Counterbalancing Cancer Growth: Harnessing Intrinsic Regulatory Pathways for Novel Anti-oncogenic Strategies

Buyun Ma

The studies presented in this thesis were performed at the Laboratory of Gastroenterology and Hepatology, Erasmus MC-University Medical Center Rotterdam, the Netherlands.

The research was funded by:
Netherlands Organization for Scientific Research (NWO); Dutch Digestive Foundation (MLDS); Daniel den Hoed Foundation; China Scholarship Council

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Cover design and layout: Optima Grafische Communicatie

Printed by: Optima Grafische Communicatie

ISBN: 978-94-6361-319-4

Counterbalancing Cancer Growth: Harnessing Intrinsic Regulatory Pathways for Novel Anti-oncogenic Strategies

Regulatoire Circuits in Kanker: verschuiving van het evenwicht

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
rector magnificus

Prof.dr. R.C.M.E. Engels

and in accordance with the decision of the Doctorate Board
The public defense shall be held on

Wednesday 11th September 2019 at 13:30

by

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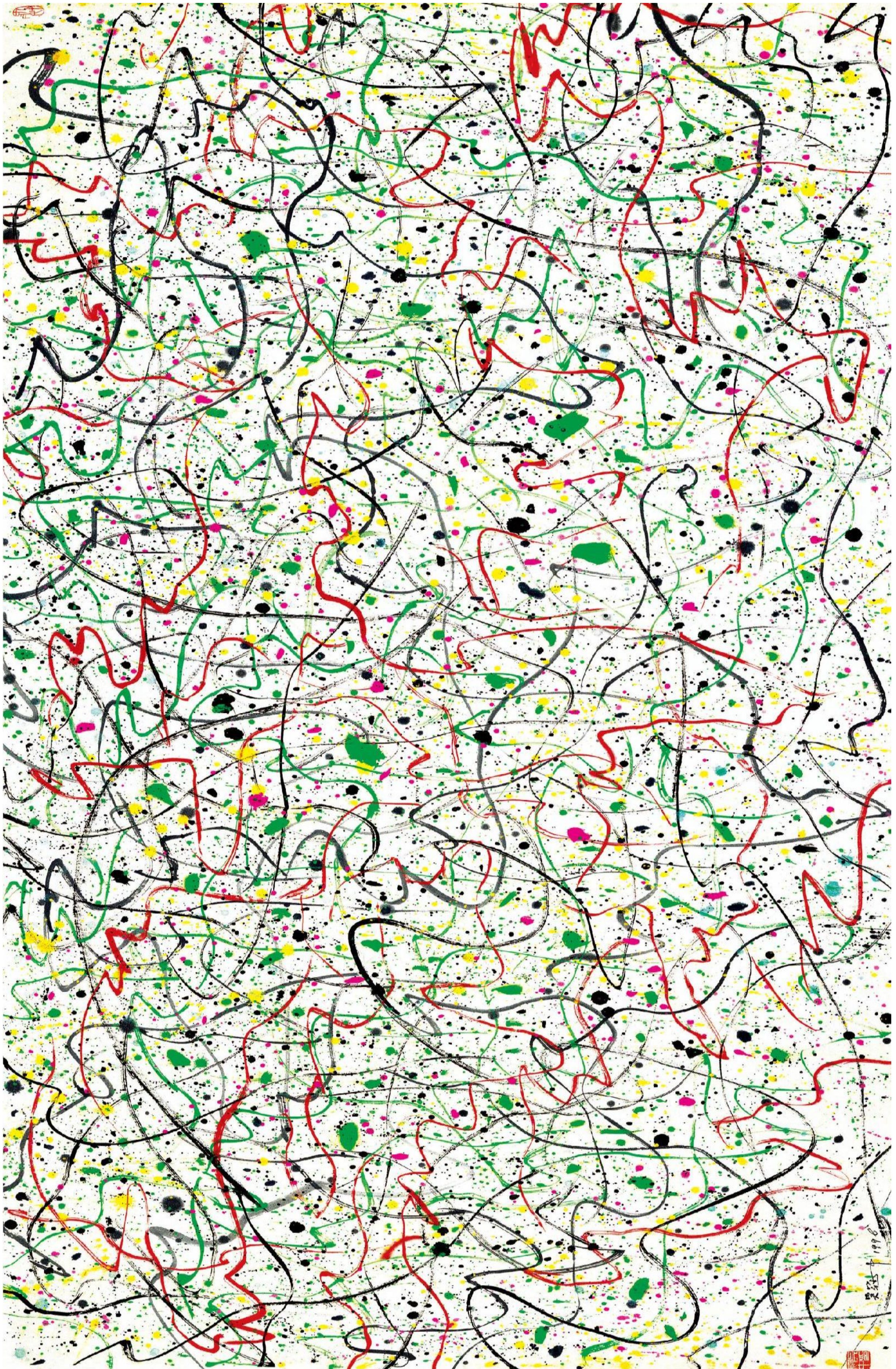
CONTENTS

Chapter 1	General introduction and outline of the thesis	9
Chapter 2	Inhibiting experimental and clinical hepatocellular carcinoma by mycophenolic acid involves formation of cytoplasmic rods and rings (Adapted from *Transplantation. 2019 May;103(5):929-937* and *Liver Int. 2017 Nov;37(11):1742-1743*)	23
Chapter 3	The two isoforms of IMPDH distinctively associate with patient outcome and exert dichotomic functions in hepatocellular carcinoma *(submitted)*	49
Chapter 4	Dichotomal functions of phosphorylated and unphosphorylated STAT1 in hepatocellular carcinoma *(J Mol Med (Berl). 2019 Jan; 97(1):77-88.)*	85
Chapter 5	Human intestinal and liver stem cells counteract telomerase-targeted anticancer therapy *(submitted)*	117
Chapter 6	Genetically engineered bacteria for treating human disease *(Trends in Pharmacological Sciences. 2017 Sep; 38(9):763-764)*	155
Chapter 7	Summary and discussion	163
Chapter 8	Dutch summary	173

Appendix

Acknowledgements	181
Publications	185
PhD Portfolio	187
Curriculum Vitae	189



Chapter 1

General introduction and outline of the thesis

1. Cancer

Cancer, often a devastating disease provoking untold human misery, has been recognized as a separate pathological condition for almost as long as written records exist, already being described in ancient Egyptian texts (*e.g.* in the almost 5000 year old Edwin Smith Papyrus). Its current name derives from a text attributed to Hippocrates in which non-ulcer forming and ulcer-forming tumors were compared to crab or crayfish (the ancient Greek word being καρκίνος)[1]. Now, cancer has become the second leading cause of death globally[2]. Generally speaking, cancer is a group of diseases characterized by uncontrolled cell growth and the ability of the cell spreading to other parts of the body[3]. The damage to the body is manifold but also physical through space occupation. Concomitant with the advance in understanding cancer, it has become clear that cancer is mainly a genetic disease with alterations in cancer cell DNA, driving the pathological process. Consequently, external agents (physical, chemical and biological carcinogens) and internal events which can disturb/interact with human genetic factors are the most important causes of cancer. It is hoped that further understanding of the cancer process will open novel avenues for rational treatment of cancer. The current thesis hopes to contribute in this respect.

Genetic changes driving cancer generally involve gain-of-function mutations in proto-oncogenes and loss of function mutations in tumor suppressor genes. The consequences of specific mutation can be highly context specific: while the transcription factor SMAD4 usually acts as a tumor suppressor[4], in the context of liver cancer it acts like an oncogene[5]. Multiple successive alterations in the genomes create genetic diversity and underlie the transformation of normal cells to cancer cells. The cancer process is complicated and different hallmarks have been proposed to understand cancer. These hallmarks include continuing proliferative signaling, evasion of growth suppressive signaling, resisting (programmed) cell death, replicative immortality, induction of angiogenesis, a capacity for invasion and metastasis, deregulated cellular energy metabolism and avoiding immune destruction[6]. Better understanding the interactions between the different elements of the cancer process will foster better comprehension of efficacy of treatment and allow better therapeutic strategies.

