in senescence in other model system (Ota, Tokunaga et al. 2006), its role in cellular senescence of HCC cells were determined. HCC cells were transduced with lentiviruses expressing shSIRT1, shSIRT2 or shCont respectively. Gene silencing of SIRT1 in SK-Hep-1 and HepG2 (p53 wild-type) resulted in cells that were enlarged in size, flattened in shape, and highly positive (~50 % of total cells) for senescence-associated β-galatosidase (SA-β-gal) staining, whereas negative staining (<10 % of total cells) was observed in Hep3B and PLC5 (p53 null or mutated) cells (Figure 3.13). Moreover, cellular senescence in SK-Hep-1 and HepG2 cells was associated with enhanced p53 acetylation on lysine 382 and induction of p21 that are known for their role in senescence induction (Figure 3.14) (Ota, Tokunaga et al. 2006). In contrast, p53 acetylation and p21 induction were not observed in Hep3B cells while enhanced p53 acetylation but not p21 induction was observed in PLC5 cells, consistent with their mutated p53 status. On the other hand, the expression of two other proteins implicated in senescence, namely p27 and p16, was not changed significantly upon SIRT1 knockdown (Figure 3.14).

To determine if SIRT1 knockdown promotes apoptosis in Hep3B and PLC5 (p53 null/mutated) cells, which failed to enter cellular senescence, western blotting analysis of poly ADP ribose polymerase (PARP) cleavage was carried out in cells transduced with lentivirues expressing shSIRT1, shSIRT2 or shCont respectively.

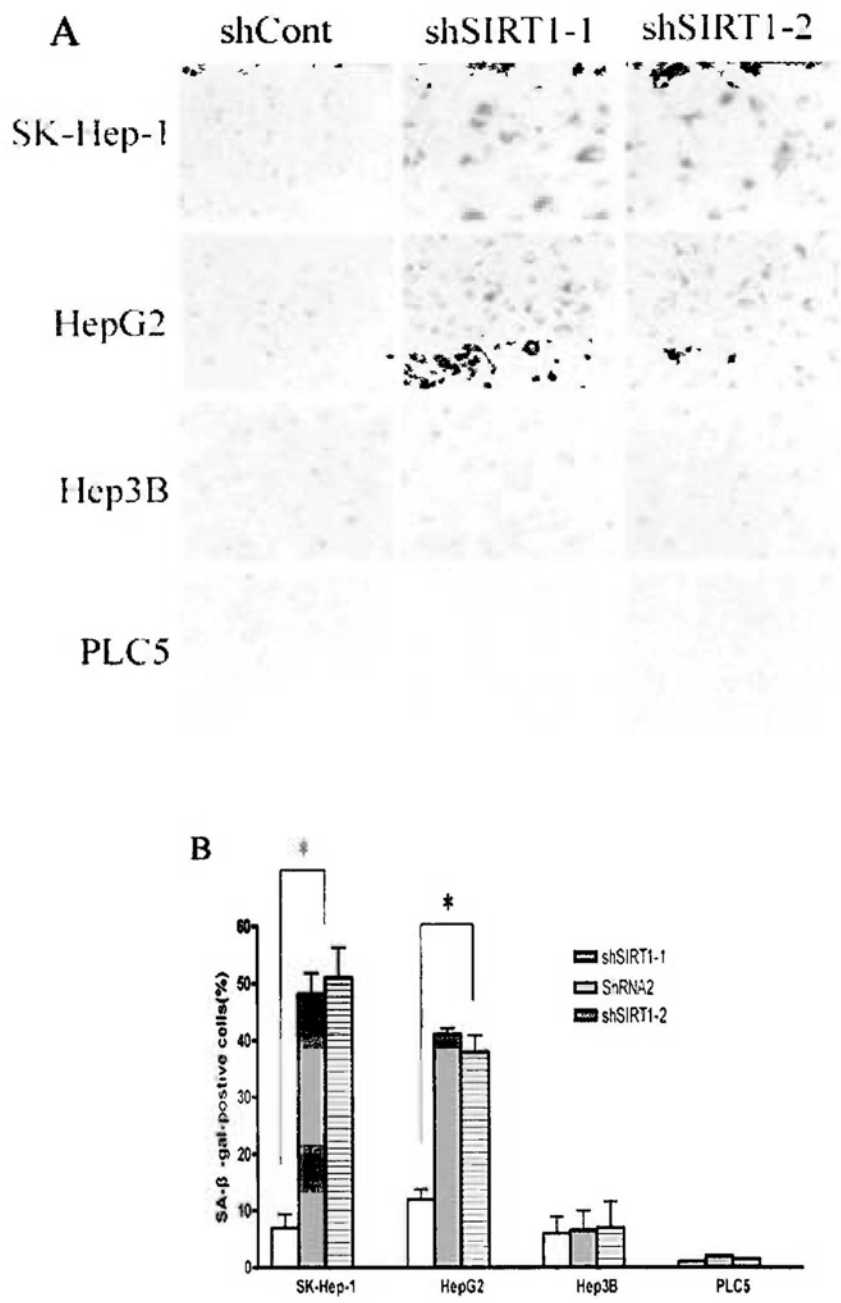


Figure 3.13 SA-β-gal staining in SIRT1 knockdown cells. A, SA-β-gal staining was performed for the analysis of cellular senescence of SK-Hep-1, HepG2, Hep3B and PLC5 cells transduced with lentiviruses expressing the indicated SIRT1 shRNAs for 6 days. **B,** Quantification of SA-β-gal positive cells. SA-β-gal–positive cells relative to total cells was obtained by counting 200 cells in three randomly chosen fields per well. Each experiment was performed in triplicate. Bar represents the mean ± SD of three independent experiments. *, $P<0.001$.

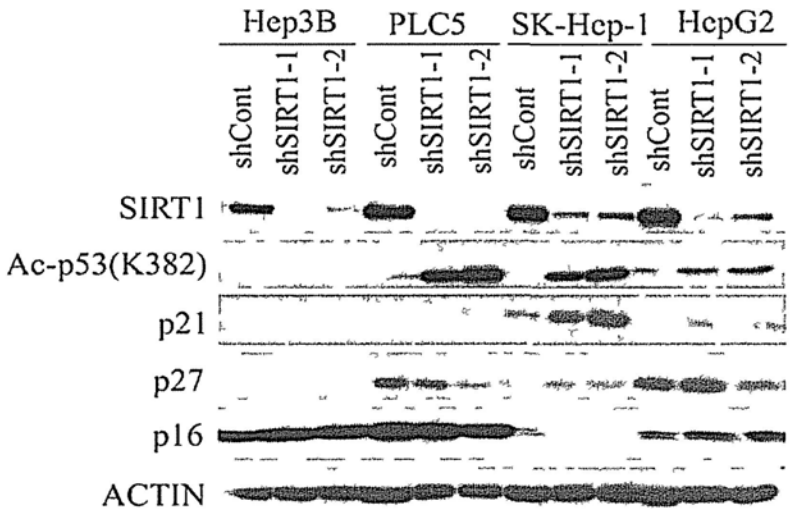


Figure 3.14 Western blotting analysis of p53 and cyclin-dependent kinase inhibitors in SIRT1–depleted cells. Cells were transduced with lentiviruses expressing expressing shCont, shSIRT1-1 and shSIRT1-2 respectively. Cells were harvested 4 days after viral transduction for western blotting analysis. β-ACTIN was used as loading control.

Enhanced PARP cleavage was observed in Hep3B and PLC5 cells compared with HepG2 and SK-Hep-1 cells (Figure 3.15), suggesting that there was an increased in propensity to apoptotic cell death in Hep3B an PLC5 cells respectively. Collectively, these data demonstrated that SIRT1 knockdown in HCC cells leads to cell senescence or apoptosis that is closely correlated with the p53 status of the cells.

3.3 The role of SIRT1 in telomeric maintenance

Telomeres are nucleoprotein structures that protect the ends of chromosomes. Mammalian telomeres are protected from being recognized as sites of DNA damage by the shelterin complex, which is composed of telomeric repeat-binding factor 1(TRF1, also known as TERF1), TRF2 (also known as TERF2), TERF1-interacting nuclear factor 2 (TIN2, also known as TINF2), protection of telomeres 1 (POT1), the POT1- and TINF2-interacting protein (TPP1, also known as PTOP) and the transcription repressor/activator protein RAP1 (also known as TERF2-interacting protein TERF2IP) (Heltweg, Gatbonton et al. 2006). Telomere length was maintained by telomerase, a specialized nucleoprotein complex that includes an RNA template (TERC) and a reverse transcriptase catalytic subunit (TERT). The loss of telomere has been considered to be the critical signal for senescence induction. It is also well accepted that telomere-dependent senescence can be induced by a change in the protective status of shortened telomere (de Lange 2005). The loss of telomere protection or any other cause of telomere dysfunction can trigger classical DNA-damage response (DDR) (Karlseder, Smogorzewska et al. 2002). (d'Adda di Fagagna, Reaper et al. 2003; Takai, Smogorzewska et al. 2003; Herbig, Jobling et al. 2004) by recruiting phosphorylated H2AX, 53BP, NBS1 and MDC1 (Gire, Roux et al. 2004). The DDR enables cells to sense damaged DNA and respond by arresting cell-cycle progression and repairing the damage if possible.

