# Epigenetic modifications and consequences of epigenetic alterations in Hepatocellular Carcinoma cell lines

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# Zusammenfassung

Das Hepatozelluläre Karzinom (HCC) ist eines der aggressivsten und am weitesten verbreitetste Malignom was beim Menschen vorkommt. Weltweit ist das HCC die dritthäufigste Todesursache bezogen auf durch Karzinome verursachte Todesfälle. Ursachen hierfür sind aufgrund fehlender Therapieoption. Die vorliegende Dissertationsarbeit beschäftigt sich mit dem steigenden Bedarf an neuen Strategien, die eine frühe Erkennung und eine effektivere Behandlung von an HCC erkrankten Patienten ermöglichen.

Eine Hauptursache des HCC ist die (komplette) Aufhebung der Methylierung, welche die Expression essentieller Gene auf der Transkriptionsebene beeinflusst. Studien haben gezeigt, dass das Chemotherapheutikum 5-Azacytidin (5-AZA), ein DNA Methyl Transferase 1 Inhibitor (DNMT1), die Aktivierung verschiedener Tumorsuppressorgene via passiver Demethylierung inhibiert. 5-AZA ist ein bekanntes epigenetisch wirksames Medikament welches als Reservetherapeutikum für die Therapie des HCCs eingesetzt wird. Ebenfalls zeigen neuere Studien, dass die aktive Demethylierung, welche durch TET-Proteine katalysiert wird, eine wichtige Rolle bei der Kontrolle globaler Methylierungsderegulationen durch Umsetzung von 5-Methylcytosin (5mC) zu 5-Hydroxymethylcytosin (5hmC) spielt. TET-Proteine sind Fe(II)/2-OG-abhängige Dioxygenase deren Funktion durch Vitamin C (L-Ascorbyl-2-Phosphat)verstärkt wird. Die vorliegende Dissertationsarbeit beschäftigt sich mit der Fragestellung, ob 5-AZA eine TET-abhängige aktive Demethylierung in HCC Zellenstimulieren kann und ob Vitamin C den Effekt von 5-AZA bezüglich der aktiven Demethylierung hervorrufen oder verstärken kann. Ein weiterer Schwerpunkt dieser Dissertationsarbeit ist der Einfluss der aktiven Demethylierung auf den Zellzyklus.

Des Weiteren steht im Fokus dieser Arbeit der Zusammenhang zwischen epigenetischer Modifikation und der Verbesserung der Biotransformation von Xenobiotika in HCC. Einige HCC-Zelllinien werden in der pharmazeutischen Industrie routinemäßig für Metabolismusstudien eingesetzt. Aufgrund ihrer guten Verfügbarkeit sind sie eine attraktive Alternative für primäre humane Hepatozyten, welche innerhalb weniger Tag in Kultur dedifferenzieren und somit ihr metabolisches Potential rasch verlieren. Viele HCC-Zelllinien zeigen eine sehr geringe metabolische Aktivität, da die entsprechenden Biotransformationsgene (CYP450) relativ gering exprimiert werden und/oder die Zellen nicht vollständig differenziert sind. Durch Verbesserung der metabolischen Aktivität einer HCC-Zelllinie, zum Beispiel durch Steigerung der Expression der CYP450-Gene, könnte diese für Studien im Bereich der Medikamententwicklung eingesetzt werdenUm die Rolle von 5hmC hinsichtlich der HCC-Entstehung besser zu verstehen, wurden verschiedene HCC-Zelllinien (Huh-7, HLE, HLF), primäre humane Hepatozyten (pHH) und Gewebe von gesunden und an HCC-erkranken Probanden untersucht. 5mhC wurde durch Immunohistochemie und Immunofluoreszenz im humanen HCC-Gewebe und in der HCC-Zelllinie analysiert. Anschließend wurden die Ergebnisse des humanen HCC-Gewebes mit den Leberproben von ‚gesundem Gewebe und die der HCC-Zelllinie mit den primären Hepatozyten verglichen. Die Menge an mRNA von IDHs (IDH1, 2) und TETs (TET1-3) wurde durch Real-Time PCR bestimmt und auf Proteinebene mittels Western Blot bestätigt. Im Vergleich zum gesunden Lebergewebe ist die Expression von 5hmC signifikant niedriger, die von 5mC im HCC-Gewebe signifikant höher. Dies ließ sich durch den Vergleich der HCC-Zelllinie mit PHH bestätigen.

Ferner haben wir den Effekt von 5-AZA auf die aktive Demethylierung und die Fähigkeit des TET-Cofaktors Vitamin C in Kombination mit 5-AZA, sowie deren

Umsetzung von 5mC zu 5hmC, untersucht. Die HCC-Zelllinien wurden mit 5-AZA (0-20 µM) und Vitamin C inkubiert. Anschließend wurde die Wirkung dieser Substanzen anhand der Viabilität der Zellen (Resazurinumsetzung), der Toxizität (LDH Ausschüttung), der Proliferation (PCNA), sowie der 5hmC/5mC Verteilung und der Expression verschiedener TET Gene bestimmt. Durchgeführt wurden zudem Knock-down Experimente der TET-Proteine in HCC-Zelllinien durch siRNA mit und ohne 5-AZA-Behandlung. Anhand der Knock-down Experimente konnten wir zum ersten Mal zeigen, dass 5-AZA sowohl die TET-abhängige aktive Demethylierung als auch das 5hmC/5mC Verhältnis in HCC-Zelllinien und in humanen primären Hepatozyten fördert. Darüber hinaus demonstrierten wir, dass Vitamin C als Kofaktor von TET, den Effekt von 5-AZA auf die Umwandlung von 5mC zu 5hmC durch Induktion der TET2- und TET3-Genexpression positiv beeinflusst.

Um den Signalweg des Zellzyklus in Abhängigkeit von Vitamin C und 5-AZA zu ermitteln, haben wir die mRNA-Expression von GADD45 und P21 (Real-Time PCR), Snail, GADD45, P21 und Cyclin-b-Protein (Western Blot) untersucht. Hier konnten wir zum ersten Mal nachweisen, dass die Behandlung von HCC Zelllinien mit einer Kombination von 5-AZA und Vitamin C zu einer Verbesserung der Downregulation der Snail-Expression, eines wichtigen Transkriptionsfaktors in EMT-Prozessen und zu einem Zellzyklusarrest führt. Die kombinierte Behandlung führt außerdem zu einer Erhöhung der GADD45-Expression welches das Gleichgewicht zwischen DNA-Reparatur, Apoptose, Seneszenz und Zellzyklusarrest beeinflusst.

Schließlich wurde der Effekt von 5-AZA und Vitamin C auf die Biotransformationsaktivität (Phase I und II) in HCC-Zelllinien durch die Messung der Enzymaktivität und Expression relevanter Gene analysiert. HCC-Zellen, die sowohl

mit Vitamin C als auch mit 5-AZA stimuliert wurden, haben eine erhöhte Aktivität von CYP450-Enzymen (z.B. CYP1A1 und CYP3A4) welche im Fremdstoffmetabolismus eine wichtige Rolle spielen. Diesbezüglich sind jedoch weitere Studien nötig, um die Effektivität dieser (5-AZA und Vitamin C) und anderer epigenetischer Substanzen in HCC-Zelllinien zu optimieren.

Unsere Ergebnisse zeigen deutlich, dass die Expression und die Aktivität von TET2 und TET3, jedoch nicht von TET1, im hepatozellulärem Karzinom beeinträchtigt sind, was zur Reduktion von 5hmC in HCC führt. Außerdem zeigt diese Dissertationsarbeit eine neue Funktion von 5-Azacytidin. 5-Azacytidin verbessert durch seine Auswirkung auf die TET-Proteine die Bildung von 5hmC, welches in Anwesenheit von Vitamin C noch gesteigert werden konnte.

Dies hat besondere Bedeutung weil Aktive Demethylierung Snail-Transkriptionsfaktoren inhibiert und die GADD45 Expression in HCC-Zelllinien verbessert. Die Wirkung von 5-AZA und Vitamin C auf den Zellzyklus beruht auf der Erhöhung der P21-Expression als Folge der Snail-Downregulation und der Induktion von GADD45-Expression. Die in dieser Arbeit gezeigten Ergebnisse, könnten die Grundlage für neue epigenetische Behandlungsstrategien von HCC darstellen. Die vorliegende Dissertationsarbeit verdeutlicht zudem, dass die Erhöhung der Biotransformationsaktivität in HCC - Zelllinien durch epigenetische Modifikation mittels Anwendung von 5-AZA und Vitamin C möglicherweise dazu führen kann, dass HCC-Zelllinien eine vielversprechende Alternative für humane primäre Hepatozyten im Medikamentenentwicklungsprozess darstellen könnten.

# Summary

Hepatocellular carcinoma (HCC) is one of the most aggressive and prevalent form of human malignancies globally and represents the third lethal cancer worldwide due to poor prognosis and lack of treatment options. This thesis highlights the urgent demand for a new strategy to achieve early diagnosis and effective treatment of HCC patients. Global deregulation of methylation is one of the crucial causes of HCC affecting essential gene expression at the transcriptional level.

It has been reported that the anti-cancer drug 5-Azacytidine (5-AZA) mediates the activation of tumor suppressor genes through passive demethylation by inhibiting DNMT1. 5-AZA is one of the most frequently used epigenetic drugs for cancer therapy including for HCC. Recent evidence suggests that active demethylation which is mediated by TET proteins may be an important mechanism to control global deregulation of methylation by converting 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC). TET proteins are Fe(II)/2-OG-dioxygenases dependent. Vitamin C (L-Ascorbyl-2-phosphate) has a crucial role in maintaining the catalytic activity of Fe(II) 2-OG-dependent dioxygenases which can promote TETs activity by enhancing 5mC to 5hmC transition. The goal of this study was to investigate whether 5-AZA could trigger an active demethylation in form of the TET-dependent process in HCC, and whether Vitamin C can induce the effect of 5-AZA on active demethylation pattern. Another focus of the study was also to evaluate the influence of active demethylation on cell cycle arrest

Moreover this study explored the relation between epigenetic modification and Xenobiotic Biotransformation capacity in HCC cell lines. HCC cell line serves as an easy alternative for primary human hepatocytes, due to their high availability for

development studies. However, HCCs usually have low metabolic activity due to a reduced level of drug biotransformation genes (CYP450). Thus, improving metabolic activity of HCC lines, i.e. elevating the level of CYP450 genes, would allow using them for drug development studies. This can be achieved by epigenetic changes through alteration of the DNA methylation status affecting gene expression of CYP450.

To investigate the role of 5hmC in the development of hepatocellular carcinoma, HCC cell lines (Huh-7, HLE, HLF), primary human hepatocytes (PHH), and tissue from both healthy and HCC patients (55 patients) were included in this study. The presence of 5hmC was determined by immunohistochemical and immunofluorescent staining in human HCC tissue and HCC cell lines and then compared with the adjacent liver tissue samples and primary hepatocytes, respectively. The mRNA level of IDHs (IDH1, 2) and TETs (TET1-3) were studied via real-time PCR and confirmed by Western Blot. Expression of 5hmC was significantly lower and 5mC was significantly higher in HCC tissue as compared to in the corresponding tumor-free liver samples. This finding was confirmed by comparing HCC cell lines to PHH. Expression of IDH1, IDH2, TET2 and TET3 was also significantly lower in HCC tissue and HCC cell lines than in non-tumor liver tissue and PHH.

The effect of 5-AZA on active demethylation pattern in DNA was not studied. Instead, the effect of 5-AZA on active demethylation and if and to what extent Vitamin C (as a TET cofactor) combined with 5-AZA can facilitate the 5mC-5hmC transition were investigated in this study. HCC cell lines were stimulated with 5-AZA (0-20 µM) and Vitamin C. The effect of these treatments was investigated with regard to viability (Resazurin conversion), toxicity (LDH release), proliferation (PCNA), 5hmC/5mC

distribution and TET expression. Knock-down experiments on TET proteins in HCC cell lines using siRNAs in the presence and absence of 5-AZA were performed. We showed here for the first time applying knock-down experiments that 5-AZA is able to trigger an active TET2-dependent demethylation process with concomitant significant changes in 5hmC/5mC in HCC cell lines and human primary hepatocytes.

We showed that Vitamin C as a cofactor for TET activity facilitates the effect of 5-AZA on the conversion of 5mC to 5hmC by an induction of TET2 and TET3 gene expression.

To understand the signaling pathways, especially cell cycling related signaling affected by Vitamin C and 5-AZA, we investigated GADD45 and P21 mRNA expression (Real-Time PCR), Snail, GADD45, P21 and cyclin B protein expression (Western Blot). We have shown for the first time in HCC that the combination of 5-AZA and Vitamin C leads to an enhanced downregulation of Snail expression, a key transcription factor governing EMT process and cell cycle arrest. The combined treatment leads to an increase in GADD45 expression. GADD45 has a key role in cell fate by controlling the balance between DNA repair, apoptosis, senescence and cell cycle arrest.

Finally, the effect of the 5-AZA and Vitamin C on the drug biotransformation activity (phase I and II) of HCC cell lines was investigated through enzyme activity measurement and related gene expression. In HCC cells treated with Vitamin C combined with 5-AZA, the increase in the level of some important CYP450 enzymes such as CYP1A1, CYP3A4 was observed. Here, further investigation is warranted. 5-AZA and Vitamin C increase the ammonia detoxification capacity of HCC cell lines.

As conclusion, our data clearly indicates that the expression and activity of TET2 and TET3 proteins but not TET1 are impaired in hepatocellular carcinoma leading to the reduction of 5hmC in HCCs. Furthermore, this study identified a novel function of 5-Azacytidine in promoting a TET-mediated generation of 5hmC which is increased by combining 5-AZA with Vitamin C. Active demethylation leads to inhibition of Snail transcriptional factor and enhancement of GADD45 in HCC cell lines. An increase in P21 expression as a consequence of downregulation of Snail accompanied by the induction of GADD45 expression are the main mechanisms leading to cell cycle arrest in HCCs by applying the combination of 5-AZA and Vitamin C. These findings may open new therapeutic strategies for epigenetic drugs to treat HCC. Finally this study exhibited that the increase of drug biotransformation activity in HCC cell lines through modification of the epigenetic status by applying 5-AZA and Vitamin C may provide HCC cell lines as a promising alternative for human primary hepatocytes in drug discovery investigations.

# 1 Introduction

## 1.1 Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is one of the most predominant types of primary liver malignancy with high rates of mortality worldwide [de Lope *et al.*, 2012]. Dramatically, over the past few decades incidence rates of HCC have been continuously increasing in the West [Cucchetti *et al.*, 2012; Siegel *et al.*, 2013].

Although it is the 6$^{th}$ most frequently diagnosed types of cancer, it is the 3$^{rd}$ cause of cancer-related deaths with approximately 700,000 deaths per year due to its poor prognosis and recurrence of tumors after therapy [Ferlay *et al.*, 2010; Yang & Roberts, 2010]. There exist geographic variations of HCC occurrence all over the world. Although the majority of cases occur in Asia and Africa, the rate of HCC incidence is increasing in developed countries such as USA and UK [Dhanasekaran *et al.*, 2012]. Also, a male predominance in the occurrence of HCC is noted with two to four times as many men as women suffering from this disease [Jemal *et al.*, 2011].

### 1.1.1 Etiologies of hepatocellular carcinoma

The etiology of hepatocellular carcinoma consists of various risk factors including: chronic infection with HBV and HCV (more than 50% of all cases), chronic alcohol abuse (the major cause of liver cirrhosis), food contamination with aflatoxin B1, tobacco use, obesity, diabetes, non-alcoholic fatty liver disease (NAFLD) and finally certain metabolic disorders such as hereditary hemochromatosis [Anestopoulos *et al.*, 2015; Bruix & Sherman, 2011] (Fig.1.1). In addition, there is a correlation between the geographic region and the cause of HCC, for example the majority causes of HCC in Eastern Asia and Africa are due to HBV and HCV infections while

alcoholic cirrhosis and obesity are the major risk factors of most of the Western countries [El–Serag & Rudolph, 2007].

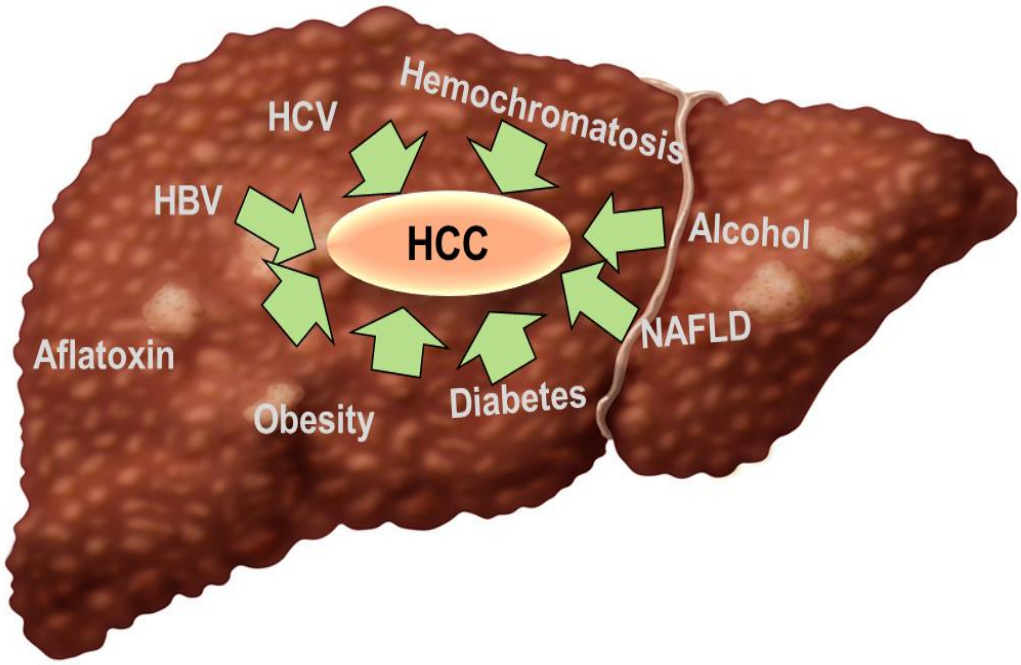



**Figure 1.1. Risk factors for developing hepatocellular carcinoma.**

The causative factors associated with HCC development are depicted. Various factors including HCV, HBC, Alcohol abuse, Obesity and Diabetes leads to progression of HCC. Abbreviations: HCV, hepatitis C, HBV, hepatitis B, HCC, hepatocellular carcinoma, NAFLD and nonalcoholic fatty liver disease.

### 1.1.2 Pathogenesis of hepatocellular carcinoma

The aforementioned risk factors cause liver injury which is followed by repair and regeneration of the damaged tissue by steady proliferation of several cell types of the liver resulting in shortening of the telomere and cell damage [Farazi & DePinho, 2006]. In addition, the hepatic stellate cells (HSC) differentiate into myofibroblasts after chronic liver injury and contribute to the fibrotic scar by secreting excessive and abnormal collagen in the injured and regenerating liver. Unattended fibrosis, leads to the formation of abnormal islands of regenerating liver nodules surrounded by fibrotic scar tissue, which is termed as liver cirrhosis [Seki & Schwabe, 2015].

Molecular mechanisms involved in the development of HCC are variable due to the variety of HCC etiology. Thus in HCC, both genetic and epigenetic alterations lead to tumor progression [Puszyk *et al.*, 2013]. For instance, in HCC caused by aflatoxin, HBV and HCV, the tumor suppressor p53 mutation or inactivation is a common characteristic [Hussain *et al.*, 2007]. In alcohol and HCV induced HCCs, liver cirrhosis and related micro environmental alteration can be seen [Farazi & DePinho, 2006]. Inflammation and necrosis are a frequent consequences of alcohol abuse, HBV and HCV (reviewed in [Farazi & DePinho, 2006]). Altogether, frequent risk factor exposure, life style and environment may lead to aberrant epigenetic status of HCCs [Herceg & Paliwal, 2011] (Fig.1.2 A).

The accumulation of epigenetic modifications such as DNA global hypermethylation, telomere shortening, inhibition of DNA damage response pathways, and chromosome segregation defects leads to increased genomic instability. The genomic instability provokes pre-malignant lesions progression into dysplastic nodules with autonomous growth capability [Farazi & DePinho, 2006; Hanahan & Weinberg, 2011].

As mentioned before, various pathways are implicated in HCC development. For example: ErBb receptor family, c-MET receptor and Wnt signaling pathways are dysregulated in HCCs. However, aberrant methylation patterns in different promoters of some cancer related genes such as E-cadherin, COX2 and $p^{16INK4a}$ indicate the important role of epigenetics in HCC progression [Farazi & Depinho, 2009]. It was shown that a large number of genetic or epigenetic modifications of critical oncogenes or tumor suppressor genes result in diverse malignant phenotypes of cancer cells [Liu *et al.*, 2014a]. Therefore, linking the hallmarks of HCC with genetics

and epigenetics may lead to a new strategy to identify promising targets for HCC therapy. Various cancer hallmarks and the underlying molecular changes during HCC progression are summarized in (Fig.1.2B).

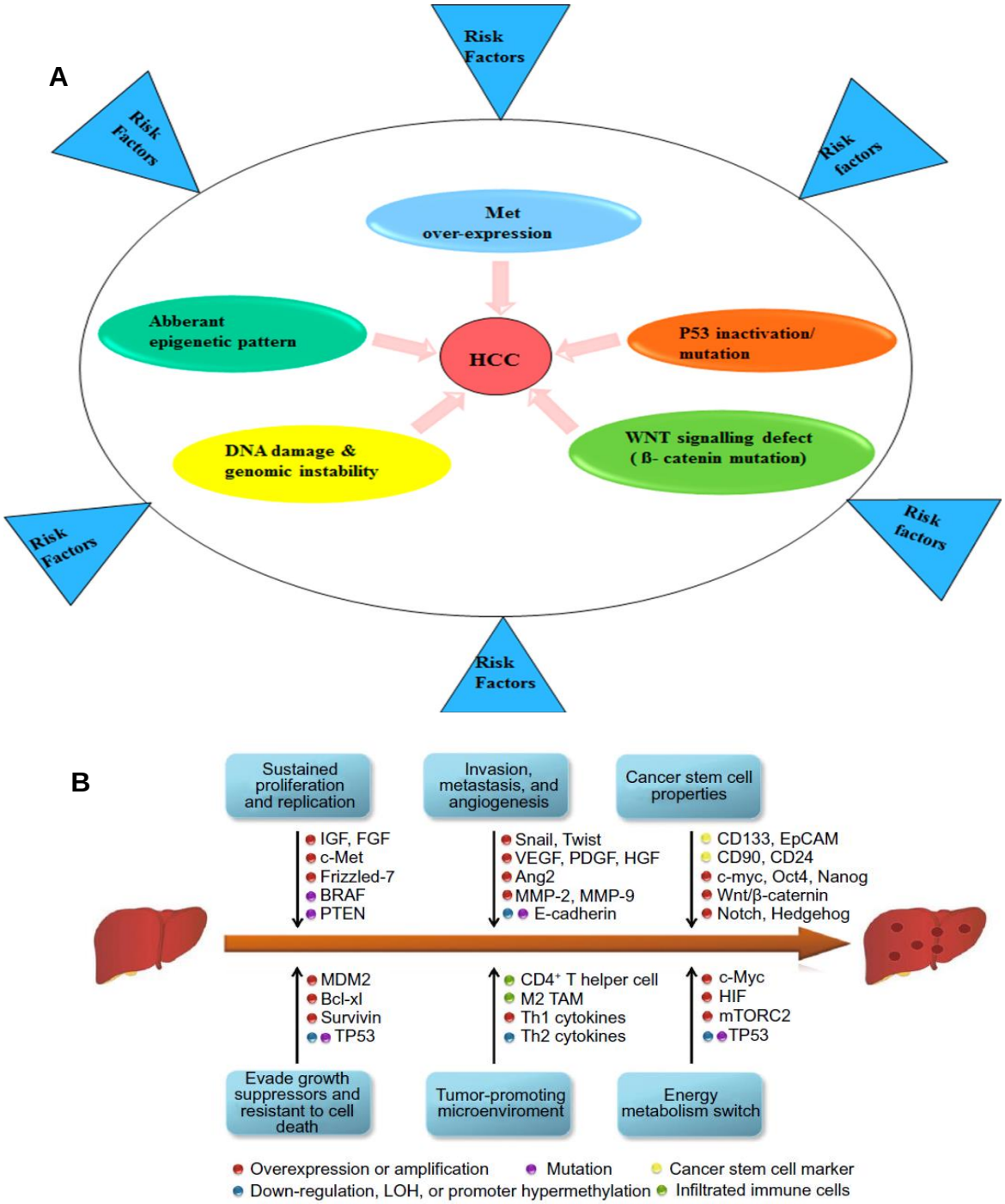



**Figure 1.2. Molecular pathways, genetic and epigenetic contributions to HCC progression.**

**A)** Frequent exposure to risk factors, life style and environment changes may lead to aberrant genetic and epigenetic status of HCCs (modified from [Farazi & Depinho, 2009]) **B)** Multiple cancer hallmarks are involved in HCC progression including sustained cell proliferation, evading growth suppressors, resistant to cell death, invasion, metastasis, angiogenesis and deregulated energy metabolism. Molecular alterations including overexpression of oncogenes, hypermethylation or mutation of tumor suppressor genes, activation of stem cells and infiltration of the immune system lead to malignant transformation of HCC [Liu *et al.*, 2014a].

## 1.2 Epigenetics and HCC

Epigenetics is defined as a permanent change of gene expression or phenotypes during cell proliferation and development, which does not cause a change of the DNA sequences [Dupont *et al.*, 2009]. Epigenetic modification can be passed through cell division to daughter cells in order to preserve 'cellular memory' therefore it is heritable from parent to offspring [Egger *et al.*, 2004]. Epigenetic regulation takes place at three separate stages: 1) nucleosome positioning, 2) histone modification, and 3) DNA methylation (Fig.1.3) [Portela & Esteller, 2010]. Chromatin complexes and non-coding RNAs together with aforementioned factors determine the chromatin structure (heterochromatin/euchromatin formation). Epigenetics has a significant role in a variety of cellular processes, cellular differentiation, maintenance of cell memory and aging [Delcuve *et al.*, 2009]. Aberrant epigenetic modification leads to repression of some particular cellular genes and genomic instability which may cause oncogenic transformation and heterogeneity in human malignancies including HCC [Herceg, 2007; Herceg & Paliwal, 2011; Tanaka *et al.*, 2002]. Therefore, the field of epigenetics has been attracting increasing attention for cancer therapy in recent years.

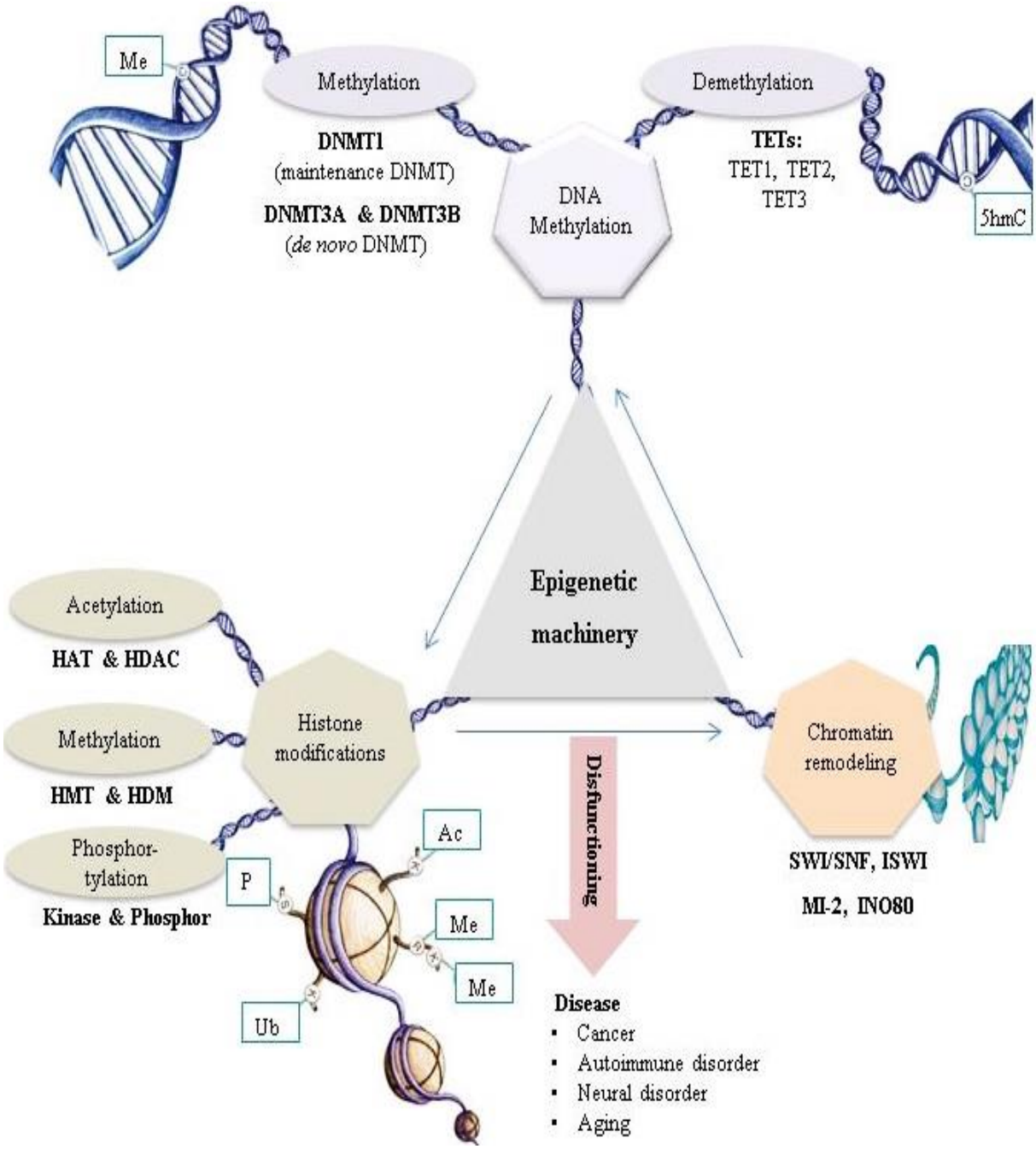



**Figure 1.3. Epigenetic machinery and interplay between epigenetic factors.**

Epigenetic regulation depends on the three following epigenetic factors: DNA methylation, histone modification, and nucleosome positioning. The interaction between these factors results in the final outcome but disturbances of this balance lead to several diseases [Sajadian & Nussler, 2015]. Abbreviations: DNMT: DNA Methyl Transferase, HAT: Histone Acetylation Transferase, HDAC: Histone Deacetylase, HMT: Histone Methyltransferase, HDM: Histone Demethyl Transferase

### 1.2.1 DNA methylation

DNA methylation has been attracting increasing attention in the past years and it plays an important role in gene regulation, particularly at the CpG site of eukaryote's DNA [Herceg & Paliwal, 2011]. DNA methylation plays an important role in different cellular processes, especially in the inactivation of the X-chromosome, in silencing of the retrotransposon (known as repetitive elements), and in the regulation of gene expression [Laird, 2003].

During the DNA methylation process, a methyl group ($CH_3$) is attached to the phenyl ring of the cytosine at the 5 carbon position yielding 5-methylcytosine (5mC). Somatic cell methylation can take place at all CpG (cytosine-phosphate-guanine) dinucleotide positions except at the CpG Island [Suzuki & Bird, 2008]. CpG islands are parts of the DNA with more than 500 bp and over 55% of GC content and accommodate approximately 60% of the mammalian gene promoters [Takai & Jones, 2002]. When DNA methylation occurs in these promoters, some crucial transcription factors (TF) are down-regulated with subsequent inhibition of the corresponding protein formation. Furthermore, it has been proven that even in regions that are poor in CpGs, such as the promoter of Oct4 gene , DNA methylation plays an important role in gene regulation [Das & Singal, 2004]. DNA methylation is catalyzed by a family of enzymes, referred to as DNA methyltransferases (DNMTs). Up to now, the following DNMTs have been discovered: DNMT1, DNMT1b, DNMT1o, DNMT1p, DNMT2, DNMT3A, DNMT3B, and DNMT3L [Das & Singal, 2004].

DNMTs such as DNMT3A, DNMT3B and DNMT3L are apparently responsible for the de-novo DNA methylation during the early embryogenesis process [Kareta *et al.*, 2006]. In order to maintain the methylation in daughter cells, a protein referred to as UHRF1 (ubiquitin-like protein with PHD and RING finger domain 1) binds to hemi-

methylated DNA. During replications, UHRF1 causes DNMT1 to methylate new cytosine from synthesized DNA strands. There exists also growing evidence which suggests that DNMT3A/B are also involved in maintaining the DNA methylation pattern in somatic cells, specifically in imprinted genes and repeated elements (Fig.1.4).

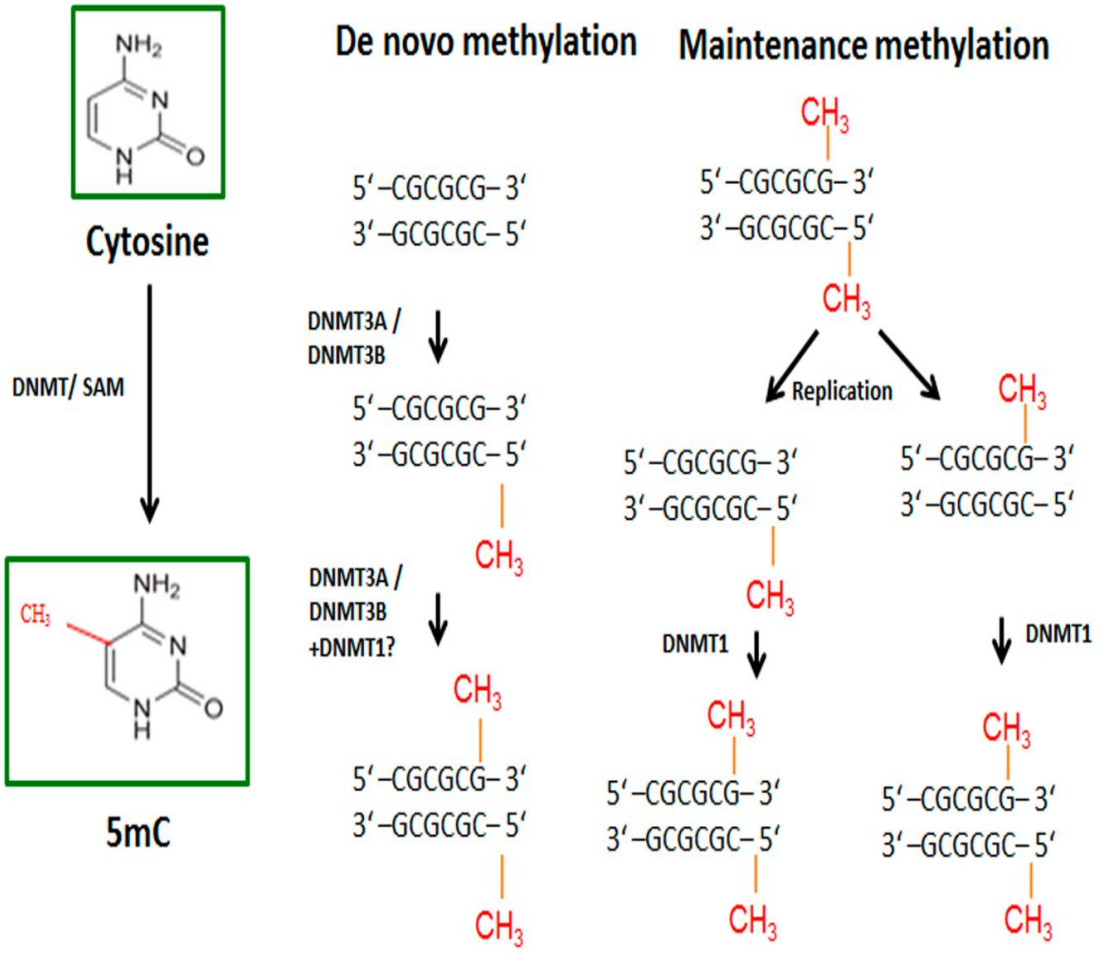



**Figure 1.4. DNA methylation through de novo and maintenance DNMT enzymes.**

De novo methylation occurs through recruiting DNMT3A and DNMT3B followed by maintenance of methylation by replication and including DNMT1. (modified from [Sasaki, 2005]).

The significance of DNMTs was demonstrated in various animal studies, in which it has been proven that mice lacking DNMTs died during their early development or

shortly after birth [Blair & Yan, 2012]. Nevertheless, the expression of these enzymes differs between various types of cancer and it is not always associated with hyper- or hypomethylation, as had been expected. Sometimes, the expression of DNMTs is regulated by miRNAs, which may explain the differences in the expression of DNMTs in various tumors [Pan *et al.*, 2010]. For instance, it has been demonstrated in several studies that DNA demethylase is post-transcriptionally regulated by mir29 and mir148 [Pan *et al.*, 2010]. Furthermore, So *et al.* have claimed that DNA methyltransferase controls stem cell aging through microRNA. They have shown that DNMT inhibition leads to a decrease of the expression of Polycomb groups (PcG) and to an increase of the expression of microRNAs (miRNAs) that target PcG [So *et al.*, 2011].

Although the methylation pathway is the same in mammals and plants, they follow different demethylation machinery. In plants, the methyl residue of 5mC is removed by DNA glycosylase, leaving an abasic site that is replaced by an unmethylated cytosine via base excision repair (BER) [Gong *et al.*, 2002]. In vertebrates, the possible pathway to remove 5mC is passive demethylation during replication. Passive demethylation takes place through dilution of symmetrically-methylated regions by inactivation of DNMT1 (maintenance DNA methyltransferase) after DNA replication. This ultimately results in hemimethylation in the daughter cells. Consecutive cell division and 5mC dilution leads to generation of two hemimethylated and two unmethylated daughter cells [Kagiwada *et al.*, 2013]. Nevertheless, 5mC is lost after fertilization in the paternal genome before replication has even started, and some non-replicating cells like neurons are demethylated. This pattern suggests the existence of an active demethylation [Branco *et al.*, 2012].

### 1.2.2 DNA demethylation

In early embryogenesis, many researchers have observed a loss of DNA methylation and subsequently have postulated the existence of several DNA demethylases and various mechanisms of the DNA demethylation (Fig.1.5) [Kohli & Zhang, 2013; Wu & Zhang, 2010]. The potential candidates for active demethylation pathway are ten-eleven-translocation proteins (TET1, TET2 and TET3), which are named after the ten-eleven-translocation (q22, q23) (10, 11) that occurs in rare cases of acute myeloid and lymphocytic leukemia [Ito *et al.*, 2010; Tahiliani *et al.*, 2009]. 5mC is oxidized by TET proteins and thereby converted to 5-hydroxymethylcytosine (5hmC). TET proteins belong to the family of Fe (II)/2-oxoglutarate-dependent dioxygenases and they are able to further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) [Ito *et al.*, 2011]. In addition to the conserved catalytic domain that is typical of Fe (II)/2-oxoglutarate-dependent dioxygenases in all three proteins of the TET family, such as TET1 and TET3 containing a CXXC domain that has an affinity for binding to CpG dinucleotides motif, which is involved in the recruitment of TET1 and TET3 to the DNA. The CXXC domain of TET2 is separated during development and broken down into separated genes that encode IDAX (inhibitor of the Dvl and axin complex; also known as CXXC4). It has been reported that IDAX, as a Wnt signaling inhibitor, regulates TET2 activity by targeting TET2 for destruction via the caspase dependent pathway (reviewed in [Williams *et al.*, 2012]). Expression of TET proteins is tissue specific. For instance, TET1 is mainly expressed in ESC while TET2 and TET3 are more or less ubiquitous [Globisch *et al.*, 2010]. Consequently, there are different levels of 5hmC (but not 5mC) in different tissues. The level of 5hmC is relatively high in ESCs, which continuously decreases during differentiation into adult cells [Koh *et al.*, 2011; Tahiliani *et al.*, 2009].

Conversion of 5mC to 5hmC takes place either by passive demethylation through inhibition of the DNMT1 activity at hydroxymethylated CpGs during replication or by active demethylation mechanisms [Valinluck & Sowers, 2007]. One potential active demethylation mechanism is further oxidation of 5hmC to 5fC and 5caC by TET enzymes [He *et al.*, 2011; Ito *et al.*, 2011]. It was reported that 5fC and 5caC can be removed by thymidine DNA glycosylase (TDG), producing an abasic site that is then repaired by the base-excision-repair (BER) machinery to cytosine [He *et al.*, 2011; Maiti & Drohat, 2011]. It was also shown that in ESCs, 5caC was converted directly to cytosine without BER interference, suggesting direct decarboxylation of 5caC as another putative demethylation mechanism. 5hmC could be also be removed via deamination machinery by recruiting AID/APOBEC deaminases [He *et al.*, 2011]. It was also reported that 5hmC could be deaminated to 5-hydroxyuracil (5hmU) by the apoliprotein B mRNA editing enzyme, catalytic polypeptide (APOBEC) and cytidine deaminase (AID) followed by TDG [Guo *et al.*, 2011b]. Apparently, multiple pathways are involved in the removal of 5mC from the genome and TET proteins play an important role in most of them. Different tissues might use different demethylation pathways to remove 5mC from the genome [Branco *et al.*, 2012]. However, it was also suggested that active demethylation can occur via the GADD45-mediated BER and NER pathways. In the BER pathway, 5mC is deaminated to thymidine via AID. Subsequently, MBD4 or TDG identify the T:G mismatch in order to remove the base site by the BER machinery [Delatte & Fuks, 2013].

Altogether, DNA methylation is reversible, either through passive demethylation via DNA replication or by active demethylation via DNA repair followed by base excision repair of modified nucleotides or DNA glycosidase [Kohli & Zhang, 2013; Tahiliani *et al.*, 2009] (Fig1.5).

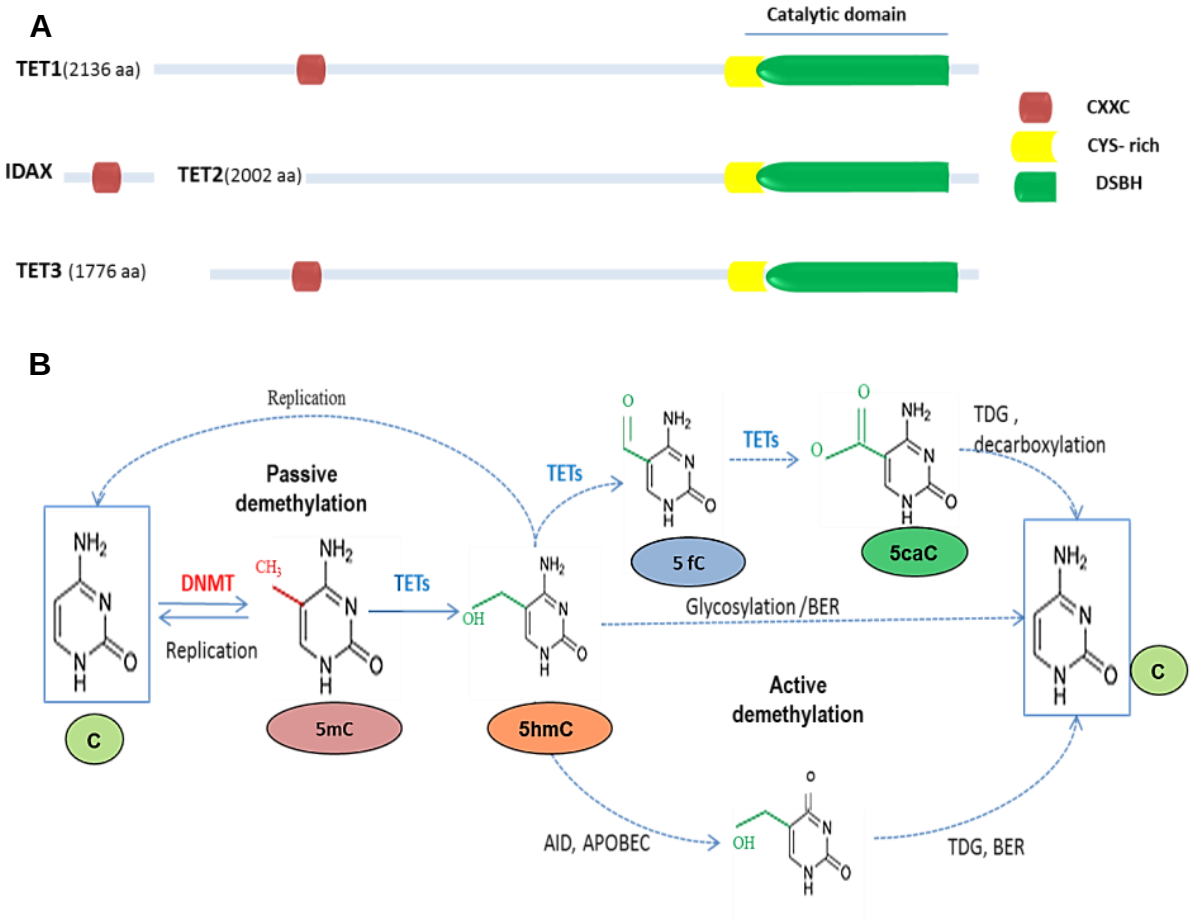



**Figure 1.5. Potential pathways for DNA demethylation.**

**A)** Domain architecture of TET proteins. TET proteins consist of 3 conserved domains including CXXC zinc finger domain, the cys-rich region and double strand β-Helix (DSBH) fold of 2OG/ Fe (II) dioxygenase domain. **B)** Genomic 5-methylcytosine (5mC) can be removed either passively via replication or by active demethylation pathways with 5hmC as intermediate by recruiting TET proteins. Following 5hmC oxidation, demethylation occurs by dilution of oxidized bases or by removal through DNA glycosidase- and DNA repair-mediated excision of modified (modified from [Kohli & Zhang, 2013]). Abbreviations: **C)** Cytosine, DNMT: DNA methyltransferase, 5mC: 5-methylcytosine, 5hmC: 5-hydroxymethylcytosine; 5fC: 5-formylcytosine, 5caC: 5-carboxylcytosine, 5hmU: 5-hydroxymethyluracil, TDG: Thymine DNA Glucosidase, BER: Base Excision Repair, APOBEC: Apolipo protein B mRNA editing enzyme catalytic peptide (Modified from [Ko *et al.*, 2013; Williams *et al.*, 2012]).

### 1.2.2.1 TET proteins and Vitamin C

TET enzymes are Fe(II)/2-oxoglutarate (2OG)-dependent dioxygenases [Ito *et al.*, 2010; Ito *et al*., 2011; Kohli & Zhang, 2013; Tahiliani *et al.*, 2009]. As described before, TET proteins contain catalytic domains consisting of a double strand β-helix (DSBH), binding to Fe(II)/2OG, which means they show their activity only in presence of Fe(II), and that it is necessary to sustain their state as an electron donor to maintain the redox state of Fe. It was shown that Vitamin C reduces the oxidized iron species. Therefore, it has a significant role in maintaining the catalytic activity of Fe(II)/2OG-dependent dioxygenases and supports the maintenance of the active form of TET proteins (Fig1.6). Vitamin C may reduce the requirement of a cofactor in TET mediated hydroxylation of 5mC to 5hmC resulting in alteration of the DNA methylation status [Monfort & Wutz, 2013]. Recently, it was shown that Vitamin C has an important role in reprogramming of somatic cells by regulating TET1 and modulating mesenchymal-to-epithelial transition (MET) [Chen *et al.*, 2013a].

Therefore, Vitamin C might be an important factor in reducing the risk of promoter hypermethylation and in maintaining the 5hmC state [Blaschke *et al.*, 2013; Monfort & Wutz, 2013; Yin *et al.*, 2013]. It was also shown that Vitamin C can facilitate the differentiation of hematopoietic stem cells by inducing TET2 [Monfort & Wutz, 2013].

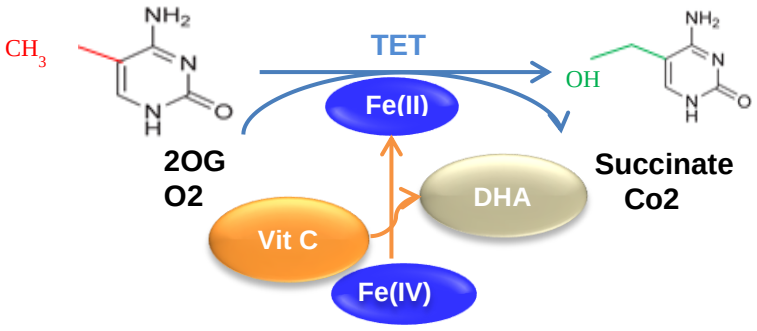



**Figure 1.6. Vitamin C is suggested as an additional cofactor in conversion of 5mC to 5hmC of TETs.**

Vitamin C can reduce Iron to Fe(II) and restore the catalytic activity of enzymes (modified from [Minor *et al.*, 2013]).

#### 1.2.2.2 Role of TET proteins

TET proteins play different roles in diverse biological processes, including epigenetic regulation of gene transcription, embryonic development, stem cell functions, and cancer. They mediate the DNA demethylation process and change the epigenetic state of DNA. A loss of 5hmC together with a down-regulation or mutation of TET proteins has been reported in numerous types of cancer, like melanoma, breast, prostate, myeloid, brain, and liver cancer [Lian *et al.*, 2012]. It has been demonstrated that in patients suffering from acute myeloid leukemia (AML), TET1 protein influences mixed lineage leukemia (MLL) development [Wu & Zhang, 2010]. Furthermore, several changes in the TET2 protein e.g. deletion and deactivation of TET2, have been identified in 30% of all patients suffering from different kinds of myeloid malignancies and the majority of the TET2 alterations include single or double copy defects and nonsense/frame shift mutations suggesting that TET2 lesions cause a loss of function [Ko *et al.*, 2013; Li *et al.*, 2011]. Therefore, it has been suggested that TET2 is a possible tumor suppressor gene that is responsible for myeloid malignancies [Li *et al.*, 2011]. Furthermore, the loss of 5hmC in correlation with clinical progression and relapse of melanoma has been reported [Lian *et al.*, 2012].

### 1.2.3 DNA methylation and HCC

Numerous studies have reported that tumor cells exhibit a major disruption in the regulation of the DNA methylation pattern in cancer including in HCC (Fig.1.7). Therefore, over the last few years, changes in DNA methylation have been particularly investigated in various cancer types [Baylin & Herman, 2000]. Earlier studies have demonstrated that the hypomethylation of interspersed repetitive DNA is a common feature of numerous types of cancer that causes genomic instability and

aberrant transcription initiation [Caiafa & Zampieri, 2005]. Recent studies have proven that the hypermethylation of focal CpG islands is elevated in cancers and may result in inhibition of various miRNAs, tumor suppressor and functional genes [Ehrlich, 2002; Esteller, 2002]. However, detailed mechanisms leading to hyper- or hypomethylation are far from being clearly understood.