2. Liver cancer

Liver cancer is the cancer that starts in liver and includes hepatocellular carcinoma (HCC), cholangiocarcinoma and hepatoblastoma. It is a major health problem, with more than 850000 new cases annually and it is the second leading cause of cancer-related death worldwide[7]. As the most common cancer of the liver cancer, HCC accounts for approximately 90% of liver cancer cases[8]. The etiology of HCC is reasonably well defined and development of this disease is linked to hepatitis virus (HBV, HCV) infection, metabolic syndrome and alcohol abuse[9]. Development of HCC is a multistep process, with most of the cases occurring in the context of cirrhosis. With the advent of high-performance genomic analyses, knowledge on the molecular pathogenesis of HCC has remarkably increased over the past decade. Accordingly, various key mutations and pathways have been identified and these include processes involved telomere maintenance, activation of Wnt signaling, inactivation of p53, chromatin remodeling, stimulation of the Ras and PI3K pathways as well as the oxidative stress pathway[7]. This increased insight has not yet translated in improved therapeutic strategies, with surgical resection (often in conjunction with liver transplantation) remaining the only curative option, whereas oral multiple kinase inhibitors such as sorafenib, turn out with moderate clinical benefit[10]. In addition, immunotherapy has now emerged as an alternative treatment approach that has been successful in many cancer types. Promising response rate and survival durations in HCC patients have also been observed with the use of immune-checkpoint inhibitors[11]. Nivolumab, a monoclonal antibody targeting programmed cell death protein 1 (PD-1), has been granted accelerated approval by the FDA as a second line treatment and is currently being tested against sorafenib in a phase III trial in the first line setting (NCT02576509)[12]. The paucity of options in this respect urgently calls for further studies in this respect and much of the work in this thesis is a reaction to that need. Especially interesting in this respect is that patients receiving orthotopic liver transplantation for HCC are being treated by immunosuppressive medication which may interact with the cancer process, especially as many of the medications involved are cell cycle-inhibiting compounds interfering with nucleotide biology. Further understanding of the biology of the interaction of such medication with the cancer may thus lead to improved therapy, in this thesis I aim to explore this angle.

3. Colorectal cancer

Colorectal cancer (CRC), which arises either from the colon or the rectum, is the third most common cancer. Risk factors for CRC are mainly aging and various lifestyle factors (meat consumption, sedentary life style, absence of NSAID use etc.), with a small population of cases due to genetic disorders that confer strongly increase risk for CRC developent[13]. Although on a molecular level the group of pathologies clustered under the denominator CRC is quite heterogeneous, three main molecular mechanisms emerge as principal mediator of CRC development, *in casu* chromosomal instability (CIN), CpG island methylator phenotype (CIMP), and the microsatellite instability (MSI)[14]. Population-wide screening efforts should be instrumental in reducing CRC burden, but if these could be combined with other efforts aimed at reducing CRC mortality, efficacy might be increased. In this thesis I shall explore both mechanistic aspects of CRC as well as novel models for prevention of this disease.

4. Tumor microenvironment and immunology

Remodeling of the microenvironment is a hallmark in the pathogenesis of cancer[15]. Co-evolution of (presumptive) tumor cells with microenvironment may create a selective landscape that drives sequentially tumor initiation, progression and metastasis. In important factor to consider in this respect is the immune system. As immune surveillance is important for the eradicating formation and progression of cancer, defect of the immune system, recognized as immunosuppression, is validated in increasing certain cancers[16]. Notably, immunosuppression is found in majority of virus-induced cancers. Studying the tumor microenvironment, including the different cell types and the crosstalk between it, would be expected to help understanding the biology of cancer.

STAT1 and IFN signaling pathways

Interferons (IFNs) are pleiotropic cytokines that protect against diseases by direct effects on target cells (cell autonomous effects) and by activating immune responses. There are three major types of IFNs, including type I IFNs (13 subtypes of IFNα, plus IFNβ, IFNε, IFNκ and IFNω), type II IFNs (IFNγ), and type III IFNs (IFNλ1, IFNλ2, IFNλ3, IFNλ4)[17]. Among them, type I IFNs are especially prominent and expressed by various cell types where they exert their effects in an autocrine or paracrine manner. In comparison, type II and type III IFNs are more

restricted, both with respect to spectrum of cells that express these cytokines and the diversity in reactions they elicit in the body. IFN-based therapy has been developed and employed for cancer treatment now for decades. All IFNs have the anti-tumor function by directly acting on tumor cells or by activating the immune cells[17]. Besides potential action on tumor cells, IFNs are important for defense of viral infection and elimination. Thus, IFNs treatment also is important for preventing cancer by limiting progression from simple infection to virus-induced cancer[18].

Virus infection including HBV and HCV, which often lead to the chronic viral hepatitis, are the major risk factors for HCC. IFNs, especially type I IFNs, have been well-studied and used in clinic for prevention and treatment of viral hepatitis-related HCC[7]. By binding to their cognate receptors on responding cells, type I IFNs signal through the key class of transcription factors, signal transducers and activators of transcription (STATs), and provoke transcription and expression of IFN-regulated genes (IRGs). STAT1 is an important member of the STAT family and form homodimers or heterodimers with other STATs upon IFN stimulation[19, 20]. Studies in different types of tumors have demonstrated that STAT1 function in tumor progression is pleiotropic, some of its effects being beneficial and other detrimental with respect to final outcome of disease. Despite the conventional view that phosphorylation of STAT1 is absolutely required for the inducing expression of downstream genes, an increasing body of evidence is emerging that demonstrates that unphosphorylated STAT1 (u-STAT1) also functions as a transcription factor, even in the absence of IFN stimulation[21]. A different subset of genes was found to be regulated by p-STAT1 and u-STAT1, which may relate to the variability in effects seen with regard to the role of STAT1 in tumor progression. ISGs selectively controlled by u-STAT1, especially STAT1 itself, are upregulated in patients after radio- and chemotherapy and this is postulated to contribute to therapy resistance[22]. Despite the previous studies showing that STAT1 functions as a tumor suppressor in HCC, exact function of p-STAT1 and u-STAT1 remains largely unknown and in this thesis endeavor to obtain better clarity as the exact functionality of STAT1 in the liver cancer process.

5. Cancer metabolism

Uncontrolled cell proliferation is a key characteristic of cancer cells and importantly is associated with reprogramming of cellular metabolism in which the main source of ATP

production becomes aerobic glycolysis. Why tumor cells rely on glycolysis even in the presence of sufficient oxygen to sustain oxidative phosphorylation remains largely obscure but it may well be required to fuel cell growth and division. This so-called “Warburg effect” has been subject to an intense research effort and has even labeled as the Achilles heel of cancer metabolism[23]. It is evident that the diverse reprogramming of metabolic activities, the corresponding genetic alterations (*e.g.* genes of metabolic enzymes), may hold promise for better therapy. Defining genes and pathways and understanding the specificity of metabolic preferences and abilities will provide new insight into cancer biology and benefit the clinical patients and also in this aspect of cancer biology I aim to make contributions with this thesis.

Role of IMPDH in cancer progression

Inosine monophosphate dehydrogenase (IMPDH) is a metabolic enzyme responsible for biosynthesis of purine nucleotides, and hence is required for DNA and RNA synthesis. It catalyzes the nicotinamide-adenine dinucleotide (NAD+)-dependent oxidation of IMP to XMP[24]. Inhibition of IMPDH results in reduction of cytoplasmic guanine nucleotide pools and also the adenylate pools. Being a rate-limiting enzyme of guanosine nucleotide synthesis, IMPDH plays a multifaceted, almost kaleidoscopic, role in cell growth and differentiation. Interruption of DNA and RNA synthesis results in rapid cell growth arrest[25]. Human IMPDH includes two isoforms, IMPDH1 and IMPDH2, with 84% sequence identity and similar properties. To date, the available evidence suggests that IMPDH1 is constitutively expressed in most cells, while IMPDH2 is subject to dynamic regulation and its upregulation associated with malignant transformation[26]. Furthermore, IMPDH has been identified as the target of mycophenolate mofetil (MMF), an immunosuppressant widely used in organ transplantation including for HCC-related liver transplant recipients[27]. Thus, IMPDH is a potential drugable target in disease. As the most prominent metabolic organ in human body, the liver contains highly active cells. Thus, based on the considerations spelled-out above I speculate that tumor transformation in the liver would be tightly associate with the metabolic reprogramming, especially the changes of the metabolic enzymes and that these enzymes and especially IMPDH is a potential target here. Thus in this thesis I aim to explore this notion and also to obtain better insight of what effect of purine metabolism inhibitors on liver cell biology might be.