3.3.1 Knockdown of SIRT1 reduced expression of TERT, PTOP and POT1

To determine whether the observed cellular senescence or cell death are

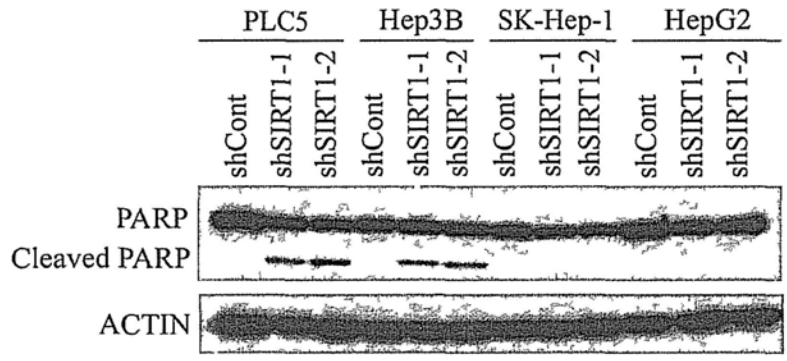


Figure 3.15 Western blotting analysis of Poly-ADP ribose polymerase (PARP) cleavage in SIRT1-depleted cells. Cells were transduced with lentiviruses expressing the indicated shRNA for 4 days before western blotting analysis. β-ACTIN was used as a loading control.

associated with telomeric dysfunction, the expression of telomerase (TERT), members of the shelterin complex, and other telomere-associated proteins (PINX1, TANK1, and KU-70) in control and SIRT1-depleted cells were determined by quantitative real-time PCR respectively. SK-Hep-1 cells expressing SIRT1 shRNAs resulted in a significant reduction of TERT mRNA (~10 fold reduction) compared to untreated or shCont-expressing cells ($p<0.01$). In addition, the expression of PTOP and POT1, which are members of the shelterin complex, was also significantly reduced in SIRT1-depleted cells ($p<0.01$). However, the expression of other members of the shelterin complex was not significant differed between SIRT1-depleted and control cells (Figure 3.16).

To further confirm the qPCR results, western blotting analysis was carried out to examine the effect of SIRT1 knockdown on expression level of TERT, PTOP and POT1 protein respectively. SIRT1 knockdown reduced the expression of TERT and PTOP protein in all hepatoma cells examined (SK-Hep-1, HepG2, Hep3B and PLC5), suggesting that SIRT1 may play an important role in PTOP and TERT expression in liver tumors (Figure 3.17). A reduction of POT1 expression upon SIRT1 knockdown were observed only in SK-Hep-1 and Hep3B cells but not in HepG2 and PLC-5 cells, implicating that the POT1 gene was subjected to a more heterogeneous regulation (Figure 3.17).

3.3.2 SIRT1 silencing-induced growth inhibition was rescued by ectopic expression of TERT or PTOP

As shown in Figure 3.17, the suppression of SIRT1 negatively regulated expression of TERT and PTOP. Rescue experiments were conducted to evaluate the contribution of each of the protein to cell growth suppression. SK-Hep1 cells were transfected with plasmid expressing TERT (pLV102-TERT), PTOP (pLV102-PTOP) or GFP (pLV102-GFP) respectively. Stable cell lines expressing either TERT, PTOP or GFP were generated after cells were cultured in medium containing puromycin for two weeks. As shown in Figure 3.18, western blot analysis revealed that gene silencing of SIRT1 reduced the expression of TERT and PTOP as expected, but the

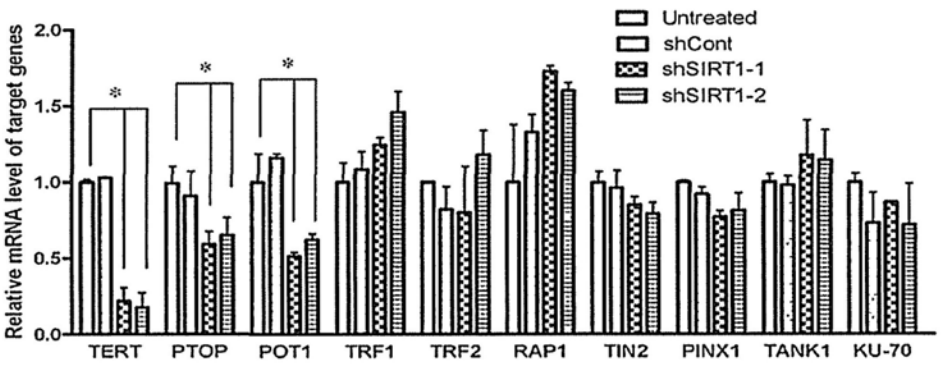


Figure 3.16 Real-time quantitative PCR analysis of gene expressions related to telomeric maintenance. The mRNA of SK-Hep-1 cells transduced with lentiviruses expressing the indicated shRNA was analyzed. β-ACTIN mRNA expression was used as an internal control. Experiment was performed in triplicate. Values represent the mean ± SD of three experiments. *, $P<0.001$

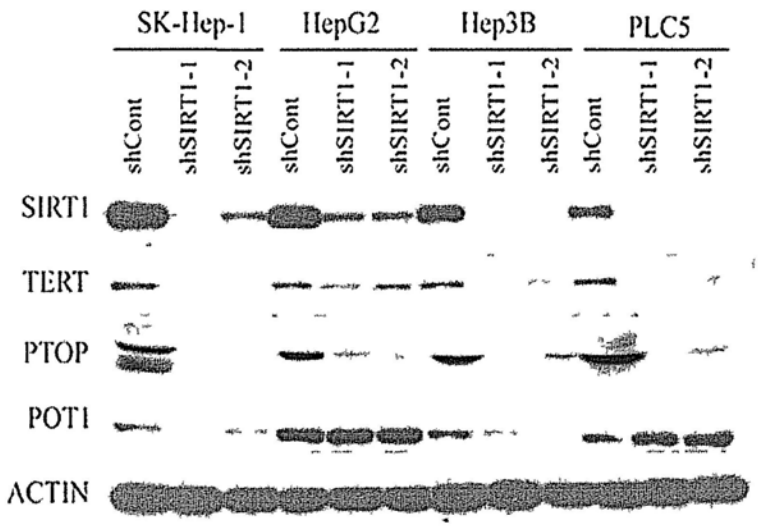


Figure 3.17 The effect of SIRT1 knockdown on TERT, PTOP and POT1 expression. Cells were transduced with lentiviruses expressing the indicated shRNA for 5 days before western blotting analysis. β-ACTIN was used as a loading control.

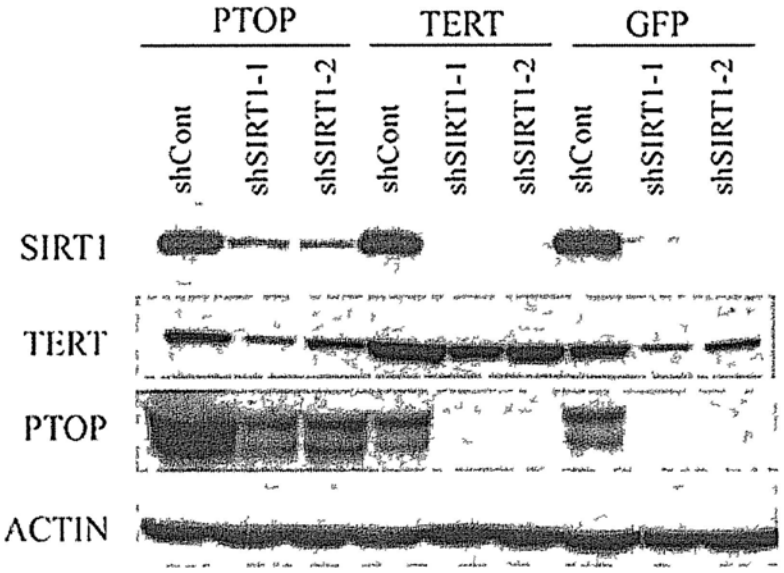


Figure 3.18 Over-expression of TERT or PTOP in SK-Hep-1 cells. SK-Hep-1 cells were transfected with TERT, PTOP or GFP expressing vector. Cells were grown 2 weeks under the selection of puromycin (0.25 μg/ml). Subsequently these cells were transduced with lentivirus expressing shCont, shSIRT1-1 and shSIRT1-2 respectively. Cells were harvested for Western blotting analysis at four days post-transduction. β-ACTIN was used as a loading control.

expression of these two proteins were rescued in cells transfected with pLV102-TERT and pLV102-PTOP respectively .

To determine whether TERT or PTOP could rescues cell growth in SIRT1-depleted cells, the effect of TERT or PTOP over-expression on cell proliferation and cell senescence was analyzed. A significant enhancement in cell growth (Figure 3.19) and a reduction in cell senescence (Figure 3.20) were observed when PTOP or TERT, but not GFP, was ectopically expressed in cells transduced with lentiviruses expressing shSIRT1-1 and shSIRT1-2 respectively. Together, these data demonstrate that PTOP and TERT depletion are responsible, at least in pat, for cell growth suppression by SIRT1 knockdown in HCC cells.

3.3.3 SIRT1 suppression induced Telomere dysfunction-induced foci (TIF)

As mentioned above, telomere dysfunction can lead to the activation of canonical DNA damage response (DDR). Mammalian cells response to lesions in their genome by two phoshpatidylionsitol 3-kinase-related protein kinases, ATM and ATR. Both kinases phosphorylate histone H2AX in a large chromatin domain surrounding the damage site and promote the local accumulation of other DNA damage response factors (MDC1, 53BP, the Mre11 complex, etc.). The cytological structure formed by the assembly of DNA damage factors at the telomeres are referred to as Telomere dysfunction Induced Foci (TIF) (d'Adda di Fagagna, Reaper et al. 2003).

PTOP is an oligonucleotide/oligosaccharide-binding fold (OB fold) protein in shelterin that forms a heterodimer with POT1 (Kim, Beausejour et al. 2004; Hockemeyer, Sfeir et al. 2005). Several lines of evidence indicated that PTOP mediates the recruitment of POT1 to telomeres to protect the chromosome end (Wang, Podell et al. 2007). Recent study also showed that PTOP plays an essential organizing function in shelterin and protect telomeres from TIF via recruiting POT1(Liu, Safari et al. 2004; Hockemeyer, Palm et al. 2007). It also recruits TERT to the telomere and regulates its activity (Kibe, Osawa et al. 2010). Therefore, SIRT1-depletion might induce TIF via reducing the expression of PTOP. To test this

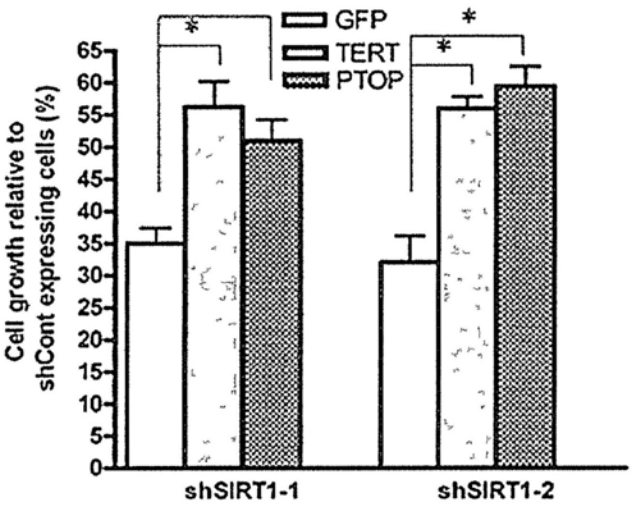


Figure 3.19 Over-expression of TERT or PTOP significantly improved cell growth. SK-TERT, SK-PTOP or SK-GFP cells were transduced with lentivirus expressing shCont, shSIRT1-1 and shSIRT1-2 respectively. The cell numbers were counted at day 6 post-tranduction using trypan blue assay. Values were expressed relative to the cell count in the shCont group, which was set to 100%. Bars represent the mean ± SD of three experiments. *, $p < 0.01$.