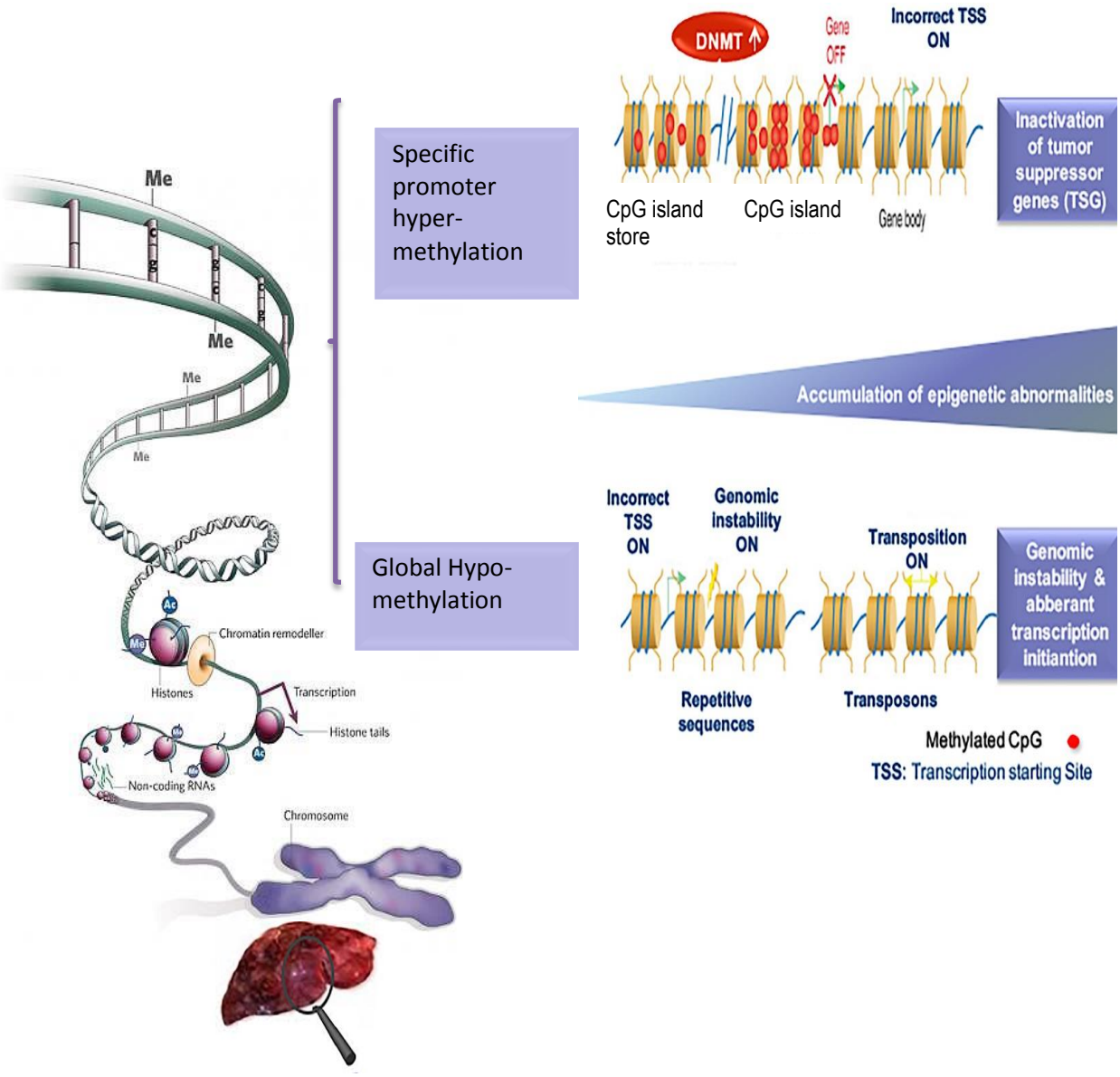



**Figure 1.7. DNA methylation in HCC.**

In HCC cells, the pattern of DNA methylation is often altered, resulting in global hypomethylation of genome associated with hypermethylation at CpG Island within the promoters of critical genes such as tumor suppressors (modified from [Qiu, 2006]).

#### 1.2.3.1 DNA hypermethylation and HCC

CpG islands are protected by various defense mechanisms by accessing the DNA methyltransferase, such as DNA replication timing, active demethylation, or active transcription. Most genes that are susceptible to hypermethylation are associated with the regulation of DNA repair, apoptosis, cell cycle, drug resistance, metastasis, and differentiation [Das & Singal, 2004]. Many tumors are hypermethylated in one or more genes. For instance, in breast cancer, it has been shown that several critical genes, which seem to be important in tumorigenesis, are hypermethylated. Among these genes, cell adhesion genes, inhibitors of matrix metalloproteinases as well as the estrogen receptor (ER) and progesterone receptor (PR) as steroid receptor genes are included and have been linked to breast cancer development [Yang *et al.*, 2001].

Hypermethylation induces inactivation of a wide range of critical genes in HCCs which leads to the progression of cancer [Anestopoulos *et al.*, 2015; Mah & Lee, 2014; Mínguez & Lachenmayer, 2011]. It was reported that DNMT1, DNMT3A and DNMT3B are up-regulated in all kinds of liver cancer [Nagai *et al.*, 2003; Oh *et al.*, 2007]. In addition, it was shown that the tumor suppressor gene p16 (CDKN2A) is hypermethylated in most HCCs and that it is related to the progression of cancer [Liggett & Sidransky, 1998]. SOCS1 gene (Suppressor of Cytokine Signaling 1) was illustrated to be hypermethylated which promotes an endless cell cycle. SPCS1 (Signal Peptidase Complex Subunit 1) is a negative regulator of the JAK/STAT pathway and it is another frequently studied prognostic marker for HCC [Yoshikawa *et al.*, 2001]. Promoter methylation of GSTP1 was also reported in HCC with HBV or HCV infection and GSTP1 methylation is correlated with age and alcohol intake [Mah & Lee, 2014]. GADD45 is another crucial gene which is deregulated in HCC

[Gramantieri *et al.*, 2005; Qiu *et al.*, 2004]. Accumulations of a number of evidences in recent years implies that GADD45 genes are implicated in various pathways such as cell cycle arrest, DNA repair, cell survival, apoptosis and epigenetic modification of genes [Cretu *et al.*, 2009] GADD45 is known as a stress sensor, mediating the complex interplay of physical interactions with proteins which are responsible for stress stimuli and cell cycle regulation including PCNA, P21, Cdc2/Cyclin B, P38 and JNK stress response kinases [Cretu *et al.*, 2009; Hall *et al.*, 1995; Harkin *et al.*, 1999; Kearsey *et al.*, 1995; Niehrs & Schäfer, 2012; Zerbini & Libermann, 2005]. In addition, it was shown that GADD45 has a role in active demethylation associated with TDG and MBD4 [Delatte & Fuks, 2013]. Interestingly, it was shown that the GADD45 gene is methylated and silenced in HCC which leads to progression of HCC [Qiu *et al.*, 2004].

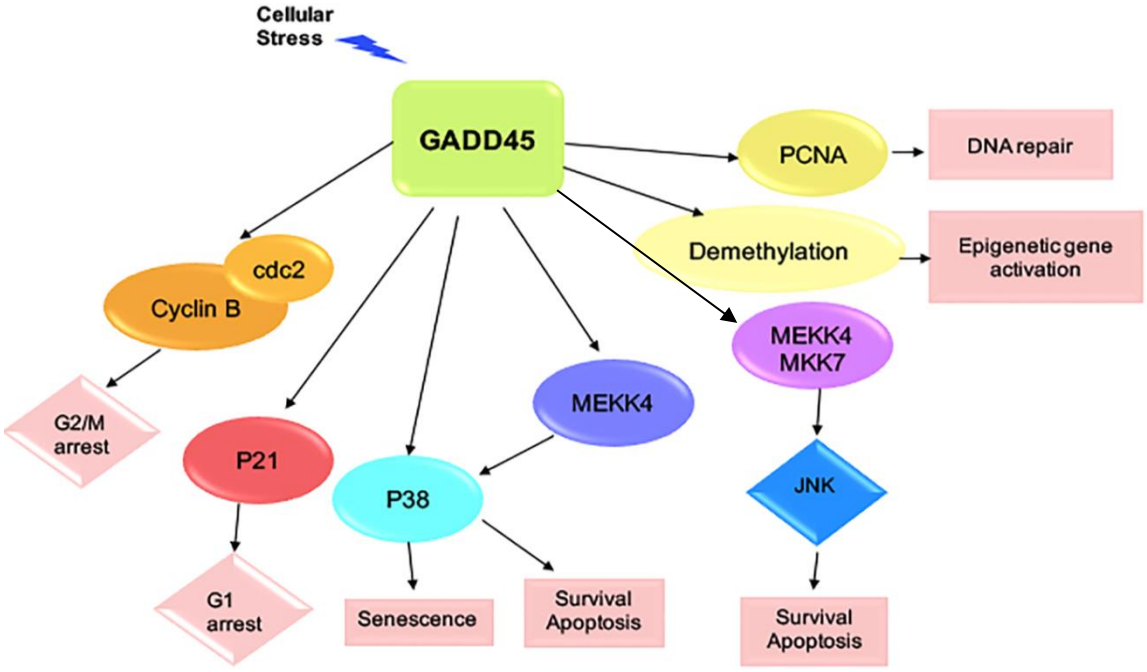



**Figure 1.8. GADD45 regulates different pathways.**

This figure shows the regulatory of GADD45 on different pathways (cell cycle, cell proliferation, cell survival, apoptosis and demethylation) (modified from [Cretu *et al.*, 2009]).

Another important mechanism affected in HCC is the Cell-Cell-Adhesion and Epithelial-Mesenchymal-transition (EMT) during the progression of HCC (Fig.1.8) [Kwon *et al.*, 2005]. E-cadherin is a member of the calcium-mediated membrane glycoprotein and it is known as an adhesion molecule which is expressed on all epithelial cells and plays an important role in the maintenance of cell polarity and environment [Sugimachi *et al.*, 2003; Tang *et al.*, 2011]. Through inactivation of E-cadherin, cells lose their adherent junctions and impairment of cell adhesiveness. Therefore, decrease of E-cadherin expression is associated with cell invasion, drug resistance, tumor progression and metastasis which are characteristics of tumor cells [De Herreros *et al.*, 2010; Lee *et al.*, 2006]. In most types of epithelial cancer, alteration of genetic and epigenetic mechanisms leads to down-regulation of E-cadherin [Tischoff & Tannapfel, 2008] Decrease or loss of E-cadherin expression is mainly caused by aberrant CpG island methylation in HCC and it was shown that loss of heterozygosity (LOH) of E-cadherin is associated with hypermethylation of CpGs [Kwon *et al.*, 2005]. Therefore, modifying the methylation status of DNA in cancer cell may lead to reactivation of E-cadherin and inhibits EMT.

Moreover, it was reported that the transcription factor Snail, a zinc-finger transcriptional repressor, directly represses the expression of E-cadherin aiding the progression of EMT, especially in epithelial kind of cancers such as HCC [Batlle *et al.*, 2000; Sugimachi *et al.*, 2003]. It was shown that the Snail gene is regulated via DNA methylation and results in E-cadherin gene inhibition which provokes EMT-MET transition in HCC [Chen *et al.*, 2013b]. Snail not only inhibits E-cadherin expression during EMT transition but also changes the phenotype of the cells and provokes the expression of mesenchymal markers [Wang *et al.*, 2013]. Snail also induces the resistance of cells towards cell death by withdrawal of survival factors like PI3K, ERK

and inhibition of cell cycle check points like p21 which is known as an inhibitor of cell cycle in either G1/S or G2/M phase [Takahashi *et al.*, 2004]. The role of Snail in different molecular pathways is summarized in (Fig.1.9).

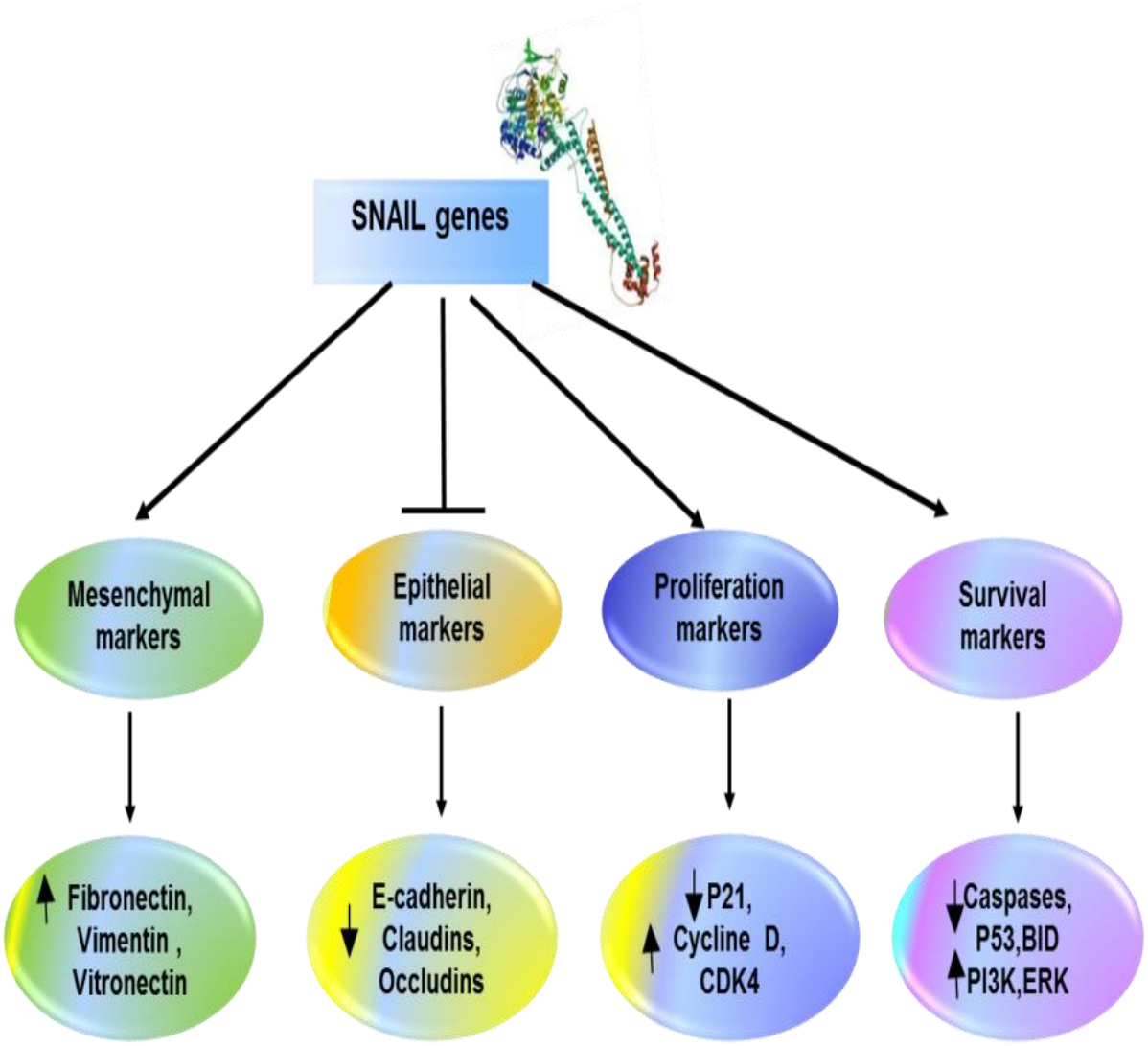



**Figure 1.9. The snail gene is an inducer if cell survival and cell movement.**

Snail gene expression evokes the loss of epithelial marker and gain of mesenchymal markers including cells morphology changes with acquisition of motility and invasive characteristic (Modified from [Barrallo-Gimeno & Nieto, 2005]).

The most important highly methylated tumor related genes in HCC are provided in Table 1.1. Taken together, therapeutic agents that can target the methylation status of DNA in order to activate the promoters of suppressed genes like tumor suppressor genes, open a new avenue of hope for HCC therapy togetherwith other treatments such as radiation therapy

Table 1.1. Promoter methylation of different tumor-related genes in HCCs.

| **Gene** | **Function** | **Ref** |
|---|---|---|
| **P169NK4a** | CDK inhibitor | [Lee *et al.*, 2013; Suzuki & Nohara, 2013] |
| **SOCS1** | Cytokine inhibitor<br>(JAK/STAT inhibitor) | [Yoshikawa *et al.*, 2001; Zhang *et al.*, 2014] |
| **GSTP1** | Detoxification | [Zhong *et al.*, 2002] |
| **CYP24A1/CYP7B1** | Drug Biotransformation | [Deng *et al.*, 2010] |
| **CASP8/TMS1/RASSSF1A** | Apoptosis | [Yu *et al.*, 2002] |
| **MGMT** | DNA repair | [Zhang *et al.*, 2003] |
| **E-cadherin/M-cadherin/H-cadherin** | Cell adhesion | [Ripoli *et al.*, 2011; Yamada *et al.*, 2007; Yu *et al.*, 2002] |
| **hMSH1/hMSH2/hMSH3** | Mismatch repair | [Park *et al.*, 2006; Wang *et al.*, 2001] |
| **P21-P53-RB** | Tumor suppressor | [Zhang *et al.*, 2008] |
| **GADD45 β** | DNA damage response | [Higgs *et al.*, 2010] |

#### 1.2.3.2 DNA hypomethylation in HCC

Hypomethylation is an alteration in DNA methylation which is frequently seen in solid tumors [Feinberg & Vogelstein, 1983; Kim *et al.*, 1994; Lin *et al.*, 2001], and is a sign of a progressive malignancy [Bedford & Van Helden, 1987]. Furthermore, it has been reported that the hypomethylation status contributes to oncogenesis through up-regulation of some oncogenes, like H-RAS and c-Myc [Feinberg & Vogelstein, 1983], as well as by chromosome instability [Tuck-Muller *et al.*, 2000]. Deregulation of a methylation state is frequently observed in HCCs. Structural-nuclear function is affected by global hypomethylation leading to chromosomal and genomic instability. DNA hypomethylation of pericentromeric satellite regions associated with overexpression of DNMT3B was reported in HCC which results in chromosomal instability [Saito *et al.*, 2002].

Nevertheless, aberrations not only occur in the DNA methylation, also in the regulation of histone modifications which also leads to cancer development [Dawson & Kouzarides, 2012]. For instance, numerous studies have demonstrated that histone deacytylases (HDACs) are overexpressed in different types of cancer. This overexpression leads to a deacetylation of the Transcription Starting Site (TSS) of important genes and subsequently to the assembly of silenced genes into more compact structures [Halkidou *et al.*, 2004; Song *et al.*, 2005].

In summary, there exists an epigenetic crosstalk between histone modification, micro-RNAs and DNA methylation. Post-transcriptionally, miR-29 and miR-148 regulate the DNA demethylases [Duursma *et al.*, 2008; Fabbri *et al.*, 2007]. Moreover, it has been proven that DNMTs interact with HDAC [Yang *et al.*, 2001]. In particular, the combination of DNMT and HDAC inhibitors is a primary target for

potential therapeutics against cancer [Unoki, 2011; Zhu & Otterson, 2003], because these inhibitors are capable of targeting the epigenetic status of DNA and histones simultaneously, which may enable the development of more effective anticancer drugs.

## 1.3 Challenges in HCC management

One of the major clinical implications in HCC therapy is the poor prognosis of cancer at its early stages. Patients are frequently diagnosed at advanced stages, which leads to more complicated therapeutic options and a frequently undesired outcome. In addition to late diagnosis, HCC is a heterogeneous kind of tumor with complications like cirrhosis, HCV or HBV infections; therefore, the management of this tumor requires a multidisciplinary approach. Up to date, there are several treatment options for HCC including surgical removal of tumor, Chemotherapy, liver transplantation and molecularly-based therapeutic such as sorafenib (Nexavar) therapy. Sorafenib is a multi-tyrosine kinase inhibitor which affects tumor angiogenesis interfering with the vascular endothelial growth factor signaling mechanism which is used to treat HCC in advanced stage [Ma *et al.*, 2014; Maluccio & Covey, 2012]. Similarly, multiple studies are focused on other drugs targeting other molecular growth pathway receptors including: the epidermal growth factor receptor, hepatocyte growth factor and platelet derived growth factor receptor [Ma *et al*., 2014].

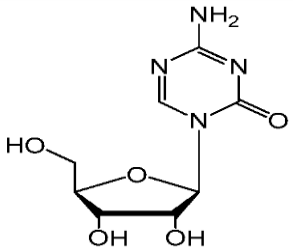
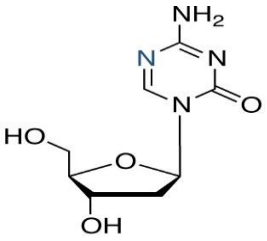
## 1.4 Epigenetic drugs and HCC treatment

In addition to molecular based and conventional therapy, epigenetic drugs provide an alternative approach through targeting the methylation status of genes like promoters of tumor suppressor genes or reversing histone modifications of altered genes (Fig.1.10).

Currently, majority of studies focus on the development of epigenetic drugs which can reverse the abnormal epigenetic state in cancer to the normal epigenetic state of cells. Up to now, the FDA has approved four epigenetic modifying drugs for the treatment of cancer: 5-Azacytidine (5-AZA) and 5-Aza-2´-deoxycytidine (5-AZA-dC) ,DNMT inhibitors, which have been used for patients suffering from myelodyplastic syndrome and from acute leukemia, as well as Vorinostat and valporic acid (HDAC inhibitors) which are used for treatment of T-cell lymphoma and some hematological tumors [Byrd *et al.*, 2005].

Although 5-AZA and 5-Aza-2´-deoxycytidine have the same therapeutic effect, their mode of action in tumor cells is different. It has been demonstrated that 5-Aza-2´-deoxycytidine is able to induce senescence in tumor cells, while 5-AZA induces cell death in tumor cells in vitro and in vivo. These two DNMT inhibitors differ from each other on the molecular and the cellular level: For instance, 5-AZA has a ribonuleoside structure and can be integrated in both DNA and RNA (20-40% and 60-80% respectively), while 5-Aza-2´-deoxycytidine can only be incorporated in DNA. Moreover, 5-Aza-2´-deoxycytidine induces more DNA damage and micronuclei formation than 5-AZA (Table 1.3) [Venturelli *et al.*, 2013].

**Table 1.2. Comparison of 5-AZA and 5-AZA-dC**

| **Characteristic** | **5-AZA** | **5-AZA-dC** |
|---|---|---|
| **Chemical Structure** |  |  |
| **DNA/RNA Incorporation** | RNA & DNA | DNA |
| **Protein Biosynthesis** | + | - |
| **DNA Strand Break** | Increase | More than 5-AZA |
| **NK Cell Activity** | Decrease of micronuclei formation | Increase of mincronuclei formation[Yu *et al.*, 2002] |
| **Mechanism** | Inhibits DNMT1 & DNMTB | Inhibits DNMT1 |

Furthermore, it has been reported that 5-AZA increases the sensitivity of the antitumor effects of Docetaxel (DTX) and Cisplation in aggressive and chemoresistant prostate cancer [Festuccia *et al.*, 2009]. In addition, it has been shown that Pyrazofurin (PF), an inhibitor of orotidylate decarboxylase improved the anti-tumor activity of 5-AZA in human leukemia cells [Cadman *et al.*, 1978]. The combination of PF and 5-AZA has been suggested being a useful treatment for patients who do not respond to standard anti-leukemic therapies [Lancet & Karp,

2009]. Furthermore, it has been proven that 5-Aza-2´-deoxycytidine inhibits the telomerase activity and reactivates p16 and c-Myc in hepatocellular carcinoma cells (HCC), such as the MMC-7721 and the HepG2 cell line [Tao *et al.*, 2012].

Other DNMT inhibitors, such as 5-fuoro-2´-deoxycytidine, in combination with other drugs are currently undergoing clinical trials for their possible use in treatment of different types of cancers [Yang *et al.*, 2010]. The DNA methyltransferase inhibitor Zebularin provoked the demethylation of tumor suppressor genes by inducing apoptosis, cell cycle regulation in HCC cell lines (Huh7, KMCH, and HepG2), and *in vivo* inhibition of tumor growth in a xenograft model [Nakamura *et al.,* 2013].

It has been discussed if the expression of specific DNMT inhibitors might influence the differentiation status in developing and dedifferentiating cells. It has been shown that 5-AZA improves differentiation in mesenchymal stem cells (MSCs) [Culmes et al., 2013; Wakitani et al., 1995; Yan et al., 2014]. Furthermore, we have reported that pretreatment of fat-derived old MSCs with 5-AZA improves osteogenic differentiation by DNA de-methylation. We have observed a reduction of 5hmC in Ad-MSCs of older donors (> 60 years) compared to young donors (< 45 years). After stimulation of cells with 5-AZA the osteogenic potential of the cells improved significantly. This was further substantiated by an improvement of AP activity and matrix mineralization with a concomitant increase of 5hmC as well as with the expression of TET2 and TET3 [Yan *et al.*, 2014]. Therefore, we have concluded that 5-AZA induce the rejuvenating process of older Ad-MSCs through alteration of pluripotency genes like Oct4 and Nanog. Furthermore, it has been shown that the incubation of human bone marrow MSCs with 1 µM TSA which have been pre-treated with hepatogenic-stimulating agents, improves the hepatogenic differentiation of these cells [Snykers *et al.,* 2007].

Moreover, a combination of TSA and 5-Aza-dc has improved the differentiation of human CD34+ to CD34/CD90 cells [Milhem *et al.*, 2004]. From our studies, we conclude that drugs that modify the DNA or histone methylation also induce changes in the pluripotency [Culmes *et al.*, 2013; Yan *et al.*, 2014].

### 1.4.1 Other epigenetic drugs

In the past years, many anticancer drugs which target the acetylation or methylation status of histones through different mechanisms have been developed (Fig.1.10), such as HDAC inhibitors of class I, II, III (Sirtuins), IV, HATs, HMTs, HDMs and various kinds of kinases. Based on their chemical properties, the HDACs are branched into the four following groups: hydroxamic acid, short fatty acids, cyclic peptide and benzamide enzymes [Rodríguez-Paredes & Esteller, 2011a]. HDACs inhibitors mainly arrest the cell cycle in G1 and G2-M phases, which in turn, induces apoptosis and cell differentiation. However, they are able to inhibit angiogenesis and metastasis, and to decrease the resistance of tumor cells to chemotherapy [Joseph *et al.*, 2004]. Recently, a new epigenetic drug called, Suberoylanilide hydroxamic acid (SAHA) has been approved for treating T-cell cutaneous lymphoma by the Federal Drug Administration (FDA) [Duvic *et al.*, 2007]. Furthermore, a large number of HDAC inhibitors, like e.g. TSA (Tricostatin A), PXD101 (Belinostat), and LBH589 (Panobinostat), are currently being developed and are undergoing clinical trials [Cang *et al.*, 2009; Khan & La Thangue, 2011].

Class III HDACs, so called Sirtuins (SIRT) are important for the regulation of senescence, survival, and cell proliferation. An increasing number of effective SIRT inhibitors have recently been generated [Rodríguez-Paredes & Esteller, 2011b]. SIRT1 and SIRT2 inhibitors are likely to stop p53 activity, thereby inducing growth arrest in cancer cells and increasing sensitivity of tumor cells towards anticancer

drugs [Bitterman *et al.*, 2002]. Salermide, another SIRT1 and SIRT2 inhibitor, induces p-53-independent apoptosis in tumor cells and reactivates proapoptotic genes by deacytelation of H4K16Ac at the epigenetic level which is mediated by SIRT1[Lara *et al.*, 2008].

Furthermore, it has been shown that curcumin, garcinol, and anacaridic acid may act as HAT inhibitors with an anti-cancer effect in ovarian and colon cancer. It has been demonstrated that all three substances are able to inhibit dose-dependently EP300-mediated p53 acetylation [Mukhopadhyay *et al.*, 2002; Shishodia *et al.*, 2005].

DZNep, Chaetocin, and BIX-01294 are known to be lysine HMT inhibitors. It has been shown that DZNep promotes apoptosis in the MCF7 cell line (breast cancer) and in the HCT116 cell line (colorectal cancer) [Tan *et al.*, 2007]. Chaetocine has an anti-cancer effect on multiple myeloma (MM) [Greiner *et al.*, 2005]. BIX-01294 deactivates the histone H3 lysine 9 (H3K9) methyltransferases G9a and G9a-like protein by promoting the apoptotic cell death [Choi *et al.*, 2013]. It has been demonstrated that BIX-01294 induces endothelial differentiation in Ad-MSCs. We have been able to demonstrate that BIX modifies DNA and histone methylation and remarkably increases the expression of various endothelial markers that are required for blood vessel formation, such as VCAM-1, PECAM, VEGFR-2, and PDGF [Culmes *et al.*, 2013].

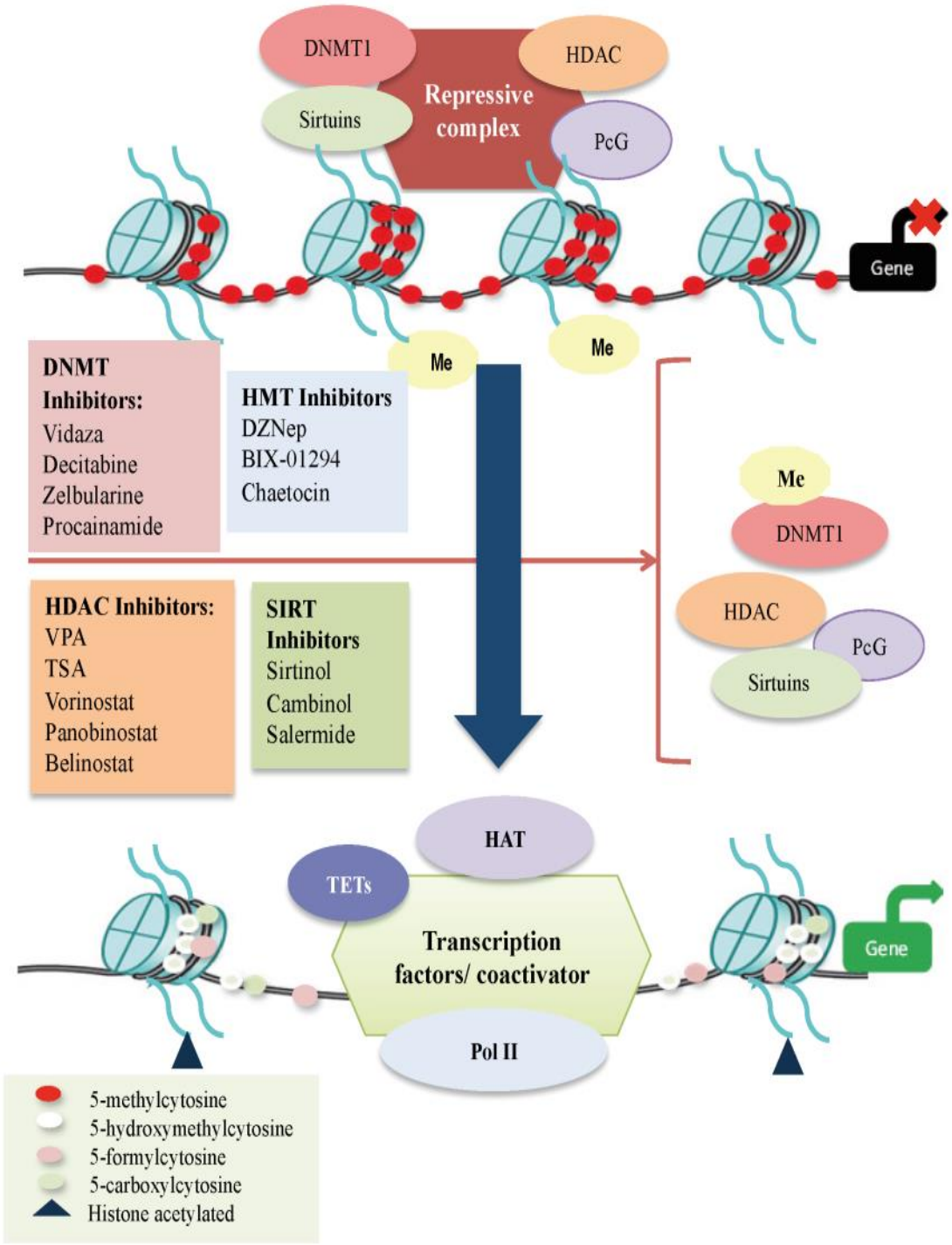



**Figure 1.10. Epigenetic drugs for cancer therapy.**

This figure illustrates some of the most important epigenetic drugs, which are classified in relation to their epigenetic target. Abbreviations: DNMT1: DNA Methyltransferase 1, HDAC: Histone Deacetylase, PcG: Polycomb Group, Me: Methylated, HAT: Histone Acetylase [Sajadian & Nussler, 2015]

## 1.5 Combination of current therapies with epigenetic drugs for HCC

Combination of epigenetic drugs along with other conventional or molecular based therapies may offer an alternative promising tool for cancer therapy. Epigenetic drugs can provoke host immunogenicity through induction of antigen presentation. It was shown that 5-AZA- 2'-deoxycitide improves CTA, a cancer testis antigen leading to increased sensitivity of cancer cells to immunotherapy. The immune-modulatory activity of 5- AZA-2'-deoxycytidine, offers a novel strategy for chemo immunotherapy for cancers which are resistant to adoptive cell transfer therapy like Melanoma. Similarly, it can be useful for treatment of HCCs with inflammatory or HBV/ HCV background where increase of IL6 and TNF-$\alpha$ are reported. The various conventional and molecular based therapies dependent on the stage of tumor are illustrated in Fig1.11.

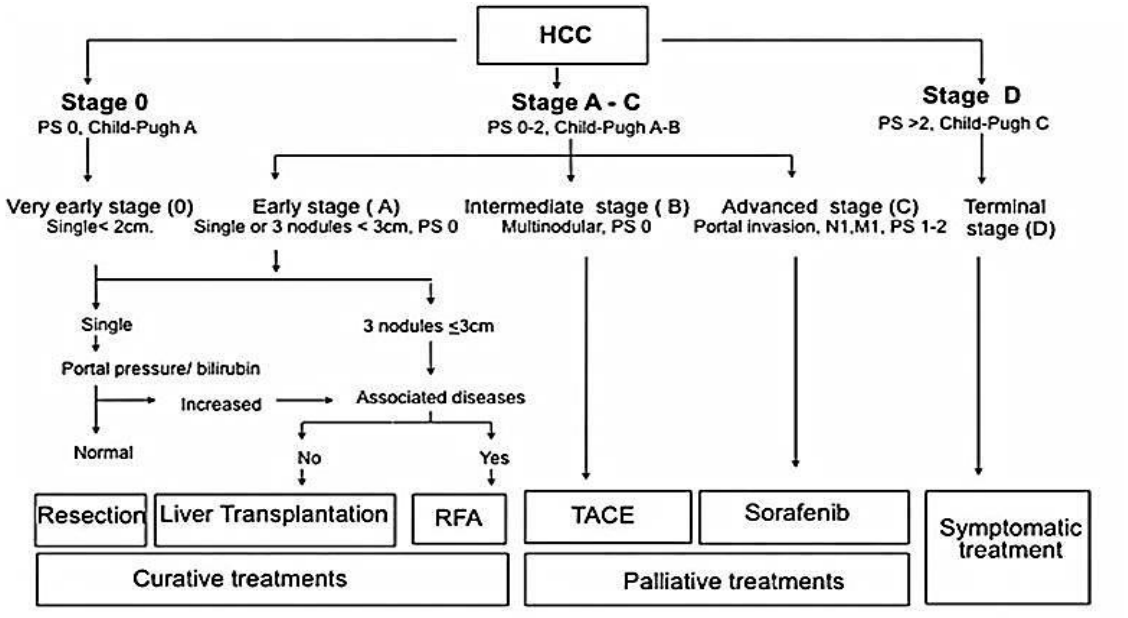



**Figure 1.11. Different strategy for HCC therapy dependent of the tumor stages base on BS**

This figure shows the different strategy for treatment of HCC dependent of the tumor stage; treatment such as liver transplantation, sorafenib and TACE [Granito & Bolondi, 2009].

## 1.6 Linking of epigenetic modification and metabolic activity in Hepatoma cell lines

It was shown that the most important hepatic genes are influenced by epigenetic regulators such as HDACi and DNMTi [Snykers *et al.*, 2009]. HDACi promotes growth arrest and upregulates C/EBPa, HNF-1a, HNF-3a, HNF-3b and HNF-4a levels in various hepatoma cells which leads to the increase of CYPs expression [Yamashita *et al.*, 2003] and ALB secretion. Therefore, modifying and triggering the epigenetic state of hepatoma cell line may induce the CYPs activity by altering the expression of genes responsible for CYPs activity. A number of studies focused on the effect of DNMTi such as 5-AZA and 5-AZA-dC on the expression of crucial phase 1 and 2 biotransformation genes and some of these studies indicated the improvement of CYP3A4, CYP3A7, CYP1B, UGT2B15 and GSTP1 gene expression [Snykers *et al.*, 2009] Therefore, in this study, we investigated the effects of the epigenetic modifiers 5-AZA and Vitamin C as a single or a combination treatment on improvement of drug biotransformation by modifying the expression of important genes such as CYPs (CYP1A1, CYP3A4, CYP2E1, CYP2C9), UGT and GST in Hepatoma cell lines (Huh7, HLE, HepG2 and AKN1) and thereby improving their function.

Drug metabolism in the liver can be divided into three phases. In Phase I a functional group (e.g., OH, SH or $NH_2$) is added to the substrate and it is characterized by oxidative, reductive, and hydrolytic pathways. In phase II, the newly introduced functional group is altered to O- and N-glucuronides, sulfate esters, various α-carboxyamides, and glutathionyl adducts and the polarity relative to the unconjugated molecules also increased [Parkinson, 2001]. This two-step transformation should

make the substrates more water soluble, so that they can be more readily excreted, and result in detoxification.

There are at least 57 isoforms of CYPs identified in the human liver [Xu et al, 2005]. Among these isoforms, CYP3A4 is known to play the important role in drug metabolism and is responsible for the metabolism of a large number of clinically used drugs. It is therefore an enzyme of interest for the study of drug metabolism processes in humans, and is particularly relevant to clinical and drug discovery research [Chiang *et al.*, 2014].

Therefore, PHH are widely used in drug discovery to study the drug metabolism due to their major role in metabolism of drugs according to their cytochromeP450 enzyme activity. However, using PHH has limitations of the scarce availability of fresh human liver samples suitable for research, complicated isolation procedures, high inter-individual variability and costs associated with their use. Indeed, isolated hepatocytes cultured in a two dimensional technique loosing quickly many liver-specific functions including cytochromesP450 (CYP450) genes and their activity [Gomez-Lechon *et al.*, 2008].

As an alternative, hepatocytes from animals (rodents) are widely used, but the drug metabolism properties of these hepatocytes do not always accurately reflect the same drug metabolism as human hepatocytes because of species difference [Chiang *et al.*, 2014]. Therefore, human hepatoma cell lines are convenient and reliable alternatives to PHH. However, their drug-metabolizing activity is very low in comparison with PHH. Many strategies have been used to induce or improve the functions of drug-metabolizing enzymes in order to provide more reliable systems: For instance, the introduction of cDNAs encoding drug-metabolizing enzymes or

growing cells in the presence of dimethyl sulfoxide (DMSO) are used in attempt to generate more metabolic competent hepatic cell lines [Gomez-Lechon *et al.*, 2008]. In three-dimensional cultures of human hepatoma (e.g. HeparG) an increase in mRNA expression of CYP1A1, 1A2, 2B6, 2D6, and 3A4 was observed [Gunness *et al.*, 2013]. In addition, the transfection of HepG2 cells with recombinant adenoviruses that encode CYP1A2, CYP2C9, and CYP3A4 has been shown to increase the activity of these enzymes remarkably to levels comparable to those detected in primary human hepatocytes [Tolosa *et al.*, 2012]. Chiang *et al.,* have shown the enhancement of CYP3A4 enzyme activity of HNF1α transfected cells [Chiang *et al.*, 2014]. However, additional studies regarding the use of viral transfection and the resulting effect on CYPs expression are needed.

## 1.7 Aim of the thesis

The aim of this thesis tries therefore to address the following questions:

I. What is the correlation of 5hmC and TETs in HCC development?
II. Does 5-AZA (DNMTi) have a role in active demethylation process?
III. Does Vitamin C play a role in inducing active demethylation?
IV. What is the consequence of DNA demethylation in HCCs?
V. Do 5-AZA and/or Vitamin C improve the metabolic activity of HCC cell lines?

# 2 Material and Methods

## 2.1 Material

| Device | Company |
|---|---|
| Auto sampler | Thermo Fisher Scientific GmbH, Dreieich, Germany |
| Balance KERN ABJ | Kern & Sohn GmbH, Balingen, Germany |
| Balance KERN PCB | Kern & Sohn GmbH, Balingen, Germany |
| Centrifuge Megafuge 40R | Thermo Fisher Scientific GmbH, Osterode, Germany |
| Clean Bench Safe 2020 | Thermo Fisher Scientific GmbH, Langenselbold, Germany |
| Digital Monochrome Printer P93DW | Mitsubishi Electric SDN. BHD, Senai, Malaysia |
| Fluorescence Microscope EVOS-fl | Peqlab Biosysteme GmbH, Erlangen, Germany |
| FLUOstar Omega | BMG Labtech GmbH, Offenburg, Germany |
| LVis Plate | BMG Labtech GmbH, Offenburg, Germany |
| Gel Stick Imager | Imaging Instruments GmbH, Chemnitz, Germany |
| Incubator BINDER | BINDER GmbH, Tuttlingen, Germany |
| Step One Plus™ Real-Time PCR System | Thermo Scientific Fisher.Inc, MA, USA |

| | |
|---|---|
| Flow Cytometry | BD Biosciences. Inc, Heidelberg, Germany |
| Ligth Microscope Primo Vert | Carl Zeiss MicroImaging GmbH, Gottingen, Germany |
| Microcentrifuge Fresco 17 | Thermo Fisher Scientific GmbH, Osterode, Germany |
| IPC peristaltic pump | IDEX Health & Science GmbH, Wertheim, Germany |
| Power Pack HC | Bio-Rad Laboratories GmbH, Munich, Germany |
| Shaker DRS-12 | LTF Labortechnik GmbH & Co. KG, Wasserburg, Germany |
| Syringe Pump Perfusor Secura | B-Braun Melsungen AG, Melsungen, Germany |
| Thermal Cycler Block ARKTIK | Thermo Fisher Scientific Oy, Vantaa, Finland |
| Thermal Cycler Block Veriti® | Applied Biosystems (Life Technologies ™), Darmstadt, Germany |
| Thermostat Lauda Alpha | Lauda Dr. R. Wobser GmbH & Co. KG, Lauda-Königshofen, Germany |
| Vortex mixer LSE™ | Corning Incorporated, Corning, USA |
| Water bath Aqualine AL25 | Lauda Dr. R. Wobser GmbH & CO. KG, Lauda-Königshofen, Germany |

| **Product** | **Company** |
|---|---|
| Cellstart® Cell Culture Flasks 25, 75 and 175 cm2 | Greiner Bio-One GmbH, Frickenhausen, Germany |
| Cellstart® Cell Culture Plates 24,48 and 96 wells | Greiner Bio-One GmbH, Frickenhausen, Germany |
| Falcon® Cell Culture Plates 6 wells | Becton, Dickinson & Co. Ltd, Franklin Lakes, USA |
| Cellstart® Tubes 15 and 50 ml | Greiner Bio-One GmbH, Frickenhausen Germany |
| Costar® Stripette 5, 10 and 25 ml | Corning Incorporated, Corning, USA |
| Cryo Tube™ vials | Nunc, Roslkilde, Denmark |
| Pasteur Pipettes 150 mm and 230 mm, glass | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Fixo gum | Marabu GmbH & Co. KG, Bietigheim-Bissingen, Germany |
| Pipette Tips 10 µl | Biozym Scientific GmbH, Oldendorf, Germany |
| Pipette Tips 200 µl | Sarstedt Aktiengesellschaft & Co., Nümbrecht, Germany |
| Ratiolab® Pipette Tips 1000 µl | Ratiolab GmbH, Dreieich, Germany |
| Rotilabo®-microcentrifuge tubes 1.5 ml | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Micro Tube 0.5 ml | Sarstedt Aktiengesellschaft & Co., Nümbrecht, Germany |

### 2.1.1 Chemicals

| Product | Company |
|---|---|
| 3-(2-(N,N-diethylamino)ethyl)-7-hydroxy-4-methylcoumarin | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| 3-(2-N,N-diethyl-N-methylaminoethyl)-7-methoxy-4-methylcoumarin | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| 3-Cyano-7-hydroxycoumarin | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| 4-Methylumbelliferone | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| 5-Carboxyfluorescein | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| 5(6)-Carboxy-2',7'-dichlorofluorescein diacetate | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| 7-Benzyloxy-4(trifluoromethyl)coumarin | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| 7-Ethoxycoumarin | Fluka Chemie GmbH, Steinheim, Germany |
| 7-Ethoxy-4(trifluoromethyl)coumarin | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| 7-Hydroxycoumarin | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| 7-Hydroxy-4(trifluoromethyl)coumarin | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| 7-Methoxy-4(trifluoromethyl)coumarin | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| Acetaminophen | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| Acetic acid | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |

| | |
|---|---|
| Acetonitrile | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Agarose | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Ammonium chloride | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Boric acid | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Bovine Serum Albumin (BSA) | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Brij 35 | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Bromophenol Blue sodium salt | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Calcium chloride | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Chloroform | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Copper (II) sulphate | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Coumarin | Fluka Chemie GmbH, Steinheim, Germany |
| D-(+)-glucose | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| Diethylpyrocarbonate (DEPC) | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Dibenzylfluorescein | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| Dimethylsufoxide (DMSO) | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Ethanol | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |

| | |
|---|---|
| Ethidium bromide | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Ethylendiaminetetraacetic acid disodium salt dehydrate (EDTA) | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Folin & Ciocalteu's phenol reagent | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| Fluorescein | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| Glucose oxidase | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| Glycerol | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Griseofulvin | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| Isopropanol | VWR, Leuven, Belgium |
| Magnesium chloride | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| Magnesium chloride 25 µM | Axon, Kaiserslautern, Germany |
| Monochlorobimane | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| Na-K Tartrate | Sigma Aldrich Chemie GmbH, Steinheim, Germany |
| N-Acetyl-L-Cysteine | Carl Roth GmbH & Co. KG, Karlsruhe, Germany |
| N-(1-naphthyl)ethylenediamine dihydrochloride | Sigma Aldrich Chemie GmbH, Steinheim, Germany |

## 2.2 Methods

### 2.2.1 RNA Isolation

Cells were treated according to the individual experiments. Culture medium was removed and PeqGOLD TriFast (PeQlab, Erlangen, Germany) was added (50 µl/cm$^2$) to the cells. RNA isolation was carried out according to the Peqlab manufacturer's protocol (Fig.2.1) (https://www.peqlab.de/wcms/de/pdf/30-2010m.pdf [19.01.2016]). Then, the cell lysate was scraped off from the culture plate and transferred to an Eppendorf tube. The cell lysate was pipetted up and down several times for complete breakdown of the cell membrane. After 5 min incubation of TriFast cell lysates at RT chloroform was added to the lysate (200 µl/ml TriFast) and mixed vigorously for 15 sec. Then, lysates were centrifuged for 15 min at 12,000 g and +4°C. The aqueous phase was transferred into a fresh Eppendorf tube. Isopropanol was added to the aqueous phase (500 µl/ml TriFast) and mixed by inverting several times. The mixtures were incubated at –20°C for 1–2 hrs, then mixtures were centrifuged for 10 min at 12,000 g/+4°C. Supernatants were discarded and pellets were washed with 1 ml 70% ethanol and centrifuged for 10 min at 12,000g and +4°C. Supernatants were again removed carefully and pellets were air dried. Then, the pellets were resolved in RNase/DNase free water (0.1% DEPC) and incubated for 20 min. on ice. Following the final steps RNA samples were frozen at -80°C until further use.