6. Cancer therapy

HCC is divided into five prognostic subclasses, based on the Barcelona Clinic Liver Cancer (BCLC) staging classification. This staging system is also used to select treatment, specific therapies offered to patients for individual stage. The treatment involved are mainly limited to surgical resection, liver transplantation, radiofrequency ablation, chemoembolization and the multi-kinase inhibitor sorafenib, while recently some patients are also receiving immune-checkpoint inhibitor therapy. Despite improvement in surveillance programs, which aim at early diagnosis, many patients have an initial diagnosis of advanced HCC and are considered to be non-curative and display a median overall survival of 1 year[10]. Thus, there is clear therapeutic need with respect to the patients involved. For CRC, colon cancer and rectal cancer are these days treated as separate entities and require different approaches depending on tumor stage. Surgery is the mainstay of treatment for patients with non-metastasized CRC and has considerable therapeutic success[28]. Neoadjuvant treatment is recommended for intermediate-stage and advanced-stage rectal cancer but not for colon cancer. Adjuvant treatment is recommended for both types of CRC and displays substantial clinical benefit. For patients diagnosed with advanced metastasized CRC, chemotherapy combinations and targeted therapies have been used and improved the overall survival of the patients, but remains depressingly lethal[13]. The lack of satisfactory treatment options for inoperable HCC and CRC requires development of novel therapies. Increased insight into the biology of cancer will prove the way forward here.

Telomerase targeted strategies for cancer therapy

A potential target for improved therapy that will receive special attention in the work described in this thesis is telomerase. Telomerase counteracts DNA loss during cell proliferation by adding a species-dependent telomere repeat sequence to the 3' end of the telomeres of the chromosomes. Without telomerase activity, chromosomes shorten during subsequent cell divisions and become finally incompatible with sustaining tumor cell biology. Accordingly, telomerase is expressed in around 90 % of human cancers and is considered an attractive therapeutic target for treating oncological disease. Different strategies targeting telomerase have been developed. GRN163L (Imetelstat) is the only telomerase inhibitor that has entered clinical development, especially for essential thrombocythemia and myelofibrosis. However, the efficacy of GRN163 on solid tumors appears limited and this

impedes broader applicability[29]. Alternative approaches are available, however, to target telomere length. 6-Thio-deoxyguanosine (6-thio-dG) is an analogue of 6-thioguanine, which can interact with telomerase and is preferentially incorporated into telomere. Incorporation of the 6-thio-dG subsequently leads to the uncapping of the telomere[30]. Telomere dysfunction caused following uncapping activates the cellular DNA damage response and subsequently arrests cell growth. However, targeting telomerase may have undesired effects on normal cells with telomerase activity, including some stem cells and progenitor cells[31]. Although only few of such cell populations exist in human body, they are considered indispensable for tissue renewal and regeneration. Thus the potential of telomerase targeting in the body requires further investigation.

Genetic modified bacteria for disease treatment

Microorganisms, including bacteria, viruses and various unicellular or multicellular eukaryotes, can live in human body and profoundly influence human health, either beneficial (*e.g.* through vitamin synthesis) or detrimental (*e.g.* by provoking diarrhea). Microbes have long been consumed in a variety of fermented food and drinks to the benefit of the host[32]. With increasing knowledge of human diseases and regulatory roles played by microbes in human health, novel living organisms have been generated that can be used therapeutically to combat human diseases. Development of the synthetic biology has further augmented the power of such living organisms as therapeutic agents, as it enables the controlled engineering of the living organisms[33].

Over the past decades, bacteria have also been harnessed to combat cancer. Many genera of bacteria have been shown to preferentially accumulate in tumors, including *Salmonella, Escherichia, Clostridium* and *Bifidobacterium*. *Caulobacter, Listeria, Proteus* and *Streptococcu,* that all have been investigated as potential anticancer agents[34]. Numerous bacterial strategies have been carried out in pre-clinical and clinical studies. Success has been observed in reducing tumor volume and increased survival. Comparing to standard cancer therapy, genetic modified bacteria holds advantages of specifically targeting tumors, intratumoral penetration, enhanced effectiveness by expressing anticancer agents[35]. With rapid development in this field, there is little standardization before it can be used in the clinic. Thus, I engaged to perform a thorough study of the literature to investigate the steps

needed to move forward in these respects, especially with the aim of defining novel therapy for gastrointestinal and hepatic cancers.

7. Aims of this thesis

Prompted by the considerations mentioned above, in this thesis I endeavor to link biology to clinical treatment for better therapy of liver cancer and also gastrointestinal cancer. My strategy is to increase understanding the molecular mechanisms of HCC development and see how these findings can relate to the potential efficacy and of existing medication, preferably those already approved for clinical use, as introduction of such medication for clinical testing is relatively straightforward. As it is not always possible to target cancer biology with existing medication, or that side effects of targeting specific targets in cancer biology may well be unacceptable (telomerase comes in mind) I decided also perform an exploratory analysis of potential of targeting treatment using genetically modified bacteria. To this end, I first explore the use of targeting liver cancer metabolism using purine synthesis inhibitors (**Chapter 2**). These inhibitors are already used for HCC patients in the context of immunosuppression following orthotopic liver transplantation, although most patients currently receive alternative medication for this purpose. As I now find that these inhibitors inhibit the cancer process – while I also characterize their effects on the hepatocyte cytoskeleton in detail – my findings imply that the use of such inhibitors for HCC-related organ transplantation would be associated with superior clinical outcome.

The insights gained from the first two chapters set the stage for an in depth analysis of the role of IMPDH isoforms in liver cancer cell biology. This analysis is provided in **Chapter 3**. As was also observed in chapter 4 (in which differential effects of u-STAT1 and p-STAT1 will be described) we see that despite the promising results obtained in chapter 2, different isoforms of IMPDH have dichotomal effects. Thus the conclusion is that fundamental new approaches will be necessary and the remaining part of my thesis explores such approaches.

Subsequently I focus on STAT1 (**Chapter 4**). Recently inhibitors that impair STAT1 phosphorylation have become available in the clinic (*e.g.* Tofacitinib), while IFNs (that stimulate STAT1 phosphorylation) have already been available for clinical use for several decades. Improved insight into the relative contribution of phosphorylated and unphosphorylated STAT1 may thus help tinkering novel therapy and hence I characterize the effects involved and this should prove instrumental in designing rational therapy.

In **Chapter 5** I characterize the effects of targeting telomerase for anticancer therapy. Use of telomerase inhibitors is being impeded by fears of unacceptable side effects on stem cell compartments. I actually show, however, that such compartment are quite telomerase inhibition resistant. Nevertheless, concerns remain over potential side effects of such therapy.

In **chapter 6** I subsequently perform an exploratory analysis on the potential use of genetically-modified bacteria to target human disease. Advantages of such a strategy would be targeted delivery, with relatively little exposure of other parts of the body to the therapeutic proteins involved. I conclude that it might be possible to execute therapy in this fashion.

Chapter 7 provides a discussion and integration of the results obtained: while I observe that the increased knowledge on the action of pharmacological compounds certainly has implications for our thinking on cancer therapy, cancer cell biology is complex and many of the effects observed have relatively little impact and can be considered incremental rather as paradigm changing. This is not true for the approach involving genetically-modified organisms, however, application of this technology is still in its infancy. Hence I am forced to include that despite now millennia of efforts in combating cancer by human kind, the battle is far from won and further research remains essential. I delineate potential avenues for such research.