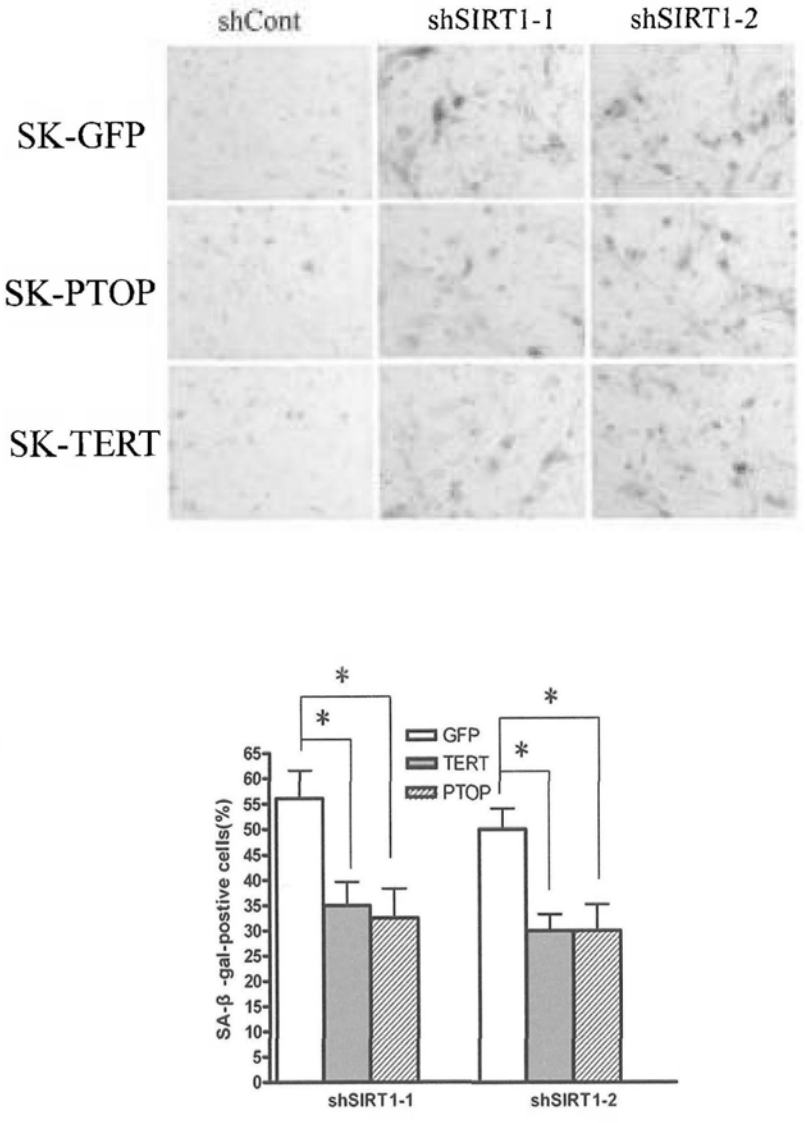


Figure 3.20 Ectopical expression of TERT or PTOP reduced senescence in SIRT1-depleted cells. A, SK-TERT, SK-PTOP or SK-GFP cells were transduced with lentivirus expressing shCont, shSIRT1-1 and shSIRT1-2 respectively. SA-β-gal staining was performed at day 6 post-tranduction. **B,** Quantification of cells showing positive staining. SA-β-gal–positive cells relative to total cells was obtained by counting 200 cells in three randomly chosen fields per well. Bars represent the mean ± SD of three experiments. *, $p < 0.01$.

hypothesis, western blotting was conducted to determine if SIRT1 suppression induces the expression of phosphorylated H2AX (γ-H2AX), which is considered to be a sensitive and selective signal for the existence of DDR. Consistent with our hypothesis, SIRT1-depletion significantly induced the formation of γ-H2AX (Figure 3.21A). To further investigate whether SIRT1 knockdown-induced γ-H2AX was associated with telomere, TIF was determined by detecting the co-localization of γ-H2AX with telomeric marker. The data showed that at 4 days after cells were infected with lentivirus, numerous γ-H2AX foci were observed in shSIRT1-1 and shSIRT1-2 expressing cells (Fig 3.21B). The γ-H2AX foci were co-localized with TRF2 immunofluorescence, suggesting DNA damage occurred at the telomeres (Figure 3.22A). Quantitive analysis revealed that about 35% γ-H2AX foci were colocalized with TRF2 in SIRT1-depleted cells, whereas only 7% these foci was observed in shCont expressing cells (Figure 3.22B).

It has been shown that TIF could lead to telomeric fusion (Celli and de Lange 2005; Attwooll, Akpinar et al. 2009) which can be revealed by analyzing metaphase spread chromosomes. However, SIRT1-depleted cells failed to undergo mitotic arrest in response to colchicine treatment, and therefore metaphase-arrested cells could not be obtained for the analysis of telomeric fusion in SIRT1-depldeted cells. Subsequent analysis revealed that genes that are critical for mitotic checkpoint signaling were significantly repressed upon SIRT1 knockdown, these includes aurora kinase A, aurora kinase B, centromere protein A, and *Bub1* respectively (Figure 3.23).

On the other hand, telomerase (TERT) is critical for telomere length maintenance. Telomere shortening coupled to cell division in the absence of telomerase activity is one of the major causes of telomere dysfunction in human cells (Harley, Futcher et al. 1990; Bodnar, Ouellette et al. 1998). Telomere eroded to a critically short length results in chromosome end-to-end fusions, and triggers cell arrest and/or apoptosis (Espejel, Franco et al. 2002; Goytisolo and Blasco 2002). To test whether the observed senescence or apoptosis was due to telomere shortening, telomere length in SIRT1-depleted cells was determined. Southern blotting analysis revealed that the overall length of the telomeres does not differ significantly between

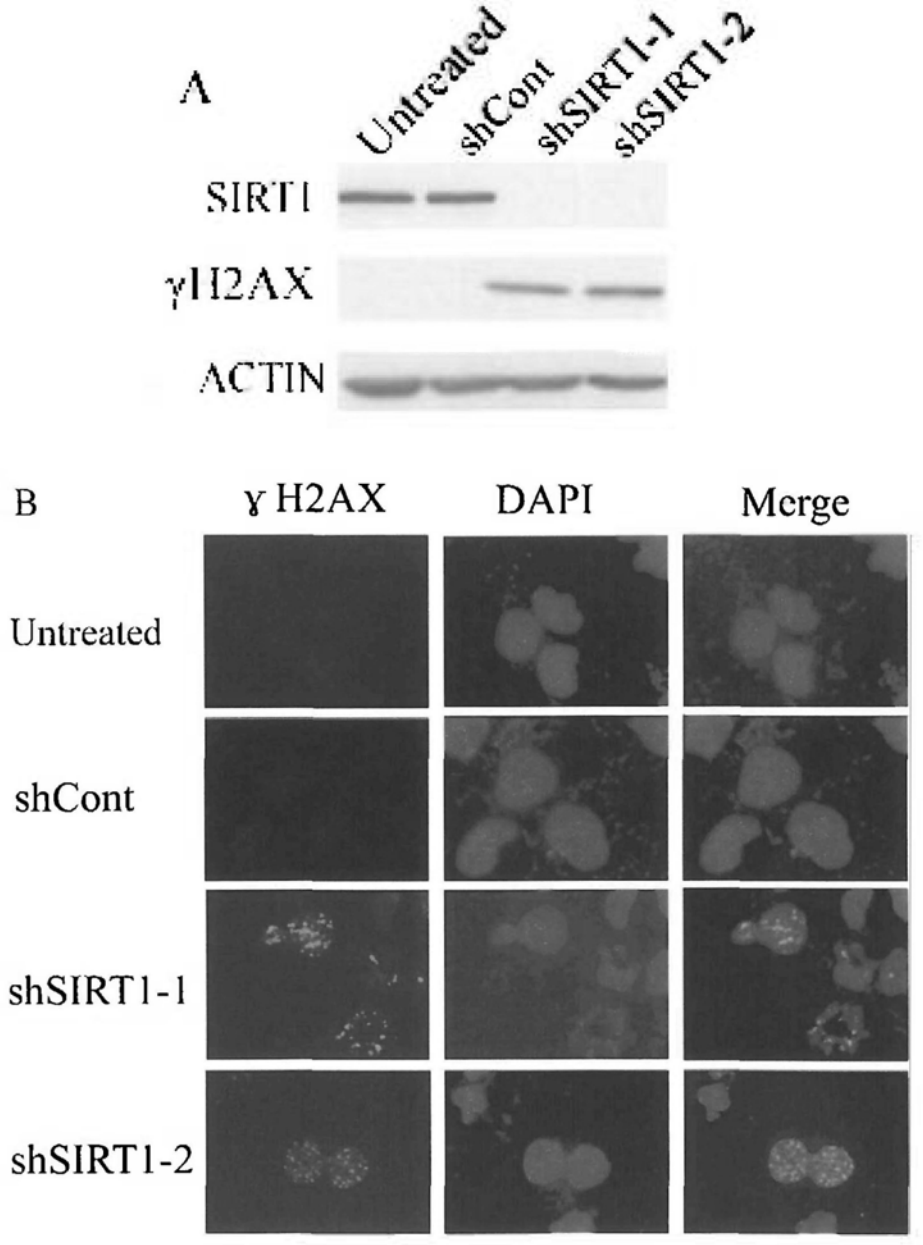


Figure 3.21 SIRT1 suppression leads to DNA damage. A, SK-Hep-1 cells were transduced with lentiviruses expressing the indicated shRNAs for 4 days and subjected to western blot analysis using γ-H2AX antibodies. β-ACTIN was used as loading control. **B,** SK-Hep-1 cells at 4 days after lentiviral transduction were stained with γ-H2AX antibodies and DAPI.