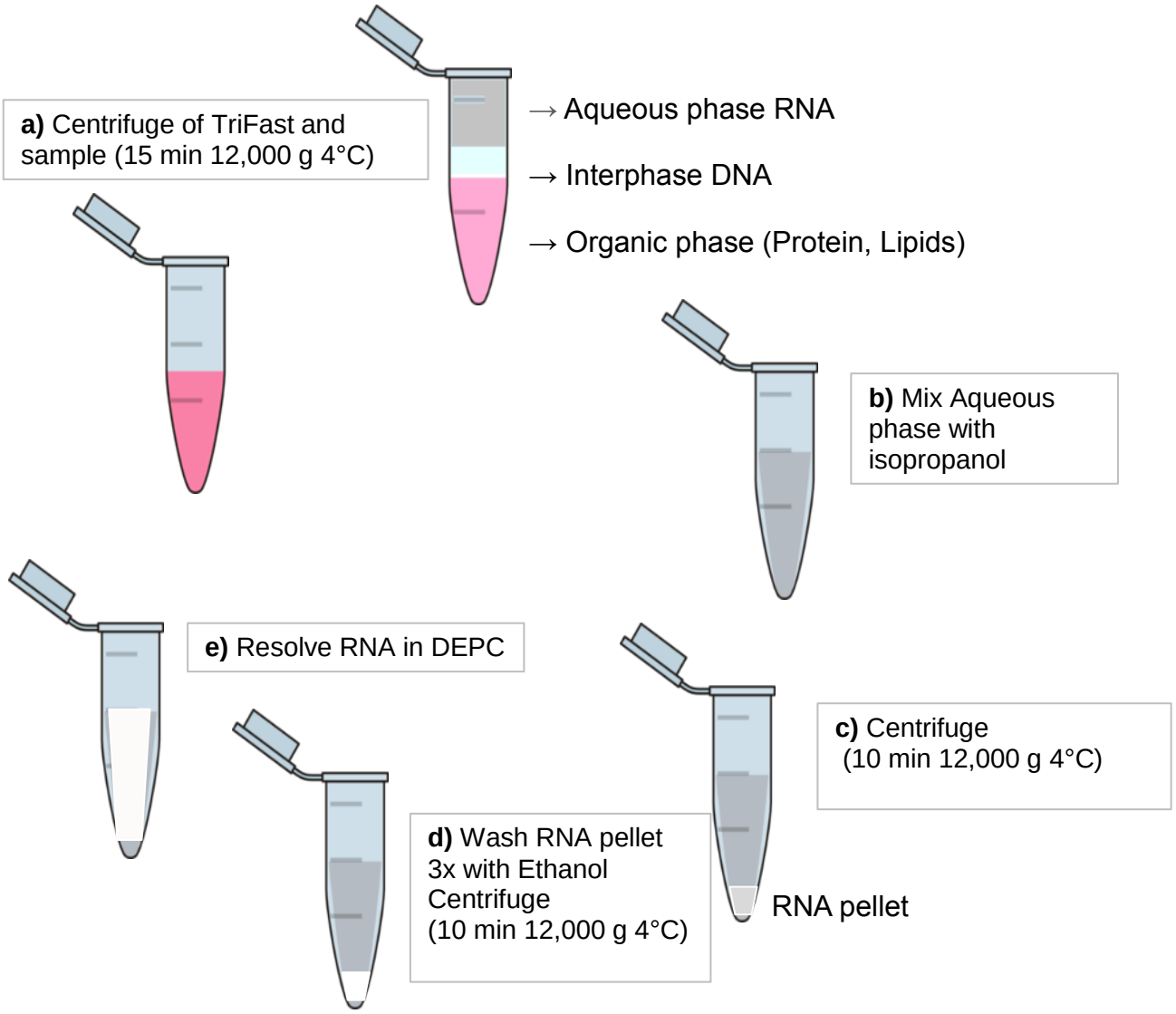



**Figure 2.1. RNA isolation procedure by Trifast**

This figure illustrates the RNA isolation step by using Trifast (from **a** to **e**)

### 2.2.1.1 Removal of genomic DNA from RNA preparations

DNase I is an endonuclease that digest single and double strand DNA. It hydrolyses phosphodiester bonds producing mono- and oligodeoxyribonucleotides with 5'-phosphate and 3'-OH groups. The enzyme activity is $Ca^{2+}$ dependent and is activated by $Mg^{2+}$ and $Mn^{2+}$ (https://www.thermofisher.com/order/catalog/product/EN0525

[19.01.2016]). RNA samples were treated with DNase 1 (Thermo Fisher scientific InC, Waltham, MA, USA) like Table (2.1) in RNase–free tube.

After 30 min incubation at 37°C, 1 µl of 50 mM EDTA was added and incubated for 10 min at 65°C. In this step RNA hydrolyses during heating with divalent cations in absence of chelating agent. The RNA integrity was checked again after removal of DNA.

**Table 2.1. Preparation of DNase I master mix.**

| **Components** | **Final Concentration** |
|---|---|
| **RNA** | 1-3 µg |
| **Dnase I** | 1µl /µg RNA |
| **10X Reaction buffer** | 1µl in 10 µl |
| **DEPC, treated water** | Up to 20 µL |

#### 2.2.1.2 RNA Quality Check (Quantification and Integrity check)

TriFast isolates the whole range of RNAs including mRNA and rRNA. The final total RNA should be free of DNA and proteins and has a 260/270 ratio of 1.6–2.0. RNA was quantified by using the LVis Plate with the omega plate reader at 230 nm, 260 nm and 280 nm (according to BMG Labtech protocol). After quantification, RNA integrity was checked by gel electrophoresis [Green & Sambrook, 2012]. 0.5 µg RNA was loaded onto an agarose gel (1.5% agarose in TBE) containing ethidiumbromide placed in TBE (90 mM TRIS, 90 mM boric acid and 2 mM EDTA). Gels ran at 90 V for 45 min and pictures of the UV induced signal were taken with an INTAS Gel Jet

Imager (INTAS Science Imaging GmbH; Göttingen; Germany). Intact RNA was showing two sharp bands representing the 18S and 28S ribosomal RNA. To investigate integrity of RNA; samples (0.5 µg/lane) were separated by electrophoresis in 1.5% gel (in TBE). Gel ran at 90 V for 45 min: Pictures of Gel was taken with GEL-Doc.

### 2.2.2 cDNA synthesis and RT-PCR

Complementary DNA (cDNA) was synthesized by Revert Aid First Strand cDNA Synthesis Kit according to manufacturer's instructor (Cat. Nr: K1612, Thermo Fisher scientific.Inc, Waltham, MA, USA). Then, reverse transcriptase PCR (RT-PCR) was performed. PCR is used to amplify a specific region of a DNA strand (the DNA target). All cDNA samples were diluted to a final concentration of 10 ng/µl. The expression of the housekeeping gene GAPDH was checked in all of cDNA samples. For PCR, KAPA Tag ready mix PCR Kit was used as a ready-to-use cocktail containing all components for PCR, except primers and template. PCR master mix was prepared according to manufacturer's instructor (Peqlab Biotechnology GmbH, MA, USA) (Table2.2).

**Table 2.2. Preparation of PCR master mix.**

| **Components** | **Final Concentration** |
|---|---|
| **2X KAPA Taq ReadyMIx (1.5 Mm $MgCl_2$ at 1X)** | 10 µL |
| **10 µM Forward Primer** | 0.5 -2.5 µL |
| **10 µM Reverse Primer** | 0.5 -2.5 µL |
| **DEPC, treated water** | Up to 20 µL |
| **Template cDNA** | As required |

After transferring the appropriate volume of PCR master mix, template and primer to individual PCR tubes, PCR was performed with the following cycle protocol: (Initialization step: 95 C, 5 min, Denaturation Step: 95°C, 30-40s, Annealing step: 52–65°C, 30–40, Elongation step: 72°C, 30–40 s, Final elongation: 72°C, 5 min. In Table 2.3, all used primers are listed with their respective size and annealing temperature. The primer annealing temperatures, number of cycles and the amount of used cDNA were optimized by testing various conditions. All generated PCR products were separated by electrophoresis gel stained with ethidium bromide. Gene expression was quantified by the ImageJ 1.45 software (National Institute of Health, Maryland, USA).

**Table 2.3. Primer sequences and optimized condition of each primer.**

| Gen | Forward / Reverse Sequences | Annealing Tm | Product Length (bp) | N of Cycle | cDNA (ng) |
|---|---|---|---|---|---|
| **DNMT1** | CCGTTTGGTACATCCCCTCC<br>CAGGTAGCCCTCCTCGGATA | 62°C | 378 | 35 | 40 |
| **DNMT3A** | GGCGAGAGCAGAGGACGA<br>ATAGATCCCGGTGTTGAGCC | 60°C | 157 | 35 | 40 |
| **DNMT3b** | AAGTCGAAGGTGCGTCGTG<br>AGGACACGGGGTTTTTCCTG | 60°C | 262 | 35 | 40 |
| **hCYP1A1** | TTCGTCCCCTTCACCATC<br>CTGAATTCCACCCGTTGC | 55°C | 302 | 35 | 40 |
| **hCYP1A2** | TCGACCCTTACAATCAGGTGG<br>GCAGGTAGCGAAGGATGGG | 60°C | 180 | 35 | 40<br>pHH 20 |
| **hCYP2C9** | CTGGATGAAGGTGGCAATTT<br>AGATGGATAATGCCCCAGAG | 59°C | 308 | 35 | 40<br>pHH 20 |
| **hCYP2E1** | GACTGTGGCCGACCTGTT<br>ACACGACTGTGCCCTGGG | 59°C | 296 | 35 | 40<br>pHH 20 |
| **hCYP3A4** | ATTCAGCAACAAGAACAAGGACA<br>TGGTGTTCTCAGGCACAGAT | 56°C | 314 | 35 | 40<br>pHH 20 |
| **hCYP3A7** | AATAAGGCACCACCCACCTA<br>AGAGCAAACCTCATGCCAAT | 58°C | 326 | 35 | 40<br>pHH 20 |
| **hUGT1A6** | CTGCCCGTATGTCCACCT<br>GCTCTGGCAGTTGATGAAGTA | 60°C | 210 | 35 | 40<br>pHH 20 |
| **hGSTA1** | TCTGCCCGTATGTCCACCT<br>GCTCCTCGACGTAGTAGAGAAG T | 59°C | 185 | 35 | 40<br>pHH 20 |

| | | | | | |
|---|---|---|---|---|---|
| **hAlbumin** | TTTATGCCCCGGAACTCCTTT<br>TGTTTGGCAGACGAAGCCTT | 60°C | 142 | 35 | 40 |
| **hOTC** | TGGCCCCCGCTGGCTAACTT<br>CACGGCCCTTCAGCTGCACTT | 62°C | 192 | 35 | 40 |
| **hARG** | TGAGAAAGGCTGGTCTGCTTGAGA<br>TGCCAAACTGTGGTCTCCGCC | 62°C | 245 | 35 | 40 |
| **hASL** | CCCTCAAGCGCAGTGCCCAG<br>CTGCACCCACAAACCGGCCA | 62°C | 182 | 35 | 40 |
| **hASS1** | ACGCCTCCAATCCCAGACGC<br>CCCTCCCGCTGGGCGATTTC | 62°C | 350 | 35 | 40 |
| **hNAGS** | TAACGTGAACCTGCCCGCCG<br>TAACGTGAACCTGCCCGCCG | 62°C | 224 | 35 | 40 |
| **hCPS1** | GGCCATCCATCCTCTGTTGC<br>GCTAAGTCCCAGTTCATCCA | 62°C | 171 | 40 | 40 |
| **hGAPDH** | GTCTCCAACATGCCTCTCTTCATCC<br>GTCAGTGGTGGACCTGACCT | 56°C | 420 | 35 | 20 |

### 2.2.3 Real Time PCR

For quantitative real time PCR (qRT-PCR), 40 ng of template cDNA was used for the expression level of each target gene (primer sequences are listed in Table 1) using SYBR Green qPCR Kit (Thermo Fisher scientific Inc., Waltham, MA, USA) and the Step One Plus® Real-Time PCR System (Thermo Fisher scientific, Inc., Waltham, MA, USA). Among different existing housekeeping genes (*e.g.*; GAPDH, ACTB, and B2M), B2M was used as an endogenous control because of the constant expression of this gene in hepatocellular carcinoma and normal tissue samples. In line with our results, Waxman *et al.* reported the dysregulation of common housekeeping genes such as GAPDH and ACTB in hepatocellular carcinoma [Waxman & Wurmbach,

2007], which may lead to wrong positive or wrong negative results. Relative expression values were calculated from Ct values using the $\Delta\Delta C_T$ method with untreated cells as a control. Fold induction was calculated according to the formula $2^{(Rt-Et)}/2^{(Rn-En)}$ [Saha *et al.*, 2001]. Real-time PCRs were performed as follows: denaturation for 10 minutes at 95°C, amplification with 40 cycles and 15 seconds at 95°C, 40 seconds at 60°C and 15 seconds at 72°C (Step One Plus™ Real-Time PCR System, Life technologies, CA, USA). The primer sequences are listed in Table.2.4. Each sample was set up in triplicates and the experiment was repeated at least twice.

**Table 2.4. The primer sequences used for Real time PCR.**

| Gene | Gene bank ID | Forward / Reverse Primer | Product length (bp) |
|---|---|---|---|
| **TET1** | NM_030625.2 | TCTGTTGTTGTGCCTCTGGA<br>GCCTTTAAAACTTTGGGCTTC | 77 |
| **TET2** | NM_001127208.2 | GAGACGCTGAGGAAATACGG<br>TGGTGCCATAAGAGTGGACA | 258 |
| **TET3** | NM_001287491.1 | CCCACAAGGACCAGCATAAC<br>CCATCTTGTACAGGGGGAGA | 129 |
| **IDH1** | NM-001282387.1 | TCCGTCACTTGGTGTGTAGG<br>GGCTTGTGAGTGGATGGGTA | 128 |
| **IDH2** | NM-002168.3 | TGAACTGCCAGATAATACGGG<br>CTGACAGCCCCCACCTC | 121 |
| **Snail** | NM_005985.3 | ACCACTATGCCGCGCTCTT<br>GGTCGTAGGGCTGCTGGAA | 115 |
| **E-cadherin** | NM_004360.3 | GTCAGTTCAGACTCCAGCCC<br>AAATTCACTCTGCCCAGGACG | 254 |
| **P21** | NM_001291549.1 | GTCACTGTCTTGTACCCTTGTG<br>CGGCGTTTGGAGTGGTAGAAA | 221 |
| **GADD45** | NM_015675.3 | GTGTACGAGTCGGCCAAGTT<br>GTCACAGCAGAAGGACTGG | 135 |
| **GAPDH** | NM_002046.4 | TGCACCACCACTGCTTAGC<br>GGCATGGACTGTGGTCATGAG | 87 |
| **B2M** | NM_004048.2 | AGATGAGTATGCCTGCCGTG<br>GCGGCATCTTCAAACCTCC | 105 |
| **HPRT** | NM_000194.2 | CCTGGCGTCGTGATTAGTGA<br>CGAGCAAGACGTTCAGTCCT | 137 |

### 2.2.4 Western Blot

#### 2.2.4.1 Collection of protein lysates

Cells were treated according to the individual experiments. Culture medium was removed and 20 µl/cm$^2$ RIPA lysis buffer (50 mM TRIS, 250 mM NaCl, 2% Nonidet P-40, 2.5 mM EDTA, 0.1% SDS, 0.5% DOC, complete protease inhibitors such as PMSF, Leupeptide, Aprotinin and 1.0% phosphatase inhibitor: NaF; Na3VO4, pH = 7.2) was added to the cells, which were scraped from the plate by rubber police-man and transferred to an Eppendorf tube and the lysate was vortexed. After incubation on ice for 20 min cell debris was centrifuged down (13,000 rpm; 10 min; +4°C).

#### 2.2.4.2 Protein measurement

Protein concentration was measured in triplicates. Concentrations were determined using Micro Lowry Assay method. The concentration of the protein is determined by a color change according to the protein concentration. This color change can then be measured with photometric techniques by OMEGA plate reader. Procedure was done according to the protocol provided by the manufacturer. Standard curves ranging from 0-10 µg/µl BSA (triplicate) were included to each measurement. ODs at λ = 690 nm were measured by OMEGA plate reader.

#### 2.2.4.3 SDS-PAGE and Immuno Blotting

SDS-PAGE was performed on gel made up for cross-linked acrylamide molecules am other components. Protein concentration was determined by micro-Lowry and 30-50 µg of total protein sample were prepared by denaturation of proteins via boiling with Lammli buffer containing, ß-Mercaptoethanol and Sodium Docdecyl Sulphate (SDS) for degradation, denaturation and providing negative charge of proteins, Bromphenol Blue as a tracking dye and Glycerol to minimize diffusion during loading.

30-50 µg of total protein was separated by SDS-PAGE and transferred to nitrocellulose membranes (Roth GmbH, Karlsruhe, Germany). Electrophoresis transfer was used to separate proteins according to their charge (Fig.2.2). After transfer the proteins on the blot, blots were stained with Ponceau S solution to control proper protein transfer. Then, membranes were blocked by 5% blocking buffer [milk powder in Tris buffered saline Tween (TBST)] for 1 hour and incubated overnight with TETs, Snail, GADD45α, P21, cyclin B1, E-cadherin and GAPDH mouse/rabbit polyclonal primary antibodies at 4°C. The following day, membranes were incubated with the corresponding HRP-labeled secondary antibodies for 1 hour at RT. Chemiluminescent signals were detected with the ChemoCam (INTAS Science Imaging GmbH, Göttingen, Germany) (Fig.2.2) [Mahmood & Yang, 2012].

The Antibodies details and correlated blocking buffers as well as western blot

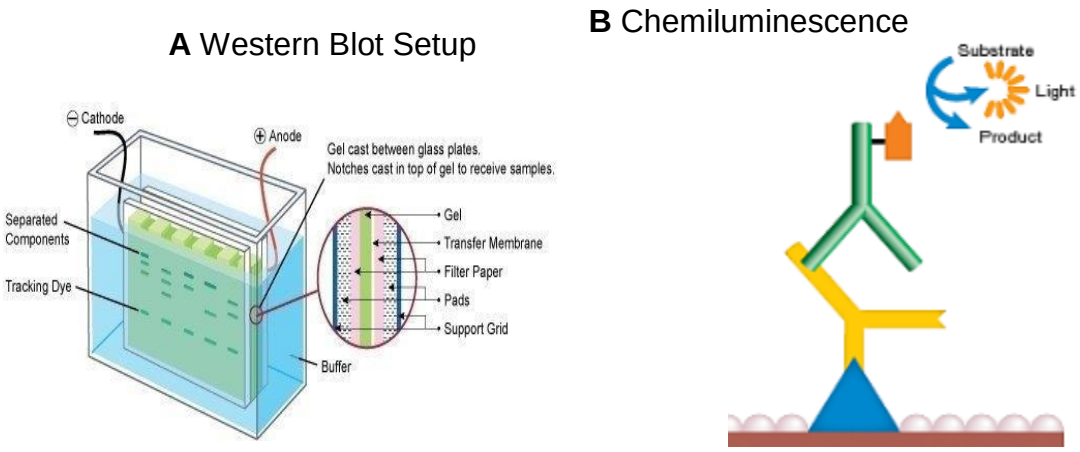



**Figure 2.2. Western Blot set up and protein detection method.**

A) Illustration of Western Blot setup. Transfer sandwich is in the following order: Supported Grid (Blue), Soaked Sheets Pads, filtered pad [Whatmann Paper] (Pink), Transfer Membrane (yellow) and Gel (Green) B) Protein detection based on chemiluminescence is the most sensitive detection methods .when the chemiluminescent substrate combined with the enzyme, produces light as a byproduct, which can be captured using CCD camera.

(*www.antibodiesonline.com*, *www.bio-rad.com* [17.01.2016]).

condition are provided in Table.2.5.

**Table 2.5. Antibodies details and condition for Western blot.**

| Antibody | Company and catalogue No | M.Wt. (K.Da) | Host | Blocking buffer | Primary Ab (Dilution) | Secondary Ab (Dilution) |
|---|---|---|---|---|---|---|
| **E Cad** | Cell signaling 14472 | 135 | mouse | 5% Milk in 0.1% TBST | 1 in 1000 | 1 in 2000 |
| **Vimentin** | Cell signaling 5741 | 57 | rabbit | 5% Milk in 0.1% TBST | 1 in 1000 | 1 in 2000 |
| **Snail** | Cell signaling 3879 | 30 | rabbit | 5% Milk in 0.1% TBST | 1 in 1000 | 1 in 2000 |
| **GADD45 beta** | Santa Cruz SC-33172 | 18-27 | rabbit | 5% Milk in 0.1% TBST | 1 in 200 | 1 in 2000 |
| **N Cad** | Cell signaling 4061 | 140 | rabbit | 5% Milk in 0.1% TBST | 1 in 1000 | 1 in 2000 |
| **Cyclin B1** | Cell signaling 4138 | 55 | rabbit | 5% Milk in 0.1% TBST | 1 in 1000 | 1 in 2000 |
| **P21 WAF1/Cip** | Cell signaling 2947 | 21 | rabbit | 5% Milk in 0.1% TBST | 1 in 1000 | 1 in 2000 |
| **P15/16** | Santa Cruz sc-377412 | 16 | mouse | 5% Milk in 0.1% TBST | 1 in 200 | 1 in 2000 |
| **Tet-2** | Sigma (SAB3500711) | 220 | rabbit | 5% Milk in 0.1% TBST | 1 in 1000 | 1 in 2000 |
| **Tet-3** | Sigma (SAB2700682) | 140 | rabbit | 5% Milk in 0.1% TBST | 1 in 500 | 1 in 2000 |
| **GADD45 alfa** | Cell signaling 4632 | 22 | rabbit | 5% Milk in 0.1% TBST | 1 in 1000 | 1 in 1000 |
| **Caspase3** | Cell signaling 9662 | 17, 19, 35 | rabbit | 5% Milk in 0.1% TBST | 1 in 1000 | 1 in 2000 |
| **cleaved PARP** | Cell signaling 5625 | 89 | rabbit | 5% Milk in 0.1% TBST | 1 in 1000 | 1 in 2000 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **PARP** | Cell signaling 9542 | 89, 116 | rabbit | 5% Milk in 0.1% TBST | 1 in 1000 | 1 in 2000 |
| **GAPDH** | Sigma | 36 | rabbit | 5% Milk in 0.1% TBST | 1 in 5000 | 1 in 2000 |
| **Beta actin** | Cell signaling 4970 | 47 | rabbit | 5% Milk in 0.1% TBST | 1 in 1000 | 1 in 2000 |

### 2.2.5 Immunofluorescence staining

Immunostaining of 5hmC and PCNA was performed using a recent published method [Szulwach *et al.*, 2011]. Briefly, cells were plated onto cover slips and treated according to the experimental setup. After 48 hrs, cells were fixed with 4% paraformaldehyde solution for 15 min at RT and then washed with PBS. For permeabilization, cells were incubated with 0.5% Triton-X-100 solved in PBS for 15 min at RT. To denature the DNA only for 5hmC staining, cells were incubated with 4M HCl for 15 min at RT, rinsed with distilled water and placed in 100 mM Tris-HCL (pH=8.5) for 10 min. Cells were rinsed with PBS, then nonspecific binding sites were blocked with blocking buffer (10% FCS, 0.1% Tween-20 in PBS) for 1 hour at RT. Then, cells were incubated with primary antibodies such as anti-5hmC rabbit polyclonal IgG (Active Motif, Inc., CA, USA) or anti-PCNA Rabbit mAB (Abcam plc, Cambridge, UK) at 1:1000 and 1:200, respectively in PBS solution containing 1% FCS and 0.1% Tween-20 overnight at 4°C. After washing with PBS, cells were incubated with secondary antibody solution (ALEXA-Fluor antibodies, Invitrogen. Inc, NY, USA), diluted 1:400 in PBS solution containing 1% FCS, 0.1% Tween-20 for 1 hour at RT. Nuclei were counterstained by incubation with Hoechst 33342 solution (2 µg/ml in PBS) for 10 min at RT. After a final washing step with PBS, the stained cells were mounted with Fluoromount G (Southern Biotech. Inc, NJ, USA). Images of the staining were taken with an EVOS fluorescence microscope (AMG, Life technologies,

Inc., MA, USA), and analyzed with Image J 1.45s software (NIH, USA) [Polzer *et al.*, 2010].

### 2.2.6 Immunohistochemical staining

Immunohistochemical stains were performed using 3.5 µm sections of TMA. Slides were deparaffinized, dehydrated by rising the sections in Roticlear for 10 min following by rinsing them in Ethanol (100% - 90% - 80% - 50%), and after antigen retrieval through rinsing and heating the whole slides in sodium citrate buffer (pH:6) in microwave with full energy for 5 minutes, the slides were placed in 2N HCL for DNA denaturation step for 5hmC/5mC staining for 60 min, rinsed with distilled water, and placed in 100 Mm Tris-HCL (pH:8.5) for 10 min. Tissue slides were incubated in DAKO Dual Endogenous Enzyme Block (DAKO GmbH, CA, USA) for 10 min. The sections were blocked in PBS solution containing 10% FCS and 0.1% Tween-20 for 1 hour. Section was incubated with either rabbit anti-5hmC at 1:1000 (Active motif, 39769) or mouse anti-5mC at 1:500 (Active motif, 39649) overnight at 4°C and then incubated with ZytoChem Plus HRP One-Step Polymer anti-Mouse/Rabbit/Rat (Cat No: ZUC053-006) from Zytomed (Zytomed System GmbH, Berlin, Germany) for 30 min at RT (100 µl per section). Tissue sections were washed and subsequently DAB Substrate Kit High Contrast (Cat: No: DAB 500 Plus) from Zytomed (Zytomed System GmbH, Berlin, Germany) was used according to the manufacturer's instruction. After rinsing, the cells were counterstained using a hematoxylin (Carl Roth GmbH, Karlsruhe, Germany) for 5 min and immediately rinsed with tap-water. Samples were air-dried and mounted with Rotimount and sections were controlled under bright field microscope.

All specimens were evaluated according to the 0-4 grading criteria (based on the percentage of 5hmC positive cells) and 0-3 grading criteria (based on the staining

intensity) [Liu *et al.*, 2014b]. The intensity was scored from 0 to 3 and defined as follows: 0: no staining, 1: weak staining, 2: moderate staining, 3: strong staining based on the staining score. In addition, the quick score (Q score) based on estimating the percentage (P) of tumor cells showing characteristic staining (0: Negative, 1: 10-25%, 2: 25-50%, 3: 50-75% and 4: > 75%) and by estimating the intensity (I) of staining was adopted for IHC scoring. The slides were scored by multiplying the percentage of positive cells by the intensity (Q = P ×I, maximum = 12). The total score was determined from 0 to 12 (http://www.ihcworld.com/ihc-scoring.htm [03.03.2016]). An overview of the IHC for all tissue sections was performed by pathologists (Prof. Dr. Sipos). Two observers evaluated the staining results independently and differences in interpretation were resolved by consensus. The statistical analysis was performed using the SAS software packages (SAS for windows, version 8.0)

### 2.2.7 Tissue samples and primary human hepatocyte isolation and cell culture condition

Tissue specimens were obtained from patients undergoing resection of HCC according to the approval of local ethics committee. A tissue microarray (TMA) contained section of HCC samples and their corresponding noncancerous liver tissue.

Primary human hepatocytes were isolated from human liver tissue according to the institutional guidelines from liver resections of tumor patients with primary or secondary liver tumor (Ethics approval number: 368/2012BO2). The isolation and purification of primary human hepatocytes was performed as previously described [Nussler *et al.*, 2009]. Culture conditions of HCC cell lines (Huh7, HLE and HLF) and human primary hepatocytes (PHH) were published previously [Dzieran *et al.*, 2013;

Lin *et al*., 2012; Nussler *et al.*, 2009]. HLE and HLF cells were purchased from ATCC and Huh7 was purchased from JCRB (Japanese Collection of Research Bioresources Cell Bank). The HCC cell as well as PHHs were plated onto collagen coated 6, 24, or 96 well tissue culture plates, treated for 24 hrs with different concentrations of 5-AZA (20, 10, 5, 1) for 48 hrs. Huh7, HLE and HLF cells were cultured in DMEM (Lonza Group Ltd, Cologne, Germany) supplemented with 2 mM glutamine (PAA Laboratories GmbH, Pasching, Austria) and 10% FCS (Invitrogen GmbH, Darmstadt, Germany) at 37°C and 5% $CO^2$ in a humidified incubator. Experiments were conducted between passage numbers 2 and 12 without full confluency throughout all experiments. Absence of mycoplasma contamination was regularly controlled using a Venor®GeMtest (Minerva Biolabs GmbH, Berlin, Germany).

### 2.2.8 Cell Viability and Cytotoxicity assay

#### 2.2.8.1 Resazurin Conversion

Resazurin conversion is a fluorescence assay which can be used to measure proliferation, viability and toxicity (indirectly) in cells. The assay is based on the ability of living cells to convert a redox dye (Resazurin) into an end product (resorufin). This conversion involves a colorimetric change which can be measured with fluorescence-based measuring instruments [Nakayama *et al.*, 1997; Nociari *et al.*, 1998]. Fluorescence was monitored at an excitation wavelength of 530-560 nm and an emission wavelength of 590 nm. The culture mediums were removed from the cells and washed twice with DPBS. Then the cells were incubated with 1:10 Resazurin stock solution in plain medium in an incubator at 37°C, 5% $CO_2$ for 2 hrs. Wells without cells were used as background subtraction from wells with cells. The

fluorescence was measured at an excitation wavelength of λ = 544 nm and at an emission wavelength of λ = 590-610 nm. The average background was subtracted from the florescence intensity of resorufin in cells.

#### 2.2.8.2 LDH leakage

Lactose dehydrogenase (LDH) is a cytosolic enzyme present in many different cell types. Plasma membrane damage releases LDH into the cell culture media. LDH catalyzes the conversion of lactate to pyruvate via $NAD^+$ reduction to NADPH. Diaphorase uses NADPH to reduce tetrazolium salt (INT) to red formazan production which can be measured at 490 nm. The level of formazan is the indicative of LDH release in medium [Decker & Lohmann-Matthes, 1988].

LDH released into cell culture media was measured using a Pierce LDH assay Kit (Cat.No: 88953, Thermo Fischer Scientific, Inc., Rockford, USA) according to the manufacturer's protocol. Briefly, cultured cells are incubated with chemical component (5-AZA and Vitamin C). 50 µl of supernatant transferred into a new plate then mixed with 50 µl of reaction mixture. After 30 min incubation at room temperature, reactions are stopped by adding a stop solution. Absorbance at 490 nm and 680 nm is measured using plate-reading spectrophotometer to determine LDH activity. Water treated cells are referred as Spontaneous LDH Activity Controls and 10X Lysis Buffer-treated cells were referred to as Maximum LDH Activity Controls.

To evaluate LDH activity, the absorbance value of 680 nm (background signal from instrument) was substrate from the 490 nm absorbance [(LDH at 490 nm) – (LDH at 680 nm)].

Percentage of Cytotoxicity was measured as following:

$$\%\ Cytotoxicity = \frac{Compound\ treated\ LDH\ activity - Spontaneous\ LDH\ activity}{Maximum\ LDH\ activity - Spontaneous\ LDH\ activity} \times 100$$

#### 2.2.8.3 Flow cytometry based apoptosis and cell cycle detection

Propidium iodide (PI) flow cytometric assay has been used for the evaluation of apoptosis. It is based on the principal that apoptotic cells are characterized by DNA fragmentation and loss of nuclear DNA content [Riccardi & Nicoletti, 2006]. PI as a fluorochrome binds to DNA and provides a rapid and precise evaluation of cellular DNA content and identification of hypodiploid cells by flow cytometric analysis. Indeed, DNA analysis is the second important application of flow cytometry. PI has a red fluorescence and can be excited at the 488 nm which was determined by flow cytometry. PI fluoresces strongly when bounds to DNA, due to the hydrophobic nature of the environment. It is possible to identify the proportion of cells that are in one of the three interphase stage of the cell-cycle by using PI and measuring their relative DNA content by flow cytometry. Cells that are in the G0/G1 phase (before DNA synthesis) have a defined amount (2x) of DNA. During S phase (DNA synthesis), cells contain between 2x and 4x DNA levels. When cells enter G2 or M phases (G2/M), they consist of 4x amount of DNA. (Fig.2.3) [Riccardi & Nicoletti, 2006]. For flow cytometry analysis, $1*10^{6}$ HCC cells per well were seeded in 6-well plates and treated with different concentrations of 5-AZA and Vitamin C. After 48 hrs, cells were fixed in 70% of ethanol for at least 1 hour and stained in a hypotonic solution with 100 μg/ml of RNase and 50 μg/ml Propidium Iodide (PI) for 15 min in RT [Riccardi & Nicoletti, 2006]. Distribution of cells in the different phases of the cell cycle based on the differences in DNA contents was determined by using flow cytometer (BD Biosciences. Inc, Heidelberg, Germany). Data were analyzed using Modfit software (ModFit LT, Verity Software House, Topsham, USA).

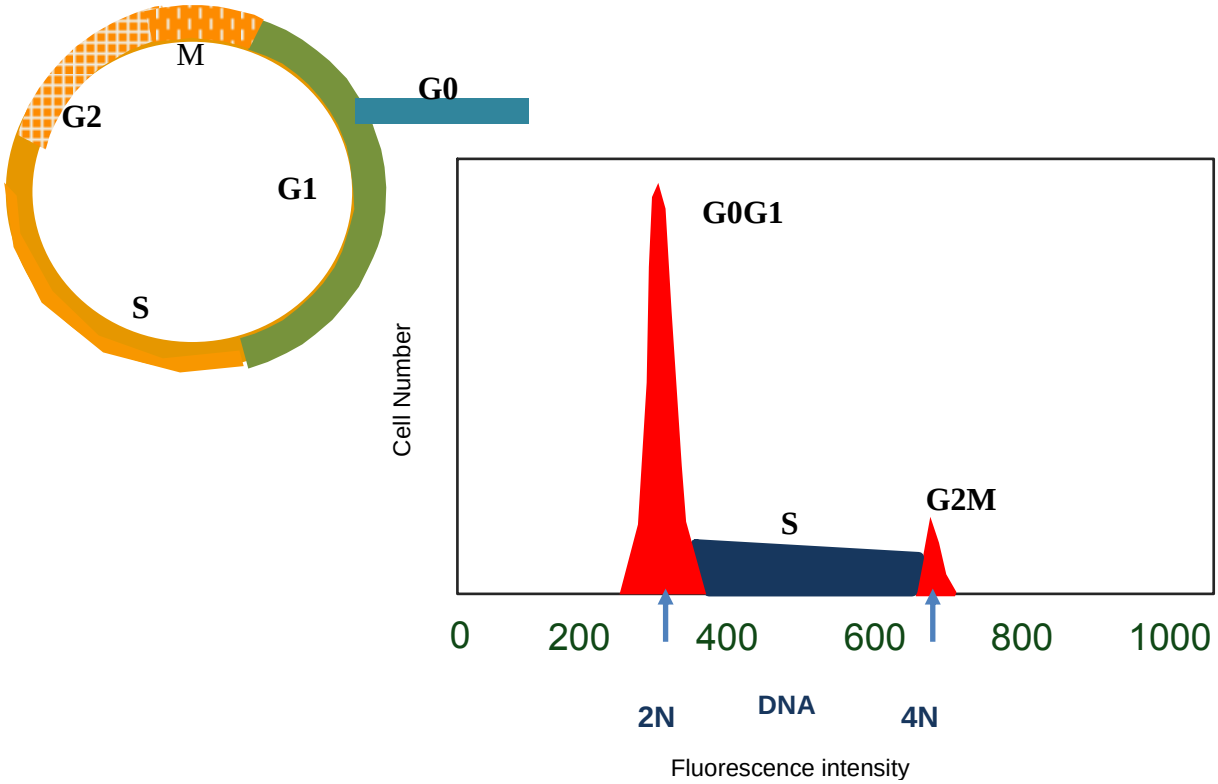



**Figure 2.3. Cell cycle analysis with PI staining by flow cytometry.**

This figure showed the relationship between the cell cycle and the DNA histogram. The histogram presents in respect to PI intensity and cell number. This is the typical result for untreated healthy cells: most cells are in G1 stage (2N), some cells undergoing DNA synthesis (2N–4N) and final population in G2/M stage (4N).

### 2.2.9 Protein depletion by siRNAs

siRNA transfection was carried out based on Thermo Fisher scientific siRNA protocol (www.thermofisher.com/de/de/home/references/protocols/rnai-epigenetics-and-gene-regulation/sirna-protocol.html). For depletion of TET2 and TET3, the siRNAs were ordered from Eurofins MWG Operon (Ebersberg, Germany) and the scrambled siRNA for negative control experiments was also obtained from Eurofins. The siRNA was transfected with lipofectamine 3000 according to manufacturer's instructor (Invitrogen Inc, Carlsbad, CA, USA). One of the most established method for transfection is lipofection in which cationic lipids are used to bring nucleic acid into cells [Dalby *et al.*, 2004]. For lipofection, cationic liposomes are used which can

easily interact with DNA. These positively charged liposomes interact with negatively charged nucleic acids (siRNA) and form stable complexes, so called lipoplexes. Then lipoplexes interact with the membrane of the cells and the siRNAs penetrate in to cells by fusion of lipoplexes cells followed by repression of the mRNA of specific gene and respective protein (Fig.2.4).

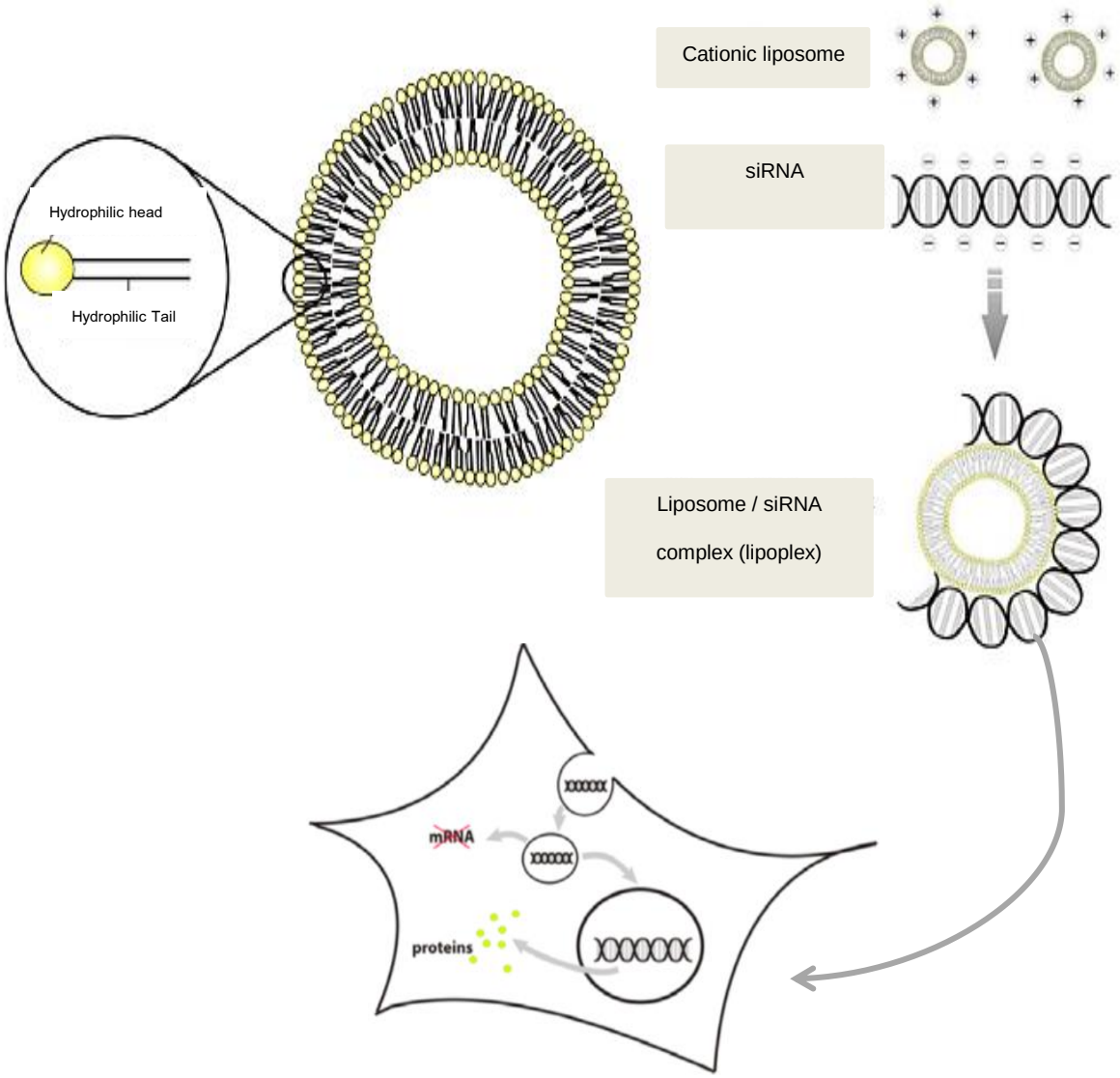



**Figure 2.4. Transfection of the cell with lipofectamine.**

This figure shows the transfection procedure with lipofectamine. The positively charged liposome binds to siRNA and formed lipolexes which perfused in to cells membrane and inhibits mRNA of specific gene expression (www.genaxxon.de)

The transfection reagents, transfected cell lines and many other parameters can affect the efficiency of the transfection. Therefore, the efficiency of transfection was measured by flow cytometry. Transfection efficiency was controlled by seeding $8 \times 10^4$ cells in 12 well plates. Cell density was 60%-70% confluent at time of transfection. Then cells were transfected with lipofectamine 3000 labeled FITC in DMEM medium without serum and antibiotics according to the following table:

**Table 2.6. Amount of components for lipofection assay.**

| **Reagent** | **Volume (12 well plate)** |
|---|---|
| **Lipofectamine 3000** | 6 µl |
| **FITC SiRNA** | 9 µl |
| **DMEM medium** | 135 µl |
| **Final Lipoplex Volume** | 150 µl |

To see the real transfection efficiency, a mock control is included, which means that the same amount of the transfection reagent without fluorescence tag is applied to cells. After 24 hrs the viability and morphology of cells were checked and compared with the control group (cells without lipofection reagents) by microscopy. Then, cells were trypsinized and washed with PBS and prepared for flow cytometry to evaluate the GFP intensity in transfected cells. The efficiency of transfection with lipofectamine 3000 was approximately 80%-90% for each siRNAs in the HCC cell lines (Fig 2.5).

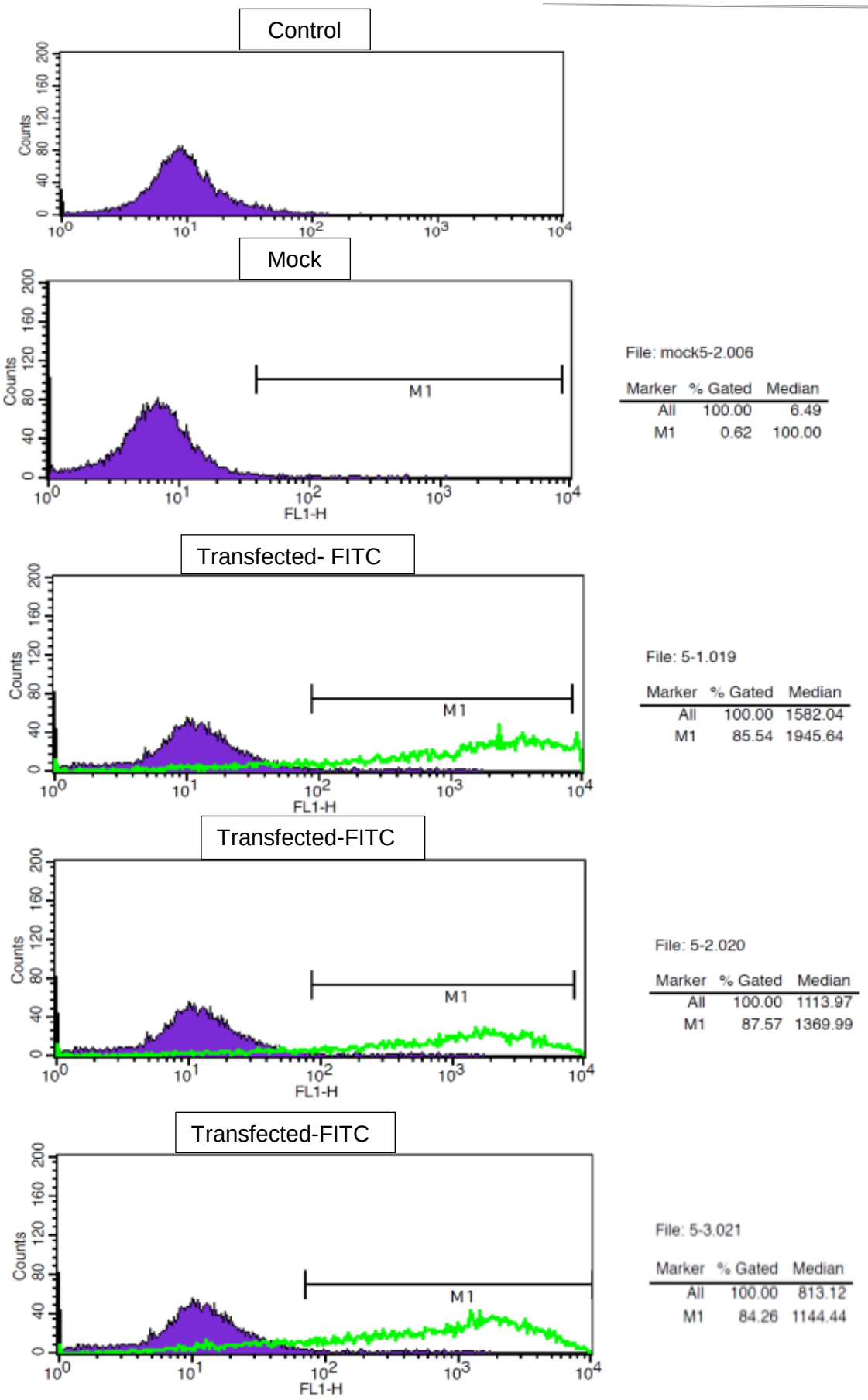



**Figure 2.5. The efficiency of transfection was tested with FITC-siRNA by flow cytometry.**

To knock down the expression of TET2 and TET3, HCC cells were seeded in 6 well plates at the density of $2 \times 10^5$ in 2 ml of DMEM medium. siRNAs were transfected with lipofectamine reagent according to the manufacturer's instruction. The final siRNAs concentration was 50 nM. Cells were transfected and then were stimulated with 5-AZA. After 48 hrs of incubation with 5-AZA, untreated cells and treated cells were harvested for RNA and protein isolation. The scrambled /nonsense siRNA was used as a negative control which indicates unintended off-targeting. The siRNA sequences are listed in Table 2.7.

**Table 2.7. The siRNA sequences for transfection.**

| Gene | Name | Target Sequence | 5' or 3' Mod |
|---|---|---|---|
| **Control** | Scrambled siRNA | 5'AACAGTCGCGTTTGCGACTGG3' | 3' FLU |
| **TET2** | TET2-siRNA | 5' CAAGACCAATGTCAGAA 3' | 3' FLU |
| **TET3** | TET3-siRNA | 5' GATGAAGGTCCATATTA 3' | 5' CY3 |

### 2.2.10 PathScan Stress and Apoptosis signaling antibody array kit (Fluorescent Readout)

The PathScan stress and apoptosis signaling antibody kit uses glass slide as the planer surface and is based upon the sandwich immunoassay principle (Cat Nr: 7323, Cell Signaling Technology, Inc., MA, USA). The array kit includes 19 signaling molecules related to stress response or apoptosis (Table 2.6.), cell lysates were prepared by using Cell Lysis Buffer (0.5 ml per 10 cm diameter) according to Pathscan protocol (http://media.cellsignal.com/pdf/7323.pdf [29.02.2016]). Lysates

were diluted to 0.5 mg/ml immediately before starting the assay. The multi-well gasket affixed to the glass slide, then 100 µl of blocking buffer was added to each well and then were incubated in RT in 15 min. After removing blocking buffer, 75 µl of diluted lysate was added to each well and incubated for 2 hrs in room temperature. Wells were washed with array washing buffer and then detection antibody cocktail was added to each well and incubated for 1 hour on orbital shaker. After washing, 75 µl 1X DyLight 680-linked Streptavidin was added to each well and incubated for 30 min and wells were washed for 4 times. Slides were removed from plastic dish and images were captured by a digital imaging system capable of exciting at 680 nm and detecting at 700 nm (INTAS Science Imaging GmbH, Göttingen, Germany). Spot intensity was quantified with Image J 1.45s software (NIH, USA). Tubulin considered as an endogenous reference. List of genes involved in the array are listed in Table 2.8.

**Table 2.8. List of Stress and Apoptosis genes involved in the array.**

| **Target** | **Site** |
|---|---|
| Positive Control | |
| Negative Control | |
| P44/42MAPK (ERK1/2) | Thr202/ Tyr204 |
| AKT | Ser437 |
| Bad | Ser136 |

| | |
|---|---|
| HSP27 | Ser82 |
| Smad2 | Ser456/467 |
| P53 | Ser15 |
| P38 MAPK | Thr180/Tyr182 |
| SAPK/JNK | Thr183/Tyr185 |
| PARP | ASP214 |
| Caspase-3 | ASP175 |
| Caspase-7 | ASP198 |
| IkB | Total |
| CHK1 | Ser345 |
| CHK2 | Thr68 |
| IkBa | Ser32/36 |
| eIF2 | Ser51 |
| TAK | Ser412 |
| Survivin | Total |
| Tubulin | Total |

### 2.2.11 Chromatin modification array

The Human Epigenetic Chromatin Modification Enzymes RT² Profiler™ PCR Array (Cat. No: 33231 PAHS-085ZA, QIAGEN, MD, USA) profiles the expression of 84 key genes encoding enzymes which affect genomic DNA and histones status to regulate chromatin accessibility and therefore gene expression. Genes which provided by the array exhibit different expression profiles in tumor cells relative to normal cells, suggesting a role for chromatin modification and remodeling in oncogenesis. Following genes are included in the array:

**DNA Methyltransferases:** DNMT1, DNMT3A, DNMT3B.

**Histone Acetyltransferase:** ATF2, CDYL, CIITA, CSRP2BP, ESCO1, ESCO2, HAT1, KAT2A (GCN5L2), KAT2B (PCAF), KAT5 (HTATIP), KAT8, KAT7, KAT6A, KAT6B, NCOA1, NCOA3, NCOA6.

**Histone Methyltransferases:** CARM1 (PRMT4), DOT1L, EHMT2, MLL, MLL3, PRMT1, PRMT2, PRMT3, PRMT5, PRMT6, PRMT7, PRMT8, SETDB2, SMYD3, SUV39H1.

**SET Domain Proteins (Histone Methyltransferases Activity):** ASH1L, MLL3, MLL5, NSD1, SETD1A, SETD1B, SETD2, SETD3, SETD4, SETD5, SETD6, SETD7, SETD8, SETDB1, SUV39H1, SUV420H1, WHSC1.

**Histone Phosphorylation:** AURKA, AURKB, AURKC, NEK6, PAK1, RPS6KA3, RPS6KA5.

**Histone Ubiquitination:** DZIP3, MYSM1, RNF2, RNF20, UBE2A, UBE2B, USP16, USP21, USP22.

**DNA / Histone Demethylases:** KDM1A, KDM5B, KDM5C, KDM4A, KDM4C, KDM6B, MBD2.

**Histone Deacetylases:** HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, HDAC8, HDAC9, HDAC10, HDAC11.

To distinguish the variation of chromatin modification among HCC cell lines and PHH, chromatin modification array was used. Four common HCC cell lines including; Huh7, HepG2, HLE, AKN1 and primary human hepatocytes (PHH) were investigated. RNA

of 5 different donors of PHH and 5 different passage numbers of HCC cell lines were isolated. The quality of the RNA was checked and quantified. The equal amounts of 5 µg of each cell types RNA were mixed together. The pooled RNA was purified with Rneasy Lipid Tissue Mini Kit according to manufacturer's instruction (QIAGEN, MD, USA).