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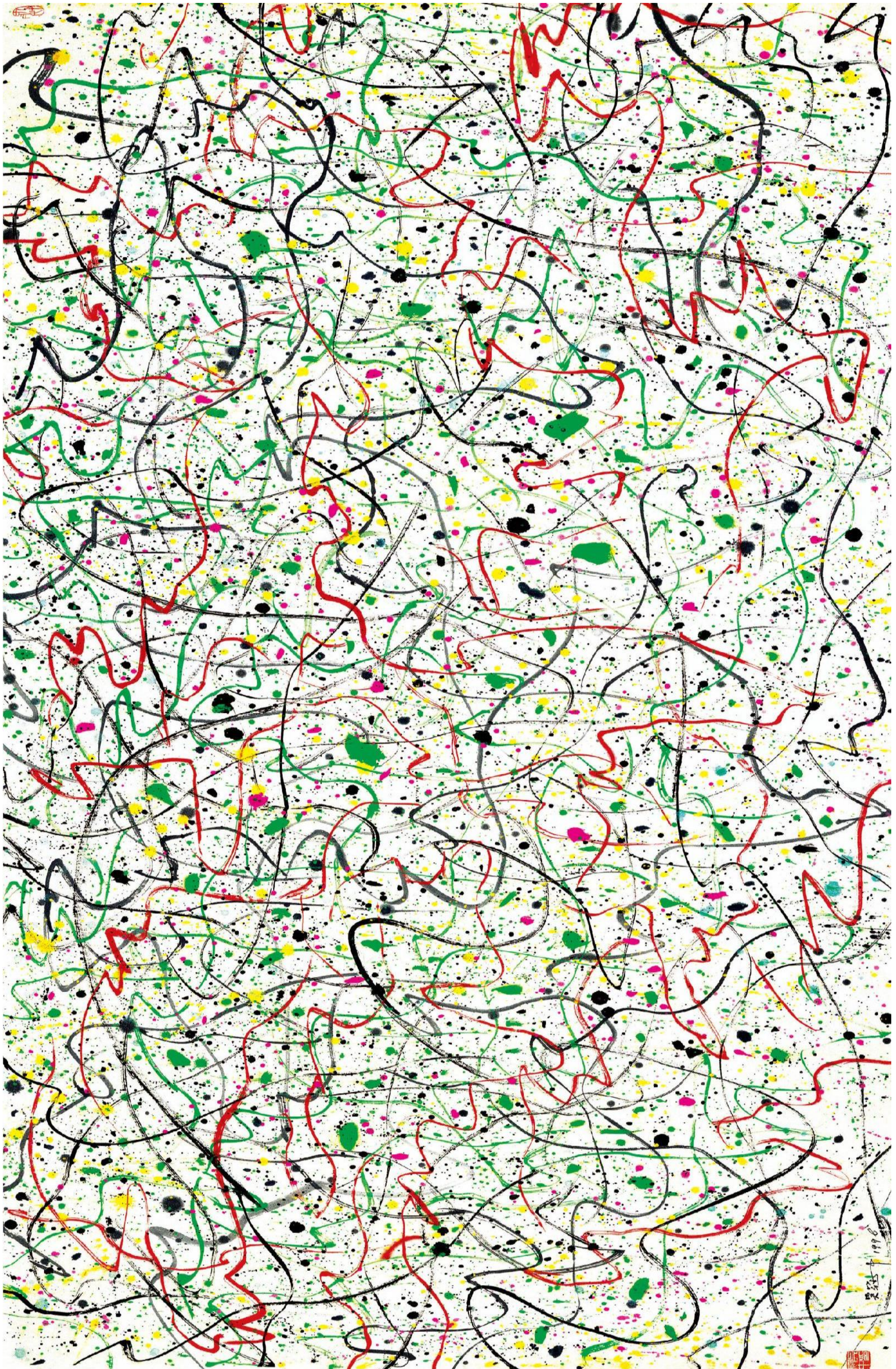

Chapter 2

Inhibiting experimental and clinical hepatocellular carcinoma by mycophenolic acid involves formation of cytoplasmic rods and rings.

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Adapted from:

Chen K, Sheng J, **Ma B**, Cao W, Hernanda PY, Liu J, Boor PPC, Tjon ASW, Felczak K, Sprengers D, Pankiewicz KW, Metselaar HJ, Ma Z, Kwekkeboom J, Peppelenbosch MP, Pan Q. Suppression of Hepatocellular Carcinoma by Mycophenolic Acid in Experimental Models and in Patients. Transplantation 2019 May;103(5):929-937.

and

Chen K, **Ma B**, Peppelenbosch MP, Pan Q. Cytoplasmic rods and rings in mycophenolic acid treatment. Liver Int. 2017 Nov;37(11):1742-1743.

Abstract

Tumor recurrence is a major complication following liver transplantation (LT) as treatment for hepatocellular carcinoma (HCC). Immunosuppression is an important risk factor for HCC recurrence, but conceivably may depend on the type of immunosuppressive medication. Mycophenolic acid (MPA) is a currently widely used immunosuppressant acting through depletion of guanine nucleotide pools by targeting inosine monophosphate dehydrogenase (IMPDH). With clinically achievable concentrations, we found that MPA effectively constrains HCC developmentin in both experimental HCC models and HCC-related LT patients. Mechanistically, MPA effectively elicited cell cycle arrest and enforced its main target IMPDH2 to form rod and ring structures in HCC cells. Most importantly, the use of MMF in patients with HCC-related LT was significantly associated with less tumor recurrence and improved patient survival. Thus, MPA can specifically counteract HCC growth *in vitro* and tumor recurrence in LT patients involves induction of IMPDH ultrastructural distribution. These results warrant prospective clinical trials into the role of MPA-mediated immunosuppression following LT of patients with HCC.

Keywords: Liver transplantation (LT), Hepatocellular carcinoma (HCC), Inosine monophosphate dehydrogenase 2 (IMPDH2)

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide[1]. Surgical resection or liver transplantation (LT) is currently the only potentially curative treatment options. LT is particularly attractive because of the radical resection of the tumor achieved. Moreover, LT cures the underlying liver disease along with the replacement of the diseased liver that remains at risk for the development of new malignant lesions when simple tumor resection is executed. However, tumor recurrence is a common threat for the success of both surgical resection and LT[2]. A unique risk factor strongly associated with recurrence in LT patients is the universal use of immunosuppressants after transplantation, which is to prevent graft rejection[3-5] but concomitantly hampers anti-cancer immunosurveillance.

Importantly, immunosuppression involves inhibition of immune cell proliferation and thus such therapy might have direct effects on the cancerous compartment as well. Besides a general impairment of the immunosurveillance system, different types of immunosuppressant could thus directly affect the malignancy process independent of the host immunity[4, 6-8]. Current research efforts in this respect are mainly focused on the mammalian target of rapamycin (mTOR) inhibitors, including rapamycin (sirolimus) and everolimus[9]. They are thought to be the only class of immunosuppressive agents that may reduce HCC recurrence, and this notion is supported by several retrospective and meta-analysis studies[10-12]. However, these studies do not provide firm evidence to establish superiority of mTOR inhibitors on HCC recurrence in comparison to other types of immunosuppression[13]. In a recent prospective study, it has been shown that sirolimus in LT recipients with HCC does not improve long-term recurrence-free survival beyond five years, although a beneficial effect between 3 to 5 years after transplantation in subgroups was suggested[14, 15]. Furthermore, higher rejection rates were reported for monotherapy of sirolimus or everolimus in HCC patients with liver transplantation[16, 17]. The differential effects of mTOR inhibitors in patients is probably related to the heterogenicity of HCC[18, 19]. It is unlikely that one immunosuppression protocol fits all cases. Therefore, the impact of other immunosuppressants also deserves to be carefully investigated, in order to define appropriate immunosuppressive regimens for management of HCC recurrence after LT.