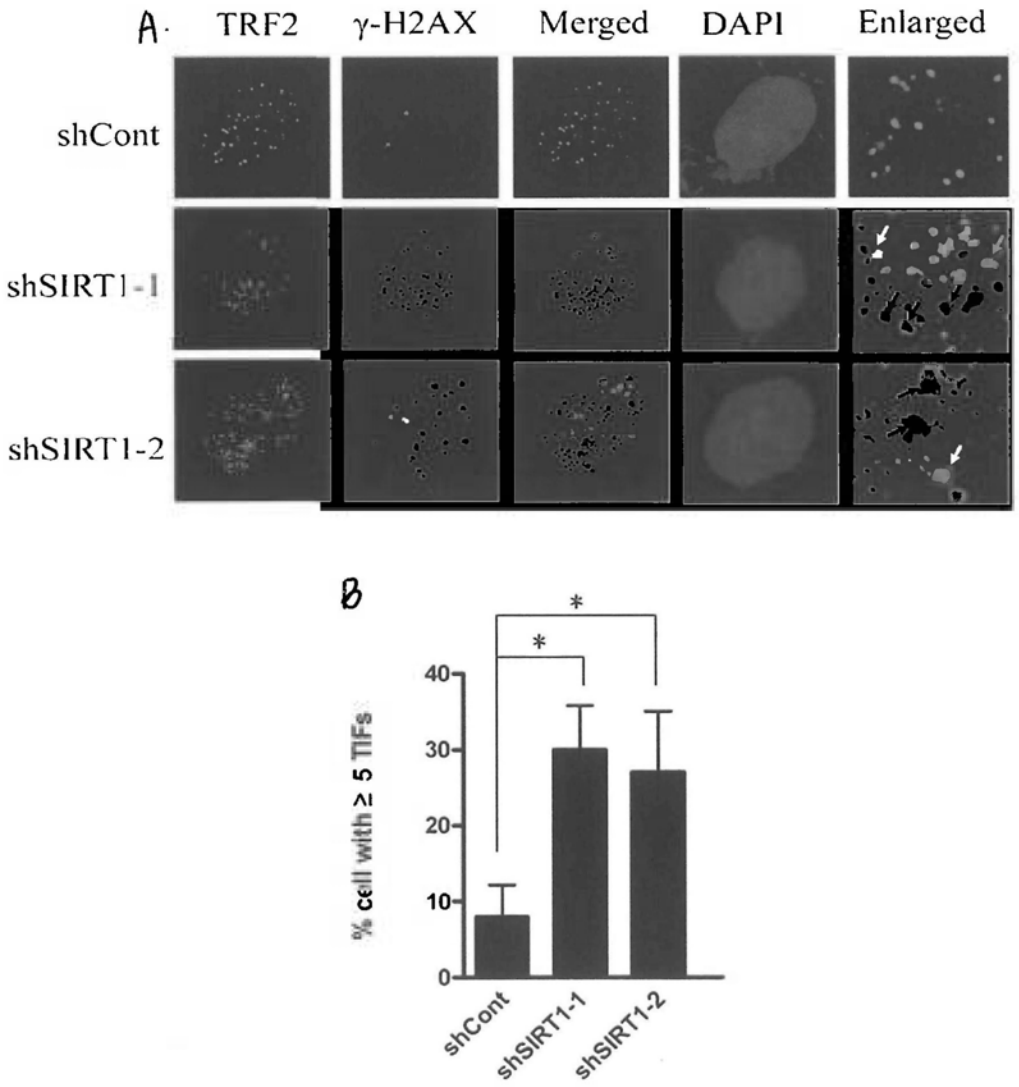


Figure 3.22 SIRT1 knockdown induce telomeric localization of γ-H2AX. A, SK-Hep-1 cells at 4 days after lentiviral transduction were analyzed using TRF2 antibodies for telomeres (TRITC, red) and γ-H2AX antibodies for DNA damage (FITC, green). **B,** Quantification of the TIF response. Cells with five or more γ-H2AX foci colocalizing with TRF2 were scored as TIF positive. 100 cells were randomly picked from 5 fields and scored. Bars represent the mean ± SD of three experiments. *, $p < 0.01$.

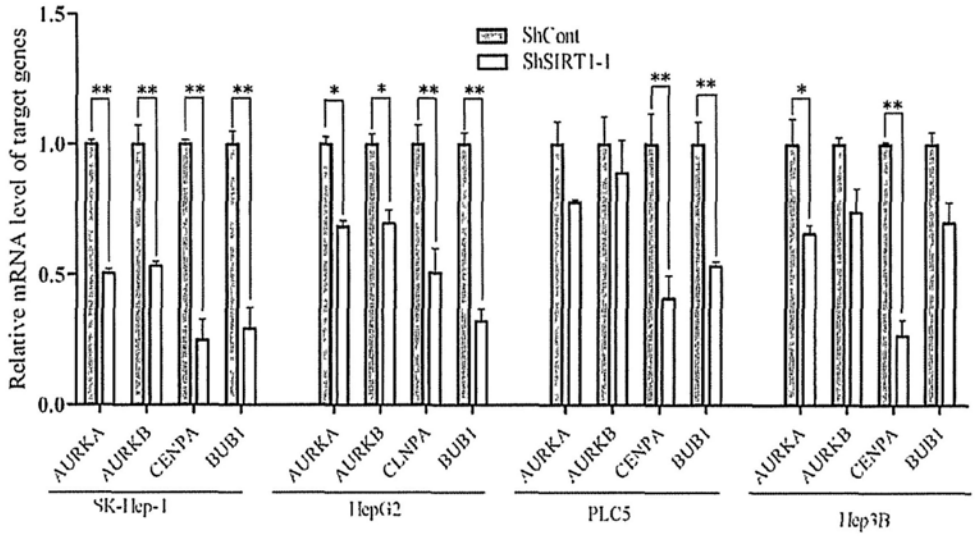


Figure 3.23 Real-time quantitative PCR analysis of gene expressions related to mitosis. Cells were transduced with lentiviruses expressing the indicated shRNA. The RNA was extracted at five days after transduction. β-actin mRNA expression was used as an internal control. Experiment was performed in triplicate. Values represent the mean ± SD of three experiments. **, $P<0.01$; *, $P<0.05$.

control and SIRT1-depleted cells as measured at 7 days after lentiviral transduction (Figure 3.24). This result demonstrated that the telomeric phenotypes in SIRT1-depleted cells did not result from telomeric attrition and TERT might contribute to cell survival via telomere-independent mechanisms.

3.3.4 SIRT1 suppression induced nuclear abnormality

In line with the observation that genes critical for mitotic checkpoint signaling were suppressed upon SIRT1 silencing, SIRT1-depleted cells exhibited severe alterations in their nuclear morphology. While most of the cells expressing ShCont contained round and oval-shaped nuclei, multinuclei and micronuclei were observed in SIRT1-depleted cells (Figure 3.25). The extents of multinuclei and micronuclei were quantified in these cells at four days after lentiviral transduction. More than 30% of SIRT1-depleted cells exhibited abnormal nuclear morphologies, in which these cells either contain micronuclei (6.7±2.6% in shSIRT1-1 expressing cells; 4.7±2.2% in shSIRT1-2 expressing cells) or are multinucleated (24±3.9% in shSIRT1-1 expressing cells; 19±4.2% in shSIRT1-2 expressing cells). In contrast, only a minority of untreated (6 ± 1.7 %) or shCont-expressing (8 ± 3.7 %) cells exhibited abnormal nuclear morphologies (Figure 3.26).

Collectively, these data suggested that telomeric dysfunction and the resulting chromosmal instability contribute to cell growth suppression induced by SIRT1 reduction.

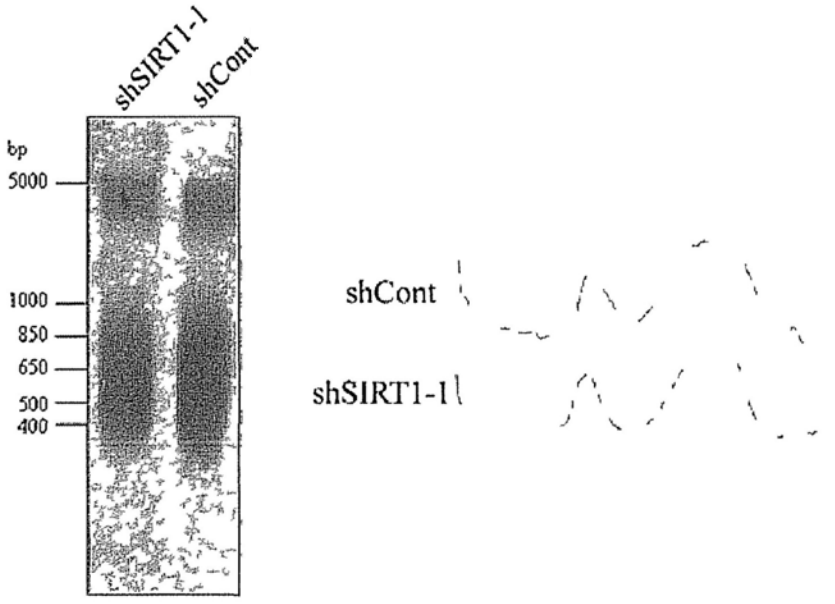
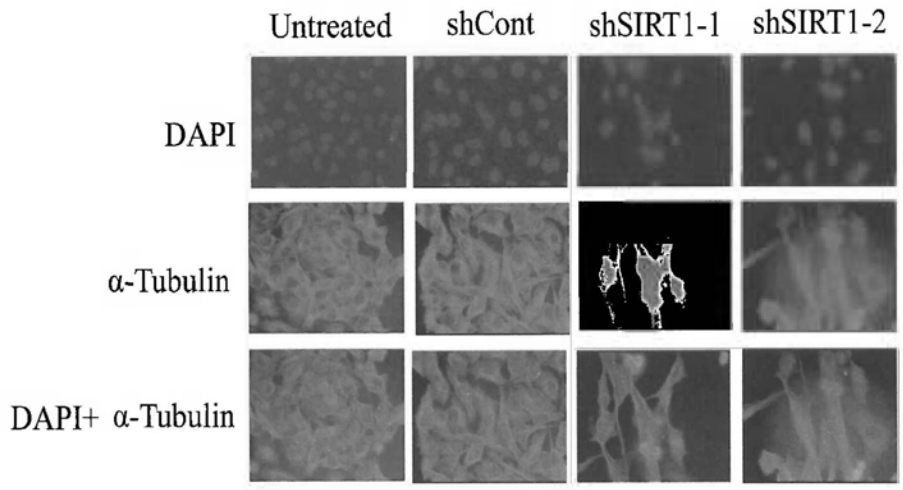