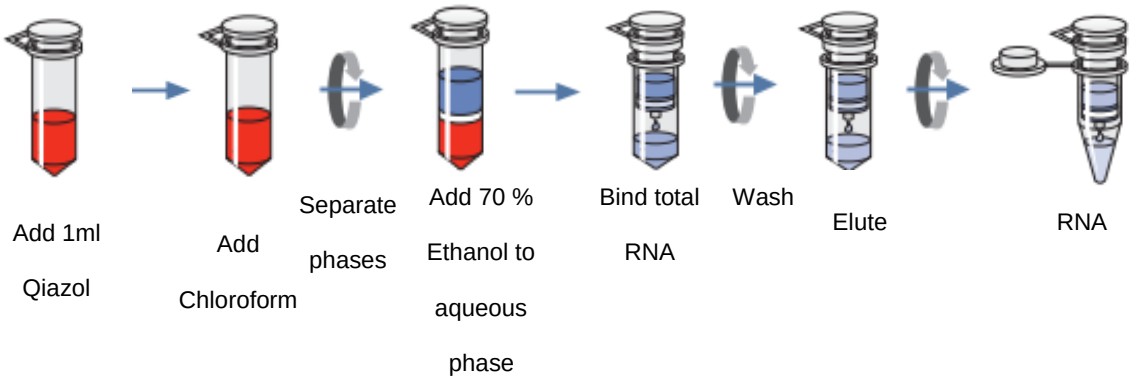



**Figure 2.6. Schematic of RNA purification.**
This figure shows the RNA purification steps by using Rneasy Lipid Tissue Mini Kit

After purification step the RNA amount was quantified and the integrity of RNA was checked. $RT^2$ First strand synthesis kit was used in order to eliminate Genomic DNA contamination and reverse transcription which was performed according to manufacturer's protocol (QIAGEN, MD, USA). Then the expression of GAPDH was tested in all samples. Then $RT^2$ SYBR Green mastermixes (QIAGEN, MD, USA) was used for performing real-time PCR for $RT^2$ PCR array according to manufacturer's protocol.

Real-time PCRs were performed as follows: denaturation for 10 min at 95°C, amplification with 40 cycles and 15 seconds at 95°C, 1 min at 60°C and 15 seconds at 72°C (Step One Plus™ Real -Time PCR System, Life technologies, CA, USA).

The $RT^2$ profiler PCR array genes abbreviations and details are included in Table 2.9.

**Table 2.9 . Gene information included in RT² Profiler PCR array.**

| N | Uni-gene | Refseq | Symbol | Description |
|---|---|---|---|---|
| A01 | Hs.491060 | NM_018489 | ASH1L | Ash1 (absent, small, or homeotic)-like (Drosophila) |
| A02 | Hs.592510 | NM_001880 | ATF2 | Activating transcription factor 2 |
| A03 | Hs.250822 | NM_003600 | AURKA | Aurora kinase A |
| A04 | Hs.442658 | NM_004217 | AURKB | Aurora kinase B |
| A05 | Hs.98338 | NM_003160 | AURKC | Aurora kinase C |
| A06 | Hs.720136 | NM_199141 | CARM1 | Coactivator-associated arginine methyltransferase 1 |
| A07 | Hs.269092 | NM_004824 | CDYL | Chromodomain protein, Y-like |
| A08 | Hs.701991 | NM_000246 | CIITA | Class II, major histocompatibility complex, transactivator |
| A09 | Hs.488051 | NM_020536 | CSRP2BP | CSRP2 binding protein |
| A10 | Hs.202672 | NM_001379 | DNMT1 | DNA (cytosine-5-)-methyltransferase 1 |
| A11 | Hs.515840 | NM_022552 | DNMT3A | DNA (cytosine-5-)-methyltransferase 3 alpha |
| A12 | Hs.713611 | NM_006892 | DNMT3B | DNA (cytosine-5-)-methyltransferase 3 beta |

| | | | | |
|---|---|---|---|---|
| B01 | Hs.713641 | NM_032482 | DOT1L | DOT1-like, histone H3 methyltransferase (S. cerevisiae) |
| B02 | Hs.409210 | NM_014648 | DZIP3 | DAZ interacting protein 3, zinc finger |
| B03 | Hs.709218 | NM_006709 | EHMT2 | Euchromatic histone-lysine N-methyltransferase 2 |
| B04 | Hs.464733 | NM_052911 | ESCO1 | Establishment of cohesion 1 homolog 1 (S. cerevisiae) |
| B05 | Hs.99480 | NM_001017420 | ESCO2 | Establishment of cohesion 1 homolog 2 (S. cerevisiae) |
| B06 | Hs.632532 | NM_003642 | HAT1 | Histone acetyltransferase 1 |
| B07 | Hs.88556 | NM_004964 | HDAC1 | Histone deacetylase 1 |
| B08 | Hs.26593 | NM_032019 | HDAC10 | Histone deacetylase 10 |
| B09 | Hs.744132 | NM_024827 | HDAC11 | Histone deacetylase 11 |
| B10 | Hs.3352 | NM_001527 | HDAC2 | Histone deacetylase 2 |
| B11 | Hs.519632 | NM_003883 | HDAC3 | Histone deacetylase 3 |
| B12 | Hs.20516 | NM_006037 | HDAC4 | Histone deacetylase 4 |
| C01 | Hs.438782 | NM_005474 | HDAC5 | Histone deacetylase 5 |
| C02 | Hs.6764 | NM_006044 | HDAC6 | Histone deacetylase 6 |
| C03 | Hs.200063 | NM_001098416 | HDAC7 | Histone deacetylase 7 |
| C04 | Hs.310536 | NM_018486 | HDAC8 | Histone deacetylase 8 |

| | | | | |
|---|---|---|---|---|
| C05 | Hs.196054 | NM_178425 | HDAC9 | Histone deacetylase 9 |
| C06 | Hs.463045 | NM_021078 | KAT2A | K(lysine) acetyltransferase 2A |
| C07 | Hs.533055 | NM_003884 | KAT2B | K(lysine) acetyltransferase 2B |
| C08 | Hs.397010 | NM_006388 | KAT5 | K(lysine) acetyltransferase 5 |
| C09 | Hs.491577 | NM_006766 | KAT6A | K(lysine) acetyltransferase 6A |
| C10 | Hs.599543 | NM_012330 | KAT6B | K(lysine) acetyltransferase 6B |
| C11 | Hs.21907 | NM_007067 | KAT7 | K(lysine) acetyltransferase 7 |
| C12 | Hs.533803 | NM_032188 | KAT8 | K(lysine) acetyltransferase 8 |
| D01 | Hs.591518 | NM_015013 | KDM1A | Lysine (K)-specific demethylase 1A |
| D02 | Hs.155983 | NM_014663 | KDM4A | Lysine (K)-specific demethylase 4A |
| D03 | Hs.709425 | NM_015061 | KDM4C | Lysine (K)-specific demethylase 4C |
| D04 | Hs.443650 | NM_006618 | KDM5B | Lysine (K)-specific demethylase 5B |
| D05 | Hs.631768 | NM_004187 | KDM5C | Lysine (K)-specific demethylase 5C |
| D06 | Hs.223678 | NM_001080424 | KDM6B | Lysine (K)-specific demethylase 6B |
| D07 | Hs.25674 | NM_003927 | MBD2 | Methyl-CpG binding domain protein 2 |
| D08 | Hs.258855 | NM_005933 | KMT2A | Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila) |

| | | | | |
|---|---|---|---|---|
| D09 | Hs.647120 | NM_170606 | KMT2C | Myeloid/lymphoid or mixed-lineage leukemia 3 |
| D10 | Hs.592262 | NM_182931 | KMT2E | Myeloid/lymphoid or mixed-lineage leukemia 5 (trithorax homolog, Drosophila) |
| D11 | Hs.744921 | NM_001085487 | MYSM1 | Myb-like, SWIRM and MPN domains 1 |
| D12 | Hs.596314 | NM_003743 | NCOA1 | Nuclear receptor coactivator 1 |
| E01 | Hs.592142 | NM_181659 | NCOA3 | Nuclear receptor coactivator 3 |
| E02 | Hs.736403 | NM_014071 | NCOA6 | Nuclear receptor coactivator 6 |
| E03 | Hs.197071 | NM_014397 | NEK6 | NIMA (never in mitosis gene a)-related kinase 6 |
| E04 | Hs.106861 | NM_022455 | NSD1 | Nuclear receptor binding SET domain protein 1 |
| E05 | Hs.435714 | NM_002576 | PAK1 | P21 protein (Cdc42/Rac)-activated kinase 1 |
| E06 | Hs.20521 | NM_001536 | PRMT1 | Protein arginine methyltransferase 1 |
| E07 | Hs.661229 | NM_001535 | PRMT2 | Protein arginine methyltransferase 2 |
| E08 | Hs.152337 | NM_005788 | PRMT3 | Protein arginine methyltransferase 3 |
| E09 | Hs.367854 | NM_006109 | PRMT5 | Protein arginine methyltransferase 5 |
| E10 | Hs.26006 | NM_018137 | PRMT6 | Protein arginine methyltransferase 6 |
| E11 | Hs.679580 | NM_019023 | PRMT7 | Protein arginine methyltransferase 7 |
| E12 | Hs.504530 | NM_019854 | PRMT8 | Protein arginine methyltransferase 8 |

| | | | | |
|---|---|---|---|---|
| F01 | Hs.591490 | NM_007212 | RNF2 | Ring finger protein 2 |
| F02 | Hs.729085 | NM_019592 | RNF20 | Ring finger protein 20 |
| F03 | Hs.445387 | NM_004586 | RPS6KA3 | Ribosomal protein S6 kinase, 90kDa, polypeptide 3 |
| F04 | Hs.510225 | NM_004755 | RPS6KA5 | Ribosomal protein S6 kinase, 90kDa, polypeptide 5 |
| F05 | Hs.297483 | NM_014712 | SETD1A | SET domain containing 1A |
| F06 | Hs.507122 | NM_015048 | SETD1B | SET domain containing 1B |
| F07 | Hs.517941 | NM_014159 | SETD2 | SET domain containing 2 |
| F08 | Hs.510407 | NM_199123 | SETD3 | SET domain containing 3 |
| F09 | Hs.606200 | NM_017438 | SETD4 | SET domain containing 4 |
| F10 | Hs.288164 | NM_001080517 | SETD5 | SET domain containing 5 |
| F11 | Hs.731691 | NM_024860 | SETD6 | SET domain containing 6 |
| F12 | Hs.480792 | NM_030648 | SETD7 | SET domain containing (lysine methyltransferase) 7 |
| G01 | Hs.443735 | NM_020382 | SETD8 | SET domain containing (lysine methyltransferase) 8 |
| G02 | Hs.643565 | NM_012432 | SETDB1 | SET domain, bifurcated 1 |
| G03 | Hs.631789 | NM_031915 | SETDB2 | SET domain, bifurcated 2 |
| G04 | Hs.567571 | NM_022743 | SMYD3 | SET and MYND domain containing 3 |

| | | | | |
|---|---|---|---|---|
| G05 | Hs.522639 | NM_003173 | SUV39H1 | Suppressor of variegation 3-9 homolog 1 (Drosophila) |
| G06 | Hs.632120 | NM_016028 | SUV420H1 | Suppressor of variegation 4-20 homolog 1 (Drosophila) |
| G07 | Hs.379466 | NM_003336 | UBE2A | Ubiquitin-conjugating enzyme E2A |
| G08 | Hs.612096 | NM_003337 | UBE2B | Ubiquitin-conjugating enzyme E2B |
| G09 | Hs.99819 | NM_006447 | USP16 | Ubiquitin specific peptidase 16 |
| G10 | Hs.8015 | NM_012475 | USP21 | Ubiquitin specific peptidase 21 |
| G11 | Hs.462492 | NM_015276 | USP22 | Ubiquitin specific peptidase 22 |
| G12 | Hs.113876 | NM_007331 | WHSC1 | Wolf-Hirschhorn syndrome candidate 1 |
| H01 | Hs.520640 | NM_001101 | ACTB | Actin, beta |
| H02 | Hs.534255 | NM_004048 | B2M | Beta-2-microglobulin |
| H03 | Hs.544577 | NM_002046 | GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| H04 | Hs.412707 | NM_000194 | HPRT1 | Hypoxanthine phosphoribosyltransferase 1 |
| H05 | Hs.546285 | NM_001002 | RPLP0 | Ribosomal protein, large, P0 |
| H06 | N/A | SA_00105 | HGDC | Human Genomic DNA Contamination |
| H07 | N/A | SA_00104 | RTC | Reverse Transcription Control |
| H08 | N/A | SA_00104 | RTC | Reverse Transcription Control |

| | | | | |
|---|---|---|---|---|
| H09 | N/A | SA_00104 | RTC | Reverse Transcription Control |
| H10 | N/A | SA_00103 | PPC | Positive PCR Control |
| H11 | N/A | SA_00103 | PPC | Positive PCR Control |
| H12 | N/A | SA_00103 | PPC | Positive PCR Control |

### 2.2.12 Biotransformation in liver (Enzymatic activity of Cytochrome P450 in Liver)

#### 2.2.12.1 Phase I (CYPs) and phase II enzymatic activity

The enzymatic activity was analyzed with a modified fluorescence-based method [Donato *et al.*, 2004; Ehnert *et al.*, 2008]. Cells were incubated for 120 min (kinetic measurement within 5 min interval) with different substrates diluted in plain medium (Tables 2.10 and 2.11). The cells converted substrates to metabolites and these change in fluorescence intensity during this process was measured with a plate reader. Moreover, in the phase I substrates, 1.5 mM (15 µl/ml DMSO) salicylamide buffer, 2 mM (10 µl/ml DMSO) probenecid buffer and 0.01 mM (1 µl/ml DMSO) buffer dicoumarol buffer (10 mM in DMSO) were added to prevent spontaneous fluorescence decay. Wells without cells with reaction solution (Medium without FCS with substances) is used as a background control. Methanol fixed cells were used as negative control. The amount of product formed was normalized to total cell number.

Total cell number was determined by SRB staining of fixed cells. The measured values were quantified by using their respective standard curves and the standard curves were consistent through all experiments.

The measured values were quantified by using their respective standard curves.

Results were expressed as picomoles of metabolite formed per minute and per $10^6$ of total cell number. Enzyme activity measurements were analyzed with the help of the GraphPad Prism software.

**Table 2.10. Substrates used during the study of phase I.**

| **Reaction** | **Substrate** | **Substrate conc.** (µM / 100µl) | **Metabolite** | **Excitation/Emission** |
|---|---|---|---|---|
| CYP1A1/2 | 7-EC | 25 | HC | 355 nm/460 nm |
| CYP3A4 | BFC | 5 | HFC | 355 nm/520 nm |
| CYP2E1 | MFC | 2 | HFC | 355 nm/520 nm |
| CYP2C9 | DBF | 5 | HFC | 355 nm/520 nm |

**Table 2.11. Substrates used during the study of phase II.**

| **Reaction** | **Substrate** | **Substrate conc.** (µM / 100 µL) | **Excitation / Emission** |
|---|---|---|---|
| UGT activity | 4-MU | 6,25 | 355 nm/460 nm |

**Table 2.12. Standard curves for phase I and phase II enzyme activity measurement.**

| Enzyme | Standard | Concentration (In 100µL) | Y (Fluorescence Intensity) = | X (µM) = | $R^2$ |
|---|---|---|---|---|---|
| **CYP1A1** | HC | 2 µM | 120.89 x +80.258 | (Y- 80.258) / 120.89 | 0.9998 |
| **CYP3A4** | HFC | 25 µM | 137.23x + 79.393 | (Y- 79.393) / 137.23 | 0.9988 |
| **CYP2C9** | FL | 1,25 µM | 1704.7x + 51.098 | (Y- 51.098) / 1794.7 | 0.9999 |
| **UGT2B7** | 4-Mu | 6,25 µM | 95.558x + 78.982 | (Y– 78.982) / 95.558 | 0.9999 |

### 2.2.12.2 Sulforhodamine B (SRB) staining

For SRB staining [Skehan *et al.*, 1990] cells were fixed to culture plastic with ice cold fixation buffer (95% Ethanol, 100 µl/well). The cells were incubated for at least 1 hour at –20°C. Then fixation buffer was removed and cells were washed with H2O. Fixed cells were stained for 30 min (dark, RT) with 0.4% SRB dissolved in 1% acetic acid (100 µl/well). At the end of the staining period SRB solution was removed and cells were washed three times with 1% acetic acid in order to washout unbound dye. After being rinsed, plates were air dried until no standing moisture was visible. Bound SRB was solubilized with 10 mM un-buffered TRIS (pH =10.5, 100 µl/well) for 10-15 min on a shaker (RT, dark). The OD at 565 nm (SRB) and 690 nm (impurities) were measured with OMEGA plate reader (BMG Labtech GmbH, Offenburg, Germany).

The SRB OD of the different number of seeded cells was determined with OMEGA plate reader. For calculation $OD_{690\ nm}$ (background and impurities) is subtracted from $OD_{565\ nm}$ (SRB concentration) and the standard curve was determined for each cell line (Table 2.11).

**Table 2.13. SRB Stand Curve for HCC cell lines.**

| **SRB Standard Curve** | **Y (Fluorescence Intensity) =** | **X (µM) =** | **$R^2$** |
|---|---|---|---|
| **PHH** | 6E-05x - 0.3433 | (Y + 0,3433) / 0.00006 | 0.00006 |
| **HLE** | 7E-05x - 0.1293 | (Y + 0,1293) / 0.00007 | 0,00007 |
| **Huh7** | 8E-05x – 0.048 | (Y + 0.048) / 0.00008 | 0.00008 |
| **HepG2** | 3E-05x + 0.0028 | (Y - 0.0028) / 0.00003 | 0.00003 |
| **AKN1** | 4E-05x + 0.0055 | (Y - 0.0055) / 0.00004 | 0.00004 |

### 2.2.13 Ammonia detoxification

The urea production by the cells was measured by the photometric quantification of urea, a decomposition product of ammonia in PHH. PHH were washed three times with DPBS. Then, the cells were incubated with 100 µl (96-well plate) of 300 mM $NH_4Cl$ in reaction buffer (1 mM MgCl2 and 1 mM sodium pyruvate in DPBS) for 24 hrs at 37ºC, 5% $CO_2$. Wells without cells were used as background subtraction from well with cells. After 24 hrs, 80 µl of culture supernatant were transferred to a new 96-well plate for urea quantification. Simultaneously, a urea standard curve was prepared in the same 96-well plate. Then, 60 µl of O-Phthalaldehyde solution (1.5

mM O-Phthalaldehyde, 4 mM Brij-35, 0.75 M $H_2SO_4$) and 60 µl of NED's reagent (2.3 mM N-(1-naphthyl) ethylenediaminedihydrochloride, 0.08 M boric acid, 4 mM Brij-35, 2.25 M $H_2SO_4$) were added in each well and incubated for 2 hrs at 37 ºC, 5% $CO_2$. After the incubation time, the absorption was measured at a wavelength of λ = 460 nm with a plate reader [Jung *et al.*, 1975; Zawada *et al.*, 2009].

# 3 Results

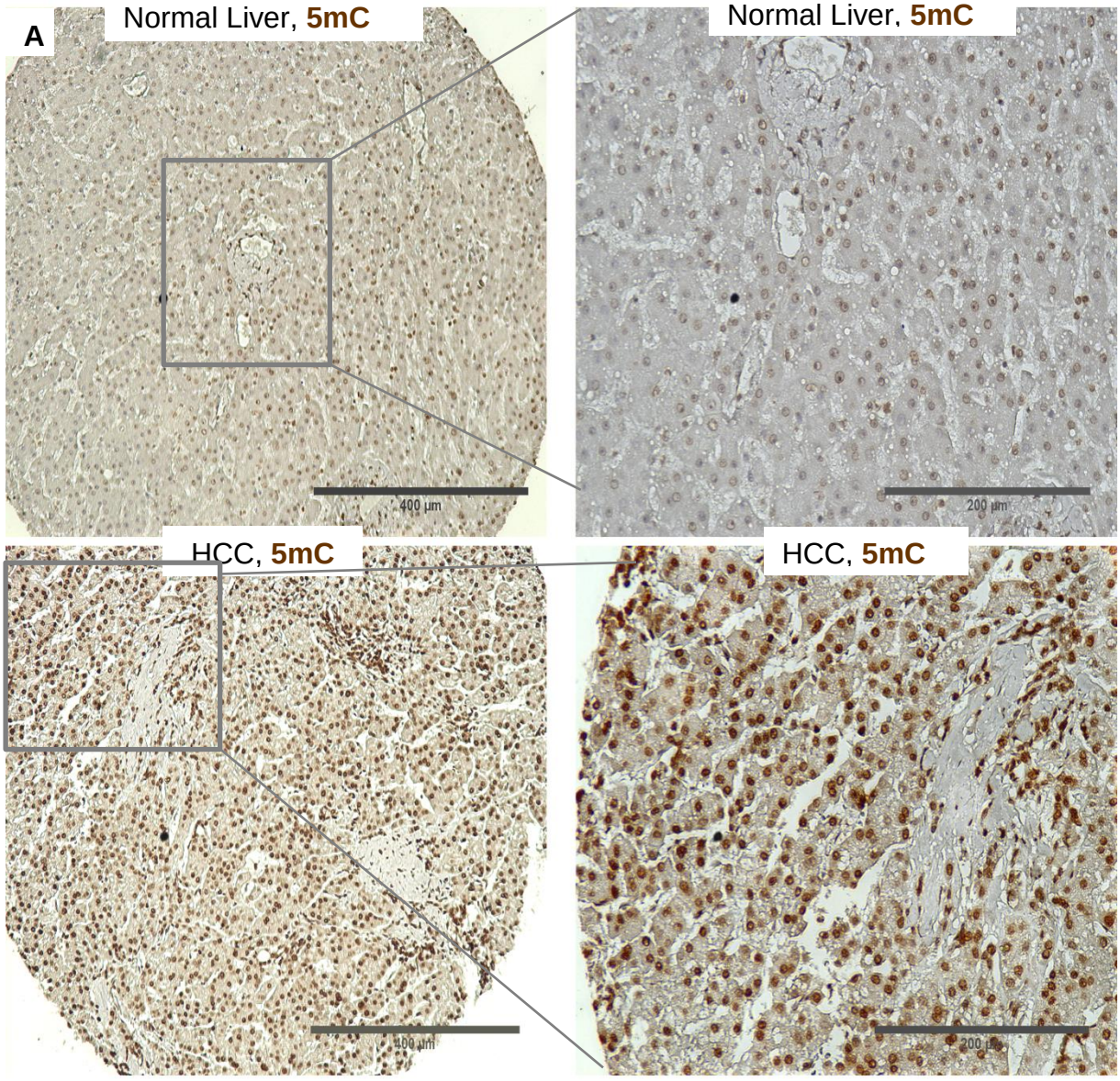
## 3.1 Alteration of DNA methylation landscape in HCCs

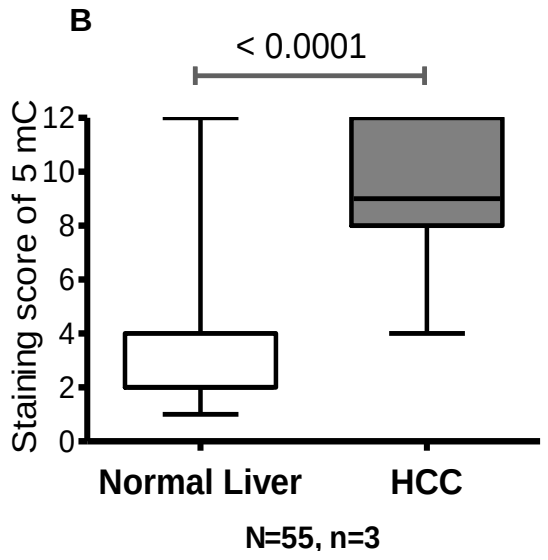
Numerous studies have reported that tumor cells exhibit a major disruption in the regulation of the DNA methylation pattern in cancers including HCCs [Bird, 1995; Santini *et al.*, 2001; Watanabe & Maekawa, 2010; Yang *et al.*, 2001; Yu *et al.*, 2002]. It has been shown that several critical genes, which seem to be important in tumorigenesis, are hypermethylated in HCCs and most importantly in CpG Island of the promotor [Yu *et al.,* 2002]. It has been reported that in various type of cancers 5hmC level drastically decreased and the level was dependent on tumor stages. Therefore, we investigated the level of 5mC and 5mC in HCCs.

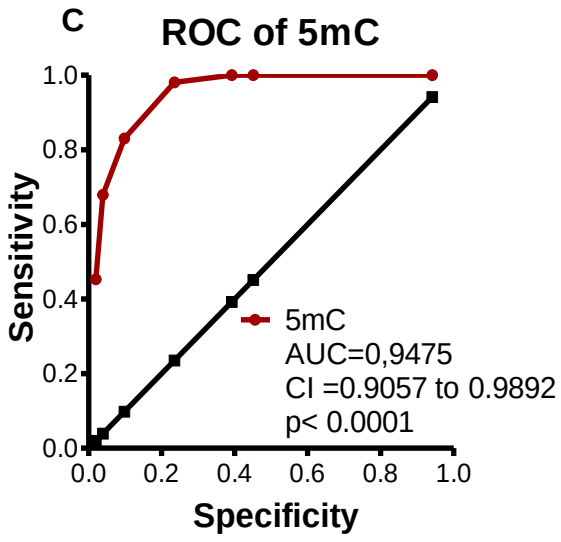
### 3.1.1 5hmC is decreased in human HCC tissues

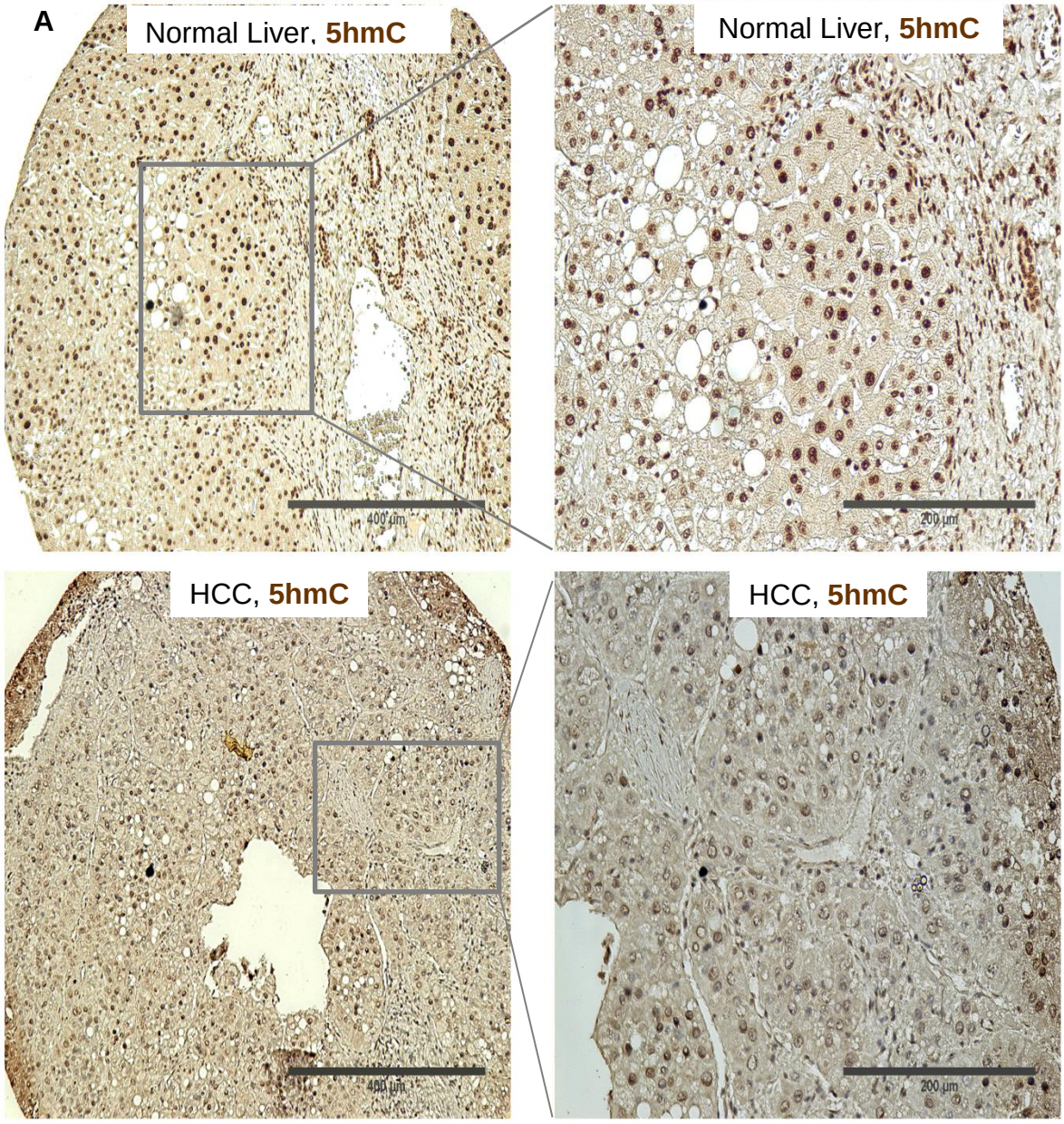
It was shown that the loss of 5hmC has a significant role in development of different types of cancer including brain, melanoma, colon and liver [Haffner *et al.*, 2011; Huang *et al.*, 2013; Ivanov *et al.*, 2013; Ko *et al.*, 2010]. The genomic distribution of 5hmC and 5mC in HCC tissue-microarray-paraffin-embedded tissues was evaluated by immunohistochemistry (IHC) staining. By evaluating 55 HCC tissue samples, a significant ($p < 0.001$) decline of 5hmC (quantified via IHC, Fig.3.1) along with a significant ($p < 0.001$) increase of 5mC as compared to noncancerous liver tissue of the same patient, was observed in HCC liver samples (Fig.3.1 A). The heterogeneous loss or reduction of 5hmC was observed in most of the HCC tissue samples and 5mC was highly generated in HCC samples as compared with corresponding normal liver. Hematoxylin was used as a nuclear counterstain. To assess the potential diagnostic value of 5hmC and 5mC, a ROC curve analysis was performed. The associated area under the curve (AUC) was used to confirm the

diagnostic value of 5hmC and 5mC. The AUC of 5hmC was 0.79 95% confidence interval [CI] 0.6915 to 0.8814, p< 0.0001) and the AUC of 5mC was 0.95 (95% confidence interval [CI] 0.9057-0.9892, p < 0.0001) (Fig. 3.1 C). As the ROC curves for HCC tissue samples show, the detection of the depletion of 5hmC and the increase of 5mC in HCC tissue provide a reliable prognosis marker for HCC therapy.


A
Normal Liver, 5mC
Normal Liver, 5mC
400 µm
200 µm
HCC, 5mC
HCC, 5mC
400 µm
200 µm


B
< 0.0001
Staining score of 5 mC
0
2
4
6
8
10
12
Normal Liver
HCC
N=55, n=3


C
ROC of 5mC
Sensitivity
0.0
0.2
0.4
0.6
0.8
1.0
Specificity
5mC
AUC=0,9475
CI =0.9057 to 0.9892
p< 0.0001


A
Normal Liver, 5hmC
Normal Liver, 5hmC
400 µm
200 µm
HCC, 5hmC
HCC, 5hmC
400 µm
200 µm

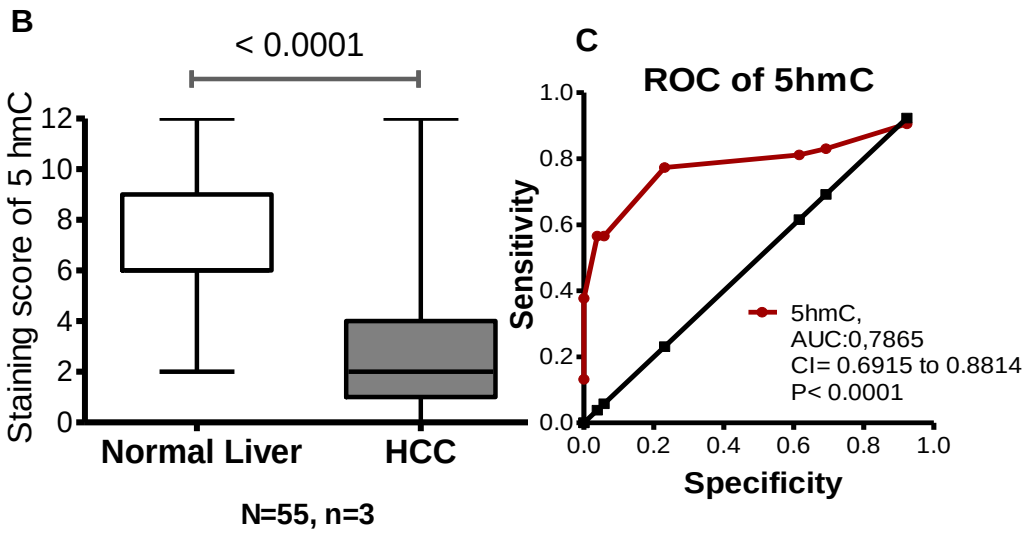
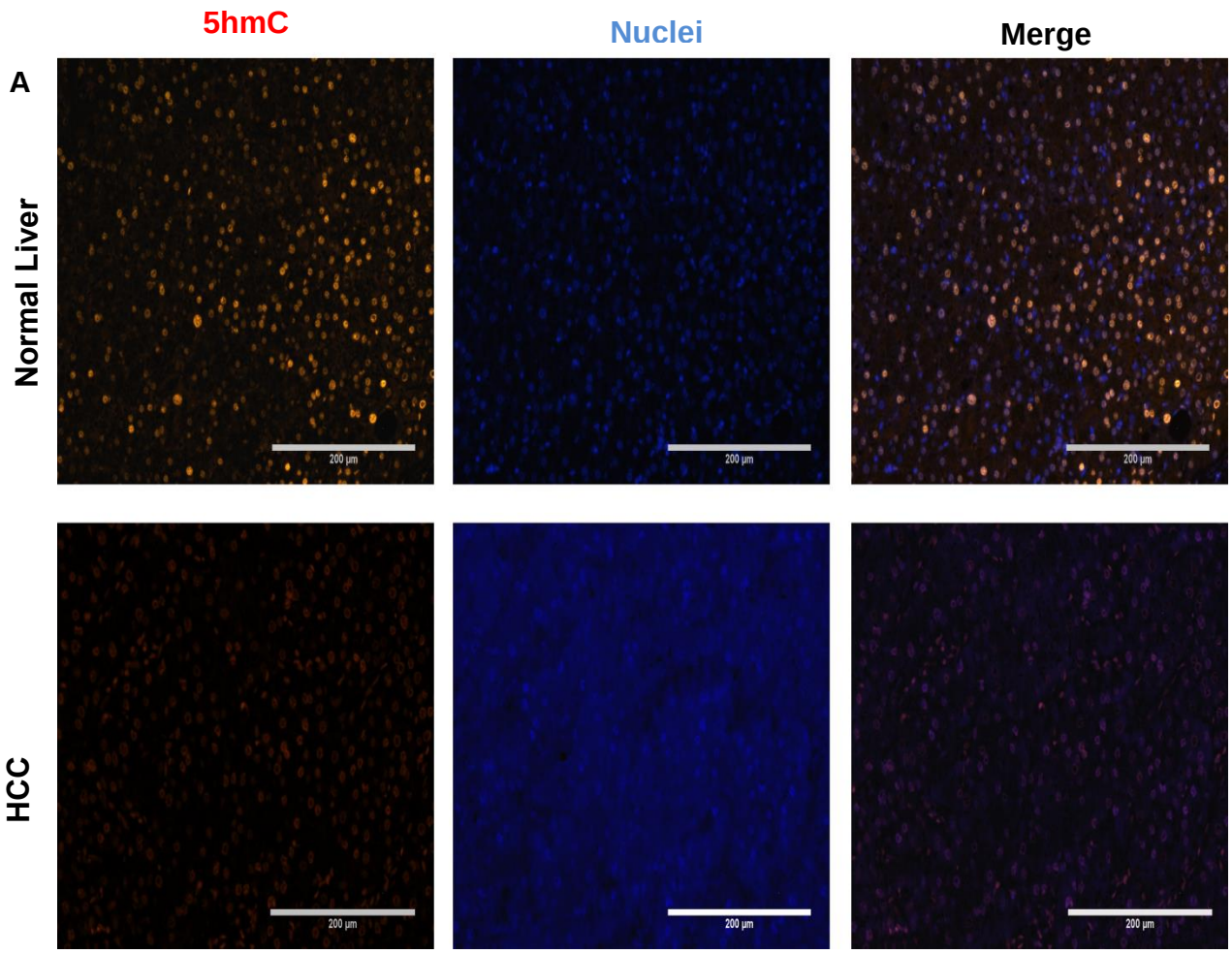

B
< 0.0001
Staining score of 5 hmC
0
2
4
6
8
10
12
Normal Liver
HCC
N=55, n=3
C
ROC of 5hmC
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
Specificity
5hmC,
AUC:0,7865
CI= 0.6915 to 0.8814
P< 0.0001

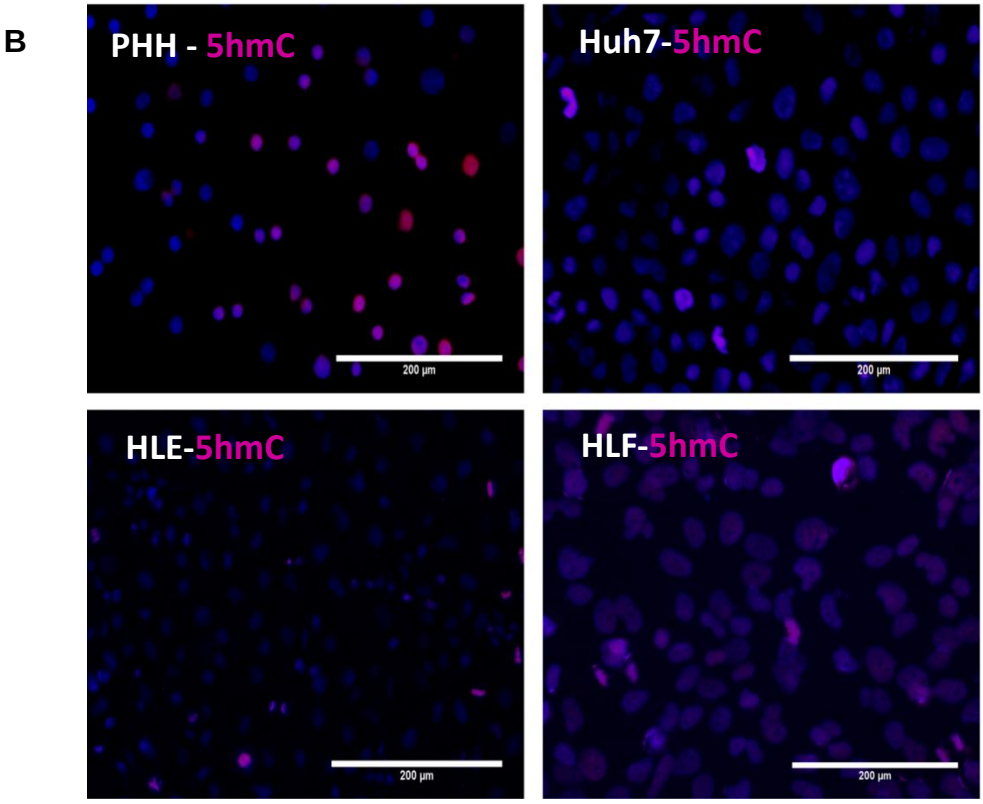
**Figure 3.1. Distribution of 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC) in HCC tissues.**

**A**) IHC was performed using antibodies against 5hmC and 5mC in human normal and HCC tissues, the mean values of the IHC are shown (each group N=55, n=3). Six pictures are presented for each condition. **B**) The reduction of the 5hmC generation and the increase of 5mC were observed in HCC samples compared with corresponding normal liver samples. Data are represented as Box Plot (Whisker, Tukey). **C**) ROC curve for 5mC and 5hmC of 55 of HCC samples showed the accuracy of the 5mC-5hmC detection. Statistical methods included Paired t-test and independent samples t-test. All statistical tests were considered significant at $\alpha = 0.05$ ($p < 0.05$).

### 3.1.2 5hmC is decreased in human HCC tissues and cell lines

To assess the differential distribution of 5hmC more particularly and quantitate in HCC cell lines as compared with primary human hepatocytes (PHH), the immunofluorescence microscopy coupled with quantitative image analysis was used. We observed a significant difference in 5hmC staining intensities between HCC cell lines and PHH. The immunofluorescence staining for 5hmC normalized to Hoechst. The quantification of 5hmC staining intensity showed the reduction of 5hmC in HCC cell lines (Huh-7, HLE and HLF) as compared to primary human hepatocytes (Fig 3.2. A).These results confirmed the loss of 5hmC in HCCs.


5hmC
Nuclei
Merge
A
Normal Liver
HCC
200 µm


B
PHH - 5hmC
Huh7-5hmC
HLE-5hmC
HLF-5hmC
200 µm

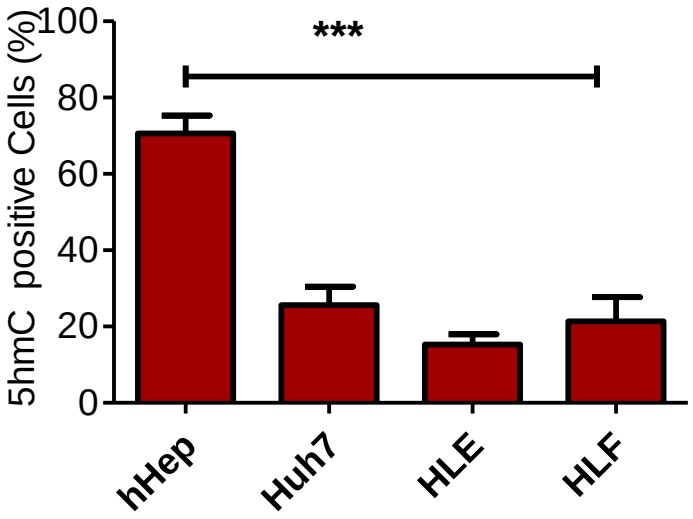



**Figure 3.2. Distribution of 5-hydroxymethylcytosine (5hmC) in HCC cell lines.**

**A**) IF staining was performed by using the antibody against 5hmC (Red) for HCC paraffin embedded tissues, HCC cell lines (Huh7, HLE and HLF) and hHep. Cell nuclei were counterstained with Hoechst 33342 (blue) (white arrowhead; scale bar, 200 µm). **B**) The quantification of 5hmC staining intensity showed the reduction of 5hmC in HCC cell lines (Huh-7, HLE and HLF) as compared to primary human hepatocytes). Cell nuclei were counterstained with Hoechst 33342 (blue) (white arrowhead; scale bar, 200 µm). For quantification, 5hmC positive nuclei were counted. Statistical methods included Paired t-test and independent samples t-test. All statistical tests were considered significant at $\alpha = 0.05$ ($p < 0.05$)

### 3.1.3 Reduction of 5hmC correlates with decreased TET2 and TET3 gene expression in HCC tissues and cell lines

To understand, the important cellular factors responsible for 5hmC reduction in HCCs; we determined the expression of IDH1, IDH2, TET1, TET2 and TET3 by qRT-PCR. Despite reports by others that TET1 is down-regulated in HCC tissue compared with 'normal' liver tissue [Liu *et al.*, 2013; Yang *et al.*, 2013], we found no TET1 expression by qRT-PCR in normal adult human liver tissue, HCC tissue as well as HCC cell lines or human hepatocytes (data not shown).

The expression of TET1 in our samples was at the detection limit with the very small difference of Ct values. In contrast, we found that the expression of TET2 and TET3 as well as the upstream genes IDH1 *and* IDH2 were significantly decreased in human HCC tissues as compared to matched 'normal' liver tissues (Fig.3.3 A). These

data were confirmed in HCC cell lines (Huh7, HLE and HLF) in comparison with freshly isolated PHH (Fig.3.3 B). Our data show that a decrease in the expression of IDHs and of TETs, suggested that these proteins could play a specific a role in the observed reduction of 5hmC in HCCs (Fig.3.3 C). After screening different housekeeping genes, we have chosen Beta-2-Microglobulin *(*B2M) whose expression was stable without much variation among the tested HCC and normal liver tissue samples for the normalization of our RT-qPCR results.

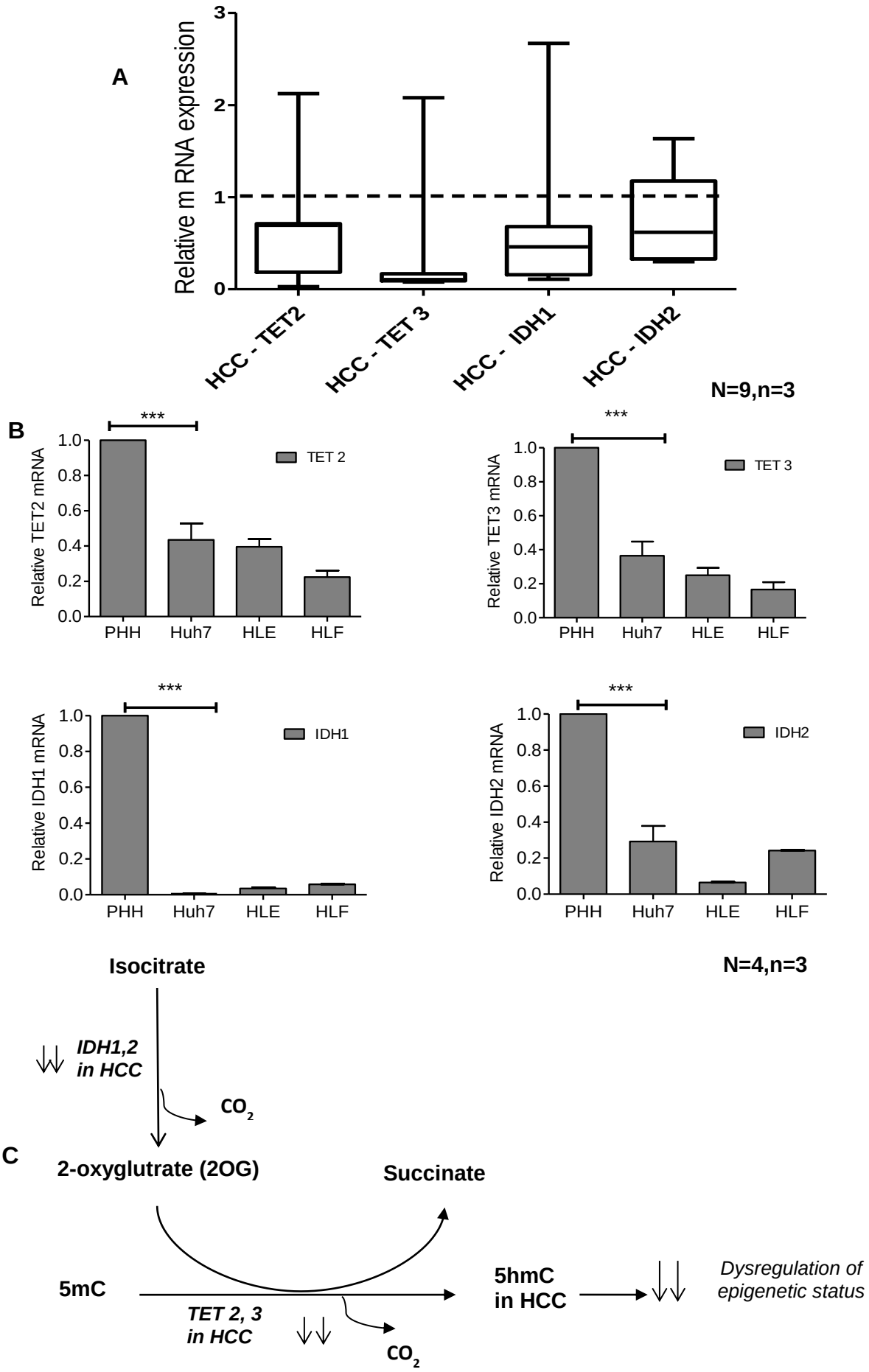

A
Relative m RNA expression
3
2
1
0
HCC - TET2
HCC - TET 3
HCC - IDH1
HCC - IDH2
N=9,n=3
B
***
Relative TET2 mRNA
TET 2
1.0
0.8
0.6
0.4
0.2
0.0
PHH
Huh7
HLE
HLF
Relative TET3 mRNA
TET 3
Relative IDH1 mRNA
IDH1
Relative IDH2 mRNA
IDH2
N=4,n=3
Isocitrate
IDH1,2
in HCC
CO2
C
2-oxyglutrate (2OG)
Succinate
5mC
TET 2, 3
in HCC
5hmC
in HCC
Dysregulation of
epigenetic status

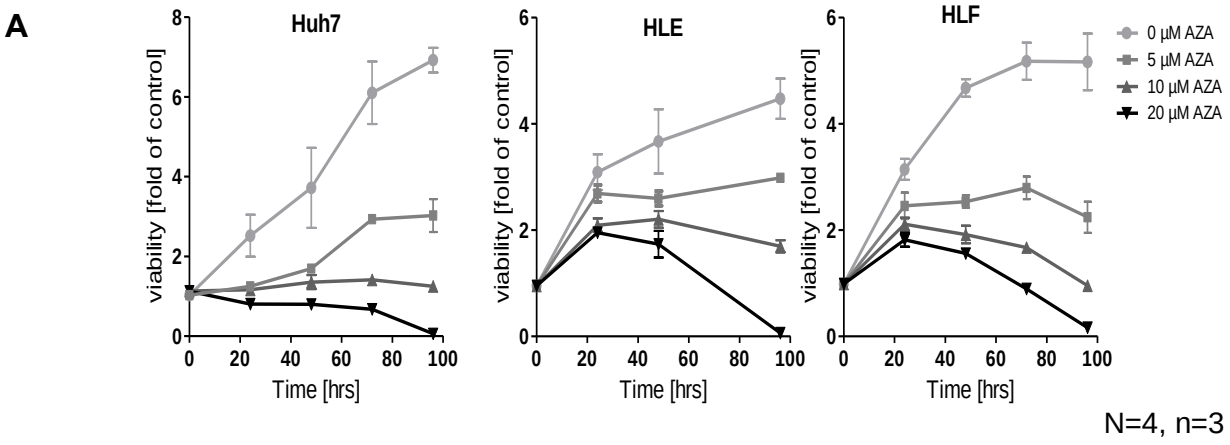
**Figure 3.3. Reduction of 5hmC is associated with the substantial reduction of IDH1 & 2 and TET2 & 3 gene expression.**

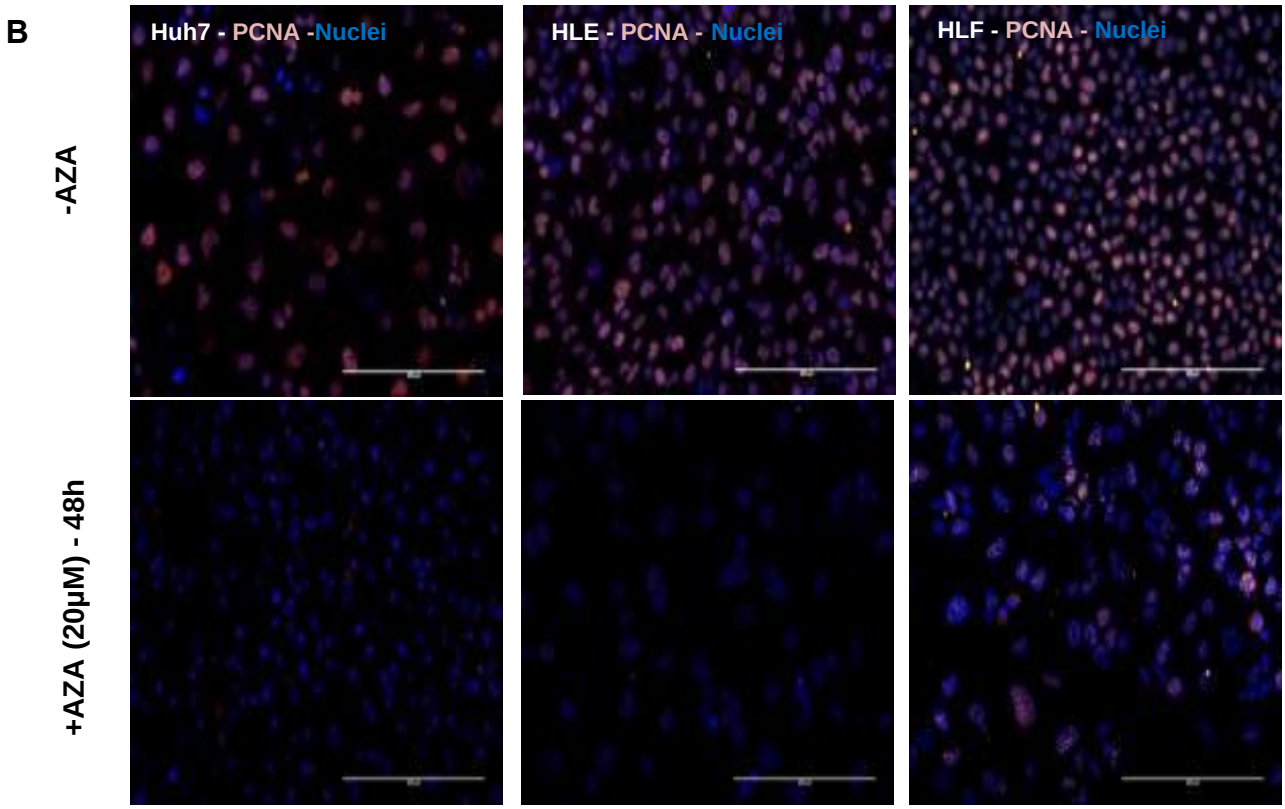
**A**) The expression of TET2, TET3, IDH1 and IDH2 decreased in HCC tissues in comparison to the matched normal liver. Real-time qRT-PCR was used to determine TETs and IDHs relative mRNA levels in HCCs while using β2M as an internal control for normalization. Data are expressed as fold change in mRNA expression compared to the normal liver control (indicated by dashed line at 1 (The Data are presented as Box Plot (N=9, n=3): Center line represents the median, box limits represent the first and third quartiles, and whiskers represent 1.5 times the interquartile range (The p value were assessed by Student's t test, bars, mean ± S.E; p*** < 0.0001 for TET2, TET3, IDH1 and IDH2). **B**) The same pattern was confirmed by comparing HCC cell lines (Huh7, HLE; HLF) to primary human hepatocytes (N=4, n=3). (P value assessed by one way ANOVA, bars, mean ± S.E) ($p < 0.001$). **C)** Shows schematic diagram of the aberrant epigenetic status due to the reduction of TETs and IDHs activity which may lead to the depletion of the 5hmC generation in HCCs.