Mycophenolic acid (MPA) and its prodrug, mycophenolate mofetil (MMF), are currently widely used for prevention of allograft rejection because of lacking nephrotoxicity[20]. These drugs act through depletion of guanine nucleotide pools by inhibition of inosine monophosphate dehydrogenase (IMPDH), in particular the isoform 2 (IMPDH2)[21]. This results in blockage of *de novo* guanine nucleotide synthesis and inhibition of lymphocyte proliferation[20]. Interestingly, MPA has been reported to be able to inhibit cancer cell proliferation in several experimental models of human solid tumors and hematological malignancies[22-25]. A large prospectively observational cohort study observed a tendency towards a lower risk of malignancy in MMF versus non-MMF treated renal transplanted patients[26]. However, this class of immunosuppressant has not been extensively studied in the setting of HCC recurrence after LT. This consideration inspired us to explore the effects and mechanism-of-action of MPA in experimental HCC models and in HCC-related LT patients.

Patients, materials and methods

Patient information

A LT database established in our previous study[5] was used for retrospective analysis of the effect of MMF on HCC recurrence. This cohort included patients transplanted between October 1986 to December 2007 at the Erasmus Medical Centre, Rotterdam, The Netherlands. All patients declared that they did not object to the use of their data in the study. Retrospective analysis of clinical data was performed in accordance with the approval and guidelines of the Medical Ethical Committee of the Erasmus Medical Center. From this database 44 out of 385 LT patients were identified as HCC-related LT and thus subjected to the analysis in this study. Their clinical information was described in Table S1.

Reagents

Stocks of MPA (AMRESCO LLC, USA) were dissolved in dimethyl sulfoxide (DMSO). The final concentrations of DMSO were ≤ 0.1%. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich. Matrigel was purchased from BD Bioscience. For the cytokines, B27 and N2 were purchased from Invitrogen; N-acetylcysteine, gastrin and nicotinamide were purchased from Sigma-Aldrich; EGF, FGF10 and HGF were purchased from Peprotech Company.

Cell culture

HCC cell lines, including HuH6, HuH7 and PLC/PFR/5 were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO Life Technologies), supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone Technologies), 100 units/mL of penicillin and 100 μg/mL of streptomycin. All the cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. For the control groups in this study, equal volumes of PBS containing the same concentration of DMSO as in the drugs were added, which were also marked as MPA at the concentration of 0 μM.

Tumor organoids culture

Single cells were isolated from liver tumor tissues of mice by using digestion solution as our previous study[27, 28]. Cells were mixed with matrigel, and then were planted into 24-well plates in a 37°C incubator for 30 min. After matrigel forming a solid gel, medium was added softly. Advanced DMEM/F12 (Invitrogen) works as the basic culture medium, supplemented with B27, N2, N-acetylcysteine, gastrin, nicotinamide, EGF, FGF10, HGF and R-spondin1 (produced by 293T-H-RspoI-Fc cell line). During the first 3 days, Noggin and Wnt3a (produced by 293T-HA-Noggin and L-Wnt3a cell lines respectively) were added. The medium was replaced every 3 days and passage was performed according to the growth of organoids.

MTT and Alamar Blue assays

Cells were seeded in 96-well plates, at a concentration of 6×10^3 cells/well in 100 μL medium. All cells were incubated overnight to attach to the bottom of the wells, and then treated with serials dilutions of MPA (3, 15, 30 and 60 μM). Cell viability was analyzed by adding 5 mg/mL MTT and then 150 μL DMSO per well. Absorbance was determined by using a spectrophotometric plate reader (Enzyme mark instrument, CytoFluor® Series 4000, Perseptive Biosystems) at the wavelength of 490 nm.

Organoids were split in the ratio of 1:10 for daily culture and seeded in 24-well plates. MPA (3 μM and 15 μM) was added to the organoids from the initial day. At the third day, organoids were incubated with Alamar Blue (Invitrogen, 1:20 in DMEM) for four hours, and medium was collected for analysis of the metabolic activity of the organoids. Absorbance was determined by using a fluorescence plate reader (CytoFluor® Series 4000, Perseptive

Biosystems) at the excitation of 530/25 nm and emission of 590/35 nm. Each treatment condition was repeated for three times and matrigel only was used as blank control.

Colony formation assay

Cells were harvested and suspended in medium, then seeded into 6-well plates (1000 cells/well). Formed colonies were fixed by 70% ethanol and counterstained with hematoxylin & eosin after two weeks. Colony numbers were counted.

For single organoid formation, organoids were digested into single cells firstly, and then the single living cells were further isolated by FACS sorter (Aria^{TM}, BD Biosciences). Propidium iodide (PI) staining was performed to exclude dead cells. Single cells were mixed with matrigel and seeded in 24-well plates (100 cells/well) for organoids initiation. Single organoids were formed after 5 days, and the sizes and numbers of the organoids were calculated.

Analysis of cell cycle

Cells (5×10^5/well) were plated in 6-well plates and incubated overnight to attach the bottom, and then serials concentrations of MPA were added. After 48 hrs, control and treated cells were trypsinized and washed with PBS and then fixed in cold 70% ethanol overnight at 4°C. The cells were washed twice with PBS and incubated with 20 µg/mL RNaseA at 37°C for 30 mins, and then with 50 µg/mL propidium iodide (PI) at 4°C for 30 mins. The samples were analyzed immediately by FACS Calibur. Cell cycle was analyzed by using Flowjo 7.6 software.

T cell isolation and [3H]-Thymidine assay

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Paque^{TM} (Life technologies). T cells were isolated with the Pan T cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Dynabeads coated with human T-activator CD3/CD28 antibodies (Life technologies) were added at a cell: bead ratio of 20: 1 T cells/well to stimulate T cell expansion and activation. T cells were cultured in round-bottom 96-well plates at the concentration of 1×10^5 cells/well in 200 µL RPMI1640 medium (GIBCO Life Technologies) supplemented with 10% FCS, at 37°C, 5% CO_2, with or without compounds. After 3 days, T cell proliferation was assessed by determination of [3H]-Thymidine (Radiochemical Central, Little Chalfont, UK) incorporation, 0.5 µCi/well was added and cultures were harvested 18 hours later.

Immunofluorescence assay

To observe the location and morphology of IMPDH2 protein, Huh7 cells treated with MPA were fixed with 4% (w/v) paraformaldehyde (PFA) for 10-15 min at RT. After three washes with PBS, cells were permeabilized with 0.1% (v/v) Triton X-100 for 10 min and washed with PBS for three times. Subsequently IMPDH2 antibody was used as primary antibody (1:200), and anti-rabbit-Alexa Fluor 488-conjuated antibody (1:1000, Cell Signaling Technology) was used as secondary antibody for staining. The cells were viewed under the LSM 510 confocal microscope (Zeiss, Jena, Germany). The images were analyzed by LSM Image Browser software.

Statistical analysis

Statistical analysis was performed by using Chi-Square test, nonparametric Mann–Whitney test, Cox regression analysis and Kaplan Meier survival analysis in IBM SPSS Statistical program (IBM Corporation, Armonk, NY, USA). Mann-Whitney U-test and T-test were performed by using GraphPad InStat software (Graph Pad Software Inc, San Diego, USA). P-values < 0.05 were considered as statistically significant.

Results

Use of MMF is associated with reduced HCC recurrence and improved survival

We investigated the effect of MPA on the outcome of LT patients indicated by HCC in a prospectively collected LT cohort[5]. We have identified 44 out of 385 patients with HCC-related LT. Twelve cases of these HCC patients were treated with immunosuppressive regimens containing MMF at any time during the follow-up and for any period; whereas 32 patients were treated with immunosuppressive regimens that did not contain MMF. There were no significant differences between these groups regarding patient characteristics, including age and sex, and regarding known prognostic factors of HCC recurrence after LT[29], including the size of tumor, the number of lesions, tumor differentiation stage, vascular invasion, the level of α-fetoprotein (AFP) before transplantation and time of follow up (Table 1).