Figure 3.24 Southern blot analysis of telomere length. SK-Hep-1 cells were transducted with lentiviruses expressing the indicated shRNA. Genomic DNAs were collected at seven days after infection. 3 μg of DNA was digested with HinfI and RasI, followed by separation on 1% agarose gel. Southern blot hybridization was conducted using digoxigenin-labeled DNA oligonucleotides of the telomeric repeat sequences 5'-$(CCCTAA)_3$-3'. Telomere length was determined by analyzing the signal using ImageJ (NIH, USA).



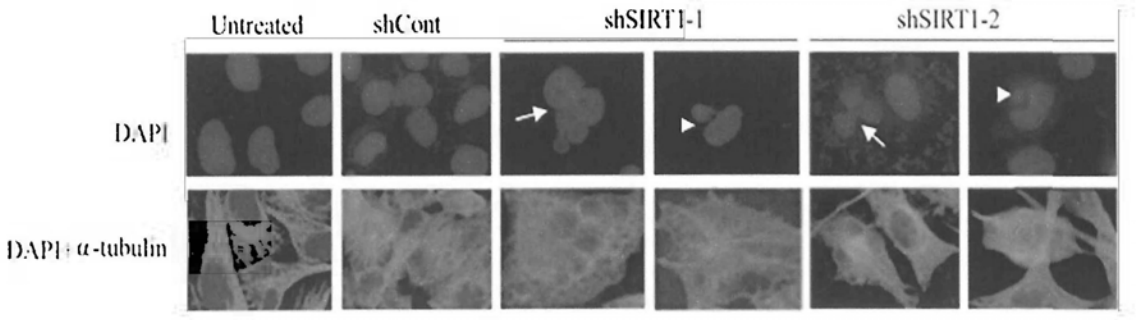


Figure 3.25 SIRT1 suppression induced nuclear abnormality. SK-Hep-1 cells were transduced with lentivirus expressing the indicated shRNAs. Four days after infection, cells were subjected to immunostaining using α-tubulin antibodies (TRITC, red) and DAPI (blue). Nuclear abnormalities were categorized into "multinuclei" and "micronuclei". Arrow denotes multinuclei, arrow head denotes micronuclei.

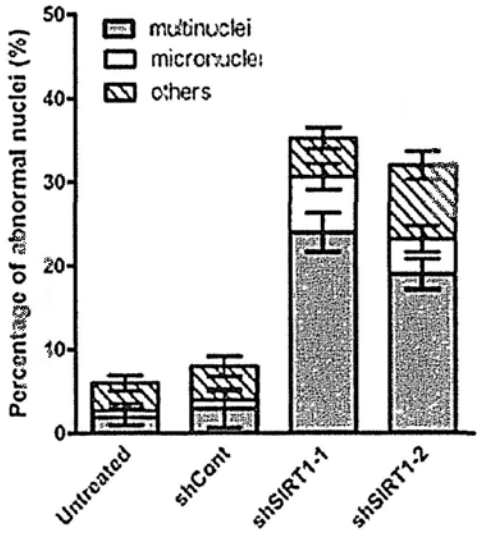


Figure 3.26 Quantitative analysis of the cells exhibiting abnormal nuclear morphologies. SK-Hep-1 cells were transduced and examined as described in figure 3.23. 100 cells were randomly picked from 5 fields and scored for abnormality of the nucleus. Bars represent the mean ± SD of three experiments.

3.4 Correlation between SIRT1, TERT and PTOP expression in human HCC

To determine the relevance of the above SIRT1-regulated pathways in human subjects, the expression of TERT and PTOP in HCC was determined by Western blotting using the same 40 paired HCC that have been used (Figure 1B) to determine SIRT1 expression. Overall, TERT was expressed at a higher levels in HCC compared to adjacent non-tumoral liver tissues, whereas PTOP was expressed at a comparable level in these tissues (Figure 3.27). Correlative analysis further revealed a significant association between tumoral induction of TERT and SIRT1 (Spearman's rank = 0.40, P=0.01) (Figure 3.28), whereas the association between tumoral induction of SIRT1 and PTOP was not apparent (Spearman's rank = 0.08, P=0.21) (Figure 3.29). These data suggested that a SIRT1-TERT regulatory axis may exist *in vivo*.

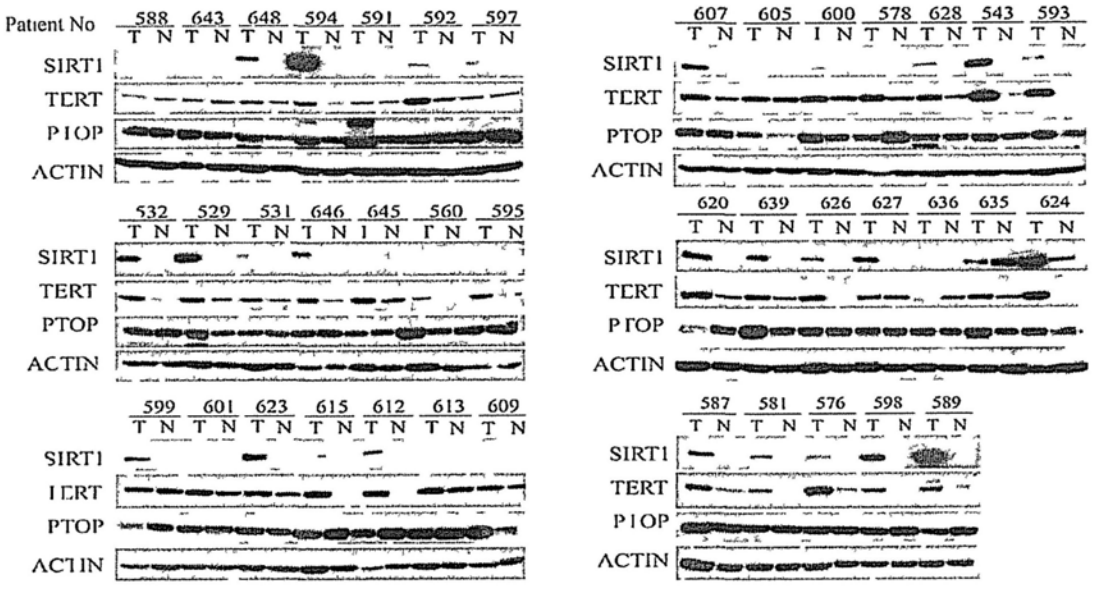


Figure 3.27 Expression of SIRT1, TERT and PTOP in 40 paired HCC and adjacent non-tumoral liver tissues.

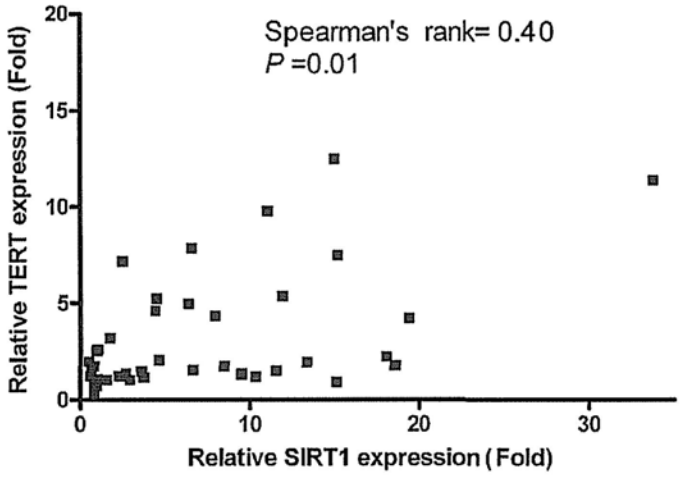


Figure 3.28 Correlation analysis between relative SIRT1 expression and TERT expression. SIRT1 or TERT expression level was first normalized by the expression level of β-ACTIN, and the fold induction of each of these proteins in HCC over non-tumoral liver in each patient was calculated. Fold induction of SIRT1 was plotted against TERT respectively, and was analyzed by Spearmans rho rank test, $p<0.05$.