## 3.2 Effect of 5-Azacytidine on HCC cells proliferation and viability

Incubation of the three tested HCC cell lines with 5-AZA showed a dose- and time-dependent reduction of mitochondrial activity, as measured by Resazurin conversion (Fig.3.4 A). HCC cell lines were treated with 5-20 µM 5-AZA for 24, 48, 72 and 96 hrs (fresh drug added every 24 hrs). Resazurin assay indicate that HCC cells survival reduced by doses of 5 µM and the viability was significantly reduced by 5-AZA at all tested concentrations. However, 20 µM of 5-AZA reduced the cell viability dramatically after 48 hrs. A time dependent inhibition of cell viability was observed upon treatment with 5-AZA and as the growth inhibition effect of 5-AZA requires time to be incorporated into genomic DNA and change the DNA methylation status. To strengthen this result, the effect of 5-AZA on cell proliferation was additionally tested by analyzing the proliferation cell nuclear antigen (PCNA) antigen with Immunofluorescence staining [Waseem & Lane, 1990]. The IF staining indicated a significant decrease of PCNA nuclear staining of treated cells compared to untreated cells after 48 hrs (Fig.3.4 B). Decrease of PCNA protein expression showed the inhibition effect of 5-AZA on HCC cell proliferation.

To determine whether 5-AZA induces apoptosis, hypodiploid DNA was measured by flow cytometric measurement. Further analyses revealed that 5-AZA induced apoptosis to a certain extent in all tested HCC cell lines (Fig.3.4 D) which is paralleled by an increased LDH enzyme release from HCCs after treatment with 5-AZA (Fig.3.4 C).



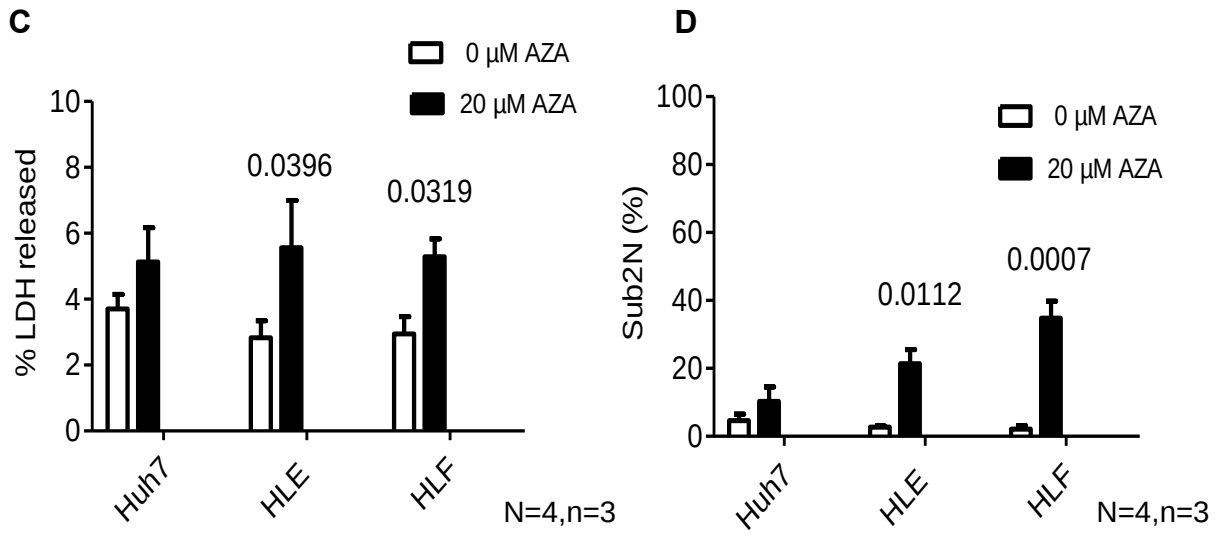



**Figure 3.4. Distinct effect of 5-AZA on HCC cell lines viability, proliferation and apoptosis**.

**A**) the mitochondria activity of cells decreased upon treatment with 5, 10, and 20 µM of 5-AZA for 24, 48, 96 and 120 hrs in time and doses dependent manner (N=4, n=3). **B**) 20 µM of 5-AZA inhibited the PCNA expression in HCC cell lines after 48 hrs of incubation (white arrowhead; scale bar, 400 µm). **C**) 20 µM of 5-AZA caused LDH released in HLE and HLF cells after 48 hrs, however LDH released in culture supernants in Huh7 cells did not increase upon treatment of 5-AZA (N=4, n=3). **D**) 5-AZA induced cell death in HLE and HLF cells. Determination of sub2N fractions as marker for apoptosis was measured after treatment of HCC cells with 20 µM 5-AZA for 48 hrs (P value assessed by Student's t test, bars show the mean ± S.E of four independent experiments).

### 3.3 5-Azacytidin triggers active demethylation in HCC cells

It is known that 5-AZA induces passive demethylation through inhibition of DNMT [Jones *et al.*, 1983], however it is not known to our knowledge if it has an influence on active demethylation through induction of TET expression. Treatment of HCC cell lines (Huh-, HLE and HLF) with 5-AZA for 24 and 48 hrs led to a significant increase in 5hmC positive cells compared to untreated HCC cells (Fig.4 A). To further investigate a potential active demethylation process through conversion of 5mC to 5hmC, the expression of TET2 and TET3 was determined. 5-AZA-stimulated cells displayed a significantly increased expression of TET2 and TET3, while the expression of IDHs is not influenced (Fig.3.5 A, C). Increased expression of TET2

and TET3 upon 5-AZA treatment was confirmed by Western blot analysis (Fig.5 B). We next needed to functionally prove if 5-AZA treatment in line with our previous work, could trigger oxidation of 5mC to 5hmC through the induction of TET2 and/or TET3 expression [Yan *et al.*, 2014] in HCC. If this would be the case, we would have strong evidence of an active demethylation process caused by 5-AZA. We were able to demonstrate the conversion of 5mC to 5hmC upon 5-AZA treatment depends on TET proteins by simultaneous knock down of TET2 and TET3 expression in Huh-7, HLE, and HLF cells, using siRNA. mRNA levels of the TET genes could be downregulated to approximately 30% for TET2 and 40% for TET3, as compared to corresponding transfected cells with control siRNAs (Fig.6 A). In control cells, TET2 and TET3 mRNA knock down decreased intrinsic 5hmC levels. Upon TET2 knock down, 5-AZA (20 µM) treatment was unable to enhance the generation of 5hmC. In contrast, 5-AZA still enhanced – although to a much lesser extent – the expression of 5hmC in TET3 knocked-down HCC cells (Fig.6 B). Overall, these results suggest that the enhancing effect of 5-AZA on 5hmC generation in HCC is mainly mediated by TET2.

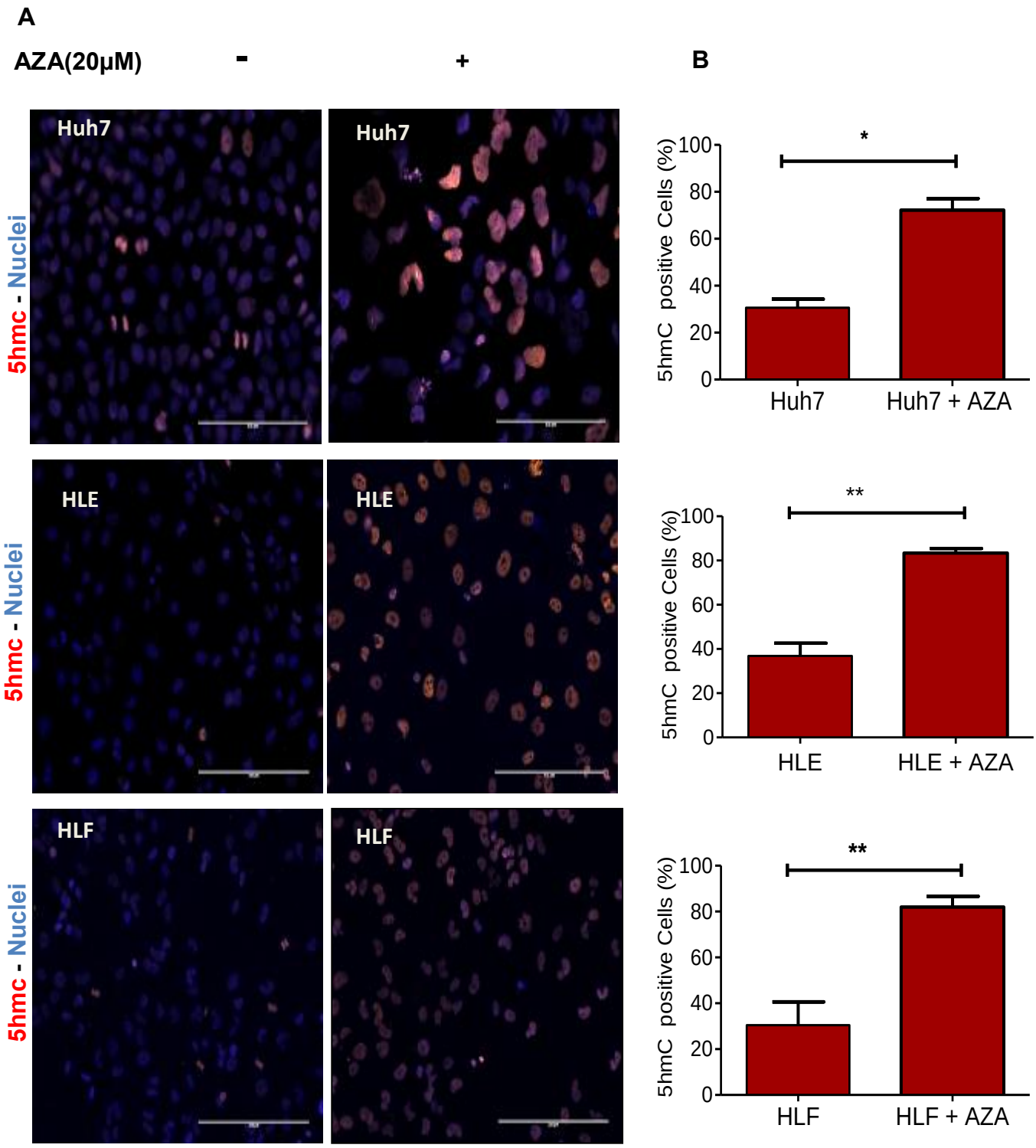



**Figure 3.5. 5-AZA enhanced the generation of 5hmC in HCC cell lines.**

**A)** Immunostaining showed that 20 μM of 5-AZA enhances the generation of 5hmC in HCC cell lines after 48 hrs (white arrowhead; scale bar, 200 μm). **B**) The semi-quantitative analysis of immunofluorescence staining indicated that the incubation of HCC cells with 5-AZA for 48 hrs increased 5hmC level. P are value assessed by Student's t test, data are represented as mean ± SE.

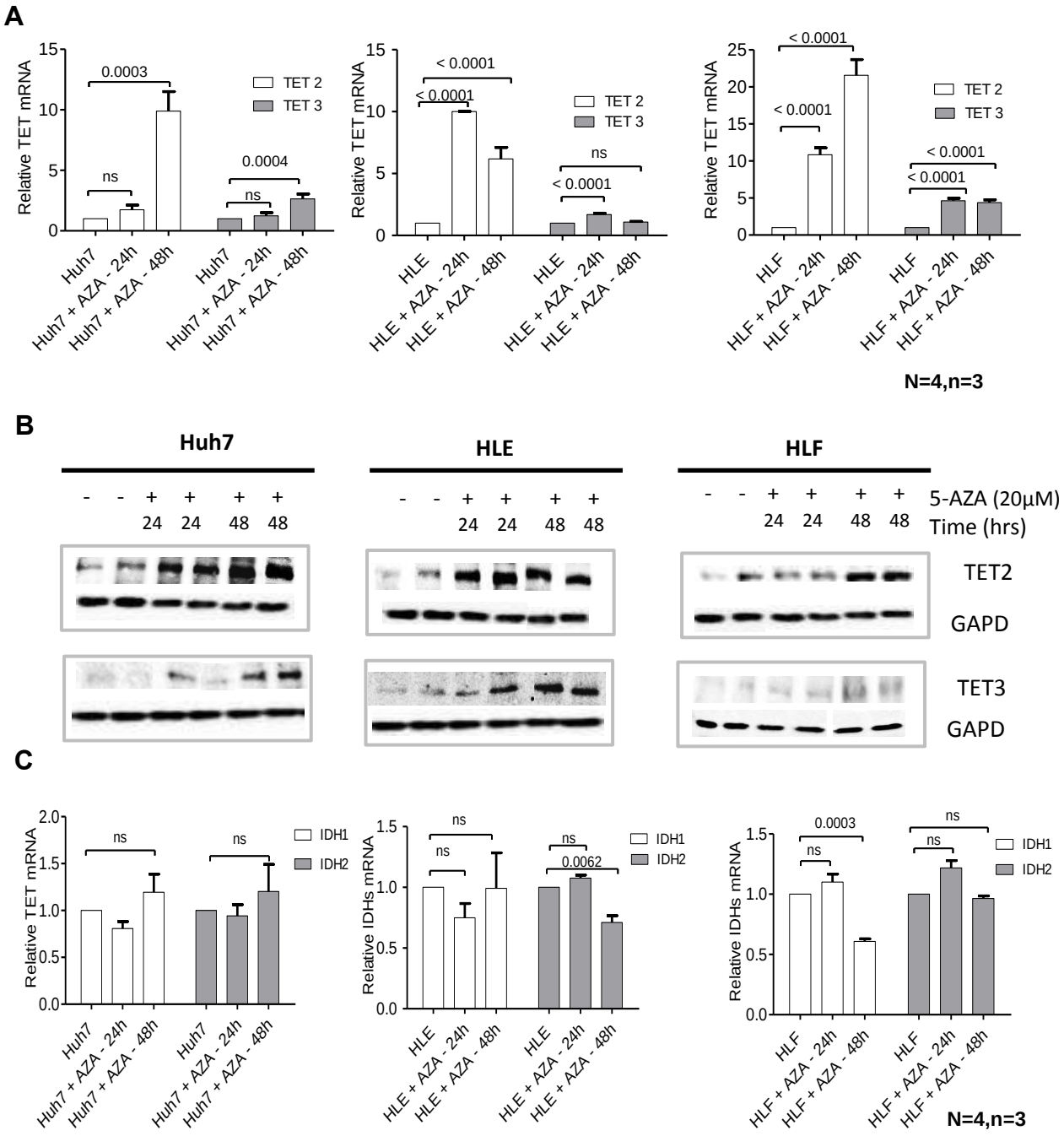
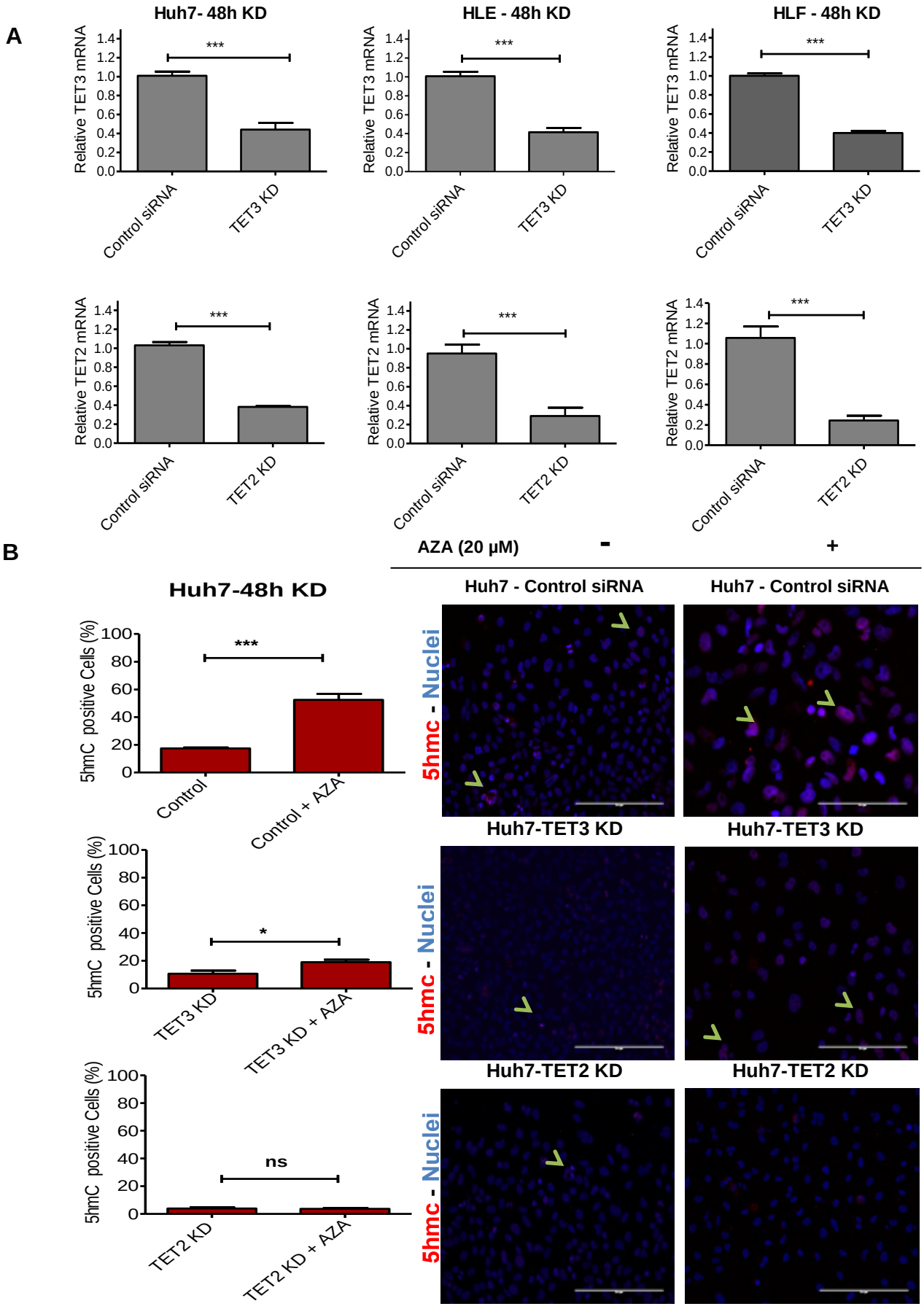



**Figure 3.6. 5-AZA enhanced the generation of 5hmC through induction of TETs expression in HCC cell lines.**

**A**) 20 µM of 5-AZA induced the expression of TET2 and TET3 in HCC cells after 48 hrs of incubation. The mRNA level of TET2 and TET3 genes were determined by quantitative real time PCR after 24 hrs and 48 hrs. P values were assessed by one way ANOVA, data represented as mean ± SE. **B)** Expression of TET2 and TET3 was examined by Western blot analysis in HCC cell lines (Huh7, HLE and HLF) after treatment with 20 µM 5-AZA for 24 and 48 hrs. **C**) 20 µM of 5-AZA didn't influence the expression of IDH1 and IDH2 in HCC cells significantly after 48 hrs of incubation. Real-time qRT-PCR was used to determine TETs and IDHs relative mRNA levels in HCC cell lines while using GAPDH as an internal control for normalization.


A
Huh7- 48h KD
HLE - 48h KD
HLF - 48h KD
Relative TET3 mRNA
Relative TET2 mRNA
***
Control siRNA
TET3 KD
TET2 KD
B
AZA (20 µM)
-
+
Huh7-48h KD
5hmC positive Cells (%)
Control
Control + AZA
TET3 KD
TET3 KD + AZA
TET2 KD
TET2 KD + AZA
*
ns
Huh7 - Control siRNA
Huh7-TET3 KD
Huh7-TET2 KD
5hmc - Nuclei

**C**

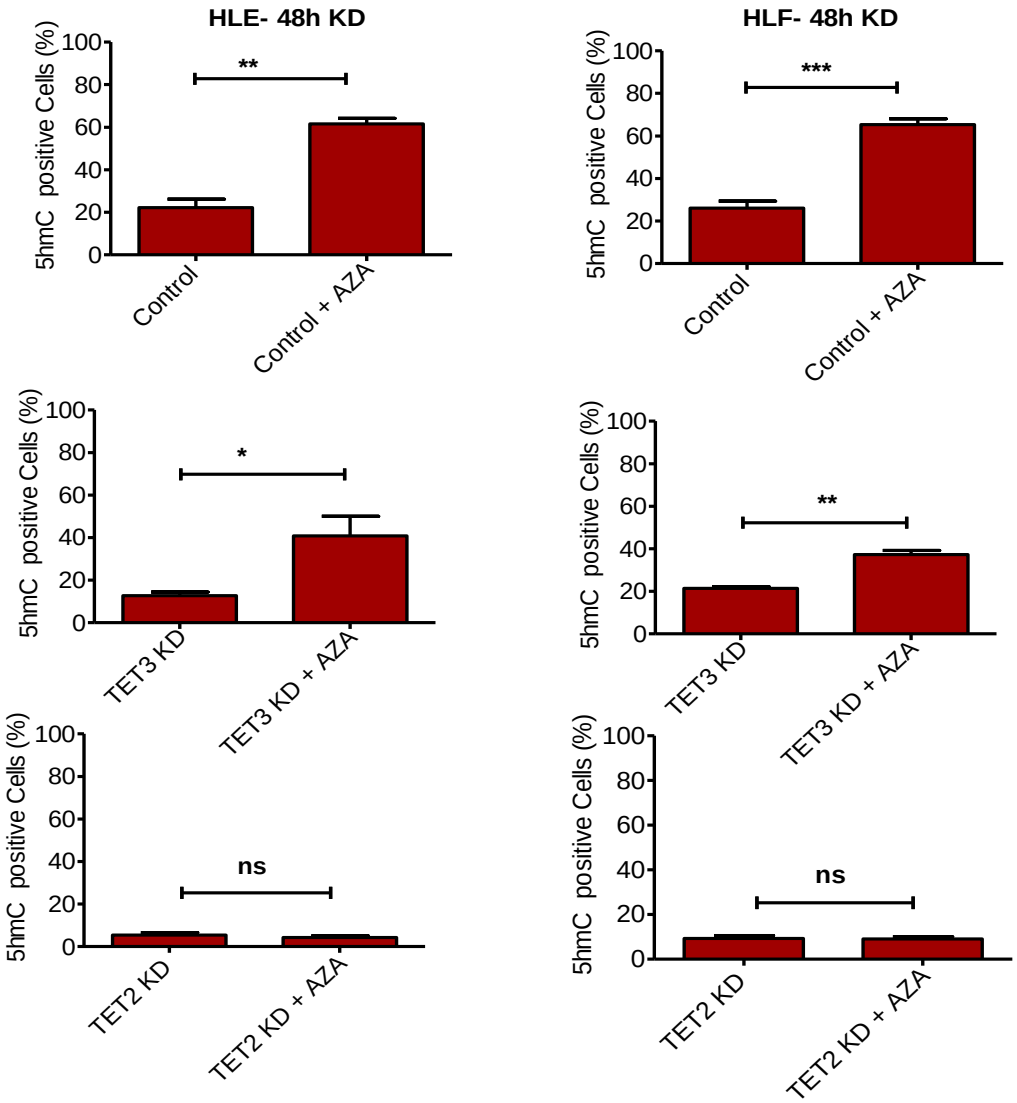



**Figure 3.7. The effect of 5-AZA is mediated by TET methylcytosine dioxygenase.**

**A)** siRNAs targeting TET 2 and TET3 decreased the level of TET mRNA to approximately 30% for TET2 and 40% as compared with corresponding cells transfected cells with control siRNAs shown by quantitative RT-PCR. Data were repeated three times and the value in columns represents mean ± SD. P values were assessed by Student's t test;* $p < 0.05$ , **$p < 0.001$ and *** $p < 0.001$ . **B** and **C**) immunostaining shows that knocking down the expression of TET genes in HCC cell lines, specifically TET2, decreased the basal level of 5hmC signal and attenuated the induction of 5hmC by 5-AZA (20 µM) treatment for 48 hrs (white arrowhead; scale bar, 200 µm).

## 3.4 Effect of 5-Azacytidin in combination with Vitamin C on HCC cell proliferation

In recent years, accumulating data provided evidence that in pharmacological doses, ascorbic acid (Vitamin C) has cytotoxic effects on cancer cells *in vitro* and *in vivo* in low micromolar-range This cytotoxicity is caused through pro-oxidative mechanism(s) which ascorbyl radicals and $H_2O_2$ catalyzed via serum components [Sinnberg *et al.*, 2014; Venturelli *et al.*, 2014]. We investigated the effect of the Vitamin C as a single or combined treatment with 5-Aza (in dose-dependent manner) on HCC cells proliferation and cells damage in order to choose an optimum dose of Vitamin C and 5-AZA for epigenetic study.

The viability of the cells was monitored after treatment with different concentration of 5-AZA and L-ascorbic acid 2-phosphate (Vitamin C) for 48 hrs. The viability of treated HCC cells was assessed as a function of mitochondrial activity by Resazurin conversion assay and compared with the non-treated control cells. A reduced mitochondrial activity, reflecting decrease in cell viability was observed in HLE and Huh7 cells treated with 5-AZA in both cell lines with and without Vitamin C, whereas Vitamin C alone did not compromise the mitochondrial function after 48 hrs (Fig.3.8 A). In Huh7, cell viability was further reduced when 5-AZA was co-incubated with Vitamin C (approximately 30%) (Fig.3.8 A). In both, HLE and Huh7, inhibition of proliferation was paralleled by increased intracellular LDH enzyme release, indicating membrane leakage after 48 hrs of treatment (Fig.3.8 B). While a very low release of LDH was observed with 5-AZA and Vitamin C individually, the combination of 5-AZA and Vitamin C showed a high rate of cytotoxicity in both cell lines.

Furthermore, flow cytometry analysis of the sub2N population as a measure of cell death revealed that the combination of 5-AZA and Vitamin C induced a higher number of cells in the Sub 2N in HLE than in solely 5-AZA treated cells (Fig.3.8 C). In Huh7 a significant increase in Sub 2N population was observed in cells treated with 5-AZA + Vitamin C, with a slight increase of LDH compared to 5-AZA single treated cells (Fig.3.8 C).

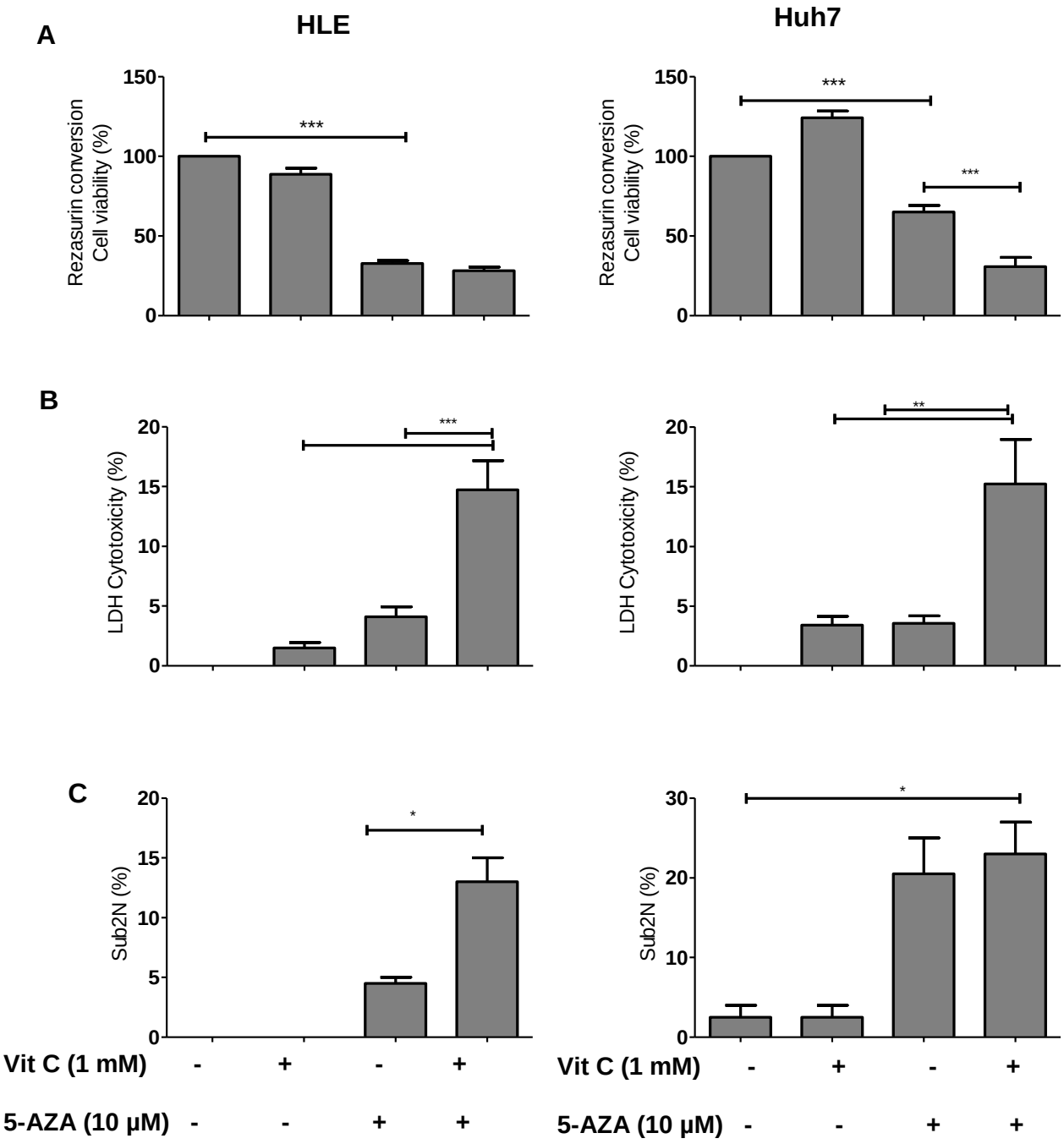



**Figure 3.8. Vitamin C enhances the cytotoxic effects of 5–AZA and inducing cell death in HCC.**

**A**) 5-AZA alone or together with Vitamin C reduced cell viability in HCC cell lines (HLE and Huh7 cells after 48 hrs. **B)** Combination of 10 µM 5-AZA and 1 mM Vitamin C caused more LDH released in HLE and Huh7 compared with the single treatment. **C**) Flow cytometric measurement of sub2N population in HLE and Huh7 showed more cell death in combined treated cells than single treated cells. Data were repeated three times and the value in columns represents mean ± SD. P values were assessed by one way ANOVA, * $p < 0.05$ , **$p < 0.001$ and *** $p < 0.001$ .

### 3.5 Inhibition of cell proliferation and induction of cell cycle arrest was enhanced by the combined treatment of 5-AZA and Vitamin C

To confirm the anti-proliferative effect of 5-AZA and Vitamin C, expression of proliferation cell nuclear antigen (PCNA) was investigated by IF staining .In comparison with the untreated control, inhibition of cell proliferation was observed in HLE and Huh7 cells treated with Vitamin C (Fig.3.9 A, B). In HLE, 5-AZA treatment induced a significantly higher inhibition, which was further enhanced with the combination treatment of 5-AZA + Vitamin C. Similarly, in Huh7, a significant inhibition of proliferation was observed with both 5-AZA and the combination of 5-AZA + Vitamin C (Fig.3.9 A, B).

Next, we determined by flow cytometry the phase of the cell cycle where the observed growth inhibition in both cell lines occurred. Cell cycle distribution analysis of HLE cells treated with 5-AZA and Vitamin C individually indicated an increase in the population of cells in G2 phase. However, a stronger increase in the S phase of the cell cycle was noted in cells treated with a combination of 5-AZA + Vitamin C (Fig.3.10 A). In Huh7, we observed an increase in the population of cells in the G1 phase of the cell cycle with 5-AZA and Vitamin C treatment. However, the number of cells in the G1 phase was highest with the combination treatment of 5-AZA and Vitamin C (Fig.3.10 B).

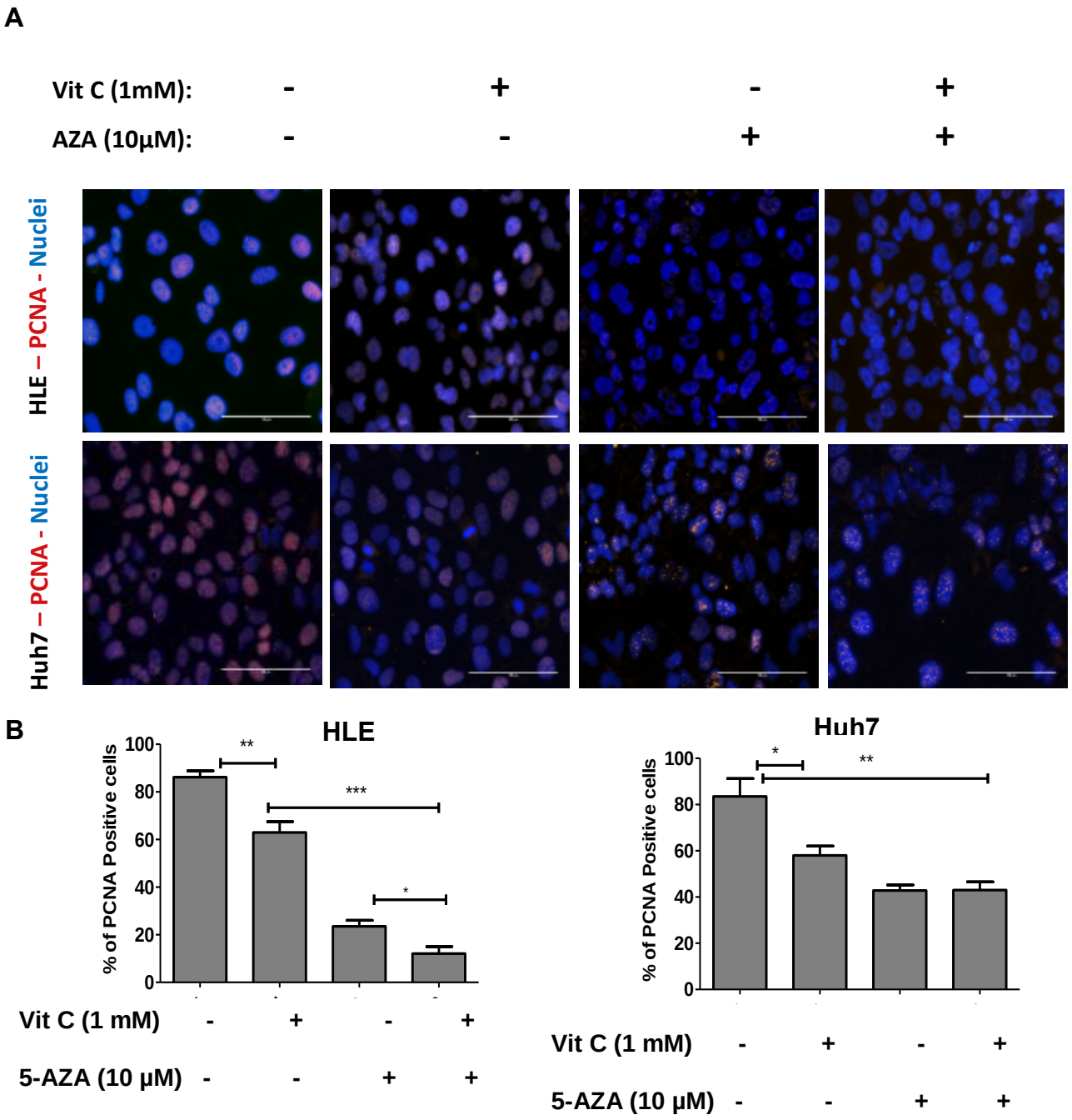
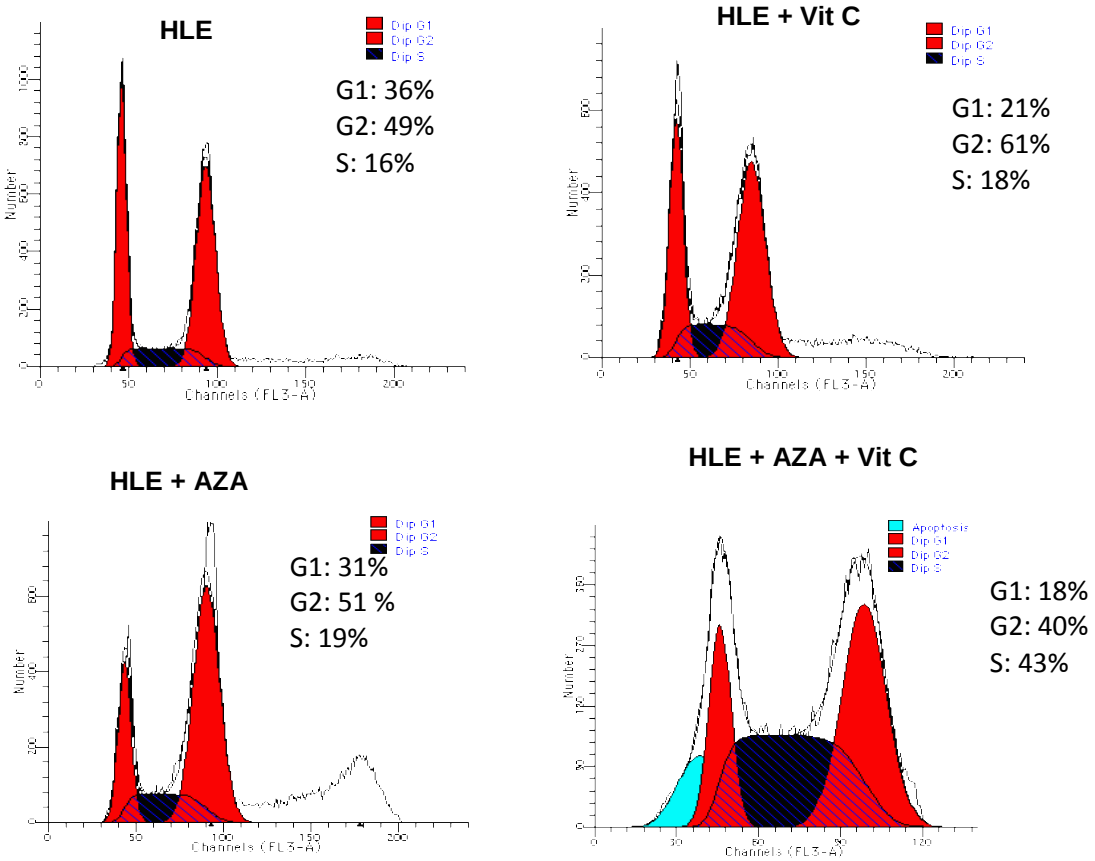
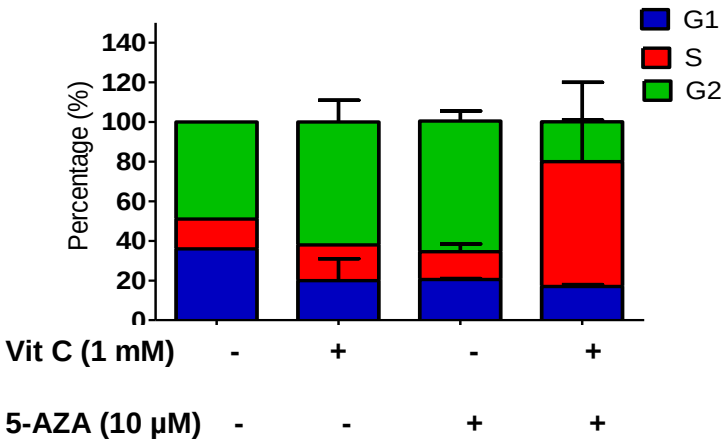



**Figure 3.9. 5-AZA and Vitamin C inhibit cell proliferation in HCC.**

**A**) PCNA nuclear staining of HCC cell lines (HLE and Huh7) illustrated the reduction of PCNA positive cells after treatment with Vitamin C and 5-AZA after 48 hrs. PCNA positive cells are seen in red, Hoechst is seen in blue (white arrowhead; scale bar, 200 µm). **B**) Graphs represent the calculation of the percentage of PCNA positive cells as an indicator of inhibition of cell proliferation in HLE and Huh7. P values were assessed by one way ANOVA, * $p < 0.05$ , **$p < 0.001$ and *** $p < 0.001$ .

**A**


HLE
G1: 36%
G2: 49%
S: 16%
HLE + Vit C
G1: 21%
G2: 61%
S: 18%
HLE + AZA
G1: 31%
G2: 51 %
S: 19%
HLE + AZA + Vit C
G1: 18%
G2: 40%
S: 43%
Channels (FL3-A)
Number


G1
S
G2
Percentage (%)
0
20
40
60
80
100
120
140
Vit C (1 mM) - + - +
5-AZA (10 µM) - - + +

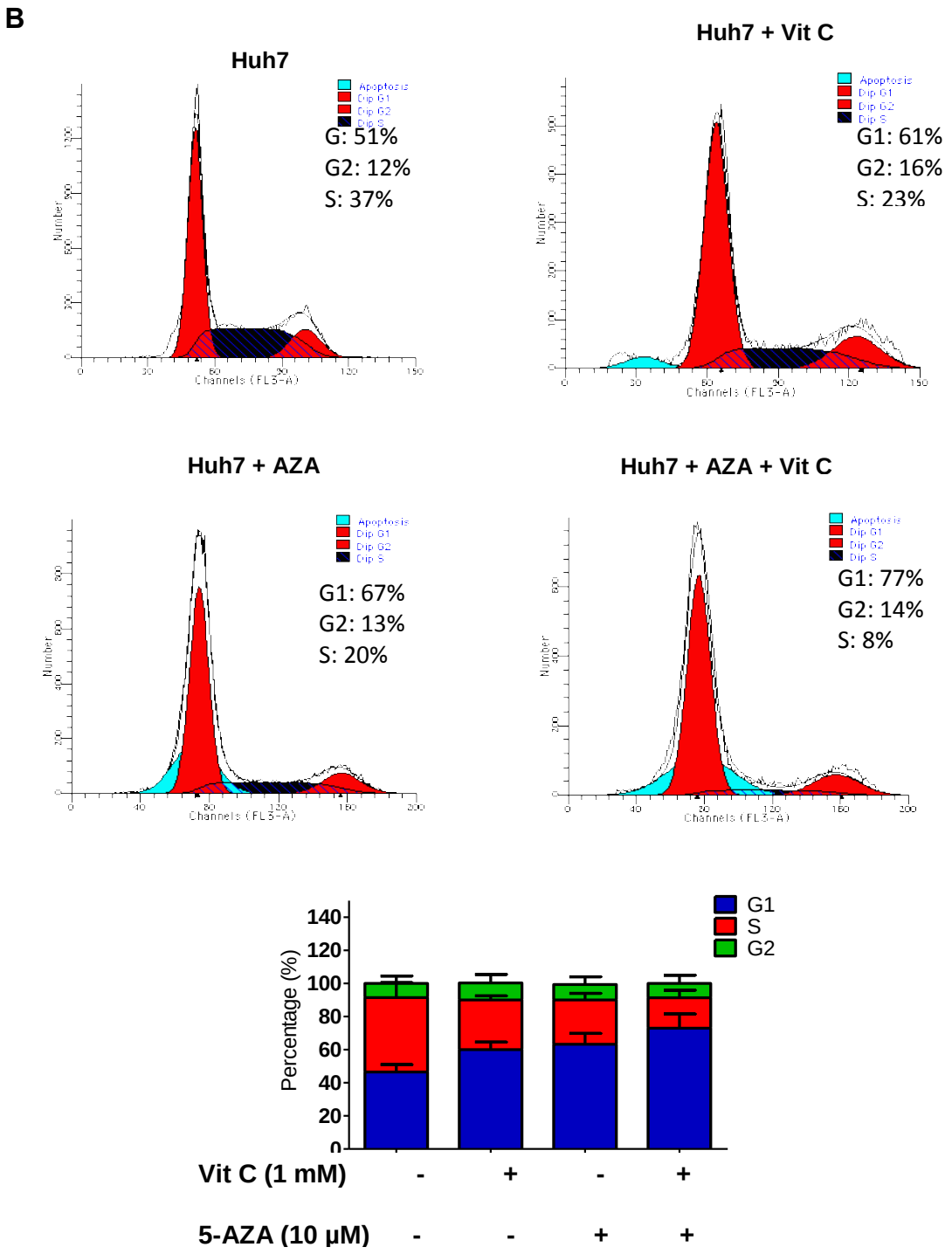


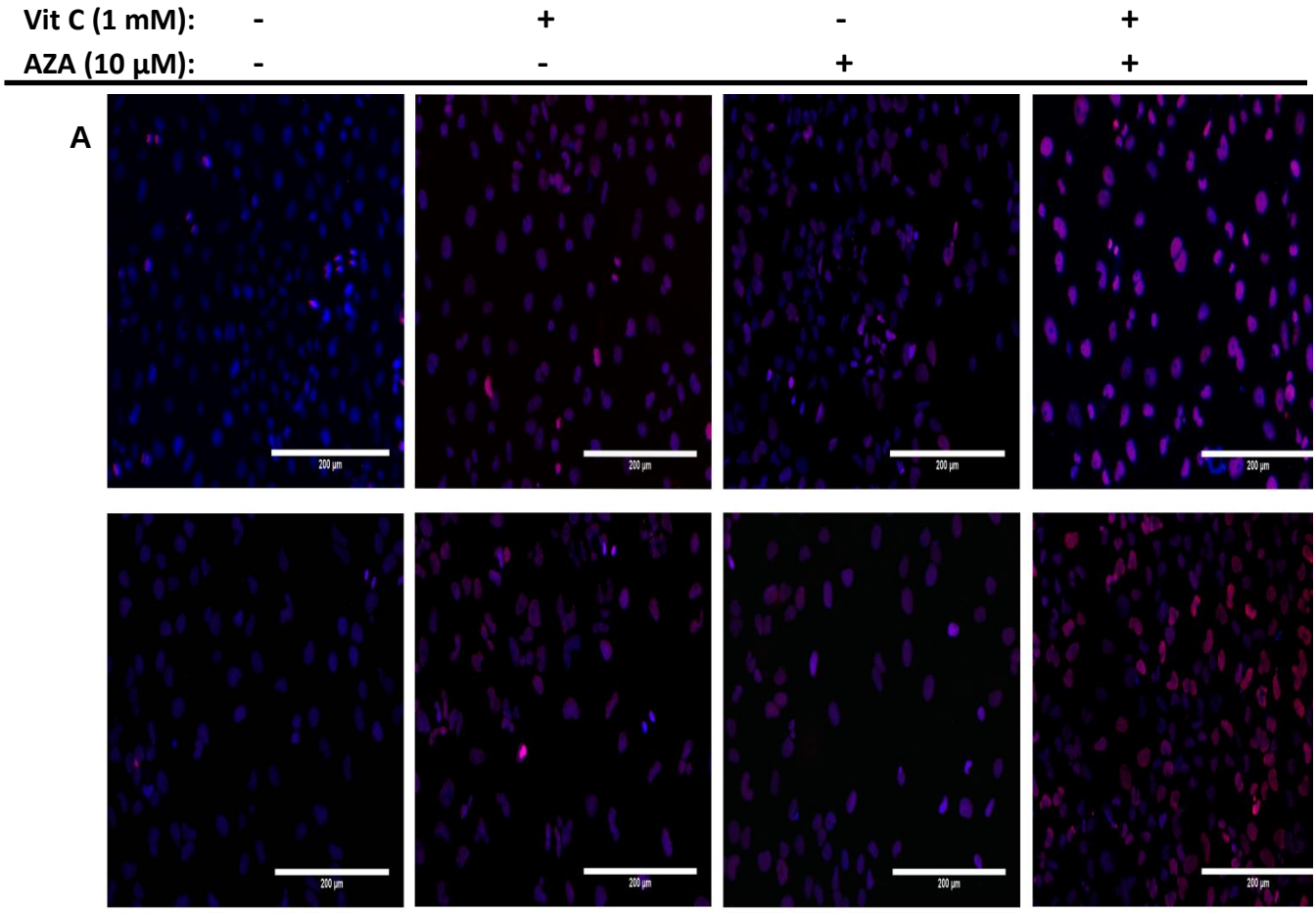

**Figure 3.10. 5-AZA and Vitamin C induce cell cycle arrest in HCC. Cell cycle analysis indicates the stage of cell cycle arrest in HLE and Huh7.**

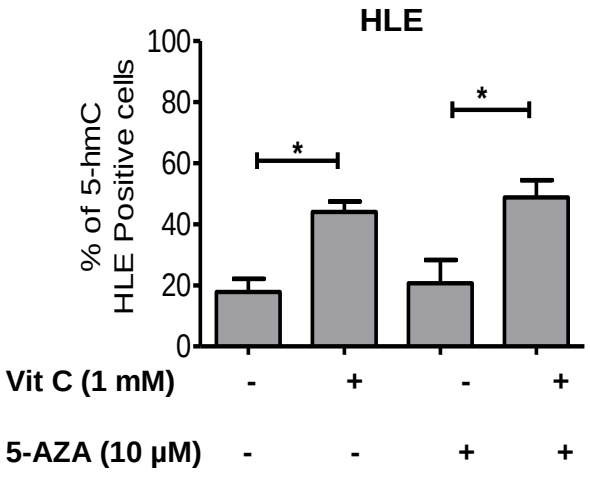
**A**) In HLE cells, the population of cells in G2 phase increased by 5-AZA and Vitamin C treatment which in combination the cell cycle was arrested in S phase. **B**) In Huh7 cells the treatment arrest cells in G1 phase. All the data present in graphics are the average of experiments (N=3, n=3).