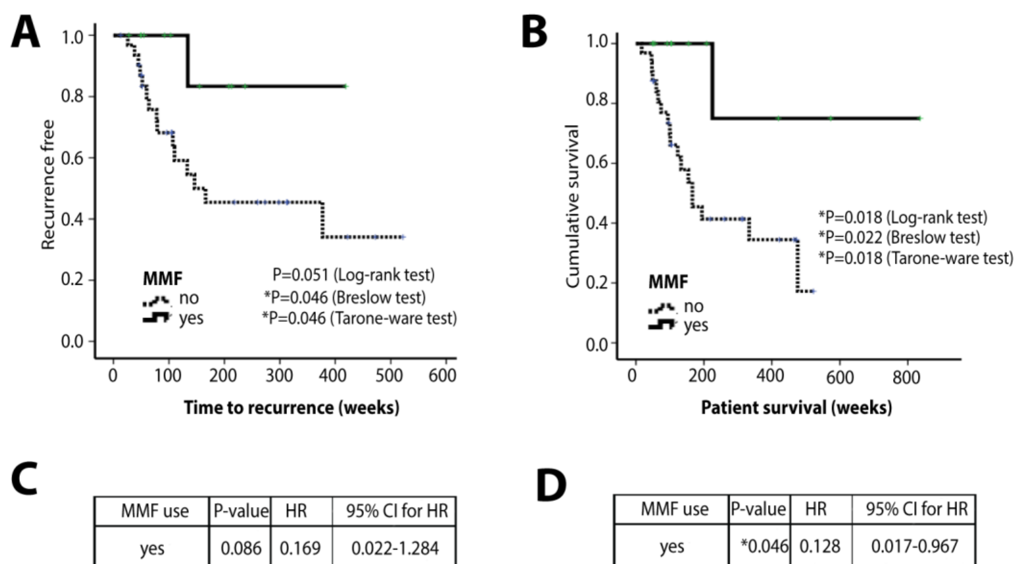
However, only one out of twelve patients (8.3%) in the MMF group developed recurrence; whereas fifteen out of thirty-two patients (46.9%) in the control group developed recurrence during follow-up. One patient died in MMF group (8.3%), but eighteen

Table 1. Patient characteristics according to MMF use

No	Characteristics	MMF use No (%/Median)	MMF use Yes (%/Median)	*P*-value[a]
1	Age	54.94	56.33	------
2	Sex (% male)	23/32 (71.9%)	10/12(83.3%)	0.446
3	Recurrence*	15/32 (46.9%)	1/12 (8.3%)	0.017*
4	Death**	18/32 (56.3%)	1/12 (8.3%)	0.004**
5	Size of tumor (>= 2 cm) [b]	18/32 (56.2%)	8/12 (66.7%)	0.542
6	Number of lesions (>= 2)	20/31 (64.5%)	8/12 (66.7%)	0.898
7	Differentiation			
	Good	9/31 (29.0%)	3/11 (27.3%)	0.798
	Moderate-Bad	22/31 (71.0%)	8/11 (72.7%)	0.789
8	Vaso - invasion	9/30 (30%)	1/11 (9.1%)	0.176
9	AFP (>25 μg/L) pre-transplantation	11/20 (55%)	4/12 (33%)	0.248

[a] Categorized parameter were compared using Pearson's Chi-Square test, mean differences were tested using Mann Whitney test.

[b] According to the Milan criteria, single lesion <= 5 cm or up to three individual lesions with none larger than 3 cm.



C

MMF use	P-value	HR	95% CI for HR
yes	0.086	0.169	0.022-1.284

D

MMF use	P-value	HR	95% CI for HR
yes	*0.046	0.128	0.017-0.967

Figure 1. MMF use is significantly associated with better clinical outcome in HCC-related LT patients. Kaplan Meier analysis (n = 44) revealed that patients using MMF display significantly longer times to HCC recurrence (*$P \le 0.05$) (A) and have a better survival (*$P < 0.05$) (B); Consistently, Cox regression analysis showed that patients using MMF have a lower risk of fast recurrence (progression) (C) and lower risk of poor survival (*$P < 0.05$) (D). HR: Hazard Ratio.

patients died (56.3%) in the control group. Thus, the use of MMF was significantly associated with lower recurrence rates ($P < 0.05$; Table 1) and higher survival rates ($P < 0.01$; Table 1). Kaplan Meier analysis confirmed that patients using MMF have significantly delayed HCC recurrence ($P \leq 0.05$; Figure 1A) and associated with better patient survival ($P < 0.05$; Figure 1B). Consistently, Cox regression analysis revealed that patients using MMF have a lower risk of fast recurrence (progression; HR = 0.169, 95% CI: 0.022-1.284; Figure 1C) and lower risk of demise (HR = 0.128, 95% CI: 0.017-0.967; Figure 1D). These results indicate that MMF use is associated with reduced HCC recurrence and improved survival in liver transplant patients.

MPA inhibited cell proliferation and colony unit formation of human HCC cells

In order to investigate whether MPA may directly affect the cellular physiology of HCC cells, the effects on cell proliferation and single cell colony unit formation (CFU) were evaluated in different HCC cell lines. Treatment of MPA inhibits cell proliferation in HuH6, HuH7 and PLC/PRF/5 cell lines at clinically relevant concentrations ($P < 0.001$; Figure 2A). In liver transplantation patients, MPA serum peak levels range from 2 to 30 μM, and the drug levels in liver will exceed those observed in serum due to accumulation[30, 31]. Sorafenib, the FDA-approved anti-HCC drug, is a small inhibitor of several tyrosine protein kinases, including VEGFR, PDGFR and Raf family kinases[32]. The potency of MPA was comparable to Sorafenib, in particular at the concentration of 3 μM, although weaker than Sorafenib at a higher concentration of 15 μM ($P < 0.01$; Figure S1A and S1B). Surprisingly, the widely used mTOR inhibitor, Rapamycin, did not show inhibitory effect on HCC cells in our experimental setting at clinically relevant or even higher concentrations (Figure S1C)[33].

In apparent agreement, MPA profoundly inhibited the number of colonies formed in the CFU assay. It appears that even at a relatively low concentration of 3 μM, MPA already impeded colony formation (Figure 2B and C). HuH7 cells were more sensitive to MPA treatment compared to HuH6 and PLC/PRF/5 cells. In this cell model, 105.70 ± 13.90 colonies were formed in untreated cultures but only 13.60 ± 11.25 colonies were formed in 15 μM MPA treated group (mean ± SEM, n = 10, $P < 0.001$; Figure 2C). We concluded that MPA strongly interferes with HCC cell expansion *in vitro*.