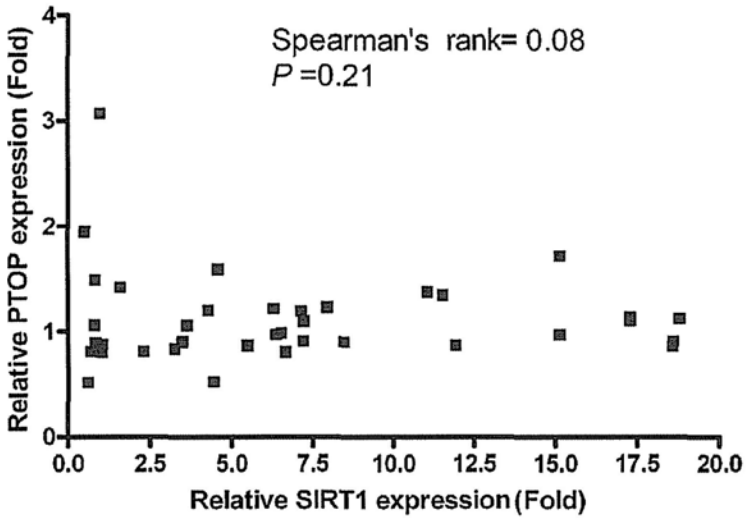


Figure 3.29 Correlation analysis between relative SIRT1 expression and PTOP expression. SIRT1 or PTOP expression level was first normalized by the expression level of β-ACTIN, and the fold induction of each of these proteins in HCC over non-tumoral liver in each patient was calculated. Fold induction of SIRT1 was plotted against TERT respectively, and was analyzed by Spearmans rho rank test, $p>0.05$.

3.5 SIRT1 knockdown enhanced cytotoxicity of doxorubicin in HCC cells

Many studies have implicated a role of SIRT1 in promoting cell survival in response to stress or chemotherapy (Wang, Podell et al. 2007; Xin, Liu et al. 2007). To further corroborate the potential of targeting SIRT1 for HCC therapy, we determined whether SIRT1 inhibition enhances the anti-tumor effect of the DNA-damaging agent doxorubicin. Cells were first transduced with lentiviruses expressing shSIRT1-1 for 2 days, before doxorubicin was added at a final concentration of 0 – 12 μM for another 48 hours. Reduced SIRT1 expression increased the chemosensitivity of SK-Hep-1 and PLC5 cells to doxorubicin treatment by 8 fold and 4 fold respectively (Figure 3.30). Concordantly, reduced SIRT1 expression also promoted doxorubicin-induced apoptosis of these cells, as evidenced by enhanced PARP cleavage (Figure 3.31). Together these data suggest the therapeutic potential of combining a SIRT1 inhibitor and doxorubicin in the treatment of a subset of HCC in which SIRT1 is up-regulated.

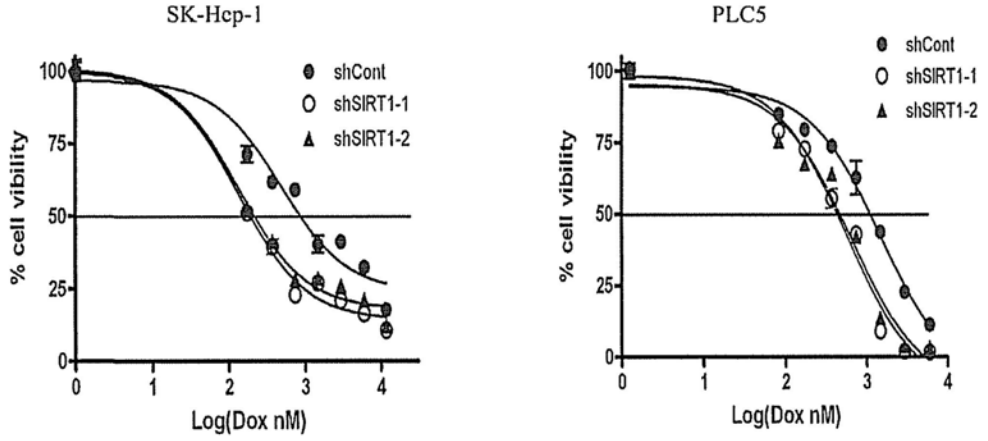


Figure 3.30 SIRT1 knockdown sensitizes HCC cells to doxorubicin treatment.

SK-Hep-1 and PLC5 cells were infected with lentivirus expressing the indicated shRNAs. Two days after infection, cells were treated with doxorubicin at various concentrations for 2 days, and then processed for MTT assay.

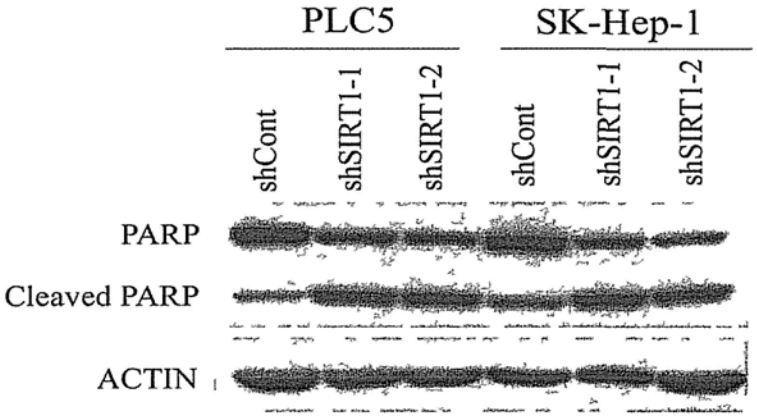


Figure 3.31 Western blotting analysis of PARP in doxorubicin-treamted cells. A, SK-Hep-1 and PLC5 cells transduced with lentivirus were treated with 0.75 μM of doxorubicin for 2 days. Cells were harvested for PARP cleavage analysis using PARP antibodies. β-ACTIN was used as loading control.

Chapter 4 Discussion

The role of SIRT1 in tumorigenesis is controversial. One of the confusing aspects of SIRT1 is that it seems to play a dual role in cell survival and cell death that is dependent of cellular context where it was studied. Most of the earlier evidence suggested that SIRT1 functions as an oncogene, which is attributed to its role in suppressing DNA damage or stress-induced apoptosis by modulating the epigenetic status of histones, as well as its ability to deacetylate nonhistone proteins such as p53 (Vaziri, Dessain et al. 2001), FOXOs (Brunet, Sweeney et al. 2004; Kobayashi, Furukawa-Hibi et al. 2005), E2F1 (Wang, Chen et al. 2006), Ku-70 (Cohen, Miller et al. 2004) and Smad7 (Kume, Haneda et al. 2007), etc. Consistent with its antiapoptotic effect, SIRT1 has been shown to be up-regulated in malignant cells or tissues from patients with prostate cancer, leukemia, glioblastoma, colorectal cancer or skin cancer. Nonetheless, recent studies indicate that SIRT1 can act as a tumor suppressor. Increased SIRT1 expression has been found to reduce tumor formation in a mouse model of colon tumor (Firestein, Blander et al. 2008) whereas SIRT1 mutant mice exhibited increased DNA instability and are more susceptible to tumor development (Wang, Sengupta et al. 2008). Reduced SIRT1 proteins levles are observed in breast tumors and human skin tumors (Wang, Sengupta et al. 2008; Ming, Shea et al. 2010). Therefore, the function of SIRT1 may be tumor-type specific and also depend on the stage of tumorigenesis being assessed.

At present, only limited reports are available describing the role of SIRT1 in human hepatocellular carcinoma. Wang *et al* found that SIRT1 mRNA is expressed at a comparable level between tumor and non-tumoral tissues by analyzing pooled microarray data from HCC samples. They further concluded that SIRT1 protein expression is reduced in HCC based on the analysis of one paired HCC specimen (Wang, Sengupta et al. 2008). In agreement with their results, my study confirmed that SIRT1 mRNA levels do not differ between HCC and non-tumoral tissues. However, contrary to their finding, my study identified up-regulation of SIRT1

expression in hepatocellular carcinoma. I have conducted the analysis on a relatively large number of HCC samples. Both the Western blotting and immunohistochemistry analysis revealed that SIRT1 protein was indeed over-expressed in a subset of HCC while its expression is low in normal and pre-malignant livers.

Although the mechanisms whereby SIRT1 expression is up-regulated in HCC cells and tissues remain to be defined, some upstream regulators have been reported to play a role in regulating the level of SIRT1. Firstly, the tumor suppressor hypermethylated in cancer 1 (HIC1) or p53 has been shown to repress SIRT1 expression (Liu, Liu et al. 2009). HIC forms a transcriptional repression complex with SIRT1, and this complex directly binds the SIRT1 promoter and represses its transcription, and thereby modulates p53-dependent apoptotic response. Since p53 is able to transactivates HIC1 transcription, it has been proposed that SIRT1-HIC1-p53 functions as a complex feedback loop (Michan and Sinclair 2007). Evidence suggested that reduced HIC1 expression may be in part responsible for the increased expression of SIRT1 in mouse prostatic adenocarcinomas (Huffman, Grizzle et al. 2007). Secondly, the oncogenic RNA binding protein HuR associates with the 3′ untranslated region of SIRT1 mRNA, stabilizing the SIRT1 mRNA, and increasing SIRT1 expression level (Abdelmohsen, Pullmann et al. 2007). In contrast, the cell cycle checkpoint kinase 2 (Chk2), a tumor suppressor, phosphorylates HuR and reduces SIRT1 expression (Abdelmohsen, Pullmann et al. 2007). Moreover, under cellular stress or DNA damage, the cell cycle and apoptosis regulator E2F1 directly induces SIRT1 transcription by binding to the SIRT1 promoter (Wang, Chen et al. 2006). Furthermore, SIRT1 binds to E2F1 and inhibits E2F1 activities, forming a negative feedback loop (Wang, Chen et al. 2006). In addition, the expression and activity of SIRT1 is also regulated by microRNAs. MiR-34a was first identified as a post-transcriptional regulator of SIRT1 in the regulation of apoptosis under cellular genotoxic stress in human colon cancer cells (Yamakuchi, Ferlito et al. 2008). Moreover, miR-34a also induces endothelial progenitor cell senescence and impedes its angiogenesis via suppressing SIRT1 (Zhao, Li et al. 2010). Interestingly, one recent study demonstrated that nuclear bile acid receptor farnesoid X receptor (FXR)

positively regulates hepatic SIRT1 expression by inhibiting the expression of miR-34a (Zhao, Li et al. 2010). Other microRNAs also target SIRT1. For example, miR-132 regulates nutritional stress-induced chemokine production through repression of SIRT1 (Strum, Johnson et al. 2009). MiR-199a was identified as a negative regulator of SIRT1 and hypoxia-inducible factor-1alpha (HIF1a), a key mediator of hypoxic response (Rane, He et al. 2009). On the other hand, aberrant expression of several microRNAs was found to be involved in human hepatocarcinogenesis. A set of 12 miRNAs (including miR-21, miR-221/222, miR-34a, miR-519a, miR-93, miR-96, and let-7c) was linked to disease progression from normal liver through cirrhosis to HCC (Pineau, Volinia et al. 2010). Interestingly, miR-34a is also downregulated in many cancers including colon cancer, leukemia, glioma, nonsmall cell lung cancer and hepatocellular carcinoma (Tazawa, Tsuchiya et al. 2007; Dijkstra, van Lom et al. 2009; Gallardo, Navarro et al. 2009; Li, Fu et al. 2009; Li, Guessous et al. 2009). MiR-34a also inhibits migration and invasion by down-regulation of c-Met expression in human hepatocellular carcinoma cells (Li, Fu et al. 2009). Considering SIRT1 mRNA levels did not differ between HCC and non-tumoral tissues, it is possible that microRNA may be responsible for the up-regulation of SIRT1 protein in HCC. However, the underlying mechanism requires further investigations.