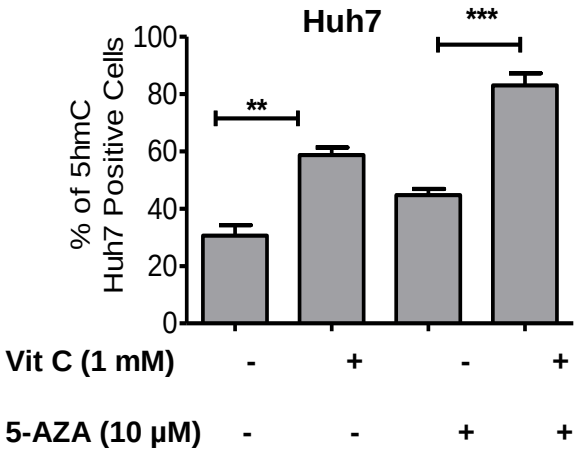
### 3.6 Vitamin C improves the efficacy of 5-AZA in TET-dependent active demethylation in HCC cell lines

In order to further evaluate the changes in the expression of genes which could have led to the cell cycle arrest, we first studied if the combination of 5-AZA and Vitamin C induces any epigenetic changes in HCC cells. Since 5-AZA and Vitamin C are both known to induce active demethylation which reflects changes in the 5hmC status [Blaschke *et al.*, 2013; Chen *et al.*, 2013a; Minor *et al.*, 2013; Sajadian *et al.*, 2015], we investigated the 5hmC content of the HCC cell lines treated with 5-AZA, Vitamin C, and the combination of both after 48 hrs. IF staining of 5hmC indicated the presence of a significantly high percentage of 5hmC positive cells with the combined treatment as compared to each single treatment in both HLE and Huh7 (Fig 3.11 A). Cells treated with Vitamin C alone also showed an increase in 5hmC as compared to 5-AZA treatment or the untreated control, underlining the importance of Vitamin C in active demethylation.

To investigate whether the effect of this increase in 5hmC intensity after treatment was correlated with changes in TET2 and TET3, the mRNA level of TET2 and TET3 was determined by real time PCR. Cells treated with the combination of 5-AZA and Vitamin C demonstrated a significantly increased expression of TET2 and TET3 as compared to the individually treated and non-treated controls in both HLE and Huh7 (Fig 3.11 B). In Huh7 cells, Vitamin C alone enhanced the expression of TET2 and TET3 while 10 µM of 5-AZA could not induce a significant increase in expression of TET2 and TET3 (Fig.3.11 B).These data confirmed by western blot too (Fig.3.11 C). These data indicate the possibility that that Vitamin C when combined together with 5-AZA could influence the conversion of 5mC to 5hmC by inducing TET2 and TET3 expression.


Vit C (1 mM): - + - +
AZA (10 µM): - - + +
A
200 µm


HLE
% of 5-hmC HLE Positive cells
100
80
60
40
20
0
*
*
Vit C (1 mM) - + - +
5-AZA (10 µM) - - + +


Huh7
% of 5hmC Huh7 Positive Cells
100
80
60
40
20
0
**
***
Vit C (1 mM) - + - +
5-AZA (10 µM) - - + +

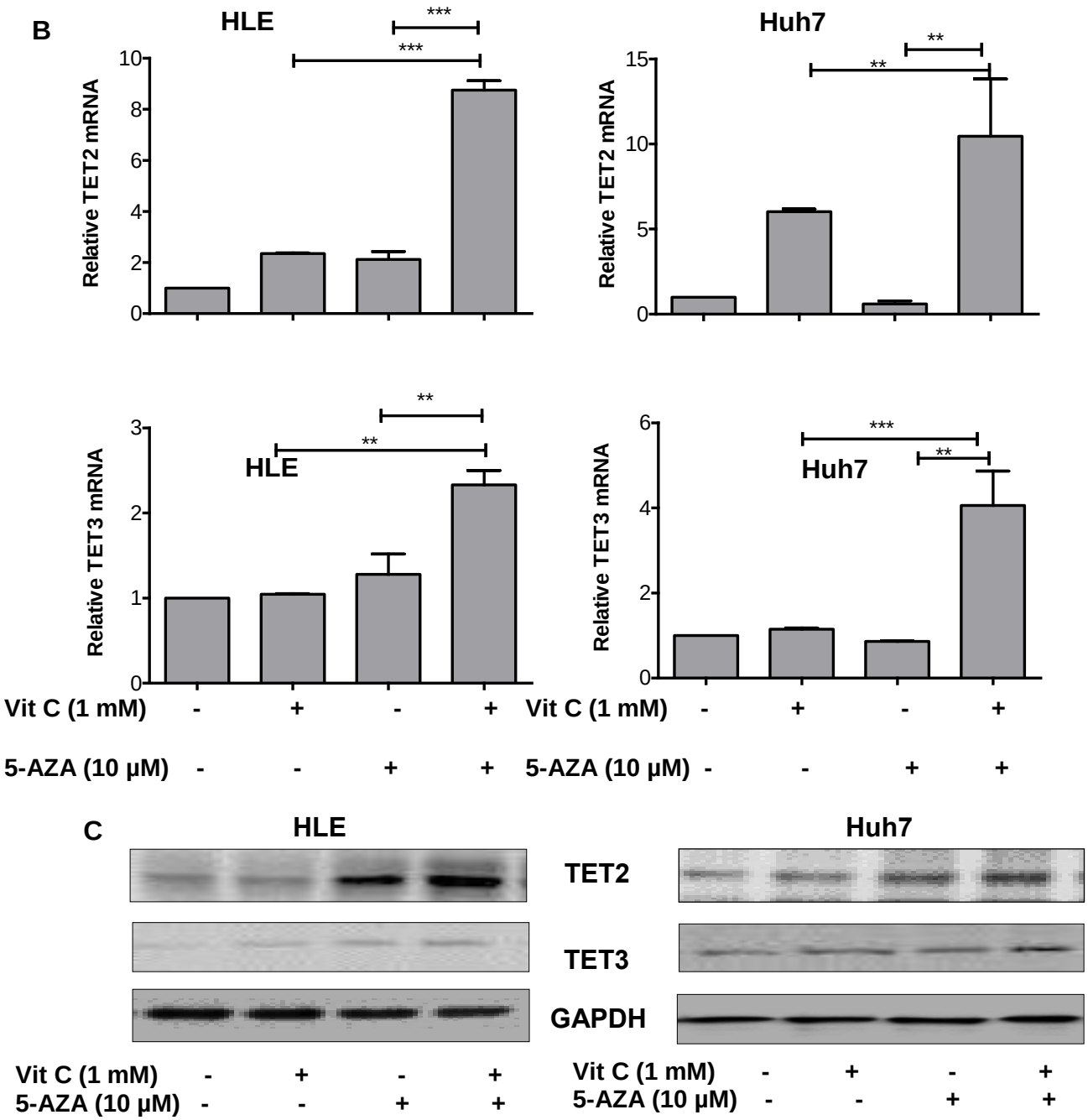



**Figure 3.11. Vitamin C enhances the efficacy of 5-AZA in inducing active demethylation and generation of 5hmc by induction of TET expression in HCC.**

**A)** 5hmC nuclear staining of HCC cell lines (HLE and Huh7) showed an increase of 5hmC intensity after treated with 5-AZA + Vitamin C. 5hmC positive cells are seen in red, Hoechst is seen in blue (white arrowhead; scale bar, 200 µm). Graphs show the calculation of the percentage of 5hmC positive cells as an indicator of active demethylation in HLE and Huh7. **B**) The Quantitative changes in the mRNA expression of TET2 and TET3 in HLE and Huh7 cells with the various treatments. Data was normalized using GAPDH expression as a reference control. All the data are average of experiments (N=3, n=3). Data were repeated three times and the value in columns represents mean ± SD. P values were assessed by one way ANOVA, * $p < 0.05$ , **$p < 0.001$ and *** $p < 0.001$ .**C**) Western Blot data confirmed the increase of TET2 and TET3 after treatment with 5-AZA and Vitamin C

## 3.7 Induction of active demethylation by 5-AZA and Vitamin C leads to downregulation of Snail and activation of GADD45

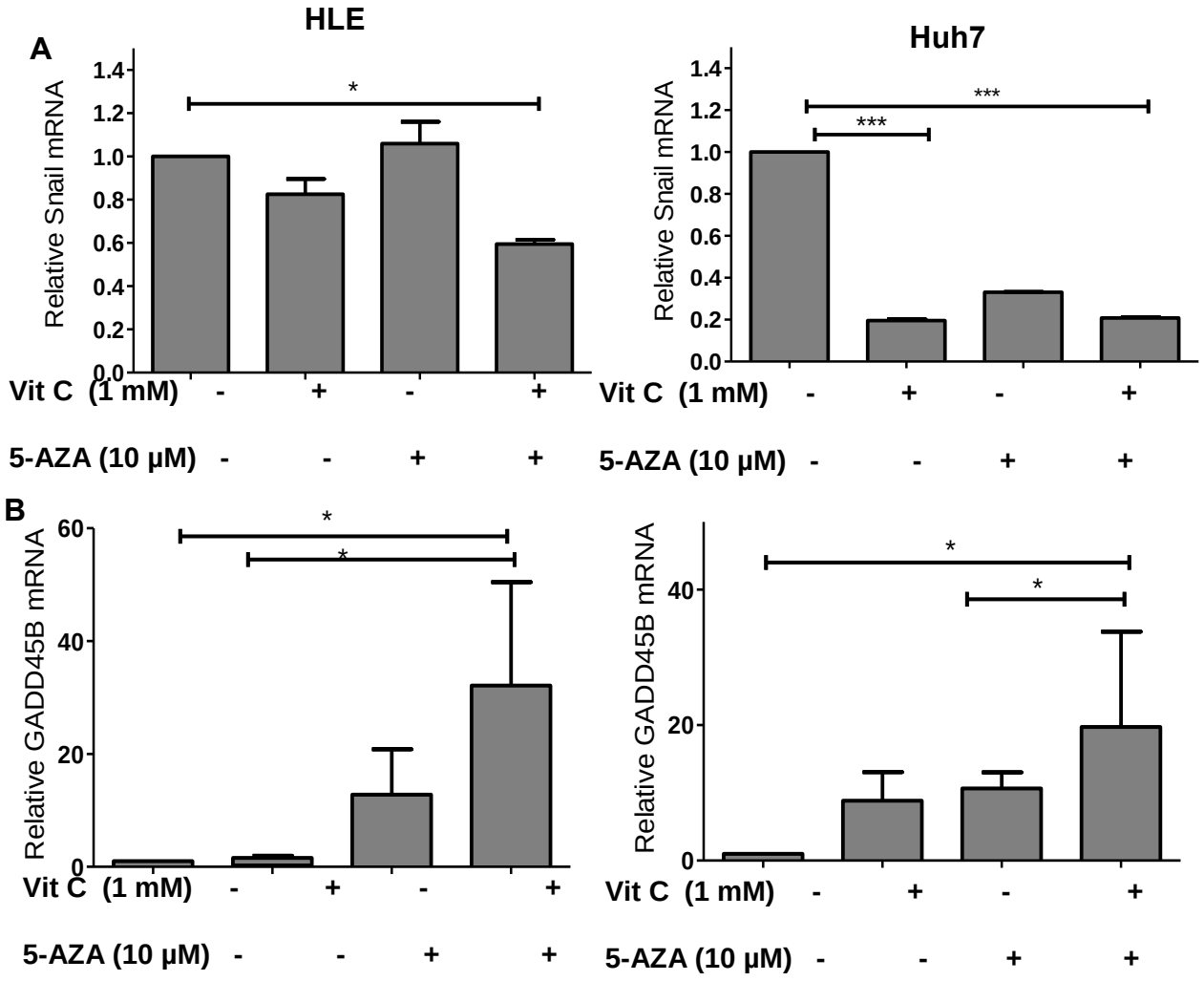
Snail is a transcription factor regulated by methylation and has an important role in mediating EMT and in inducing tumorigenesis. Therefore, we first evaluated the effect of 5-AZA and Vitamin C on Snail expression.

Our results show that HLE cells treated with Vitamin C or 5-AZA individually, show only small changes in the expression of mRNA and protein, while combination of both treatments result in a significant reduction (approximately 50 percent) of both Snail mRNA and protein levels (Fig.3.12 A and C). In Huh7 cells, a significant decrease in the mRNA was noted with Vitamin C and 5-AZA independently, and with a combination of 5-AZA + Vitamin C a decrease of 80% of mRNA level of Snail was observed in compared with non-treated Huh7 cells. The attenuation of Snail protein was also observed after treating Huh7 cells with the combination of 5-AZA and Vitamin C for 48hrs (Fig.3.12 A and C).

We next studied the expression of the DNA damage-inducible gene GADD45 which is involved in G1 or G2 cell cycle arrest [Taylor & Stark, 2001] and which has been implicated in or has been linked to the progression of HCC [Qiu *et al.*, 2004; Zerbini & Libermann, 2005]. We investigated whether the combined treatment of 5-AZA and Vitamin C resulted in an altered expression of GADD45.

In both HLE and Huh7, an increase in GADD45 transcripts and protein was observed with Vitamin C and 5-AZA independently, but the increase was significant only with the combined treatment of 5-AZA + Vitamin C (Fig.3.12 B and C). The combination

of 5-AZA and Vitamin C induced GADD45 mRNA and protein in both the HCC cell lines (Fig.3.12 B and C).Further, to investigate whether the observed changes in the expression of Snail and GADD45 are TET-dependent, quantification of Snail and GADD45B transcripts was done in TET2 and TET3 KD of HLE and Huh7 treated with 5AZA + Vitamin C. While Snail expression is down regulated in the SiRNA control, in the TET2 and TET3 KDs an increase in the expression of Snail in HLE and Huh7 was observed with the treatment (Fig.3.12 D). Similar results were observed with GADD45B, where an, increase in GADD45B expression was seen in the SiRNA controls treated with 5AZA+Vitamin C, however within the TET2 and TET3 KD's an increase in the expression of GADD45B expression was seen with the treatment with 5-AZA+Vitamin C (Fig.3.12 E).



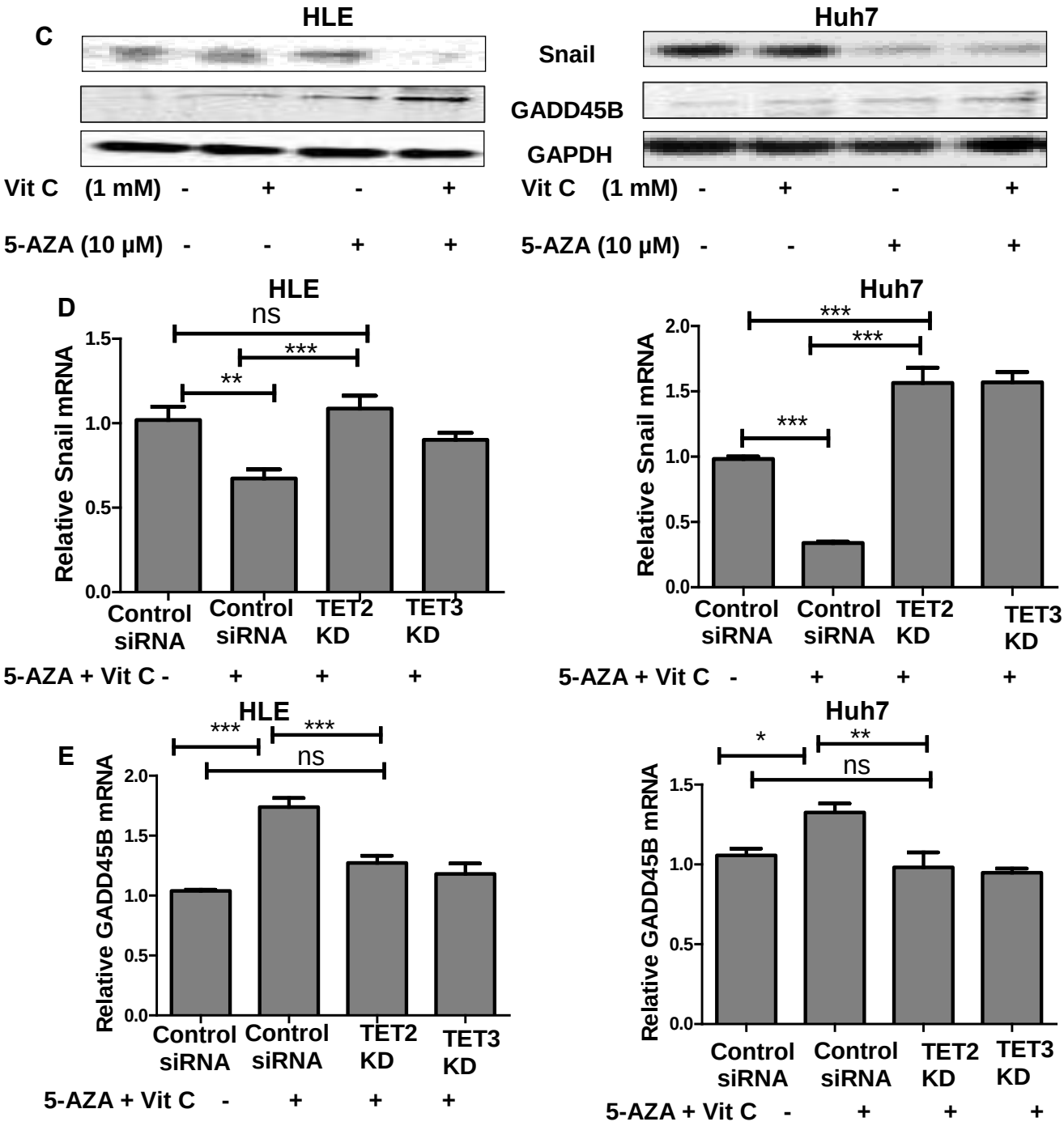



**Figure 3.12. Vitamin C enhances the downregulation of Snail and upregulation of GADD45 expression induced by 5-AZA in HCC.**

**A**) Real-time PCR quantification illustrated a decrease of Snail mRNA in HLE and Huh7 treated with 5 5-AZA + Vitamin C compared to untreated control after 48 hrs. **B**) 5-AZA together with Vitamin C increased the GADD45 mRNA level in HLE and Huh7 after 48 hrs. Data was normalized using GAPDH expression as a reference control (N=3, n=3). **C**) Western blot analysis of HLE and Huh7 showed a decrease of Snail protein and an increase of GADD45 proteins in HLE and Huh7 with combination of both treatments. Data were repeated three times and the value in columns represents mean ± SD. P values were assessed by one way ANOVA, * $p < 0.05$ and *** $p < 0.001$ .

### 3.8 Upregulated p21 and downregulated Cyclin B1 expression induce cell cycle arrest

In HLE and Huh7 cells, a significant upregulation of P21 mRNA was noted by the combination of 5-AZA + Vitamin C as compared to 5-AZA or Vitamin C individual treatments (Fig.3.13A and B). In HLE, 5-AZA and 5AZA + Vitamin C treatments induced a significant increase in p21 protein, whereas in Huh7 the increase in p21 protein was higher in the combination treatment of 5-AZA +Vitamin C than with 5-AZA alone. Since Snail represses p21[Takahashi *et al.*, 2004], it is possible that the increase in p21 may be attributed to a corresponding decrease in the Snail protein in both HLE and Huh7 leading to the arrest of the cells in G1, S and or G2 phases of the cell cycle.

We also investigated changes in Cyclin B1 protein expression as a downstream target of P21, which is essential for the progression of cells from G2 to the M phase of the cell cycle (Fig.3.13 B and C). In HLE, we observed that the expression of Cyclin B1 decreased with independent Vitamin C treatment but increased with independent 5-AZA treatment. However again, only the combination of 5-AZA + Vitamin C reduced the expression of Cyclin B1 of 60% in compared to non-treated HLE cells. As a consequence of Cyclin B1 decrease, cells in the G2 phase are inhibited to progress to the M phase, which means that cell arrest takes place in the G2/M phase of the cell cycle. In Huh7 cell line, however, we observed a significant decrease (approximately 40%) of Cyclin B1 with 5-AZA treatment alone.

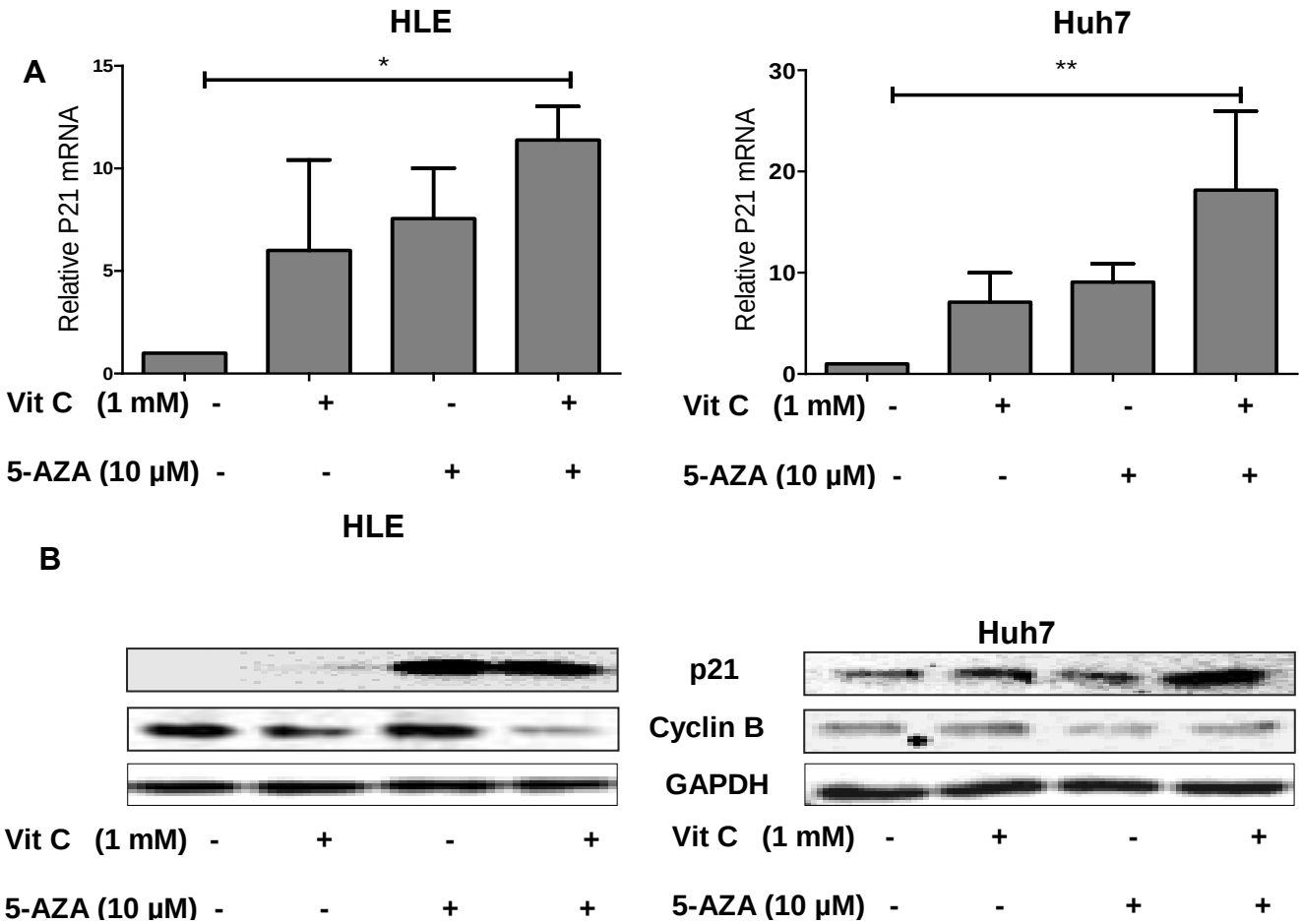



**Figure 3.13. Increased P21 and reduced cyclin B1 by 5-AZA and Vitamin C in HCC lead to cell cycle arrest.**

**A)** Quantitative analysis of Real-time PCR shows an increase of P21 mRNA level in HLE and Huh7 cells treated with Vitamin C, 5-AZA and 5-AZA + Vitamin C compared to untreated controls after 48 hrs. Data was normalized using GAPDH expression as a reference control. Data were repeated three times and the value in columns represents mean ± SD. P values were assessed by one way ANOVA, * $p < 0.05$ and ** $p < 0.01$. B) Western blot analysis showed an increase of P21 in 5-AZA treated cells which induced in combined treated cells and a decrease of Cyclin B1 proteins in HLE and Huh7 with 5-AZA + Vitamin C treatment.

### 3.9 Increased E-cadherin expression indicates a possible shift of cells towards epithelial phenotype

Snail is a direct repressor of E-cadherin and induces EMT in HCC [Qiu *et al.*, 2004; Van Zijl *et al.*, 2009]. In addition, it was reported that Vitamin C induces MET by induction of TET proteins which may prevent cancer cells from attaining further invasive traits [Chen *et al.*, 2013a]. Thus, we were interested whether or not Vitamin C and/or 5-AZA influence E-cadherin expression in HCC lines. In both HLE and Huh7 cell lines treated with Vitamin C, 5-AZA, and 5-AZA + Vitamin C, we observed an increase in E-Cadherin mRNA and protein expression as compared to the untreated controls (Fig 6 A and B). In both HCC cell lines, the combination of 5-AZA + Vitamin C showed a higher expression than 5-AZA alone. Further, it was interesting to note that in both cases Vitamin C independently was also able to induce a high expression of E-cadherin (3.14 A and B)

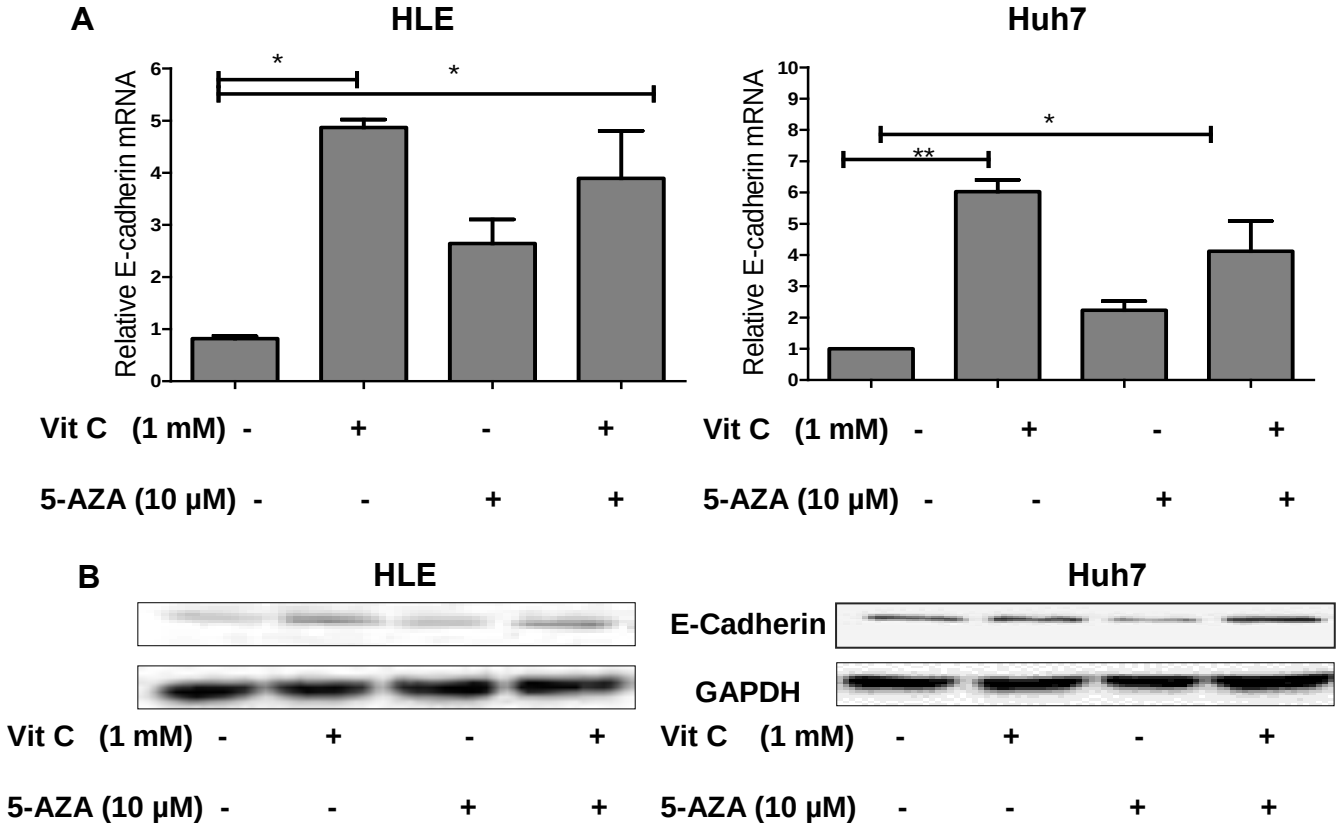



**Figure 3.14. Increased E-cadherin expression in HCC treated with 5-AZA and Vitamin C.**

**A)** Quantitative Real-time analysis showed an increase in *E-cadherin* mRNA expression in HLE and Huh7 cells treated with Vitamin C compared to untreated controls after 48 hrs. Data were normalized using *GAPDH* expression as a reference control (N=3, n=3). Data were repeated three times and the value in columns represents mean ± SD. P values were assessed by one way ANOVA, * $p < 0.05$ and ** $p < 0.01$. **B)** Western blot analysis of E-cadherin proteins in HLE and Huh7 cells with various treatments after 48 hrs. GAPDH was used as a loading control.

### 3.10 Effect of 5-AZA and Vitamin C on level of NF-κB and P16 protein expression in HLE and Huh7 cell lines

The combination of 5-AZA and Vitamin C attenuated the NF-κB protein with a concomitant increase of P16 protein level in Huh7 cells after 48 hrs. In HLE cells, 5-AZA and Vitamin C reduced the expression of NF-κB however, no effect on the expression of P16 protein was noted (Fig.3.15).

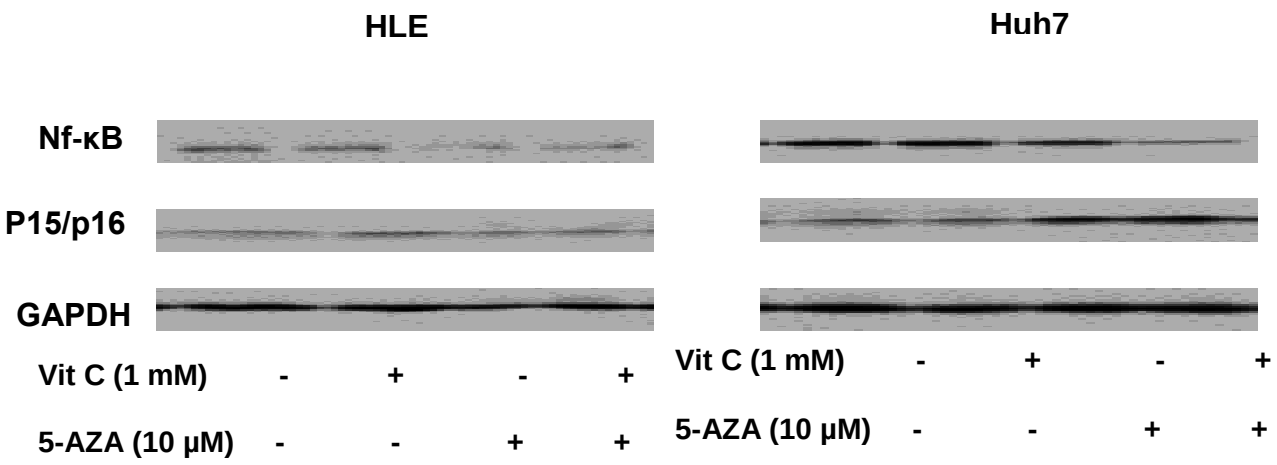



**Figure 3.15. Protein level expressions of Nf-κB and P15/P16 in Huh7 and HLE after treatment with 5-AZA and Vitamin C for 48 hrs.**

Western Blot data show the reduction of NfKB in both cell lines after treatment and a concomitant enhancement of P15/ P16 in Huh7 cells.

Altogether, we have shown that Vitamin C enhances the demethylation activity of the epigenetic drug 5-AZA and induces cell cytotoxicity. Inhibition of Snail expression leads to upregulation of p21 and simultaneous activation of GADD45 which are considered as the major effectors in inducing cell cycle arrest [Cayrol *et al.*, 1998; Kearsey *et al.*, 1995; Takahashi *et al.*, 2004; Zhao *et al.*, 2000], as shown in the HCC cell lines treated with 5-AZA and Vitamin C (Fig. 3.16).

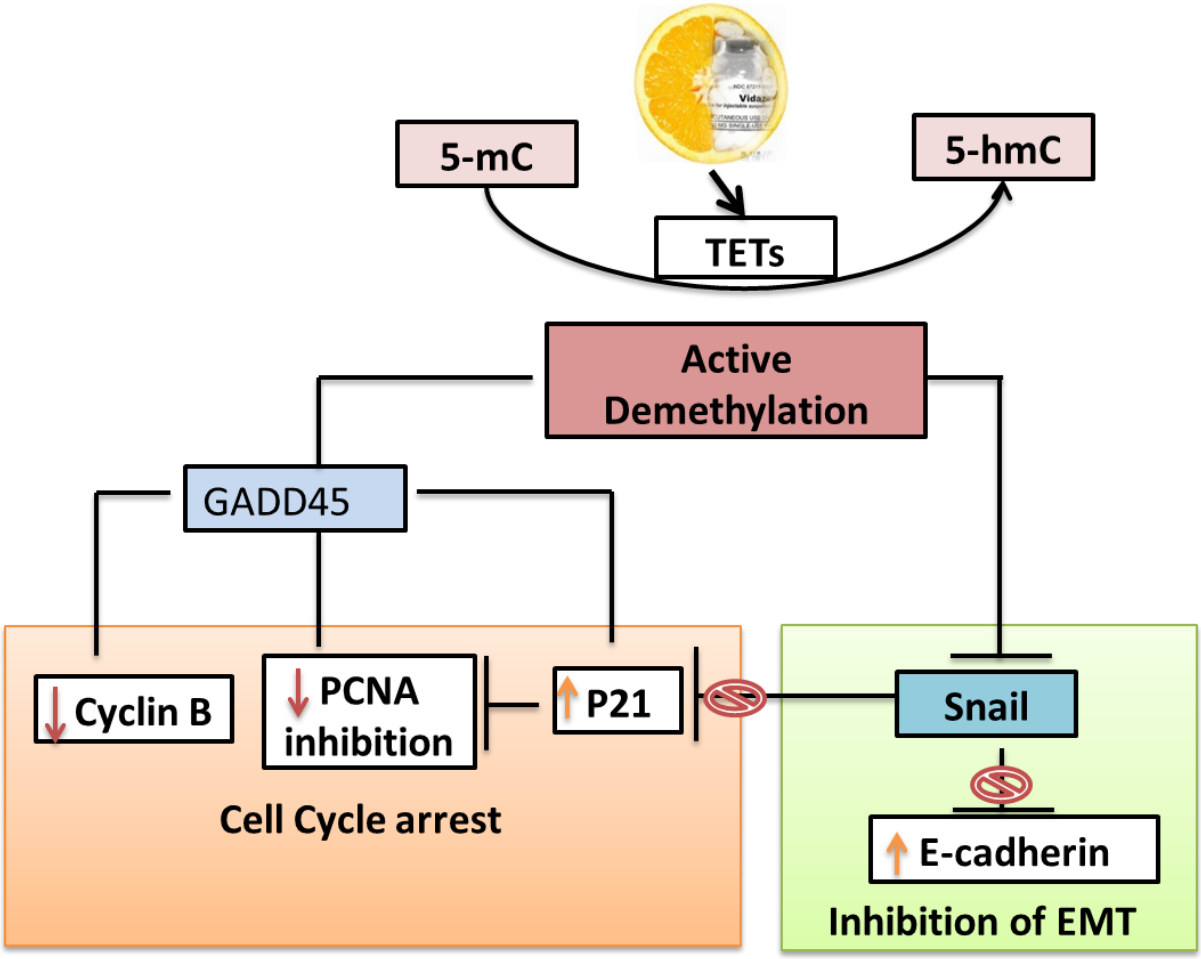



**Figure 3.16. Proposed model of epigenetic changes induced by 5-AZA and Vitamin C leading to cell cycle arrest and inhibition of EMT in HCC.**

Vitamin C enhances active demethylation induced by 5-AZA, in converting 5-methyl cytosine to 5-hydroxymethyl cytosine by increasing the expression of TET2 and TET3. Induced demethylation leads to decrease in Snail expression and upregulation of GADD45. Reduced Snail expression leads to upregulation of P21. The network of interactions between GADD45, P21, Cyclin B1 and PCNA lead to the arrest of cells in G1, S or G2/M phases of the cell cycle. On the other hand, increased expression of E-cadherin by reduction of Snail leads to the inhibition of EMT.

## 3.11 Apoptosis and Stress array for HCCs

The pathscan apoptosis array based upon sandwich immunoassay principle provides simultaneous detection of 19 signaling molecules which are involved in the regulation of the stress response and apoptosis pathway.

The analysis of pathscan array showed that the epigenetic modifiers 5-AZA and Vitamin C have an effect on the regulation of apoptosis and stress response pathway. 5-AZA and Vitamin C enhance the cleavage of caspase-3, caspase-7 and PARP in HLE cells but not in Huh7 cells. 5-AZA and Vitamin C increase the p38 MAPK and p53 but decrease TAK1 and ikBa ser 32/36 in both cell lines. Reduction of SAPK/JNK was observerd in HLE cells through combined treatment (Fig.3.17).

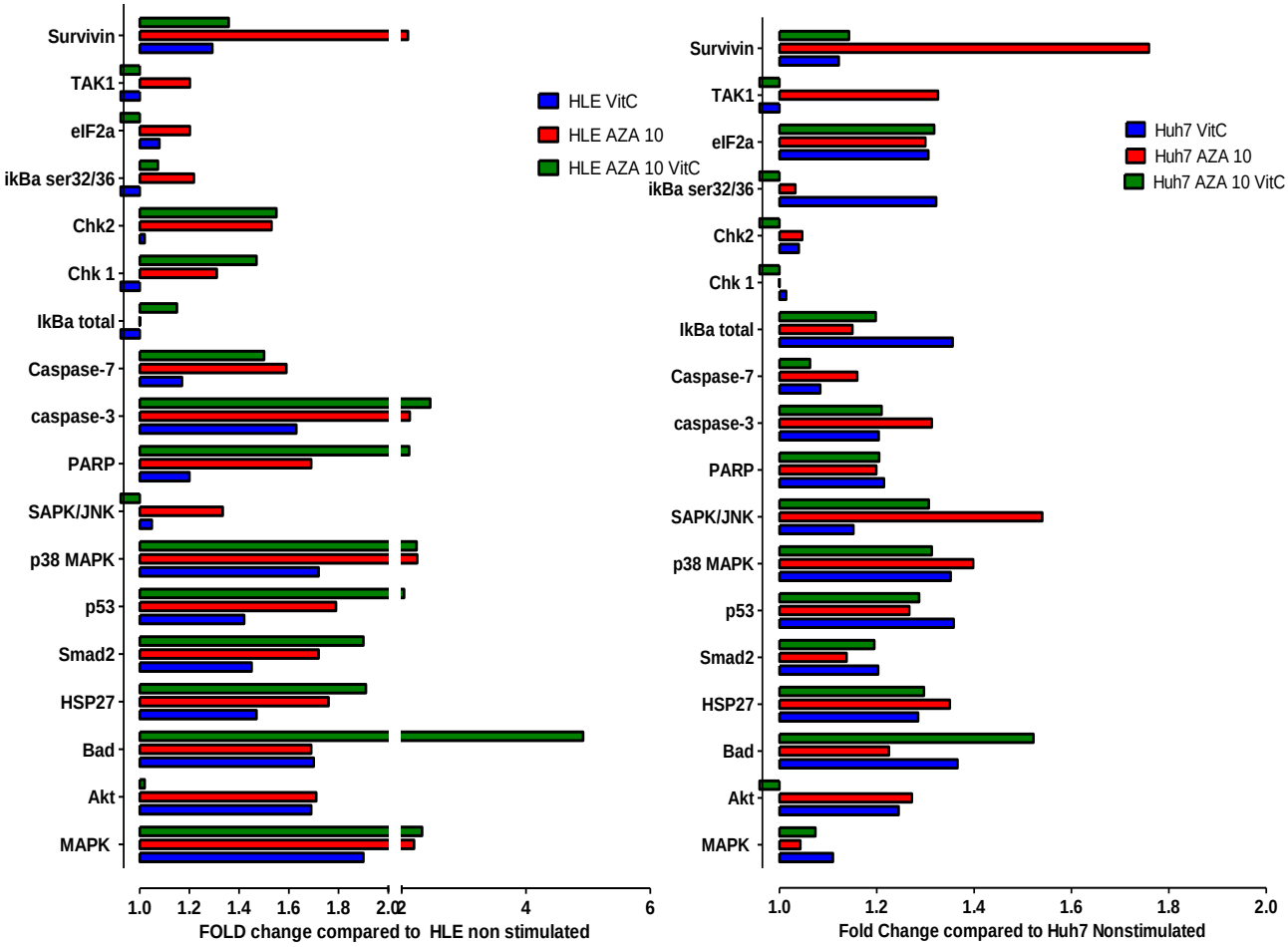
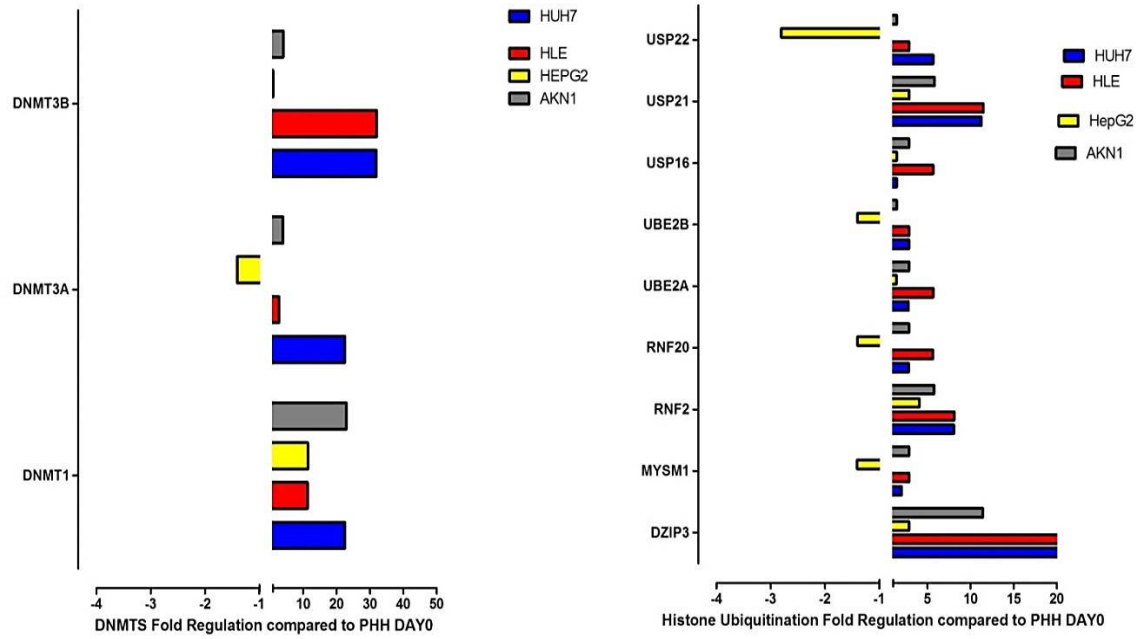


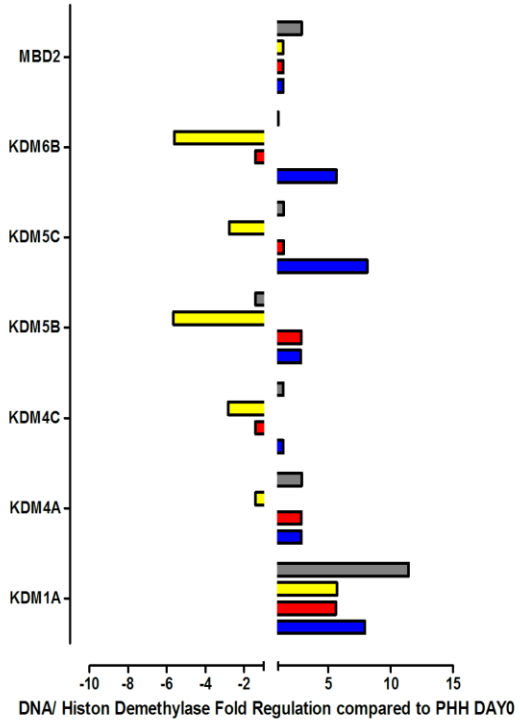

**Figure 3.17. Pathscan apoptosis and cell stress array in HLE and Huh7 cells.**

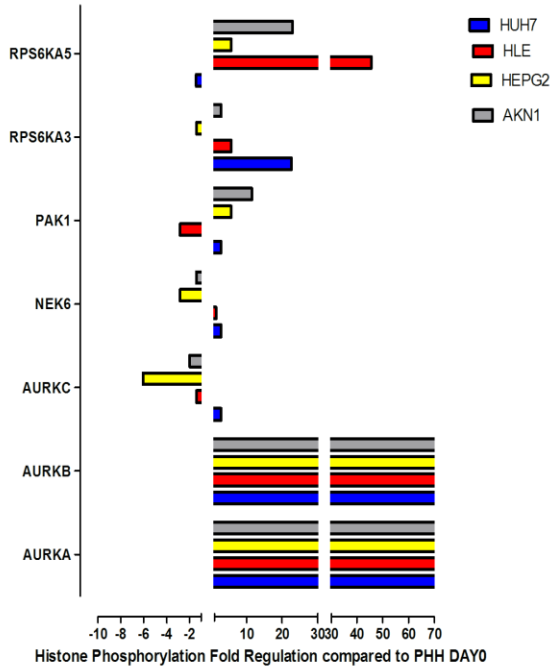
5-AZA and Vitamin C have a different regulatory effect on the expression of apoptosis and cell stress response proteins. The data were compared to an untreated cell which is considered as 1.

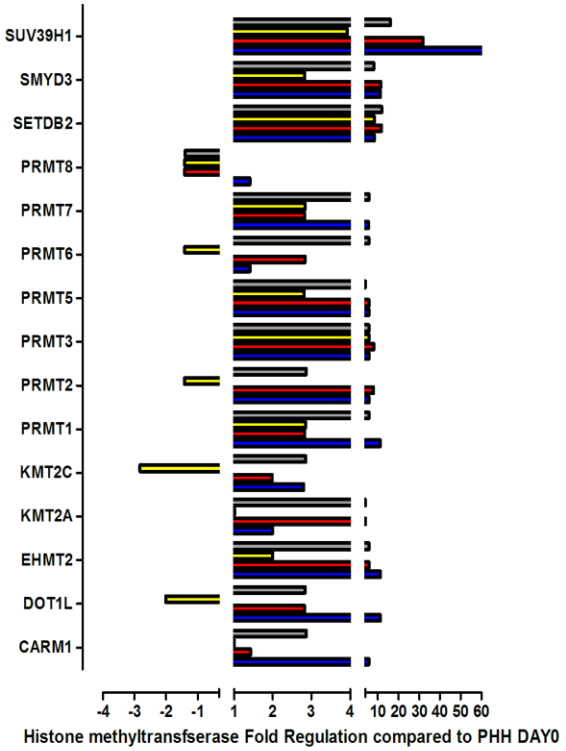
### 3.12 Human Epigenetic Chromatin Modification Enzyme array for HCCs

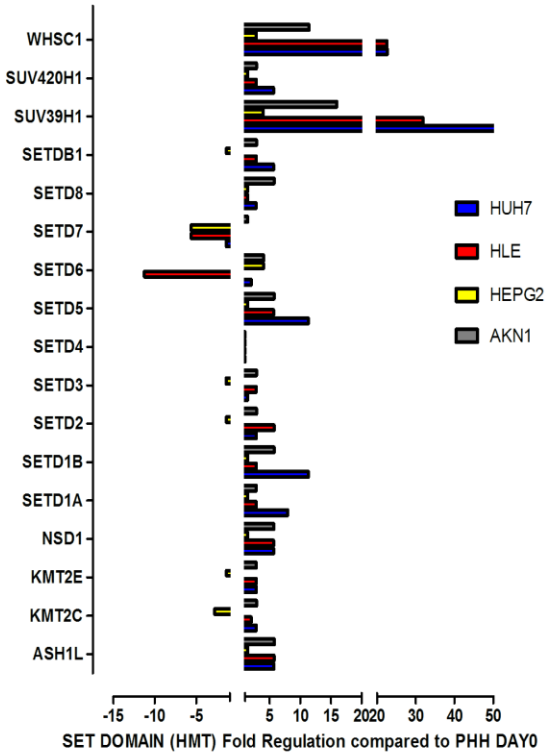
The PCR array provides the profile of the expression of 84 key genes (mentioned in the material and method section) encoding enzymes known or predicted to modify genomic DNA and histones to regulate chromatin accessibility and therefore gene expression (http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-085A.html). The de-novo and maintenance DNA methyltransferases, and the enzymes responsible for demethylation of CpG dinucleotides are represented by the array. Enzymes catalyzing histone acetylation, methylation, phosphorylation, and ubiquitination are also included on the array as well as the enzymes deacetylases and demethylases. Analyzing real-time PCR, results indicated that each of HCC cell lines consists of individual chromatin modified genes compared with primary hepatocytes (Fig 3.18). The largest differences of the chromatin status were observed in Huh7 cells and fewer differences were seen in HepG2 compared to PHH. Among four tested HCC cell lines 10 genes were upregulated which contained DNMTs (DNMT1, DNMT 3A) (Fig 3.19).