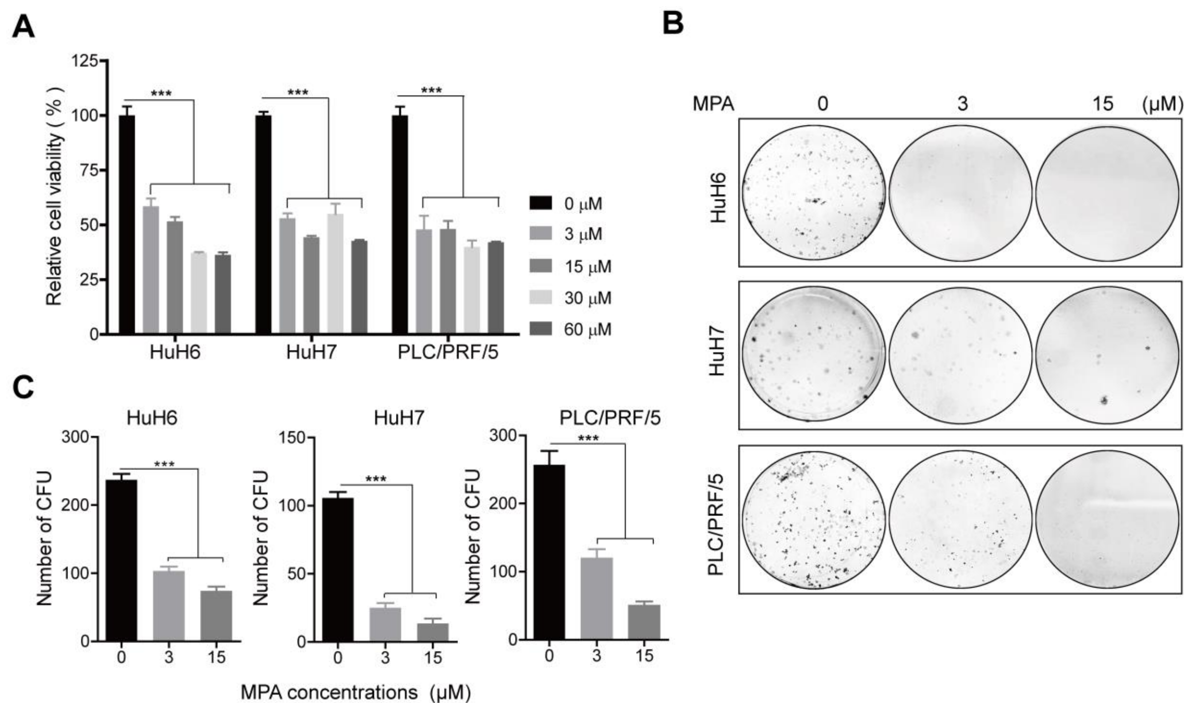


Figure 2. MPA inhibits cell growth in HCC cell lines. (A) With clinically achievable concentrations, MPA potently inhibited cell proliferation, determined by MTT assay (mean ± SEM, n = 6, ***P < 0.001); (B) and (C) MPA inhibited the ability of colony formation in HuH6, HuH7 and PLC/PFR/5 cell lines respectively. (mean ± SEM, n = 9 or 10, respectively, ***P < 0.001). Shown is results from at least 3 independent experiments.

MPA effectively inhibited the initiation and growth of mouse liver tumor organoids

3D culture of primary tumor organoids has been recently demonstrated as advanced liver cancer models[27, 28, 34]. Therefore, we have investigated the effects of MPA on the initiation and growth of tumor organoids derived from primary mouse liver tumors. MPA effectively inhibited the growth of formed organoids shown by morphological appearance (Figure 3A). Alamar Blue assay demonstrated 79.03% ± 0.01 and 82.75% ± 0.01 inhibition at 3 μM and 15 μM, respectively (mean ± SEM, n = 3, P < 0.001; Figure 3B). Furthermore, MPA robustly inhibited the initiation of organoids from the dissociated single organoid cells (Figure 3C). The numbers of initiated organoids were 27.67 ± 4.51, 8 ± 1.00 and 4.67 ± 1.70 at 0, 3, and 15 μM of MPA, respectively (mean ± SEM, n = 3, P < 0.001; Figure 3D). The size of formed organoids was inhibited by 82.00% ± 0.08 and 89.09% ± 0.06 at 3 μM and 15 μM of MPA, respectively (mean ± SEM, n = 9, P < 0.001; Figure 3E).

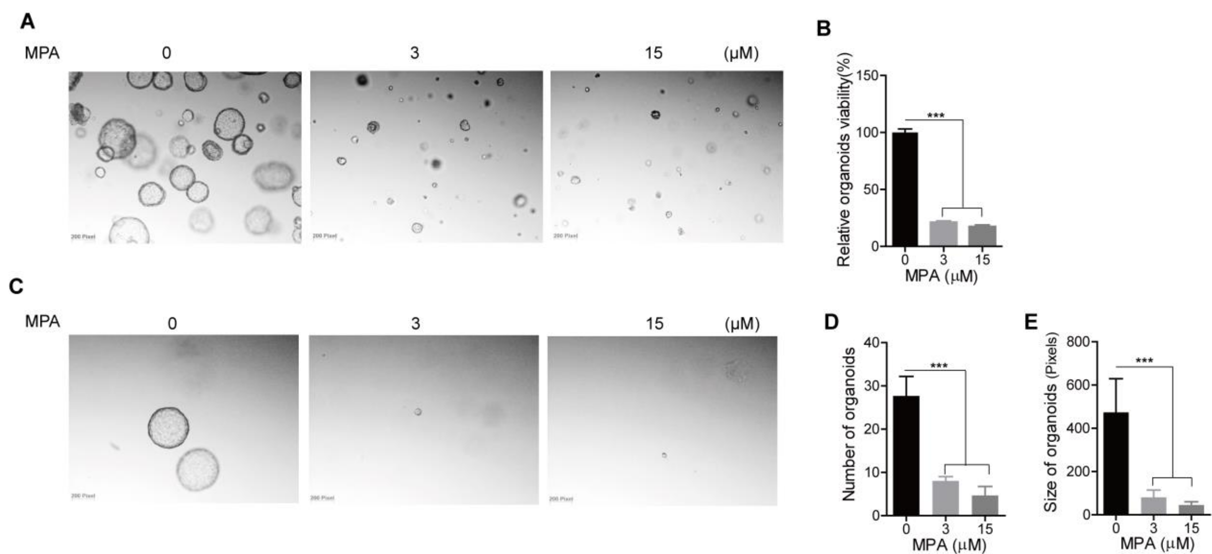


Figure 3. MPA inhibits the initiation and growth of organoids established from mouse primary liver tumors. (A) The appearance of organoids under 3-day MPA treatment; (B) MPA treatment significantly inhibited the growth of organoids, as determined by Alamar Blue assays after 3 days (mean ± SEM, n = 3, ***P < 0.001); (C) The appearance of single organoids expansion under 5-day MPA treatment; (D) The number of organoids (mean ± SEM, n = 3, **P < 0.01). (E) The size of organoids after 5 days (mean ± SEM, n = 3, ***P < 0.001). Shown is results from at least 3 independent experiment.

The cell cycling of HCC cells was arrested at S-phase by MPA treatment

To further understand how MPA acts on HCC cell growth, an assay for quantifying cell cycling was performed in HuH7 cells. Treatment of MPA dose-dependently increased the proportion of S phase by 25.83% ± 0.20 and 131.42% ± 0.32 at the concentrations of 3 and 15 μM, respectively. This concomitantly decreased the proportion of cells in the G2/M phase by 67.82% ± 0.23 and 87.28% ± 0.09 at the concentrations of 3 and 15 μM, respectively (mean ± SEM, n = 3, P < 0.05; Figure 4). These data suggested that MPA inhibits HCC cell growth by arresting the cell cycle.