The analysis of HCC patients consist of a cohort of 150 patients revealed that the positivity of SIRT1 staining is significantly associated with poorly differentiated histology (p=0.01). Cellular de-differentiation in HCC is regarded as a sign of tumor progression and increased malignant potential (Kojiro and Nakashima 1999). In glioblastoma, SIRT1 represses the expression of differentiation-associated genes and retards cell differentiation (Liu, Yuan et al. 2006). Consistent with my observation, SIRT1 expression is significantly elevated in mice with poorly differentiated adenocarcinomas compared with those with less-advanced disease (Huffman, Grizzle et al. 2007). Together my data support the notion that SIRT1 over-expression may play a role in HCC tumorigenesis.

Recent studies also suggested that inhibition of SIRT1 might have anti-cancer

potential. SIRT1 silencing induces growth arrest and/or apoptosis in human epithelial cancer cells (Ford, Jiang et al. 2005), and reactivated tumor suppressor genes (Pruitt, Zinn et al. 2006). Cambinol, a SIRT1 inhibitor, induced apoptosis of Burkitt lymphoma cells and suppressed the growth of tumor xenografts *in vivo* (Heltweg, Gatbonton et al. 2006). I found that SIRT1 inhibition by two independent shRNAs induced senescence-like growth arrest phenotypes in p53 wild-type HCC cells. These results are consistent with previous studies showing that overexpression of SIRT1 antagonizes premature senescence through deacetylation of p53 in mouse fibroblasts (Langley, Pearson et al. 2002). Moreover, another sirtuin inhibitor, sirtinol, also induces senescence-like growth arrest in human breast cancer and lung cancer cells (Ota, Tokunaga et al. 2006). A recent study also demonstrates that miR-217 induces endothelial cell senescence via inhibition of SIRT1 (Menghini, Casagrande et al. 2009). The senescence growth arrest can be established and maintained via the p53-p21 or p16-pRB tumor suppressor pathway. These two pathways may interact but can also halt cell-cycle progression independently (Campisi and d'Adda di Fagagna 2007). I first investigated the alterations of p53 and p21 in SIRT1-silencing cells. Cellular senescence in SK-Hep-1 and HepG2 cells was associated with enhanced p53 acetylation on lysine 382 and induction of p21 expression and enhanced p53 acetylation but no p21 induction was observed in PLC5 cells, consistent with its mutated p53 status. Since SIRT1 functions as an NAD-dependent p53 deacetylase, inhibition of SIRT1 in turn increased the acetylation of p53, which is indispensable for its activation (Tang, Zhao et al. 2008). Active p53 establishes the senescence growth arrest in part by inducing the expression of p21 which is a cyclin-dependent kinase (CDK) inhibitor. On the other hand, the expression of two other proteins that are implicated in senescence, namely p16 and p27, were not changed significantly upon SIRT1 knockdown. Furthermore, increased propensity to apoptotic cell death but not cellular senescence was observed in Hep3B cells (p53-null) and PLC5 cells (p53-mutant), compared with HepG2 and SK-Hep-1 cells. Collectively, theses results suggested that HCC cells senescence mediated by SIRT1 silencing is mostly attributed to the activation of the p53-p21 pathway.

Cellular senescence is a process leading to terminal growth arrest with characteristic morphological features. This process can be mediated by many stimuli including telomere-dependent, oncogene-induced and ROS-induced pathways. Telomeres are highly conserved nucleoprotein structures that cap the ends of linear chromosomes and are essential for chromosomal stability. Human chromosome telomere ends are composed of TTAGGG repeats (5-20 kb) in a DNA-protein complex formed by six telomere-specific proteins named "shelterin". Telomeres are added to the end of chromosome with a complex containing the RNA template TERC and the reverse transcriptase TERT (Cong, Wright et al. 2002). Telomeres are shortened on every DNA replication cycle, because of replication complex's inability to copy the ends of linear DNA (Olovnikov 1973). Incremental loss of telomeric DNA ultimately results in the synthesis of chromosomes that are not protected at their ends by functional telomeres, resulting in end-to-end chromosome fusion, activation of DNA damage response pathways, leading to cell cycle arrest, senescence, or apoptosis (Stewart and Weinberg 2006). Telomere shortening also serves as a tumor suppressor mechanism to limit the proliferation of transformed cells. Progressive telomere shortening is also a key feature of chronic liver disease and cirrhosis (Urabe, Nouso et al. 1996; Miura, Horikawa et al. 1997; Stewart and Weinberg 2006). Telomere shortening of hepatocytes promotes chromosomal instability and contributes to the initiation of HCC (Plentz, Caselitz et al. 2004). In addition, similar to other cancers, reactivation of telomerase is also found in over 80% of HCC cases (Tahara, Nakanishi et al. 1995; Kojima, Yokosuka et al. 1997). Moreover, the level of telomerase activity in HCC or surrounding non-cancerous liver tissue has been identified as a prognostic marker for HCC-recurrence after hepatectomy (Kobayashi, Kubota et al. 2001; Kobayashi, Sugawara et al. 2002). Re-activation of telomerase is therefore thought to be one of the events underlies the multigenetic process of hepatocarcinogenesis (Okuda 2000). Accordingly, the inhibition of the telomerase limits the growth of telomerase-positive human cancer cell lines by the induction of telomere shortening, apoptosis and cell cycle arrest (Corey 2002). TERT antagonists also inhibit tumor growth in a xenograft animal model of HCC (Djojosubroto, Chin et

al. 2005).

I showed for the first time that the expression of both TERT mRNA and protein was downregulated by SIRT1 depletion. I concluded that SIRT1 controls TERT expression via regulating mRNA stability and/or through transcriptional and post-transcriptional regulations. The expression of hTERT is tightly regulated by a minimal promoter that contains numerous binding sites for various transcription factors (Horikawa, Cable et al. 1999; Takakura, Kyo et al. 1999; Wick, Zubov et al. 1999). Among others, c-Myc and Sp1 play a critical role in the activation of hTERT transcription via binding to the E- and GC-boxes respectively (Wu, Grandori et al. 1999; Kyo, Takakura et al. 2000; Xu, Popov et al. 2001; Ducrest, Szutorisz et al. 2002). In addition, transcription factors such as AP-2, HIF-1, WT-1 and MZF-2 also play a role in the process (Oh, Song et al. 1999; Fujimoto, Kyo et al. 2000). It is therefore possible that SIRT1 might regulate the transcription of TERT via modulating the activity of the transcription factors involved. On the other hand, hTERT expression is subjected to epigenetic regulation. It has been shown that the level of CpG methylation, together with the relative abundance of active (acetyl-H3K9 and dimethyl-H3K4) versus inactive (trimethyl-H3K9 and trimethyl-H3K27) marks of the histones around the transcription start site, plays an essential role in hTERT expression (Zinn, Pruitt et al. 2007). Concordantly, SMYD3 and LSD1, a methyltransferase and demethylase for H3K4 respectively, also regulates hTERT expression (Liu, Fang et al. 2007; Zhu, Liu et al. 2008). To elucidate the role of SIRT1 in epigenetic regulation of hTERT expression, bisulfite sequencing could be conducted to determine the methylation status of CpG sites. In addition, chromatin immunoprecipitation (ChIP) followed by quantitative PCR amplification of the promoter region to determine the abundance of active versus inactive chromatin marks could be carried out.

In this model system, ectopic expression of TERT significantly rescued the proliferation of SIRT1-depleted HCC cells, suggesting that TERT is responsible for SIRT1 depletion-induced cell growth inhibition. Intriguingly, others have shown that inhibition of SIRT1 induces TERT protein expression and telomerase activity in HeLa

cells (Narala, Allsopp et al. 2008). Another study using human diploid fibroblasts as model suggested that SIRT1 depletion cooperates with TERT to promote cell growth under normal and nutrient limiting conditions, but alternation of SIRT1 expression level alone had no effect on the lifespan of the cells (Narala, Allsopp et al. 2008). A unifying hypothesis regarding the role of SIRT1 in TERT regulation is lacking at present. It is possible that SIRT1 may play a growth-promoting or growth-suppressive function in a tissue-specific or tumor type-specific manner.

TERT is best known for its role in telomere maintenance. Ectopic expression of TERT in somatic cell is sufficient to prevent telomere shortening, and thereby promotes infinite proliferation (Bodnar, Ouellette et al. 1998). Moreover, re-activation of endogenous telomerase in telomerase-deficient cells leads to cellular immortality. However, evidence suggested that TERT may also play a telomere-independent function in tumorigenesis. Oncogenic transformation of human fibroblasts by telomerase can be genetically separated from its ability to maintain telomere length (Stewart, Hahn et al. 2002). Telomerase expression also conferred increased resistance to specific DNA damaging agents (Sharma, Gupta et al. 2003; Kang, Choi et al. 2004) and decreased apoptosis (Fu, Killen et al. 2000; Zhang, Chan et al. 2003; Del Bufalo, Rizzo et al. 2005; Massard, Zermati et al. 2006). TERT overexpression also promotes the proliferation of resting stem cells in the skin epithelium independent of its activity in synthesizing telomere repeats (Sarin, Cheung et al. 2005). In my study, although TERT plays a pivotal role in tumorigenesis and the proliferation of SIRT1-depleted HCC cells was rescued by TERT expression, I believed that telomere phenotypes in SIRT1-depleted cells did not result from telomeric attrition. This is because telomere shortening is a gradual process that requires a substantial number of cell division cycles for its effect on cell growth to become apparent, but cell growth arrest induced by SIRT1 silencing has become apparent with 6 days. Therefore, the acute cell growth suppression effect as a result of SIRT1 knockdown argues against telomere attrition as the major mechanism. Indeed, telomeric length did not differ significantly between control and SIRT1-depleted cells as measured at 7 days after lentiviral transduction. Therefore, TERT might contribute

to cell survival via telomere-independent mechanisms, so that reduced TERT expression in SIRT1 knockdown cells suppresses cell proliferation that can be rescued by its re-expression.