MBD2
KDM6B
KDM5C
KDM5B
KDM4C
KDM4A
KDM1A
-10 -8 -6 -4 -2 5 10 15
DNA/ Histon Demethylase Fold Regulation compared to PHH DAY0


RPS6KA5
RPS6KA3
PAK1
NEK6
AURKC
AURKB
AURKA
HUH7
HLE
HEPG2
AKN1
-10 -8 -6 -4 -2 10 20 30 30 40 50 60 70
Histone Phosphorylation Fold Regulation compared to PHH DAY0


SUV39H1
SMYD3
SETDB2
PRMT8
PRMT7
PRMT6
PRMT5
PRMT3
PRMT2
PRMT1
KMT2C
KMT2A
EHMT2
DOT1L
CARM1
-4 -3 -2 -1 1 2 3 4 10 20 30 40 50 60
Histone methyltransfserase Fold Regulation compared to PHH DAY0


WHSC1
SUV420H1
SUV39H1
SETDB1
SETD8
SETD7
SETD6
SETD5
SETD4
SETD3
SETD2
SETD1B
SETD1A
NSD1
KMT2E
KMT2C
ASH1L
HUH7
HLE
HEPG2
AKN1
-15 -10 -5 5 10 15 20 20 30 40 50
SET DOMAIN (HMT) Fold Regulation compared to PHH DAY0

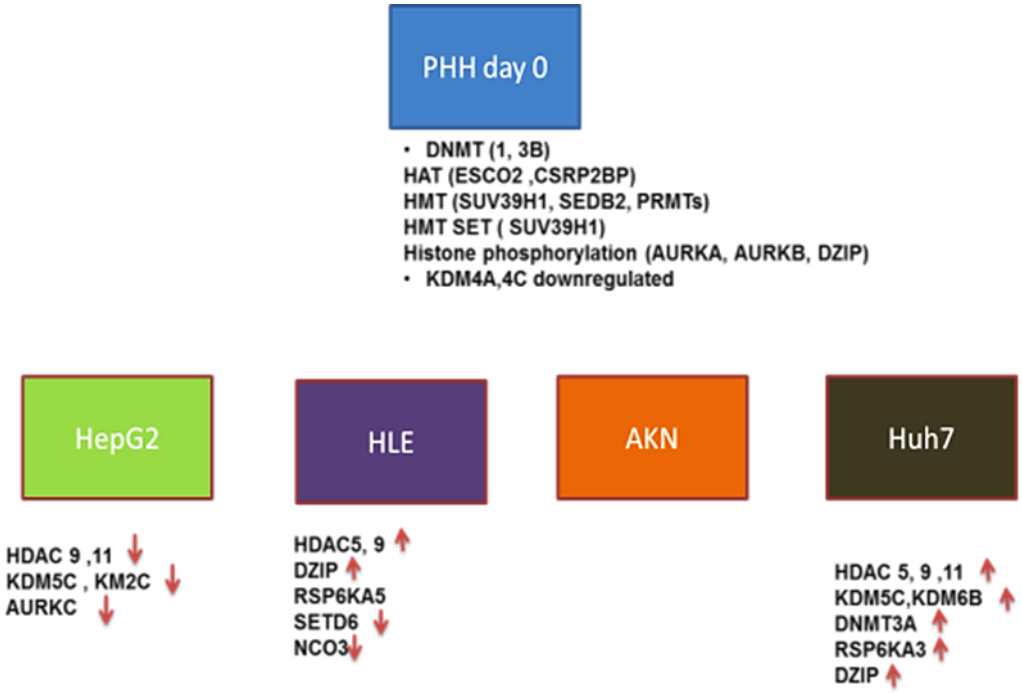



**Figure 3.18. Chromatin modification enzyme profile array.**

**A)** Relative Fold regulation of Histone methyltransferases, SET Domain, Histone phosphorylation, Histone ubiquitination, Histone demethylases and DNA methyltransferases gene expression compared with PHH. **B)** Summary of alteration of chromatin modifier regulator gene expression in HCC compared to PHH ( : downregulated, : upregulated)

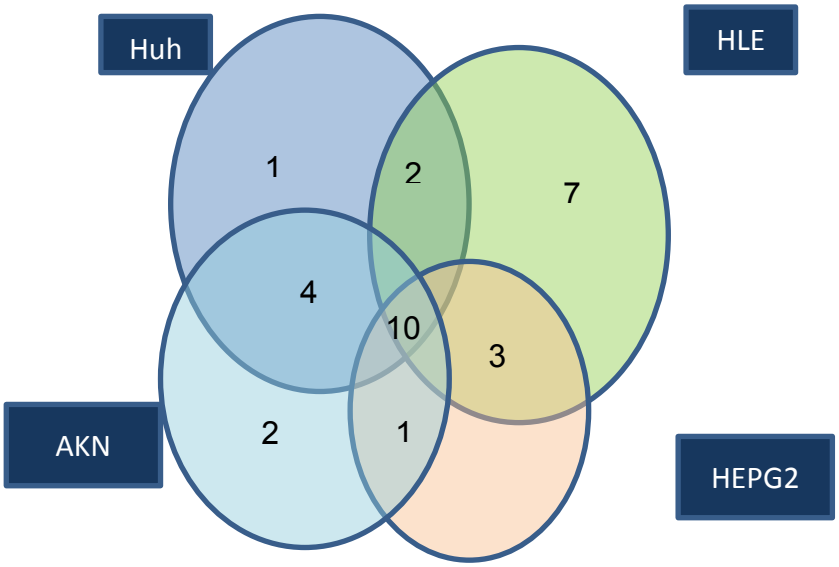



**Figure 3.19. Chromatin modification array in HCCs compared to PHH.**

Proportional Venn diagram depicting the overlap between upregulated chromatin modification enzymes identified among Huh7, HLE, AKN and HepG2 compared with human primary hepatocytes. The Venn diagram showed that ten common genes were upregulated in all 4 cell lines compared to Primary hepatocytes including DNMT1 and DNMT 3B.

## 3.13 Enzymatic activity of CYP450

### 3.13.1 Gene Expression

The expression of CYP450 was investigated in HCC cell lines after 48 hrs of treatment with 5-AZA and/or Vitamin C in 3 different passages (3 – 5 – 9). Results of mRNA expression indicated that the mRNA expression profile of CYP450 enzymes was altered depending on the confluency and the passage number of cell lines. Therefore, the passage number which showed the highest improvement of CYP enzyme gene expression was investigated in further experiments (Fig 3.20 - 3.21).

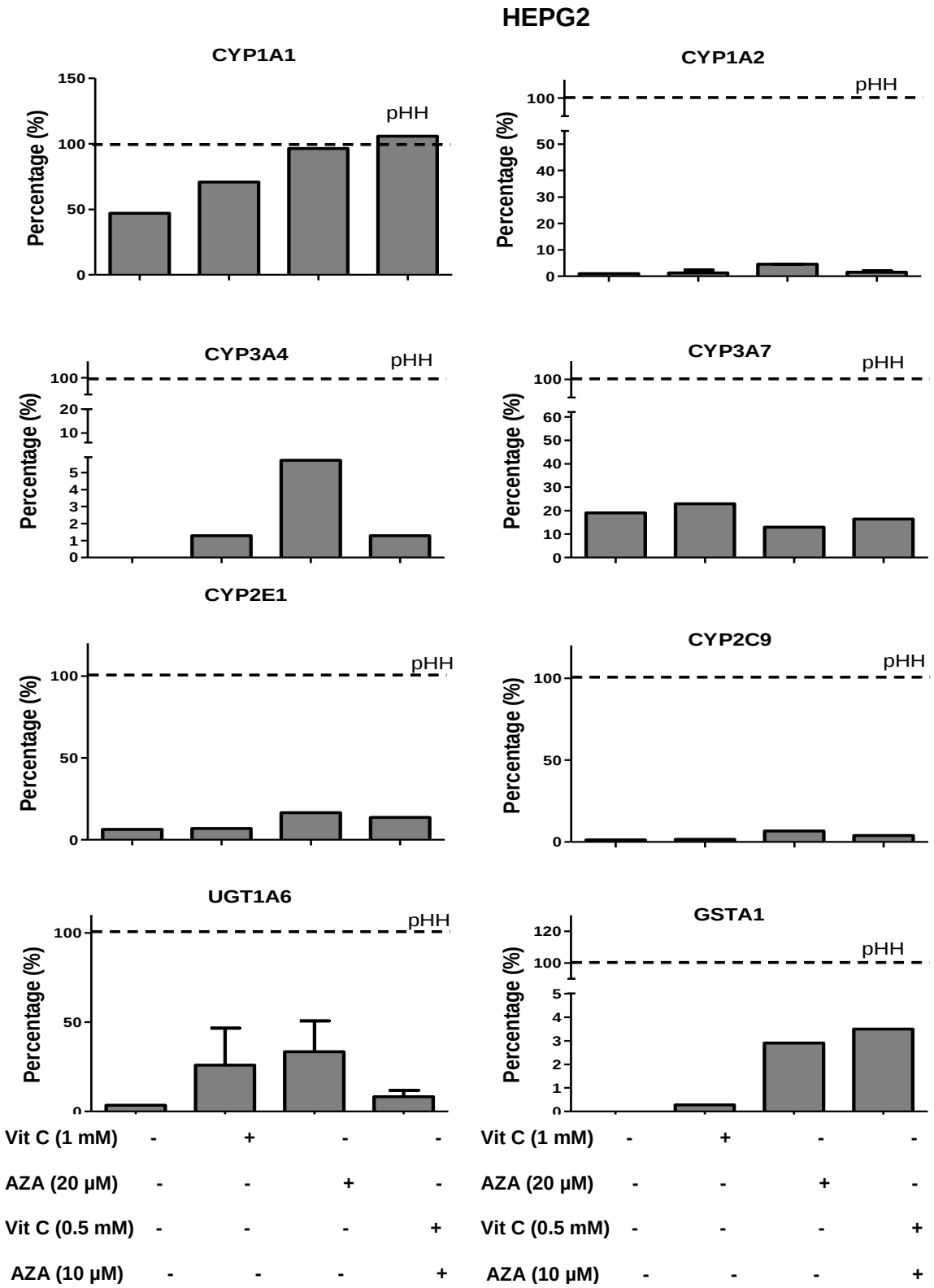



**Figure 3.20. Phase 1 and Phase 2 metabolic mRNA Expression in HepG2 cell line.**

5-AZA and Vitamin C change the expression of CYP450 genes expression after 48 hrs of treatment. CYP3A4 gene expression increased 20% more in 5-AZA treated cells than unstimulated cells. Vitamin C and 5-AZA increase the UGT mRNA level 40% more than untreated HLE cells. The dash line is the indicator of human primary hepatocytes (GAPDH used as reference gene).

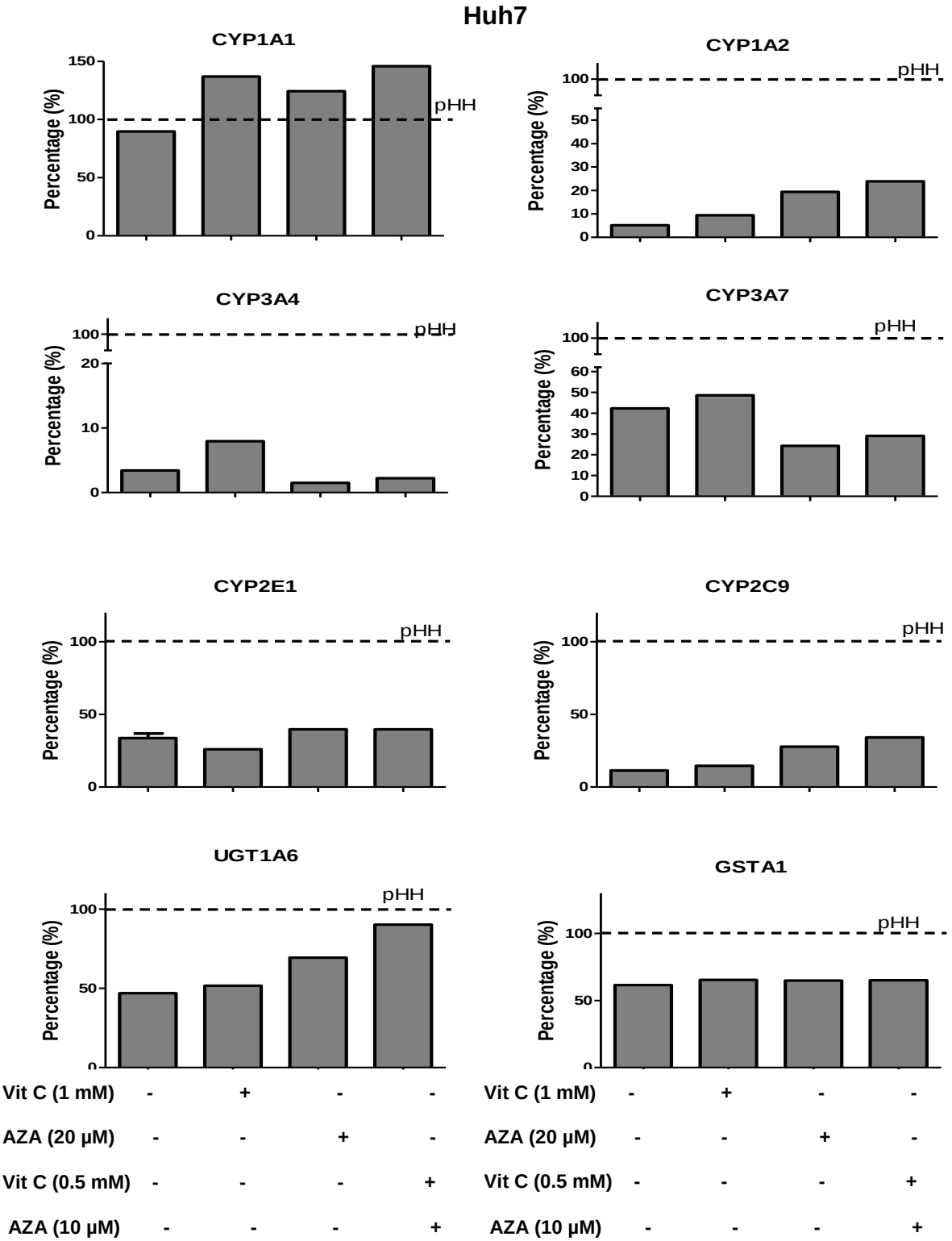



**Figure 3.21. Phase 1 and Phase 2 metabolic mRNA Expression in Huh7 cell line.**

5-AZA and Vitamin C change the expression of CYP450 genes expression after 48 hrs of treatment. CYP1A1 gene expression increased 50% more in 5-AZA + Vitamin C treated cells than unstimulated cells. Vitamin C enhances the expression of CYP3A4 and CYP3A7 in Huh7 cells. 5-AZA and Vitamin C increases the UGT mRNA level 40% more than untreated Huh7 cells. The dash line is the indicator of human primary hepatocytes (GAPDH used as reference gene).

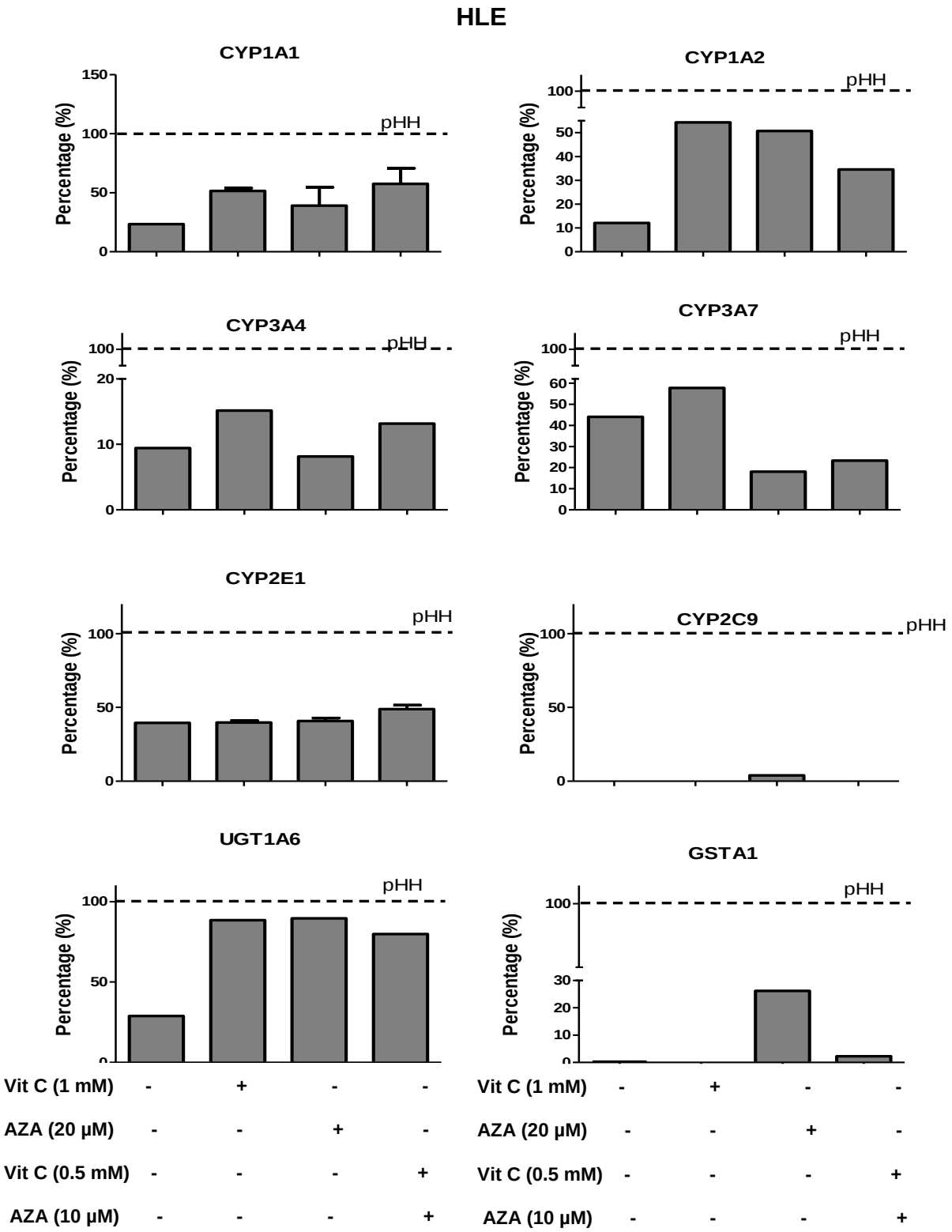



**Figure 3.22. Phase 1 and Phase 2 metabolic mRNA Expression in HLE cell line**.

5-AZA and Vitamin C change the expression of CYP450 genes expression after 48 hrs of treatment. CYP1A1 gene expression increased 50% more in the combined treatment than unstimulated cells. Vitamin C and 5-AZA increase the UGT mRNA level 40% more than untreated HLE cells. The dash line is the indicator of human primary hepatocytes. (GAPDH used as reference gene).

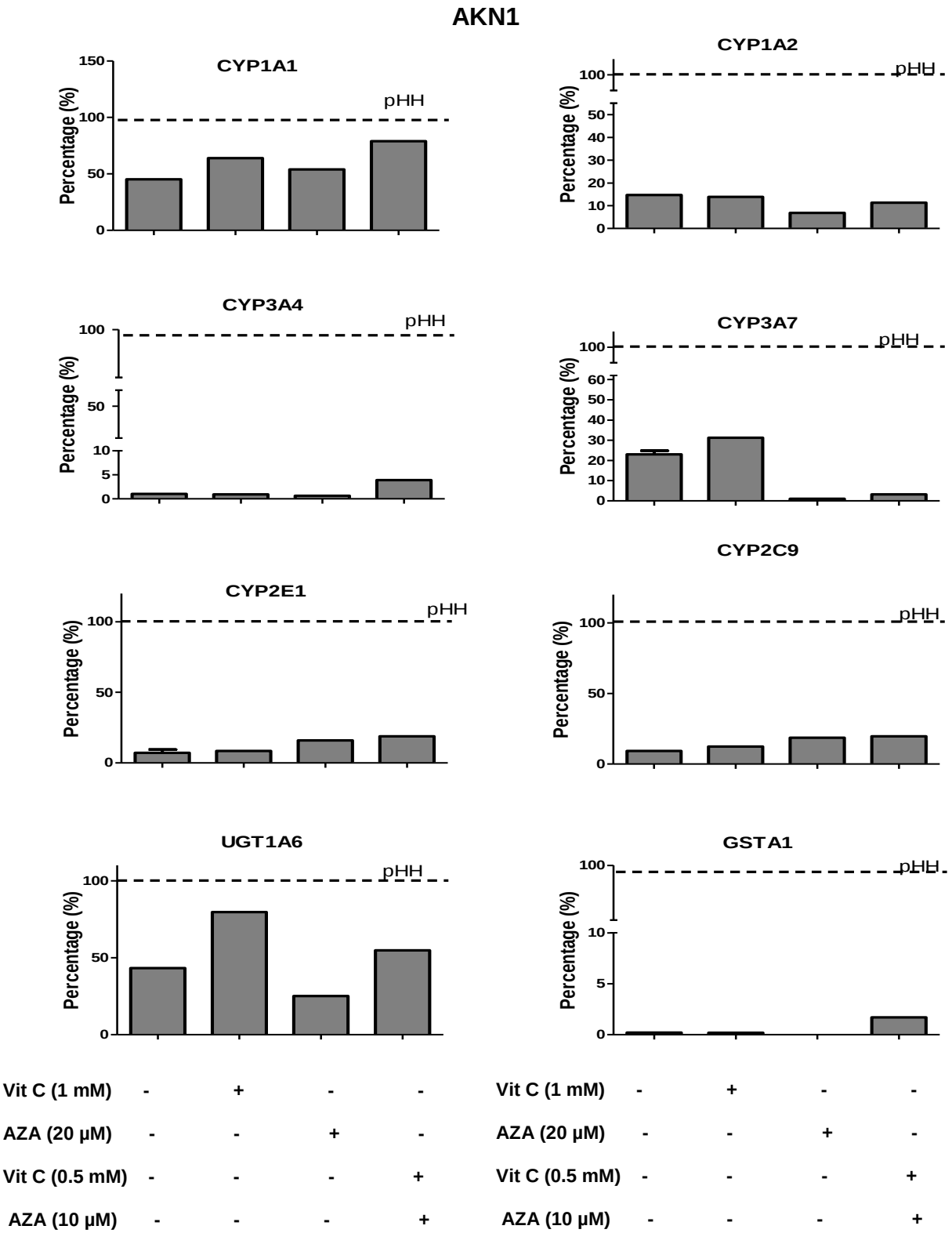



**Figure 3.23. Phase 1 and Phase 2 metabolic mRNA Expression in AKN1 cell line**.

5-AZA and Vitamin C change the expression of CYP450 genes expression. After 48 hrs of treatment CYP1A1 gene expression increased 30% more in the combined treatment than unstimulated cells. Vitamin C enhances the expression of UGT 40 % more than untreated cells. The dash line is the indicator of human primary hepatocytes (GAPDH used as reference gene).

### 3.13.2 Phase I and Phase II of enzyme activity

Phase I enzyme activity was measured by fluorescence-based assays after 48 hrs of treatment. The results showed that 20 µM of 5-AZA enhanced the CYP1A1 activity in all four HCC cell lines. CYP3A4 enzyme activity seems to be induced by 5-AZA while, Vitamin C enhanced the effect of 5-AZA on CYP3A4 enzyme activity in AKN 1 and HLE. 5-AZA increased the CYP2E1 enzyme activity in all four cell lines, interestingly in HePG2, Huh7 and AKN cells treated with 5-AZA, the enzyme activity of CYP2E1 overtakes Primary Hepatocyte enzyme activity and Vitamin C and 5-AZA can both enhance the level of CYP2C9. The UGT induced more in the combined treated cells than single treated. GST enzyme activity induced more by 5-AZA than combined treatment. However, the extension of the effect of Vitamin C and 5-AZA on CYP450 activity was different among the investigated HCC cell lines (Fig 3.24 - 3.26).

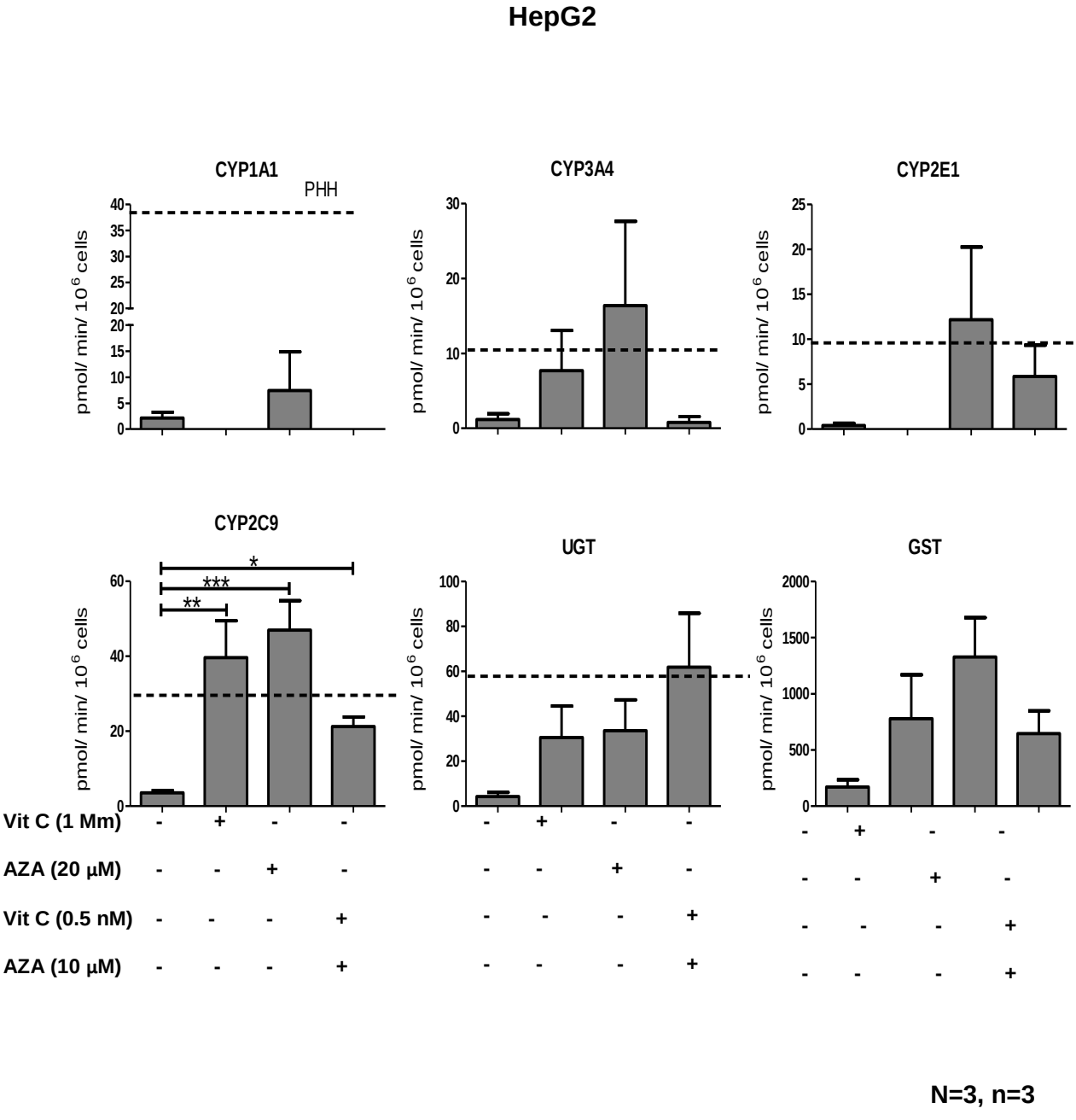



**Figure 3.24. Phase 1 and Phase 2 metabolic enzyme activity in the HepG2 cell line.**

5-AZA treatment increases the CYP1A1 and CYP3A4 enzyme activity approximately by 10% and for CYP2C9 by 50% compared to untreated HepG2 cells after 48 hrs of treatment. UGT and GST also increased after 5-AZA treatment plus Vitamin C. The dash line shows the primary human hepatocyte enzyme activity as a gold standard. Data were repeated three times and the value in columns represents mean ± SD. P values were assessed by one way ANOVA, * $p < 0.05$ , ** $p < 0.001$, *** $p < 0.001$.

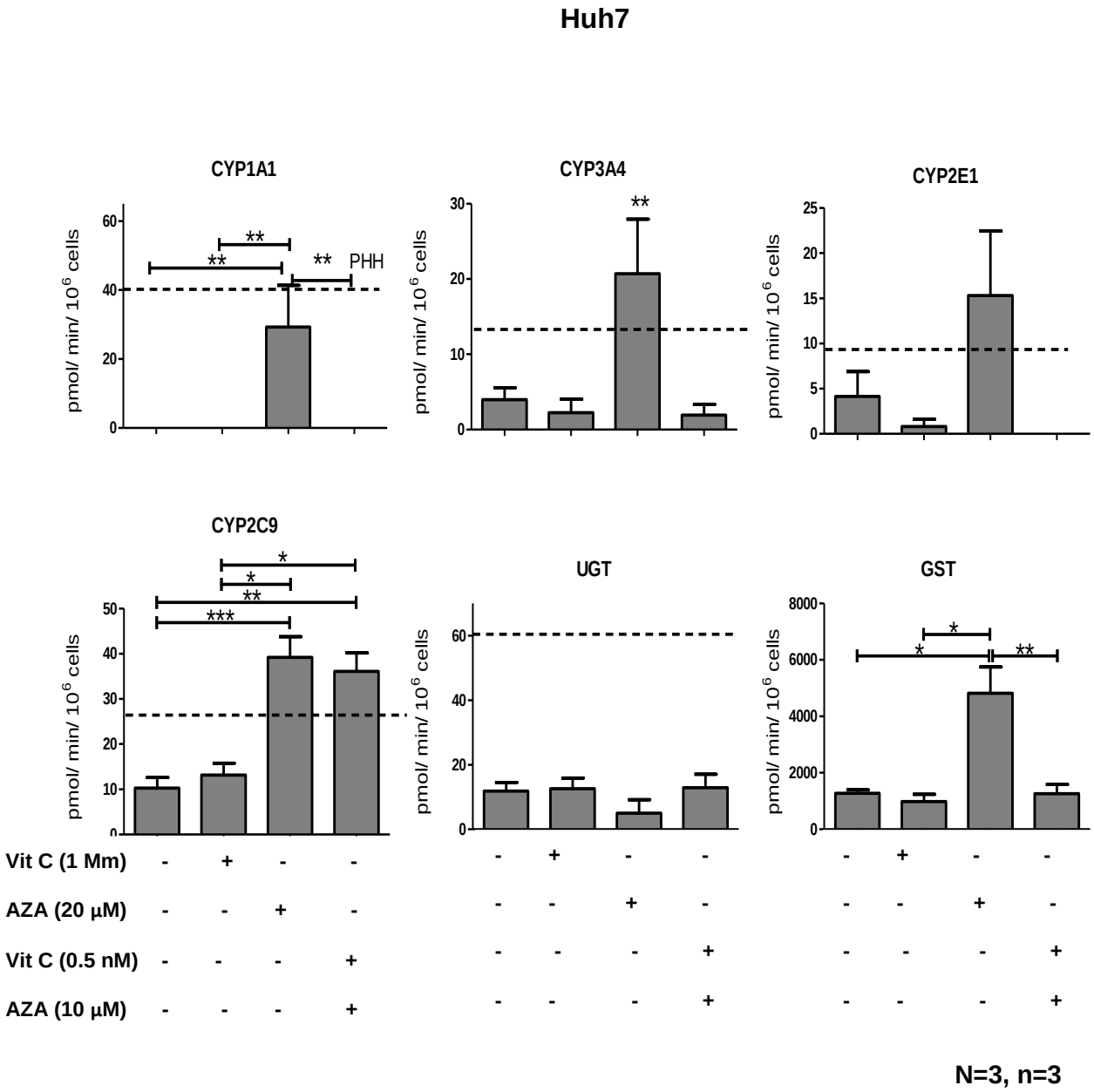



**Figure 3.25. Phase 1 and Phase 2 metabolic enzyme activity in Huh7 cell line.**

5-AZA increases CYP1A1 (30%) and CYP3A4 (20%) CYP2E1 (10%) and CYP2C9 (30%) more than untreated Huh7 cells after 48 hrs of treatment. GST increased after treatment with 5-AZA and Vitamin C. The dash line shows primary human hepatocyte enzyme activity as a gold standard. Data were repeated three times and the value in columns represents mean ± SD. P values were assessed by one way ANOVA, * $p < 0.05$ , ** $p < 0.001$, *** $p < 0.001$.

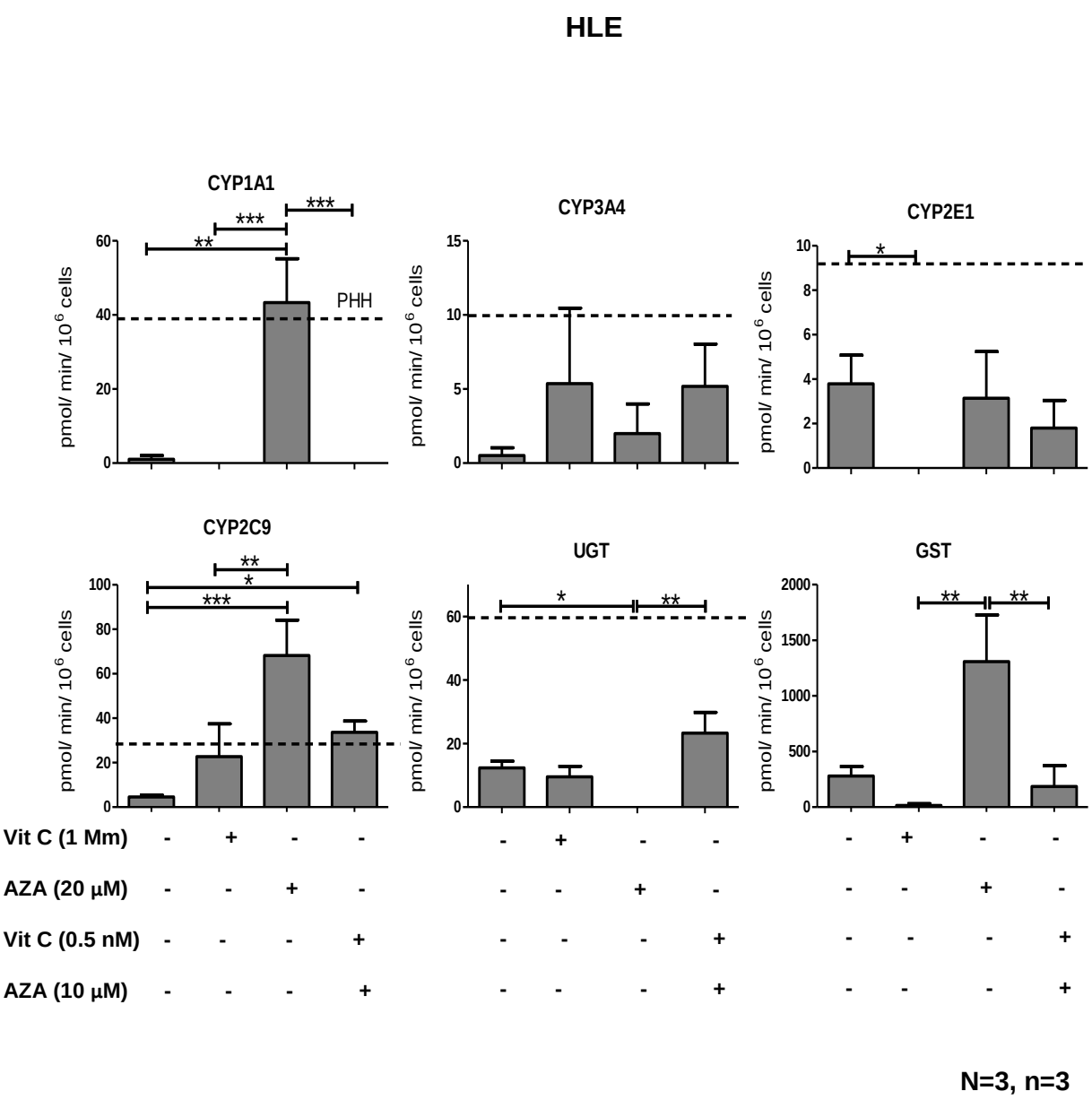



**Figure 3.26. Phase 1 and Phase 2 metabolic enzyme activitiy in HLE cell line.**

5-AZA increases CYP1A1 and CYP2E1 enzyme activity and CYP2C9 50% more than untreated Huh7 cells after 48 hrs of treatment. UGT increased after treatment with 5-AZA and Vitamin C. The dash line shows primary human hepatocyte enzyme activity as a gold standard. Data were repeated three times and the value in columns represents mean ± SD. P values were assessed by one way ANOVA, * $p < 0.05$ , ** $p < 0.001$, *** $p < 0.001$.

**AKN1**

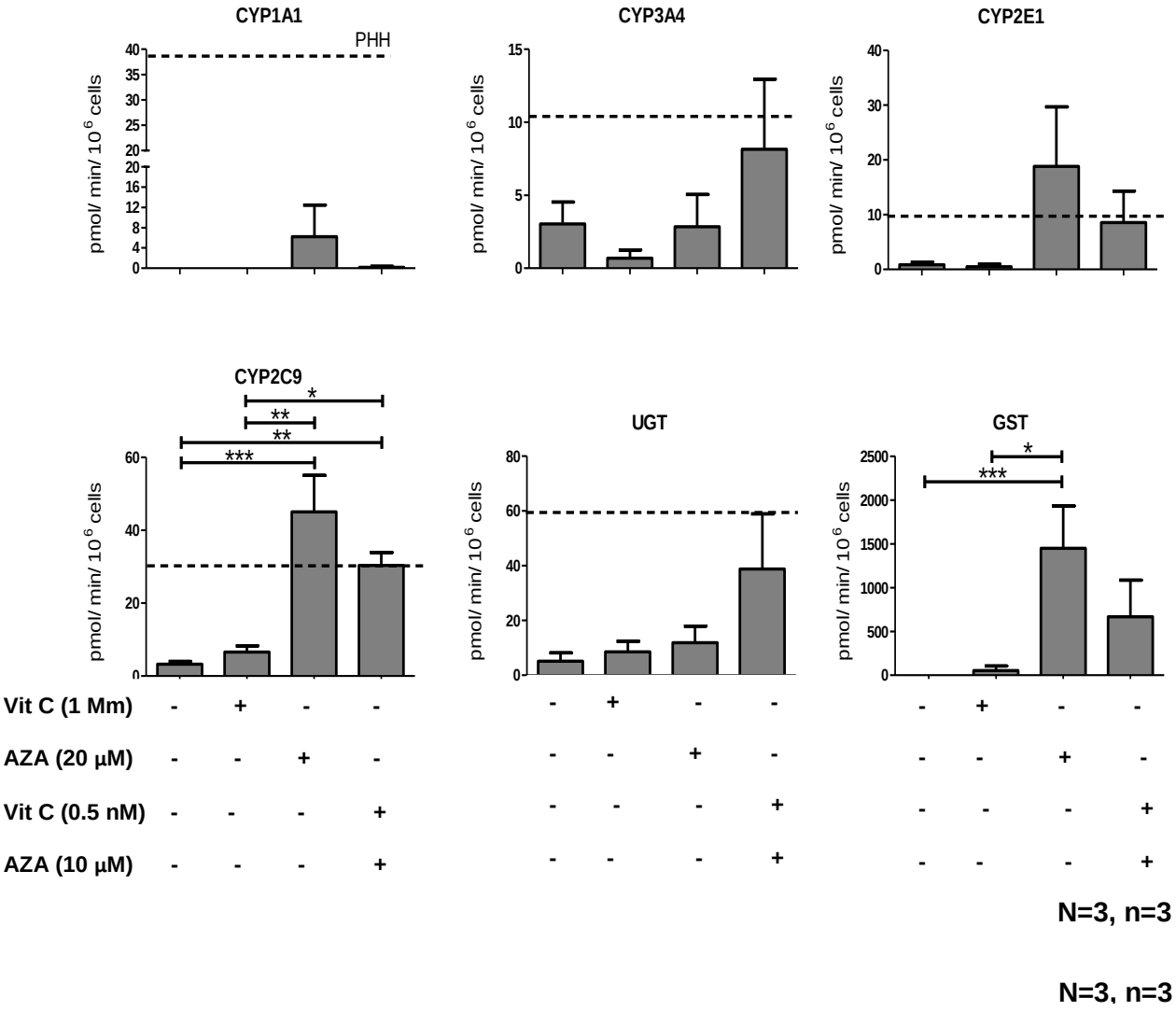



**Figure 3.27. Phase 1 and Phase 2 metabolic enzyme activity in AKN1 cell lines.**

The CYP1A1, CYP3A4 and CYP2E1 enzyme activity increased after treatment with 5-AZA for 48 hrs. CYP2C9 enzyme activity level improved more than 30% of the untreated cells. dash line shows primary human hepatocyte enzyme activity as a gold standard Data were repeated three times and the value in columns represents mean ± SD. P values were assessed by one way ANOVA, * $p < 0.05$ , ** $p < 0.001$, *** $p < 0.001$.

### 3.14 Ammonia detoxification

Detoxification of ammonia usually takes place in liver, but HCC cells lines lose the ammonia detoxification capacity. Therefore, we had interest to investigate the effect of epigenetic modifiers on ammonia detoxification pathway. HCC Cell lines were treated with 5-AZA and Vitamin C and the urea production was measured by florescence based assay. Our results show that 5-AZA enhanced urea production in all four cell lines which is induced by ornithine. In Huh7 the urea production reached the level of urea production of PHH after treatment of cells with 5-AZA for 48 hrs (Fig.3.28 and Fig. 3.29).

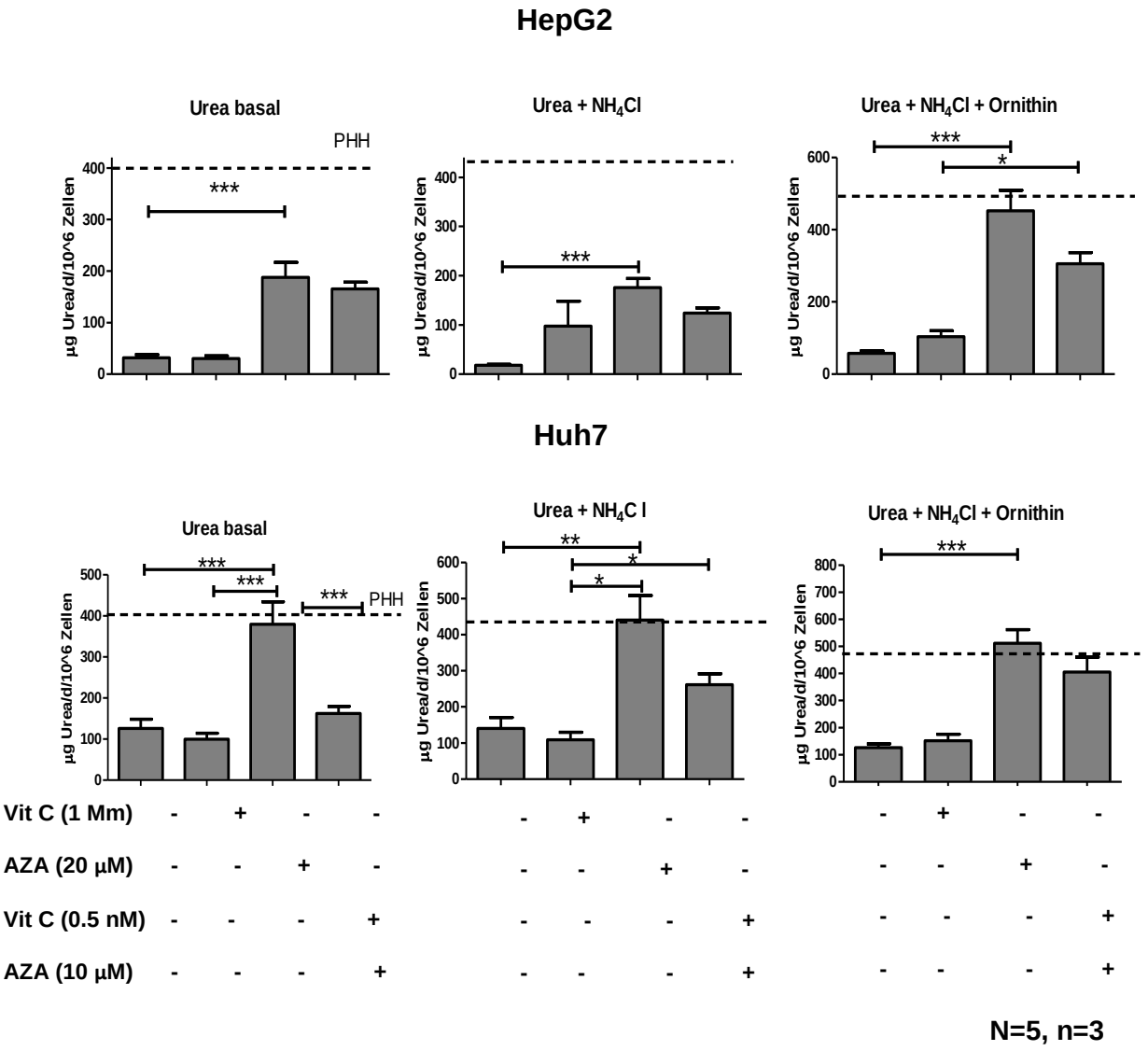



**Figure 3.28. Detoxification of ammonia in HepG2 and Huh7 cell lines after 48 hrs of treatment with 5-AZA plus Vitamin C.**

The absorbance was measured by plate reader. 5-AZA and Vitamin C have an effect on urea production which it increased by adding NH4Cl and Ornithine (N=5, n=3). Red line shows primary human hepatocyte enzyme activity as a gold standard Data were repeated three times and the value in columns represents mean ± SD. P values were assessed by one way ANOVA, * $p < 0.05$ , ** $p < 0.001$, *** $p < 0.001$.

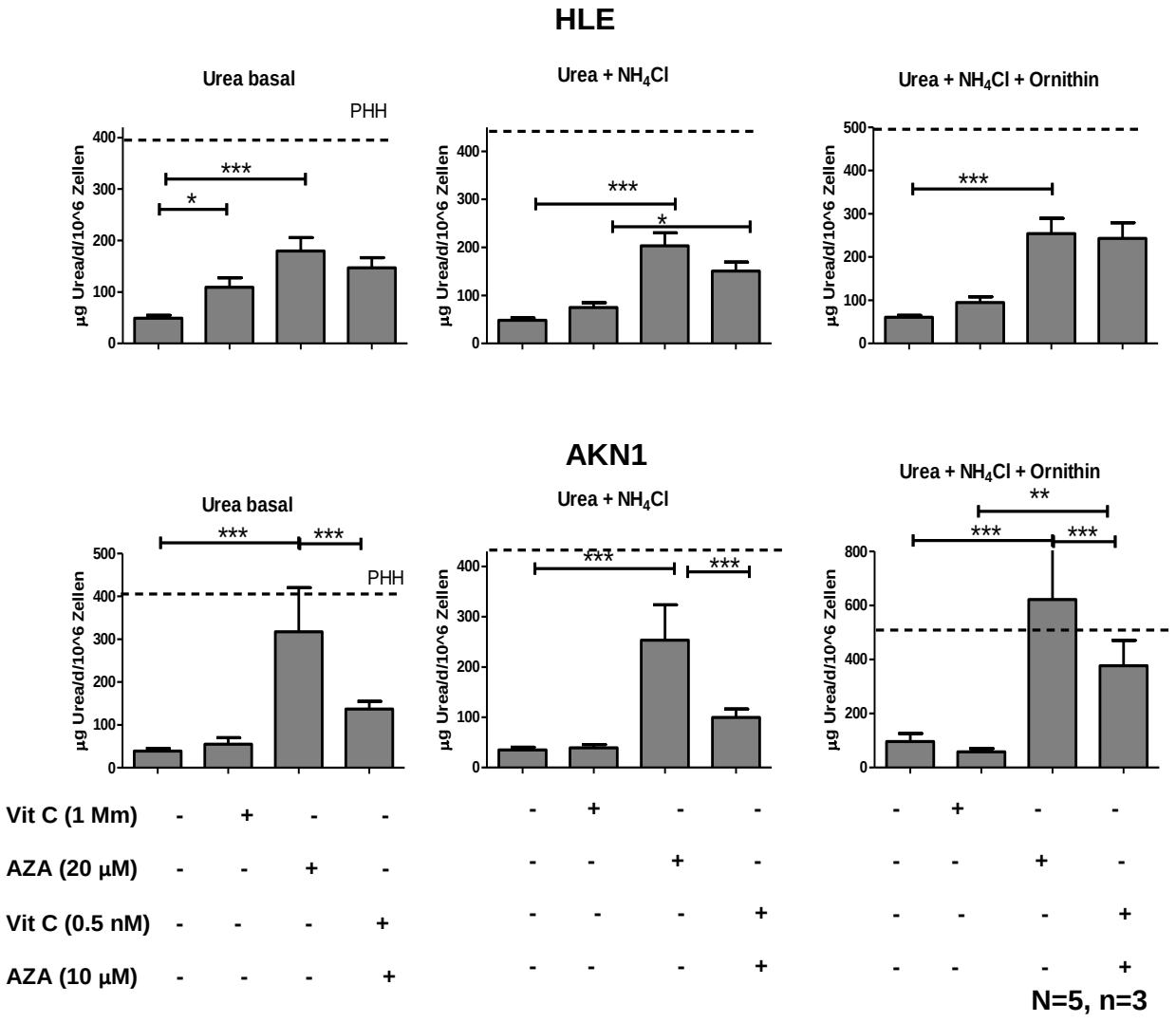



**Figure 3.29. Detoxification of ammonia in HLE and AKN1 cell lines after 48 hrs of treatment with 5-AZA and Vitamin C.**

The absorbance was measured by plate reader.5-AZA and Vitamin C have an effect on urea production which it increased by adding NH4Cl and Ornithine (N=5, n=3). Red line shows primary human hepatocyte enzyme activity as a gold standard. Data were repeated three times and the value in columns represents mean ± SD. P values were assessed by one way ANOVA, * $p < 0.05$ , ** $p < 0.001$, *** $p < 0.001$.