The human telomere binding protein POT1 binds to the most distal single-stranded extension of telomeric DNA, as well as associating with the double stranded telomeric DNA binding proteins TRF1 and TRF2 through the bridging proteins PTOP and TIN2. PTOP functions to recruit POT1 to telomeres and improves its ability to bind to single-stranded DNA in vitro (Wang, Podell et al. 2007). PTOP deletion results in the release of POT1 from chromatin and loss of these proteins from telomeres (Kibe, Osawa et al.). Downregulation of POT1 in human cells causes apoptosis or senescence, as well as an increase in telomere associations and anaphase bridges, telltale signs of telomere instability (Veldman, Etheridge et al. 2004). Deletion of PTOP induced telomere dysfunction phenotypes and chromosomal abnormalities that were consistent with the consequences of POT1 loss (Kibe, Osawa et al.). POT1 can also prevent the activation of the phosphatidylinositol 3-kinase ATR and contributes to the repression of the nonhomologous end-joining pathway (NHEJ) at newly replicated telomeres (Denchi and de Lange 2007). Deletion of POT1 from mouse cells leads to TIFs and elicits cell cycle arrest (Denchi and de Lange 2007). The ability of POT1 to repress the ATR kinase signaling cascade is dependent on its association with PTOP (Denchi and de Lange 2007; Hockemeyer, Palm et al. 2007). Inhibition of PTOP gives rise to a DNA damage response at telomeres that is indistinguishable from the response to POT1 deletion (Hockemeyer, Palm et al. 2007; Xin, Liu et al. 2007). The PTOP-POT1 complex also recruits TERT and is essential for its activity (Wang, Podell et al. 2007). Over-expression of telomere-binding proteins is also found in many cancers (Poncet, Belleville et al. 2008; Cookson and Laughton 2009). Consistent with these notions, telomere dysfunction and nuclear abnormalities were observed in SIRT1 knockdown cells and these observations are most probably due to the depletion of PTOP and POT1. I concluded that depletion of telomeric-binding proteins, including PTOP and POT1, causes defects in telomere protection and activates DNA-damage response, leading to a DNA damage signal and

inappropriate DNA repair reactions at chromosome ends. Finally chromosomal and nuclear abnormalities resulting from telomere dysfunction, lead to cell growth inhibition.

The emergence of drug resistance presents a major obstacle in cancer chemotherapy for HCC. If SIRT1 indeed functions as a tumor promoter, the SIRT1 inhibitor might be useful as a chemopreventive agent. Many studies have implicated a role of SIRT1 in promoting cell survival in response to stress or chemotherapy drug treatment. Up-regulation of SIRT1 expression has been observed in doxorubicin-resistant cancer cell lines, including neuroblastoma SKN-SH, osteosarcoma SaOS2 and breast cancer MCF-7 cells, as well as in cisplatin-resistant ovarian cancer A2780 and IGROV1 cells (Chu, Chou et al. 2005). Importantly, SIRT1 knockdown increased sensitivity of doxorubicin-resistant SaOS2 cells to the drug and reduced the expression of multi-drug resistance P-glycoprotein (Chu, Chou et al. 2005). On the other hand, treatment of lung cancer cell line NCI H460 with SIRT1 inhibitor, cambinol, results in a dose-dependent sensitization to DNA-damaging agents in p53-independent manner and downregulation of SIRT1 expression by siRNA mimicked the sensitizing effect caused by cambinol (Heltweg, Gatbonton et al. 2006). A recent study further confirmed inhibition of SIRT1 by sirtinol or siRNA-mediated downregulation of SIRT1 expression enhanced chemosensitivity to camptothecin and cisplatin in prostate cancer cells, resulting in a significant reduction of viable cells due to apoptotic cell death (Kojima, Ohhashi et al. 2008). Consistent with these observations, I found that reduced SIRT1 expression increased the chemosensitivity of HCC cells to doxorubicin treatment, resulting in increased doxorubicin-induced apoptosis in these cells. Together these data suggest the therapeutic potential of combining a SIRT1 inhibitor and doxorubicin in the treatment of a subset of HCC in which SIRT1 is up-regulated. It is of great importance to dissect the molecular mechanisms of enhanced chemosensitivity mediated by SIRT1 inhibition, which can be the direction in future study.

At present, a number of SIRT1 inhibitors have been discovered. These include the 2-hydorxynaphthaldehyde derivative of sirtinol, the physiological sirtuin

inhibitor nicotinamide, the coumarin derivative of splitomicin, cambinol (Heltweg, Gatbonton et al. 2006), and the tenovins and its derivative (Lain, Hollick et al. 2008). The only SIRT1 inhibitors that have been tested in animal models of cancer are cambinol and tenovins. Treatment of BCL6-expressing Burkitt lymphoma cells with cambinol induces apoptosis, which was accompanied by hyperacetylation of BCL6 and p53, and enhances chemotherapy-induced apoptosis in cancer cells (Heltweg, Gatbonton et al. 2006). In nude mice xenografted with Burkitt lymhoma cells, monotherapy with cambinol suppresses tumor growth (Heltweg, Gatbonton et al. 2006). Tenovin 6, which inhibits both SIRT1 and SIRT2, also suppresses tumor growth of melanoma cells-derived xenograft tumors (Lain, Hollick et al. 2008).

The current understanding of the pro-survival function of SIRT1 is its role to direct the cells towards DNA repair via modulating the activity of MRE11-RAD50-NBS1, p53, Ku70, and FOXO proteins, etc (Liu, Liu et al. 2009). Recent studies using $SIRT^{-/-}$ mice have revealed its role in telomere maintenance and promoting genomic stability in normal cells (El Ramy, Magroun et al. 2009; Palacios, Herranz et al. 2010). My finding extends the current understanding of the function of SIRT1 to include telomeric maintenance. The disruption of telomeric maintenance via targeting telomerase is one of the emerging anti-tumor strategies. However, one major limitation of telomerase inhibitors in tumor therapy is the long lag time for its action, during which the tumor burden may have already increased substantially (Djojosubroto, Chin et al. 2005; Shay and Keith 2008). My work suggested that targeting SIRT1 might be a more efficient strategy to induce telomeric dysfunction and tumor cell death in the subset of HCC patients who over-expressed this protein.

Chapter 5 Summary and future plan

In this study, I provided evidence for the involvement of SIRT1 in HCC. My finding first showed that SIRT1 was significantly over-expressed in HCC cell lines and in a subset of HCC. Nuclear SIRT1 staining was associated positively with tumor grade. Down-regulation of SIRT1 consistently suppressed the proliferation of HCC cells via the induction of cellular senescence or apoptosis. Strikingly, two novel SIRT1-modulated genes were identified, including telomerase reverse transcriptase (TERT) and PTOP, which is a member of the shelterin complex. SIRT1 depletion-mediated repression of TERT and PTOP leaded to telomere dysfunction-induced foci and nuclear abnormality, which are responsible for SIRT1 depletion-induced grow inhibition of HCC cells. There was also a positive correlation between the level of induction of SIRT1 and PTOP in human HCC. Finally, SIRT1-silencing sensitized HCC cells to doxorubicin treatment. My data revealed a hitherto undiscovered role of SIRT1 in telomere maintenance, and that inhibition of SIRT1 activity might serve as a potential target for the development of anti-HCC therapeutics.

To further elucidate the function of SIRT1 in HCC, several research directions appear interesting. Firstly, it would be important to characterize the mechanism of SIRT1-dependent hTERT and PTOP regulation. To this end, our group has generated a luciferase reporter gene construct driven by the hTERT promoter and found that SIRT1 did not affect the promoter activity of hTERT. Furthermore, our group also determined the CpG methylation status at the transcription site of TERT gene using bisulfite sequencing. The methylation status of TERT DNA also did not differ significantly between SIRT1-depleted cells compared to control cells. Based on these observations, it is hypothesized that SIRT1 might control TERT expression via regulating the stability of its RNA, and therefore it will be important to continue investigation along this line. Secondly, it will be important to evaluate the effect SIRT1 silencing on the growth of liver tumor *in vivo*. HCC cells stably expressing

tetracycline-responsive shRNA targeting SIRT1 or control shRNA could be xenografted into the flank of athymic nude mice. Tumor growth could be determined and paraffin sections of tumors could be stained for proliferative (Ki67), apoptotic (caspase 3, TUNEL), and differentiation (alpha-fetoprotein, cytokeratin 8, and cytokeratin 7) markers respectively. Besides, the expression of SIRT1, hTERT and PTOP of these tumors could also be determined. As mentioned above, besides SIRT1, other members of sirtuins have been shown to play a role in tumorigenesis. The latest data from our laboratory suggested that SIRT2 is also overexpressed and localized to the nucleus in a subset of HCC. In addition, SIRT3 has been implicated a role in breast tumor, and SIRT6 is involved in maintaining genomic stability. Therefore, it will be also important to extend the study by conducting a comprehensive analysis on other members of the sirtuins family to determine if they also play a role in the pathogenesis of HCC. Clinicopathological correlation between the expression of other sirtuin members and HCC could be conducted. Dependent on the data obtained from above analysis, the role and the underlying mechanisms of sirtuins in HCC cell proliferation could be further investigated. These studies holds value in providing a better understanding on the role of sirtuins in HCC tumorigenesis, in the discovery of novel prognostic or diagnostic markers for HCC, and will impact on the future development of SIRT inhibitors as HCC therapeutics.

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