# 4 Discussion

## 4.1 Epigenetic modification in HCC

### 4.1.1 Reduction of 5hmC in HCC

Fine tuning the balance of DNA methylation vs. demethylation in relation to epigenetic modification has become a main focus of cancer research in recent years [Esteller, 2008]. The loss of 5hmC as an indicator of demethylation has been suggested as prognostic marker in different types of cancer, and even to play a crucial role in the pathogenesis of cancers [Huang & Rao, 2014; Wang *et al.*, 2014]. It was reported that 5hmC, in addition to its role as a DNA demethylase has a role in the regulation of genes involved in development, pluripotency, and the regulation of RNA splicing processes [Tan & Shi, 2012; Yan *et al.*, 2014]. It was shown that 5hmC is a major epigenetic modification mark in an adult human liver, playing an important role in changes in hepatic gene expression in hepatocytes [Ivanov *et al.*, 2013; Seeliger *et al.*, 2013].

In this thesis, we demonstrated that the generation of 5hmC is significantly reduced in hepatocellular carcinoma tissues and cell lines compared with healthy liver tissue and human hepatocytes. In line with other papers, our data clearly support the link between 5hmC ablation and tumor development [Baylin & Jones, 2011; Khan & La Thangue, 2011]. Furthermore, our results indicate that 5mC levels are enhanced in HCC tissues as well as in HCC cell lines. Altogether, our study provided a large scale screening of HCC tissues for detection of 5hmC and underlines that 5hmC could be a crucial diagnostic marker for HCCs which could be explored further for new HCC therapy strategies.

### 4.1.2 Downregulation of TET2 and TET3 leads to loss of 5hmC in HCCs

TET proteins (TET1, TET2 and TET3) mediate the oxidation of 5mC to 5hmC. TET proteins were identified as dioxygenases that utilize two substantial factors: Fe(II) and 2-Oxyglutarate (2-OG), in order to oxidize the methyl group ($CH_3$) of 5mC to form 5hmC, thereby mediating active DNA demethylation [Griffiths & Gore, 2008; He *et al.*, 2011; Ito *et al.*, 2010; Tahiliani *et al.*, 2009]. The expression of these three TETs varies not only in different organs but also in the development of organs [Baylin, 1997; Ito *et al.*, 2010]. In various tumor types, a different TET family member has been reported to function as tumor suppressor [Bestor *et al.*, 1988; Costello & Plass, 2001; Das & Singal, 2004; Wu & Zhang, 2011]. Hence, identification of the most effective TET protein leading to cancer in the corresponding tissue is crucial to be investigated in different tumors. Recent evidence suggests that there is a remarkable correlation between decreased 5hmC levels and TET expression, resulting in progression of tumor and metastasis. This suggests that TET proteins might serve as a tumor suppressor in certain types of cancers [Haffner *et al.*, 2011; Xu *et al.*, 2011]. Recently, it was reported that the gene encoding TET2 but not TET1 and TET3 is frequently mutated in various tumors and was identified as the relevant tumor suppressor gene, which is mutated in leukemia [Delhommeau *et al.,* 2009]. However, in breast cancer and prostate cancer, low expression of TET1 correlated with advanced cancer staging [Hsu *et al.*, 2012] however, in other tumors such as colorectal cancer (CRC), the reduction of all three TET proteins has been reported [Guo *et al.*, 2011a; Necula *et al.*, 2015]. In addition, it was shown that the enzymes IDH1 or IDH2 are mutated in several cancers and play a crucial role in the reduction of 5hmC in cancer tissue [Lian *et al.*, 2012; Liu *et al.*, 2014b; Ye *et al.*, 2013]. In HCCs the published results are contradicting each other regarding the role of TETs.

Liu *et al*. reported that a reduction of 5hmC is associated with the down-regulation of the TET1 protein in HCC [Liu *et al.*, 2013] while Gao *et al.* claimed a down-regulation of TET1 and TET2 but no change in TET3 gene expression with a concomitant decrease of 5hmC in HCC [Gao *et al.*, 2014]. Moreover, in another study by Yang *et al.*, the decrease of all three TET genes was reported however in only three pairs of frozen human hepatocellular carcinoma tissue compared to matched normal liver tissue [Yang *et al.*, 2013]. However, these three studies dealing with limited amounts of study samples.

In our study we clearly found that only TET2 and TET3 but not TET1 genes are significantly reduced in HCC tissue and in hepatocellular carcinoma cell lines. These changes were paralleled by a concomitant decrease of both IDH genes and 5hmC as well as a significant increase of 5mC. The expression of TET1 in our healthy liver samples was in almost all samples undetectable. Recently, it was shown that TET1 plays a crucial role in the early development of self-renewal pattern in embryonic stem cells and has been implicated in epigenetic regulation of stem cells and genomic imprinting [Baylin, 1997; Huang *et al.*, 2013; Ito *et al.*, 2010; Yamaguchi *et al.*, 2013]. These findings suggest a more crucial role for TET1 in embryogenesis. In the study by Neri *et al.* it was shown that TET1 is regulated by the stemness factors such as; Oct 3/4, Nanog and c-Myc. Therefore, it is implicated in pluripotency of transcriptional network of ESC. They showed that TET1 is switched off in adult cells and tissues. The TET1 downregulated through H3K27me3 histone mark deposition which leads to genome-wide reduction of 5hmC [Neri *et al.*, 2015]. Therefore, this thesis focused on the effect of TET2 and TET3 on HCC development.

The changes in 5hmC and 5mC in tumor tissues are confirmed by other studies [Liu *et al.*, 2013; Yang *et al.*, 2013], including our results. There are conflicting reports regarding to decisive TET in HCCs. But what might be the reasons for these conflicting results? An additional explanation could be simply the use of specific analytical tools. It has been reported that the expression of typical housekeeping genes include glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-actin (ACTB), could also be affected in the course of HCC development and a significant difference in these housekeeping genes was reported in HCC tissues compared to normal liver [Waxman & Wurmbach, 2007]. The use of inappropriate genes for normalization can lead to an over- or under-estimation of the fold-changes or to misinterpretation of the results. Therefore, using inconstant genes like ACTB for the normalization of the q-PCR may lead to different interpretation of the TET gene expression in HCCs tissue samples. We have observed the different expression of GAPDH and ACTB in HCC tissue samples which is known to be deregulated in HCCs caused by viral infections such as HBV and HCV [Waxman & Wurmbach, 2007]. We used ß2M among 4 tested housekeeping genes including; GAPDH, ACTB, HPRT, ß2M as a reference gene because the expression of ß2M was the only which was constant among all tissue samples.

It is well established that 5-AZA causes the reduction of the DNA methylation status by inhibiting DNMT through passive demethylation pathway [Christman, 2002]. Recently, the active demethylation of DNA through oxidation of 5mC to 5hmC mediated by TET proteins was discovered [Tahiliani *et al.*, 2009]. To our knowledge, our study was the first to demonstrate that 5-AZA reduces the methylation status of DNA not only by triggering the passive demethylation pathway of DNA [Christman, 2002] - but also the active demethylation pathway conversion of 5mC to 5hmC by

inducing TET2 and TET3 proteins. Our finding was further substantiated when 5-AZA was unable to induce 5hmC inTET2 knocked-down HCC cell lines suggesting the effect of 5-AZA on TET-2 and also suggesting a crucial role for TET2 in 5hmC induction and in the pathogenesis of HCC. This was further underlined by our findings that 5-AZA inhibited HCC tumor cell growth; a decline of 5mC and a strong increase of 5hmC. However, a detailed mechanism of how 5-AZA affects the activity of TET proteins remains to be further elucidated [Sajadian *et al.*, 2015].

#### 4.1.2.1 Vitamin C facilitates the effect of 5-AZA on oxidation of 5mC to 5hmC

In this study we have shown that Vitamin C enhances the demethylation efficacy of 5-AZA by increasing the expression of TET2 and TET3 in HCC. Our result showed that Vitamin C is able to enhance the epigenetic activity of 5-AZA, thus compensating for a higher dose of 5-AZA (20 μM) in which it can facilitate active-demethylation.. As it was mentioned before, the catalytic activity of TET dioxygenases for oxidation of 5mC depends on two crucial factors, Fe(II) and 2-oxoglutarate and they are only active when iron is present in Fe(II) status [Pastor *et al.*, 2013]. In the absence of substrate, iron left in a high level of oxidation state, which inhibits further catalysis [Monfort & Wutz, 2013]. Vitamin C as an electron donor for reducing iron atom is able to rescue the dioxygenase enzyme and restore the catalytic activity for the next cycle. Several histone and DNA modifying enzymes belong to Fe(II) and 2-oxoglutarate dependent family including, JumonjiC-domain- containing histone demethylases (JHDMs) which can facilitate multiple function in adjustment or change of chromatin remodeling and TET DNA hydrolyses that catalyze the oxidation of methyl groups from 5mC [Monfort & Wutz, 2013]. In a study by Venturelli *et al.*, it was shown that a pharmacological doses of Vitamin C (8 mM) inhibited the DNMT but not

HDAC activity in a melanoma cell line and up-regulated 32 miRNA mainly involved in tumor suppression and drug resistance pattern [Venturelli *et al.*, 2014]. These studies suggest a crucial role of Vitamin C in chromatin remodeling which may prove beneficial in future cancer therapy.

### 4.1.3 Effect of the 5-AZA and Vitamin C on cell death pathway and stress signaling

We investigated the level of the expression of caspase 3, caspase 9 and PARP proteins to see the effect of treatment on apoptosis cascade. We didn't detect cleavage of caspase 3 and PARP protein in HCCs after 48 hrs of treatment with 5-AZA and Vitamin C. Balance of survival and death signals under normal and pathological conditions determines cell fate. Therefore, we used Pathscan stress and apoptosis signaling antibody array kits which included 19 signaling molecules that are involved in the regulation of the stress response and apoptosis mechanisms (Pathscan Array, Cell Signaling, MA; USA) to investigate signaling pathways molecules which are affected by 5-AZA and Vitamin C treatment and drives cell cycle arrest.

A slight increase of the cleavage of caspase 3 and 7 as well as BAD was observed after treating HLE cells with 5-AZA and Vitamin C but not in Huh7 cells. Survivin acts as anti-apoptotic molecule and inhibits caspase 3 activity [Shin *et al.*, 2001]. Pathscan array analysis showed that 5-AZA elevated the expression of the survivin protein in both cell lines, however when 5-AZA was combined with Vitamin C, the level of survivin was reduced which renders HCC cells more susceptible to death pathways [Sugawara *et al.,* 1999]. On the other hand the combined treatment causes the increase in BAD protein expression (BCL2 Associated Death receptor) which is a caspase independent pro-apoptotic member of BCL-2 gene protein. Our results

suggest that the increase of the BAD protein by 5-AZA and Vitamin C treatment might be caused by attenuation of NF-κB and AKT proteins. AKT inhibits apoptosis by activating anti-apoptosis proteins such as NF-κB and through inactivation of pro-apoptotic proteins such as BAD [Kamada *et al.*, 2007; Ozes *et al.*, 1999].

TAK1 is a member of MAPKKK family and has an activatory effect on [Ninomiya-Tsuji *et al.*, 1999]. Combined treatment of 5-AZA and Vitamin C results in a reduction of TAK1 (TGFβ-activated kinase 1) which is in association with the reduction of NF-κB in HCC cells after treatment. In addition, it was shown that Snail activation is correlated to NF-κB activation and NF-κB signaling pathway and inhibits the expression of Snail transcription[Julien *et al.*, 2007]. Apparently our data suggests that Vitamin C together with 5-AZA are implicated in the influencing snail expression through NF-kB signaling [Zhang *et al.*, 2010]. Apparently our data suggests that Vitamin C together with 5-AZA are implicated in the NF-κB signaling pathway and inhibits the expression of Snail transcription Interestingly, our results indicate that the combination of 5-AZA and Vitamin C is able to enhance p38MAPK but inhibits SAPK/JNK which leads to increase of p53 [Jing & Anning, 2005]. In addition, P38MAPK has an inhibitory effect on ERK expression resulting in the inhibition of cell survival. Recently it was shown that high level of MAPK signaling (ERK) leads to an increased miR-29b expression. miR-29b attenuates TET1 expression which drives decreased of 5hmC level resulting in enhancement of DNA methylation. This finding suggests that drug treatment that target TET may synergize with MAPK suppression [Taylor *et al*., 2016].

Furthermore, our results show that a combination of 5-AZA and Vitamin C is able to activate ATM/CHK1/CHK2/P53 signaling pathways in HLE cells which lead to S/G2

cell cycle arrest and increase of caspase 3 and caspase 7 but not Huh7 cells. However, in Huh7 cells the combined treatment of 5-AZA and Vitamin C leads to an increased expression of p16 and p21 which might lead to the inhibition of cyclin D/E [Harper *et al.*, 1995] and reduction of PCNA expression resulting in G0/G1 arrest [Flores-Rozas e*t al*., 1994]. Figure 4.1 summarizes the death and stress response factors which are triggered by 5-AZA and Vitamin C through epigenetic changes which are based on the findings of this study and literature.

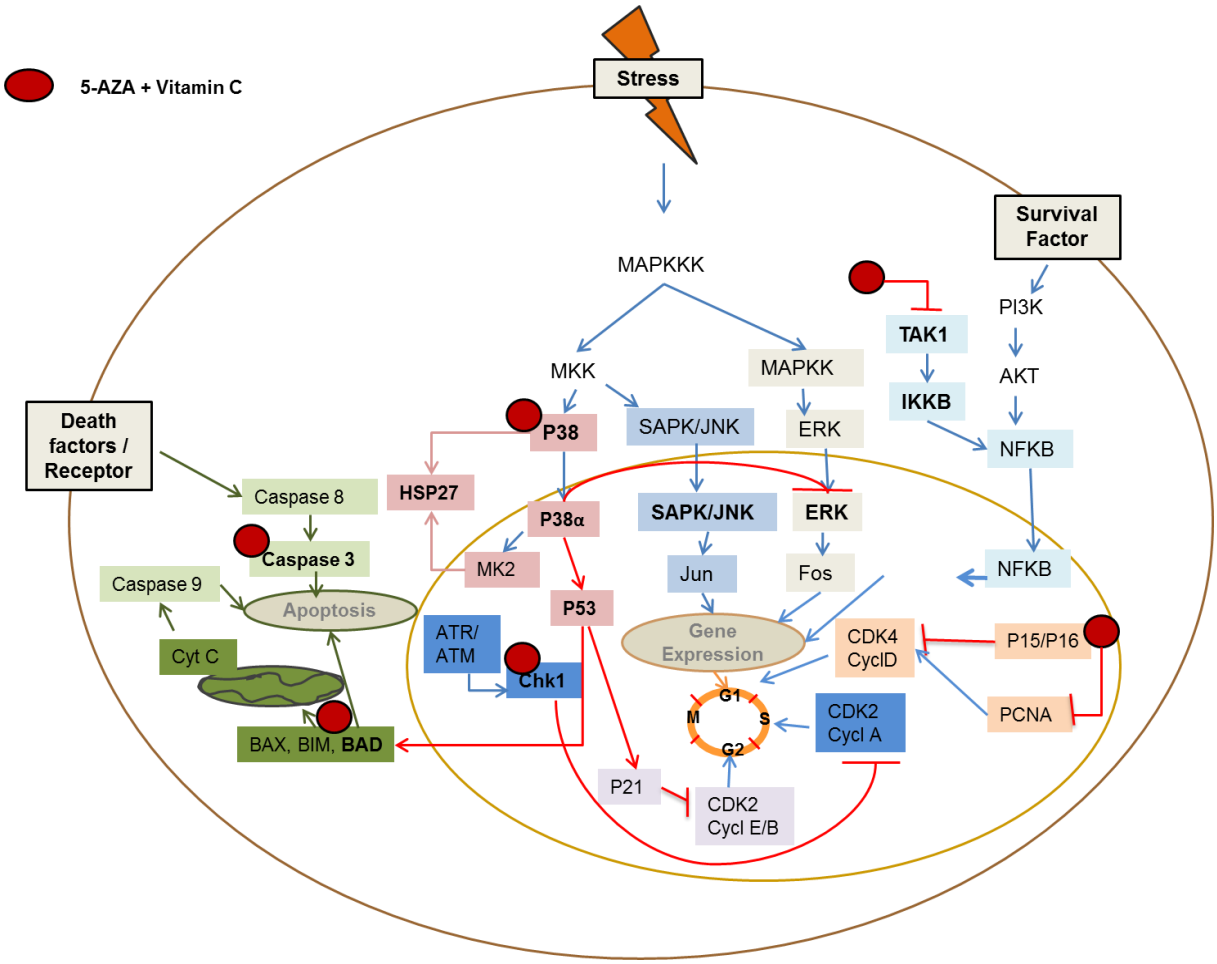



**Figure 4.1. Effect of 5-AZA and Vitamin C on Cell cycle pattern of cells.**

5-AZA and Vitamin C are able to trigger different crucial cell cycle checkpoints, tumor suppressors and genes which sensitize the cells to death (based on this study findings, pathways regulation is based on literatures)

## 4.2 Consequences of active demethylation in HCCs

As a consequence of induction of DNA demethylation by using 5-AZA and Vitamin C in our HCC cell lines, an inhibition of some oncogenes like Snail and reactivation of some tumor suppressors like GADD45 and p21 was observed. The enhanced expression of GADD45 combined with the changes in expression of PCNA and Cyclin B1 induced cell cycle arrest in HCC in the G1 and G2 phase, respectively.

Dynamic epigenetic changes mark EMT by the inducible expression of Snail [Javaid *et al.,* 2013]. Snail governs cell cycle progression by repressing P21 *[Takahashi et al.,* 2004]. While Snail expression is essential for tumorigenesis, reduction in Snail expression in cancer cells is considered essential in limiting tumor cell progression by inducing cell cycle arrest [Sugimachi *et al.*, 2003; Wu & Zhou, 2010]. A recent study reports that 5-AZA inhibited the inducible Snail expression in cultured hepatocytes and also suggests the possible involvement of miR29b in Snail regulation [Cicchini *et al.*, 2015]. Based on these studies and our recent report [Sajadian *et al.*, 2015], we propose that the downregulation of Snail expression is mainly attributed to an increase in TET activity by a combination of 5-AZA and Vitamin C in an indirect manner involving negative or positive regulators. The upregulation of Snail repressors such as NF-κB or some miRs (*e.g.:* miR337), could play a significant role in limiting Snail expression [Cicchini *et al.*, 2015]. In addition, it was shown that miR337 has an inhibitory effect on Snail and can increase HNF-4α, which is required for controlling many genes involved in liver development and metabolism [Lemaigre, 2009; Schulz *et al.*, 2013]. We observed the downregulation of NF-κB which might be related to the reduction of TAK1 in HCCs after treatment with 5-AZA and Vitamin C. However, the details of this mechanism need to be further investigated.

The growth arrest and DNA damage-inducible GADD45 genes are central players that are upregulated during cellular stress. Activation of GADD45 results in several processes of growth arrest, DNA repair, survival or apoptosis [Tamura *et al.*, 2012]. Hypermethylation of the GADD45 promoter is found in various cancers including HCCs, which leads to downregulation of GADD45 expression and promotes tumor progression [Tamura *et al.*, 2012; Zerbini & Libermann, 2005]. It was reported that 5-AZA enhances the expression of GADD45 in colon cancer cells resulting in the induction of apoptosis [Schneider-Stock *et al.,* 2005]. GADD45 proteins are also reported to induce cell cycle arrest by direct interaction with P21, PCNA and Cyclin B1 (through CDK1/Cyclin B1 complex) thereby inducing cell cycle arrest at the various phases of the cell cycle [Liebermann & Hoffman, 2003; Liebermann & Hoffman, 2007; Zhao *et al.*, 2000]. P21 is also known to have a PCNA binding domain towards its carboxyl terminal and to inhibit the replication of DNA at the S phase [Flores-Rozas *et al.*, 1994]. In the same line of evidence, it was also reported that binding of P21 to PCNA induces cell cycle arrest at the G1 or G2 level [Cayrol *et al.*, 1998]. In agreement with the above reports, we have seen an enhanced expression of GADD45 in our HCC cells treated with a combination of 5-AZA and Vitamin C. It has been reported recently that GADD45 could also induce active demethylation processes via nucleotide excision repair or base excision repair pathway [Delatte & Fuks, 2013] which might also be associated with the induction of active demethylation via 5-AZA and Vitamin C, as shown in this study**.** The possible pathways that were affected by 5-AZA and Vitamin C though triggering GADD45 and Snail were summarized in Fig 4.4.

The most common problem associated with tumor cells is their increased resistance to programmed cell death. Induction of E-cadherin expression is considered as an

important step in sensitizing tumor cells towards apoptosis [Qiu *et al.*, 2010]. E-cadherin modulates apoptosis by coupling with the death receptors DR4/DR5. Consequently, increased expression of E-cadherin sensitizes cancerous cells to cell death [Lu *et al.*, 2014]. Therefore, reactivation of E-cadherin could be an important target for epigenetic therapy in HCC. 5-AZA, when used either alone or in combination with other drugs, was shown to enhance the expression of E-cadherin in HCC cell lines and lung epithelial cells, respectively [Qiu *et al.*, 2010]. In differentiated hepatocytes, 5-AZA not only maintains the expression but also inhibits downregulation of E-cadherin [Cicchini *et al.*, 2015]. In the present study, since Snail is a direct repressor of E-cadherin [Cano *et al.*, 2000], reduction of Snail was expected to lead to an enhanced expression of E-cadherin. In line with this, we observed an increase in the expression of E-cadherin by a combination of 5-AZA plus Vitamin C. An increase in the number of cells undergoing cell death with the combined treatment is probably due to both the arrest of cells by reduced Snail and the increased sensitivity of the cells towards cell death by the enhanced expression of E-cadherin.

An increase in E-cadherin expression upon independent treatment with Vitamin C further highlights the role of Vitamin C in epigenetic regulation. Use of Vitamin C could therefore support the maintenance of epithelial morphology of the cells and thus prevent the MET, which is involved in cancer cell invasion and progression [Chen *et al.*, 2013b]. Figure 4.2 proposed signaling pathways which may affected by 5-AZA and Vitamin C treatment in HCC cells with an alteration of GADD45 and Snail protein based on the findings of this study.

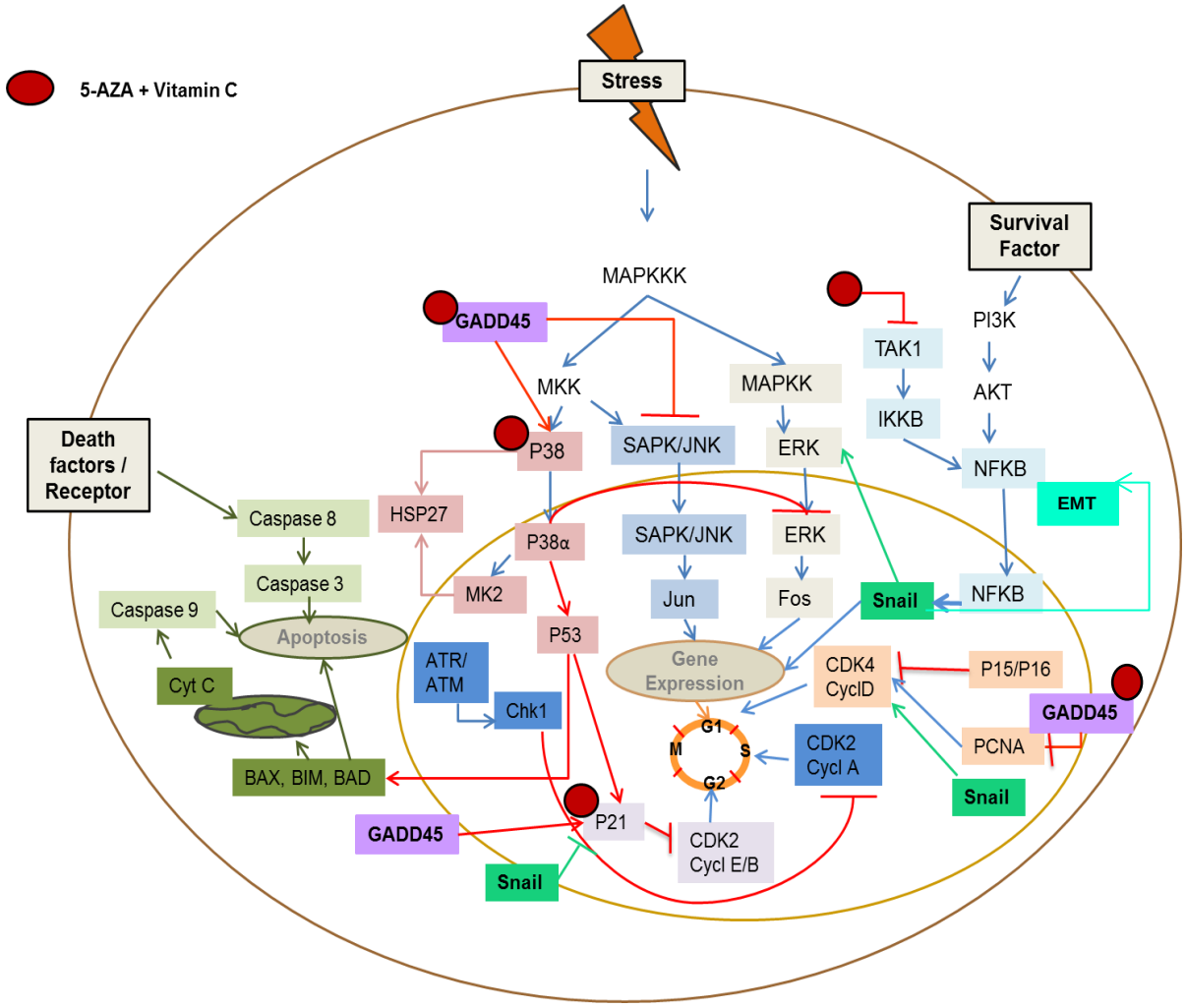



**Figure 4.2. Effect of 5-AZA and Vitamin C on Snail and GADD45 and downstream regulators.**

The combination of 5-AZA and Vitamin C inhibits Snail protein expression in HCCs and induced GADD45 expression**.** **Snail** and **GADD45** are both crucial transcriptional regulator which can change the fate of cells and make them more sensitize to death and inhibits the endless proliferation of HCCs (the effect of 5-AZA and Vitamin C on different pathways and the contribution of these pathways are based on findings of this study)

## 4.3 Induction of metabolic activity of HCC cell line by alteration of epigenetic landscape

The hepatic function of hepatocytes, in particular, the xenobiotic biotransformation capacity is reduced during isolation and culture. This is mainly attributed to the loss of the differentiated hepatic phenotype during *in-vitro* culture [Snykers *et al.*, 2009]. The stabilization of a differentiated hepatic phenotype frequently relies on the binding

of DNA binding protein like: liver-enriched transcription factor (LETFs). Histones and other proteins assist chromatin fibers to condense the DNA, therefore, modification of chromatin status through DNA methylation and histone modification is substantial for the activation of transcriptional factors for stabilization of hepatic phenotype. It was shown that inhibition of HDAC and DNA methylation were contributed to down-regulation of genes which are involved in cellular proliferation but an up-regulation of genes responsible for xenobiotic metabolism [Snykers *et al.*, 2009]. Therefore, regulation of epigenetic modification may play a substantial role in gene transcription, with maintenance of differentiated characteristics (geno/phenotype) of primary hepatocytes. In our study we have shown that 5-AZA and Vitamin C inhibits the proliferation of HCC cell lines with an enhancement of active demethylation pathway associated with increase of TET proteins expression which may contributes to activation of some crucial transcription factor which are responsible for metabolic activity of hepatoma cell lines.

### 4.3.1 CYP metabolic activity

PHH in culture and hepatoma cell lines are the most common standards in *in vitro* systems to evaluate drug metabolism and toxicity. Unfortunately, cultured hepatocytes lose their drug metabolic capacity rapidly in culture and they have large batch-to-batch variations. In contrast, hepatoma cell lines have an unlimited life span and they consist of a more stable phenotype than primary hepatocytes, are constantly available, but they have a low CYP metabolic activity [Rodriguez-Antona *et al.*, 2002]. Different factors are suggested to be responsible for a reduction of CYP activities including inactivation of CYP enzymes, instability of apoprotein or decreased of mRNA production [Rodriguez-Antona *et al.*, 2002]. Indeed, It was shown that the expression of key inhibitory (LIP, HNF-3α) and activatory (HNF4α,

RCR-αLAP) transcription factors and liver enriched transcription factors (LETFs) are modified in hepatoma cell lines which is linked to the reduction of CYP expression and functionality of these cells [Rodriguez-Antona *et al.*, 2002]. Furthermore, various transcription factors such as PXR, CAR and HNF4α can modulate the regulation of specific CYP450 genes like CYP3A4 and CYP2E1 [Snykers *et al.*, 2009]. In the same line of evidence, it was shown that Snail has an inhibitory effect on HNF4α which is crucial for liver development and for controlling the expression of many genes contributed to drug metabolic pathway including such as CYP450 enzymes, UDP-glucoronosyltransferase and sulfotransferases [Gonzalez, 2008].

Multiple studies investigated the effect of epigenetic modifiers like DNMTi and HDACi on alteration of CYP activity in HCC cell lines [Snykers *et al.*, 2009]. Therefore, in the present study we investigated the effects of 5-AZA and Vitamin C as epigenetic modifiers on CYP activities which can suppress the cell proliferation and differentiation with the most likely hepatocyte characteristics and higher level of drug metabolism enzymes. We have shown that 5-AZA and Vitamin C have an inhibitory effect on Snail transcription factor either by inhibiting TAK/NF-κB pathway molecules or inducing some miRs, which requires further investigation. Therefore we conclude that inhibition of Snail by 5-AZA and Vitamin C may lead to an increase of CYP450 activities especially of CYP3A4.

Screening for chromatin modification enzymes showed that each cell line has an individual chromatin status which may lead to discrete CYP catalytic activities of cells after treatment with the epigenetic modifiers; 5-AZA and Vitamin C. For instance, the chromatin enzyme array of HepG2, which is commonly used to investigate drug metabolism, is more similar to primary hepatocytes and in opposite, Huh7 illustrated

the most alteration of genes responsible for chromatin formation in compared with primary hepatocytes.

Our results also showed that 5-AZA and Vitamin C have stronger effects on CYP450 catalytic activities in the HepG2 cell line than in other investigated HCC cell lines. Previously, we have shown that for each CYP isoenzyme, all hepatoma cell lines have a cell certain passage in which maximum CYP activity was seen [169]. Interestingly, our results showed that the CYP expression is dependent on the confluency and passage number which is in line with our previous data [Lin *et al.*, 2012]. Regarding to the cell confluency, in another study it was also shown that when Huh7 cells are confluent, the CYP3A4 isoenzyme activity increased which is related to increase of PXR and HNF4$\alpha$ mRNA expression. When the confluency of cells is high, they avoid proliferating and resulting in formation of dense layer of cells with more hepatocyte cells characteristic including higher level of albumin, glucose-6-phosphate, drug metabolizing enzymes and transcriptional factors [Sivertsson *et al.*, 2010]. They have shown that treatment of confluent Huh7 cells with rifampicin, a well-known inducer of CYP3A4 through its interaction with PXR, increased the CYP3A4 activity in 4-weeks confluent cells. This finding indicates the role of PXR receptor in CYP transcription activity.

We observed the variant regulation of CYP isoforms mRNA level in HCC cells over the whole culture period. For example, HepG2 indicated better CYP transcription level at passage 8, while Huh7 cells showed highest CYP isoenzyme activity at passage 9, AKN cells also in passage 9 and in HLE cells the best CYP activity was observed at passage 5. Our results suggest that the maximum increase of CYP catalytic activity though epigenetic modification of hepatoma cells (5-AZA and Vitamin C) is after 48 hrs of treatment. However, the mechanism(s) behind of this

increase on CYP catalytic activity through epigenetic modification in HCCs needs further investigation.

### 4.3.2 Ammonia detoxification

Acute liver damage results in dys-synthesis of urea, which causes high blood ammonia concentrations and even hepatic coma [Patzer II *et al.*, 2002]. The number of patients with liver function failure has steadily increased over the years [Tang *et al.*, 2008], therefore it is crucial to discover a way to reduce ammonia toxicity for clinical therapy aspect. Bio-artificial Liver (BAL) therapy has been developed to bridge the need for liver transplantation and liver regeneration [Nussler *et al.*, 2011; Patzer II *et al.*, 2002]. However, lack of stable and conveniently usable human hepatocyte is the major problem of BAL therapy [Nussler *et al.*, 2006]. Primary hepatocytes have the limitation of availability, losing their functionality after culture or cryopreservation damaging. Porcine hepatocytes were used as a source of hepatocytes in BAL therapy [Tang *et al.*, 2008]. However, the major problems with use of animal hepatocytes are xenotransplantation-related risks and infection risks [Nussler *et al.*, 2006; Nussler *et al.*, 2011]. Hepatoma cell lines would be a possible good alternative for primary hepatocytes but their ammonia detoxification capacity is extremely low [Mavri-Damelin *et al.*, 2008; Nibourg *et al.*, 2012; Tang *et al.*, 2008]. Therefore developing a method which improves the ammonia detoxification of HCC cell lines would be highly desirable. We were able to induce the detoxification of ammonia significantly in all four HCC cell lines by inducing epigenetic modifications on chromatin by using Vitamin C and 5-AZA which was most significant in AKN1 cells.

# 5 Conclusion and outlook

The data presented in this study indicates the reduction of TET2 and TET3 expression and activity with concomitant decrease of 5hmC in Hepatocellular Carcinoma tissues and cell lines. Therefore, epigenetic drugs that mediate conversion of 5mC to 5hmC through modulating TET activity may find a broad use in the treatment of HCC. This study identifies a novel function of 5-AZA in promoting TET-mediated generation of 5hmC suggesting that availability of 5-AZA in cancer cells will have different effects on various epigenetic marks. However, the mechanism of how 5-AZA mediates TET expression needs to be investigated. Apparently, the TET genes possesses the CpGs island in their promoter region which undergoes demethylation by 5-AZA and so bisulfite sequencing of the promoter of TET genes may address this question.

We have shown that Vitamin C enhances DNA demethylation effect of the epigenetic drug 5-AZA by modulating TET activity. Our study has further added value to the growing evidences of Vitamin C as an epigenetic player. Increased TET expression leading to a concomitant increase in demethylation by a combination of an epigenetic drug with cofactors such as Vitamin C might prove to be a beneficial and attractive strategy for cancer therapy in the future. *In vivo* studies may provide an overview of how HCC tissue can be treated by the combination of 5-AZA and Vitamin C in tumor micro-environment.

Epigenetics plays a crucial role in tumorigenesis by modulating EMT and cell cycle proliferation pathways. Inhibition of Snail transcription factor with simultaneous activation of GADD45 are considered as major effectors in inducing cell cycle arrest, as shown in the HCC cell lines when treated with 5-AZA and Vitamin C. Our data

suggest that 5-AZA in combination with Vitamin C is implicated in the NF-κB signaling pathway, thereby inhibiting the expression of Snail transcription. Reduction in Snail expression in HCC cells is considered as an essential limiting factor in tumor cell progression by inducing cell cycle arrest through activation of p21. Further study on miRs, which affect the Snail (miR 337) expression through treatment, should be investigated to elucidate the complete mechanism which is triggered by the epigenetic changes. In order to address further, the potential effect of 5-AZA and Vitamin C on crucial transcription factors such as Snail and GADD45, an evaluation of the siRNA knockdown study is important.

Furthermore, we observed an increase in the expression of E-cadherin by a combination of 5-AZA plus Vitamin C. An increase in the number of cells undergoing cell death with the combined treatment is probably due to both the arrest of cells by reduced Snail and the increased sensitivity of the cells towards cell death by enhanced expression of E-cadherin. An increase in E-cadherin expression upon independent treatment with Vitamin C further highlights the role of Vitamin C in epigenetic regulation. Use of Vitamin C could therefore support the maintenance of epithelial morphology of the cells and thus prevent MET. To confirm the anticancer effect of the epigenetic treatment on HCCs, EMT-MET transition should be analyzed more in detail. It would be also interesting to look at the differentiation and drug resistance characteristic of the cells which might be correlated to EM-MET transition.

Moreover, it was shown that inhibition of HDAC and DNA methylation down-regulates genes involved in cellular proliferation and up-regulates the genes responsible for xenobiotic metabolism. In this study, we have shown that 5-AZA and Vitamin C inhibits the proliferation of HCC cells through TET-mediated active demethylation,

which may contributes to activation of some of the crucial transcription factors which are responsible for metabolic activity of hepatoma cells. Our results also showed that 5-AZA and Vitamin C have stronger effects on CYP450 catalytic activity in the HepG2 cell line compared to other investigated HCC cell lines.

Though we were able to induce the detoxification of ammonia significantly in all four HCC cell lines by changing the epigenetic pattern of chromatin using Vitamin C and 5-AZA, the results were found to be more significant in AKN1 cells. However, detailed analysis of genes and transcription factors which are responsible for CYP450 activity needs to be done in order to understand the mechanism/s which is/are implicated by epigenetic modifications. Therefore, the siRNA knock down study of transcriptional regulators of CYP450 genes such as HNF4α should be completed. Furthermore, it would be also interesting to investigate if the CYP450 activity is getting improved by using the combination of methylation inhibitors (*e.g.*: 5-AZA, Vitamin C) and Histone deacetylases inhibitors (*e.g*.: TSA) in HCC cell lines.

Our result showed the combination of 5-AZA and Vitamin C increased the ammonia detoxification. The effect of epigenetic modification on urea pathway needs to be investigated more in detail, especially screening of genes and transcription factor which are implicated in urea pathway. In addition, *in vivo* confirmation using Animal model would be a promising way to address the potential effect of epigenetic modification on ammonia detoxification.

# 6 Abbreviations

| | |
|---|---|
| **7-AAD** | 7-amino-actinomycin D |
| **AID** | Cytidine Deaminase |
| **AML** | Acute myeloid leukemia |
| **ANOVA** | Analysis of Variance |
| **AP** | Alkaline phosphatase |
| **APOBEC** | Apolipo protein B mRNA editing enzyme catalytic peptide |
| **5-AZA** | 5-Azacytidine |
| **5-AZA-dC** | 5- Azacytidine-2'-deoxycitidine |
| **BER** | Base Excision Repair |
| **BFC** | 7-Benzyloxy-4(trifluormethyl)coumarin |
| **bp** | Base pare |
| **BSA** | Bovine serum albumin |
| **C** | Cytosine |
| **5caC** | 5 carboxylcytosine |

| | |
|---|---|
| **CDK-2** | Cell division kinase 2 |
| **CDNA** | Complementary DNA |
| **Cou** | Coumarin |
| **CRC** | Colorectal cancer |
| **DBF** | Dibenzylfluoresceine |
| **DMEM** | Dulbecco’s Modified Eagle’s Medium |
| **DMSO** | Dimethyl sulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **dNTP** | Desoxynukleotidtriphosphat |
| **DNMT** | DNA Methyl Transferase |
| **DPBS** | Dulbeccos's Phosphate Buffered Saline |
| **EMT** | Epithelial Mesenchymal Transition |
| **ER** | Estrogen receptor |
| **FACS** | Fluorescent Activated Cell Sorting |
| **FBS** | Fetal Bovine Serum |
| **FC** | Flow cytometry |

| | |
|---|---|
| **5fC** | 5 formylcytosine |
| **FITC** | Fluorescein Isothiocyanate |
| **FACS** | Fluorescent Activated Cell Sorting |
| **FBS** | Fetal Bovine Serum |
| **GAPDH** | Glyceraldehyde 3-phosphate dehydrogenase |
| **GFs** | Growth Factor(s) |
| **GSH** | Glutathione conjugate |
| **H3K9** | Histone H3 lysine 9 |
| **HAT** | Histone Acetylation Transferase |
| **HBV** | Hepatitis B |
| **HCC** | Hepatocellular carcinoma |
| **IDAX** | inhibitor of the Dvl and axin complex; also known as CXXC4 |
| **IDH** | Isocitrate dehydrogenase |
| **IF** | Immunofluorescrnce |
| **IHC** | Immunohistochemistry |
| **IDH** | Isocitrate dehydrogenase |

| | |
|---|---|
| **IF** | Immunofluorescrnce |
| **Kda** | Kilodalton |
| **LDH** | Lactose dehydrogenase |
| **MAPK** | Mitogen activated protein kinase |
| **5mC** | 5 methylcytosine |
| **MET** | Mesenchymal-epithelial transition |
| **MSC** | Mesenchymal Stem Cells |
| **4-MU** | 4-Methylumbelliferone |
| **NAFLD** | non-alcoholic fatty liver disease |
| **$NH_4OH$** | Ammonium Hydroxide |
| **nM** | Nanomolar |
| **2OG** | 2-oxoglutarate |
| **$O_2$** | Oxygen |
| **PAGE** | Polyacrylamide Gel Electrophoresis |
| **PBS** | Phosphate-Buffered Saline |
| **PCNA** | Proliferating cell nuclear antigen |

| | |
|---|---|
| **PCR** | Polymerase Chain Reaction |
| **PE** | Phycoerythrin |
| **Pen/Strep** | Penicillin/Streptomycin |
| **PI** | Propidium Iodide |
| **PHH** | Primary Hepatocytes |
| **pM** | Picomolar |
| **PMSF** | Phenyl-Methyl-Sulphonyl-Fluoride |
| **PR** | Progesterone receptor |
| **qRT-PCR** | quantitative Real Time Polymerase Chain Reaction |
| **RNA** | Ribonucleic Acid |
| **Rpm** | Revolution Per Minute |
| **RT** | Room Temperature |
| **SCF** | Stem Cell Factor |
| **SDF** | Stromal Derived Factor |
| **SDS** | Sodium Dodecyl Sulphate |
| **Si-RNA** | Short interference RNA |

| | |
|---|---|
| **SOCS1** | Suppressor of Cytokine Signaling 1 |
| **SPCS1** | Signal Peptidase Complex Subunit 1 |
| **TCA** | Trichloro Acetic Acid |
| **TEMED** | N, N, N', N'-tetramethylene diamine |
| **TET** | Ten- eleven-translocation |
| **TGF-β** | Transforming Growth Factor Beta |
| **Tm** | Melting Temperature |
| **TNF** | Tumour Necrosis Factor |
| **Tris** | Tris-hydroxymethylaminomethane |
| **TIMP** | Tissue inhibitor of metallo protinases |
| **TDG** | Thymidine DNA Glycosylase |
| **TSA** | Trichostatin A |
| **UHRF1** | Ubiquitin-like protein with PHD and RING finger domain 1 |
| **µg** | Microgram |
| **µl** | Microliter |
| **µM** | Micromolar |

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# Eidesstattliche Erklärung

Ich versichere hiermit, dass ich die vorliegend Dissertation selbständig und ohne unzulässige fremde Hilfe erbracht habe. Ich habe keine anderen als die angegebenen Quellen und Hilfsmittel benutz sowie wörtliche und sinngemäße Zitate kenntlich gemacht.

Diese Arbeit wurde zu keinem früheren Zeitpunkt weder im Inland noch im Ausland einer anderen Hochschule zum Zwecke der Promotion vorgelegt. Frühere Promotionsversuche bestehen nicht.

Tübingen

# Curriculum Vitae (CV)

## Personal Information

**Name**: Sahar Olsadat Sajadian

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## Education

**06/2013 - 06/2016** **PhD thesis** at Technical University of Dortmund / Eberhard Karls University of Tübingen, Siegfried Weller Institute for Trauma Research.
**PhD thesis**: "The importance of the TET protein family for the development of Hepatocellular Carcinoma and the role of epigenetic drugs in improvement of active DNA Demethylation in HCC".
Supervisors: Prof. Andreas K Nüssler, Prof. Jan G. Hengstler

**11/2012 – 05/2013** Internship at Friedrich-Alexander-University Erlangen-Nürnberg, Dept. of Microbiology.

**2009-2012** **Master of Science** in **Cellular and Molecular Biology** at University of Tehran, Toxicology & Pharmacology Dept. of Tehran University of medical science (MSc)

**2004-2008** **Bachelor of Science** in **Cellular and Molecular Biology** at Tehran Medical University( BSc)

## Employment

**2011-2012** Research assistant in Stem Cell Department, Royan Institution of Stem Cell Biology and Technology, Tehran, Iran.

Projects:

## Publications

- Nussler AK**, Sajadian S**. *Adult Stem Cells*. Stem Cell Nano Engineering. 2015: 1: 1-23.
- **Sajadian S**, Nussler AK. DNA Methylation: *A Possible Target for Current and Future Studies on Cancer? Epigenetic Diagnosis & Therapy*. 2015;1 (1): 5-13.

- **Sajadian S**, Vatankhah M, Majdzadeh M, Kouhsari SM, Ghahremani MH, Ostad SN. *Cell cycle arrest and apoptogenic properties of opium alkaloids noscapine and papaverine on breast cancer stem cells*. Toxicology mechanisms and methods. 2015; Volume:1-8.
- **Sajadian S**, Ehnert S, Vakilian H, Koutsouraki E, Damm G, Seehofer D et al. *Induction of active demethylation and 5hmC formation by 5-Azacytidine is TET2 dependent and suggests new treatment strategies against Hepatocellular Carcinoma*. Clinical Epigenetics. 2015; 7(1): 1-14.
- **Sajadian S,** Chaturvedula T, Samani S, Rouss M, Dooley S, Baharvand H et al. *Vitamin C enhances epigenetic modifications induced by 5-Azacytidine and cell cycle arrest in hepatocellular carcinoma (Accepted) .*
- Tripura Sundari Chaturvedula, Srinivas Gunda, Avinash Raj Thatipalli, Gauri Tendulkar, **Sahar Olsadat Sajadian**, Jayashree Chiring Phukon, Nitin Tupperware, Mahesh Jerald, Gopal Pande, Andreas K Nussler. *Fibrosis associated hepatic extramedullary hematopoiesis (EMH) in mice: potential role of stellate cells in EMH (Submitted)*

## Workshops attended

- 5th PhD Berlin-Brandenburg School for Regenerative Therapies (BSRT) symposium, Berlin, 3-5 December 2014
- FALK workshop (Viral Hepatitis from bench to bench) in Klinikum recht Isar, Munich, January 30- February 1, 2015
- 5th International Clinical Epigenetic Society meeting, Düsseldorf, 5-6 March, 2015

## Conferences and Meetings (Poster/ Oral Presentation)

- **Saharolsadat Sajadian,** Sabrina Ehnert, Anastasia Bachman, Bence Sipos, Andreas K Nüssler (Tübingen, DE). 5-Azacytidin induces DNA demethylation actively through increasing 5-hydroxymethylcytosine and TETs that decreased in Hepatocellular Carcinoma. *18. Annual Meeting on Surgical Research,* Hannover, Germany, **Poster**October 9 - 11, 2014, Langenbecks Arch Surg, 399 **(IF: 2,191).**
- **Saharolsadat Sajadian,** Sabrina Ehnert, Anastasia Bachman, Bence Sipos. Andreas K. Nüssler. 5-Azacytidine as an antitumor agent induces active DNA demethylation in Hepatocellular Carcinoma cells via TET proteins. *5th BSRT Ph.D. Symposium*, Berlin, Germany, **Oral presentation** & **Poster**, December 3 - 5, 2014.
- **Saharolsadat Sajadian,** Sabrina Ehnert, Anastasia Bachman, Bence Sipos, Andreas K Nüssler. Targeting active demethylation; a new possible mechanism in treatment of hepatocellular carcinoma*. FALK workshop (Viral Hepatitis from bench to bench) in Klinikum recht Isar*, Munich, Germany, **Poster,** January 29 - 30, 2015.
- **Saharolsadat Sajadian,** Sabrina Ehnert, Anastasia Bachman, Bence Sipos, Andreas K Nüssler. Inducing active demethyalation through 5-Azacytidine open a new aspect for treatment of Hepatocellular Carcinoma. *GASL Meeting in Klinikum recht Isar*, Munich, Germany. **Poster**, January30-February1, 2015. Zeitschrift für Gastroenterologie, 53(01), **(IF: 1.05).**

- **Saharolsadat Sajadian,** Sabrina Ehnert, Anastasia Bachman, Bence Sipos, Andreas K Nüssler. Targeting active demethylation through epigenetic drugs – a possible mechanism in HCC. *Clepso International Meeting*, Dusseldorf, Germany, **Poster**, March 05 - 06, 2015.
- **Saharolsadat Sajadian,** Sabrina Ehnert, Anastasia Bachman, Bence Sipos, Andreas K Nüssler. Induction of active demethylation by administration of 5-Azacytidine opens up new possibilities for the treatment of Hepatocellular Carcinoma, *Kongress der Deutschen Geselschaft für Chirugie*, Munich; Germany, **Oral presentation,** April 28 - 01 May, 2015.
- **Saharolsadat Sajadian**, Tripura Chaturvedela, Andreas K Nüssler. Vitamin C enhances epigenetic modifications induced by 5-Azacytidine on cell cycle arrest in Hepatocellular Carcinoma. *International Liver Congress 2016 (EASL),* Barcelona, Spain**, Poster,** April 13 - 17, 2016, Journal of Hepatology.

## Languages

- Persian: Native
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## References

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