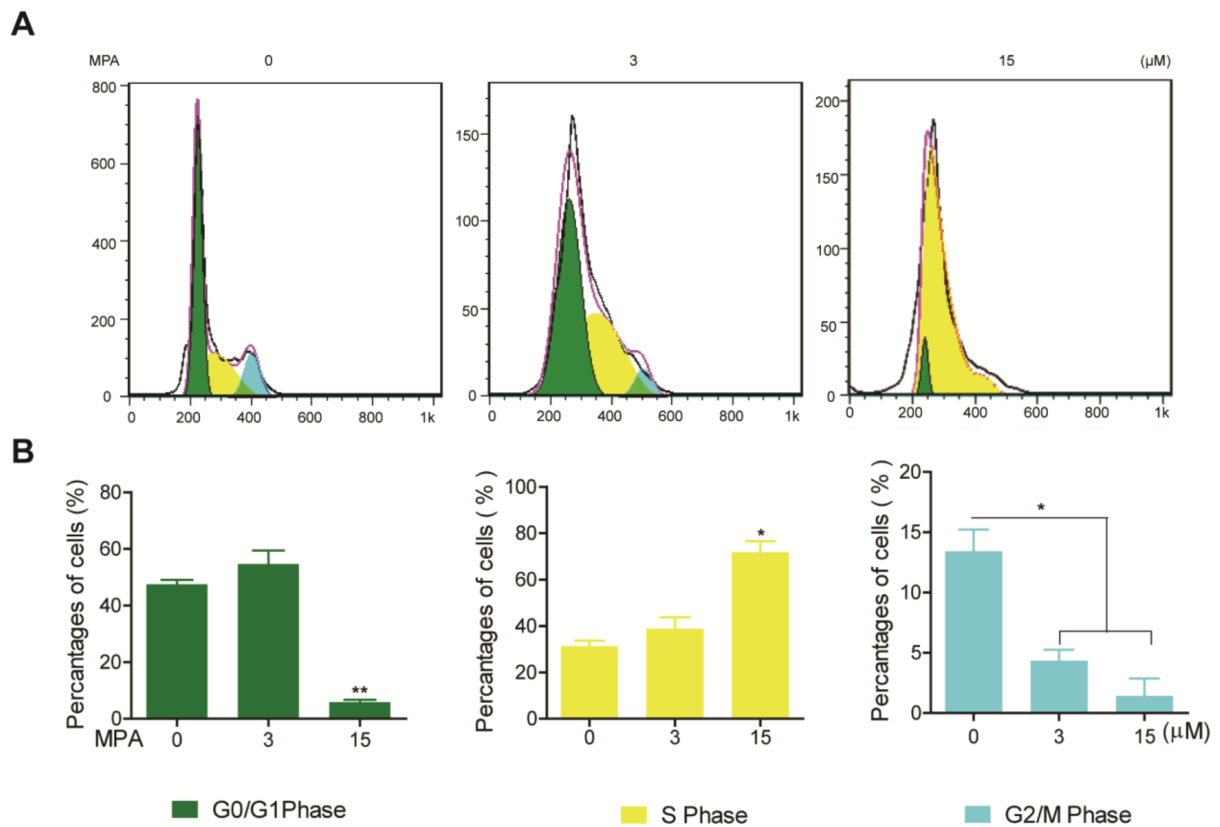


Figure 4. MPA arrests cell cycling. (A) HuH7 cells were arrested in the S phase by MPA treatment (FACS analysis); (B) Quantification of cell cycling analysis (mean ± SEM, n = 3. *P < 0.05; **P < 0.01).

Exogenous nucleotide supplementation partially counteracts the anti-growth effect of MPA

Depletion of intracellular nucleotide pool is the key immunosuppressive mechanism employed by MPA to inhibit lymphocytes proliferation. Supplementation of exogenous guanosine nucleotide indeed partially counteracted the anti-proliferative effects of MPA on HCC cell lines, but this effect is related to the cell type and dosage (Figure 5A). This effect was also observed in colony formation assay. The numbers of colonies were 102.17 ± 19.63, 31.17 ± 14.02 and 107.67 ± 27.73 in HuH6, HuH7 and PLC/PRF/5 cell lines with MPA (3 μM) treatment, respectively. Supplementation of exogenous guanosine nucleotide (25 μM) increased the colony numbers to 134.83 ± 29.49, 71.50 ± 9.95 and 145.67 ± 28.91 in HuH6, HuH7 and PLC/PRF/5 cell lines, respectively (mean ± SEM, n = 6, P < 0.05 or P < 0.001; Figure 5B and C). However, high doses of MPA out-compete exogenous guanosine nucleotides, especially in HuH7 and PLC/PRF/5 cells (Figure 5A, B and C).

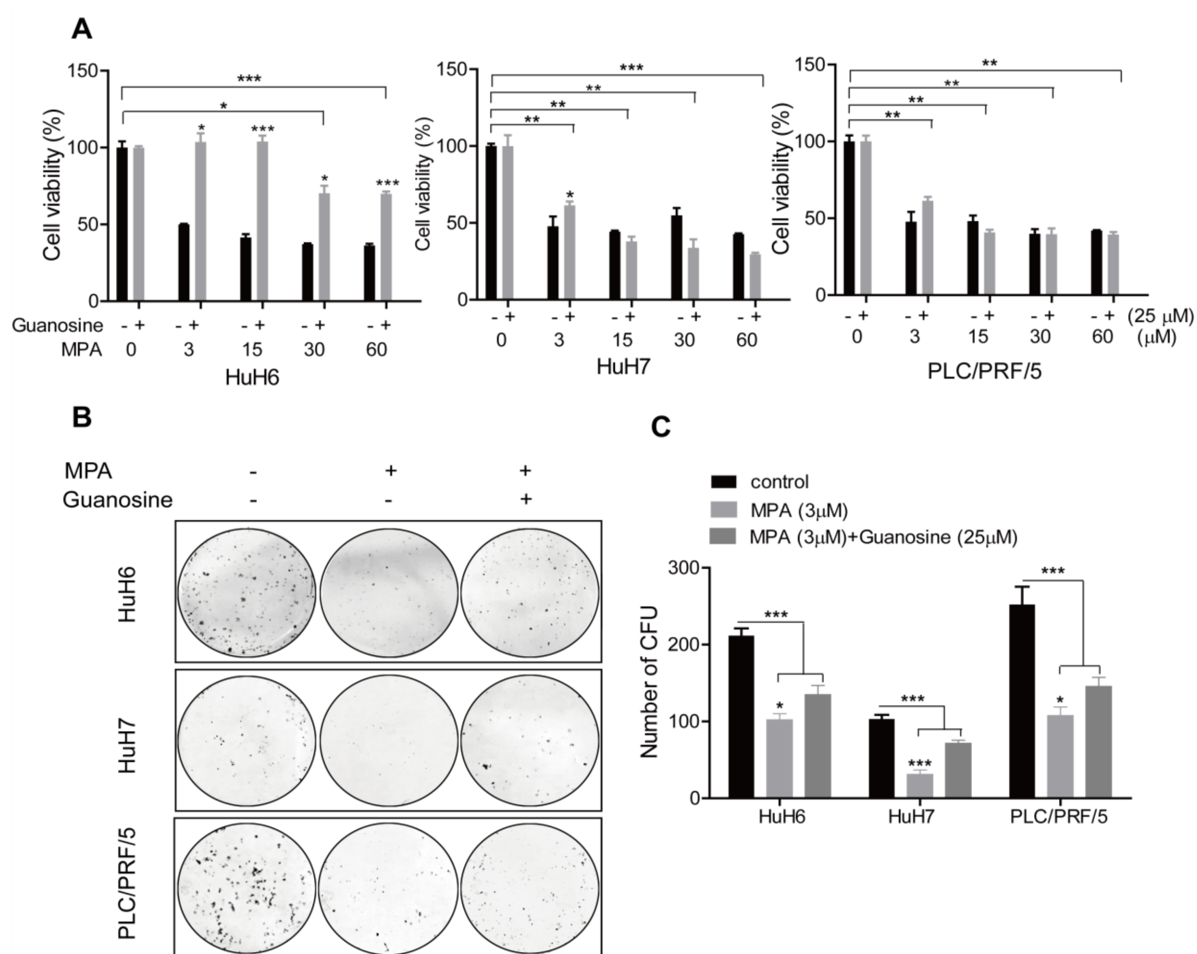


Figure 5. Guanosine supplementation partially counteracts effects of MPA. MTT assay of HuH7, HuH6 and PLC/PFR/5 cell lines (A) and CFU assay of HuH6 and PLC/PRF/5 cell lines treated with MPA or/and guanosine (B) showed that exogenous guanosine could partially counteracted the effect of MPA; (C) Quantification of CFU assay (mean ± SEM, n = 6, ***P < 0.005). Shown is results from at least 3 independent experiments.

New IMPDH inhibitors have potential immunosuppressive and/or anti-HCC properties

We explored the possibility to develop new IMPDH inhibitors exhibiting superior anti-HCC activity as compared to MPA but with comparable immunosuppressive activity, which may constitute improved treatment choices following HCC-indicated LT. Twenty-three IMPDH inhibitors were developed and profiled. Their immunosuppressive capability was evaluated in a T cell proliferation assay. Fifteen of them were more potent than MPA in inhibiting T cell proliferation after 72 h treatment (mean ± SEM, n = 9, P < 0.01; Figure 6A). Intriguingly, four out of these compounds (1351, 1353, 1382 and 1407) were identified as more potent inhibitors of HuH6 cells proliferation than MPA (mean ± SEM, n = 9, P < 0.05; Figure 6B). Collectively, three compounds (1351, 1353 and 1382) were found possessing both stronger

immunosuppressive and anti-tumor activity (Figure 6C). Interestingly, three compounds significantly inhibit HuH6 cells proliferation (1393, 1400 and 1407) (compounds vs CTR, mean ± SEM, n = 9, P < 0.001) without affecting T cell growth (Figure 6C), which suggests that these compounds may have potential as new generation of anti-HCC drugs in a non-transplant setting that does not require immunosuppression.

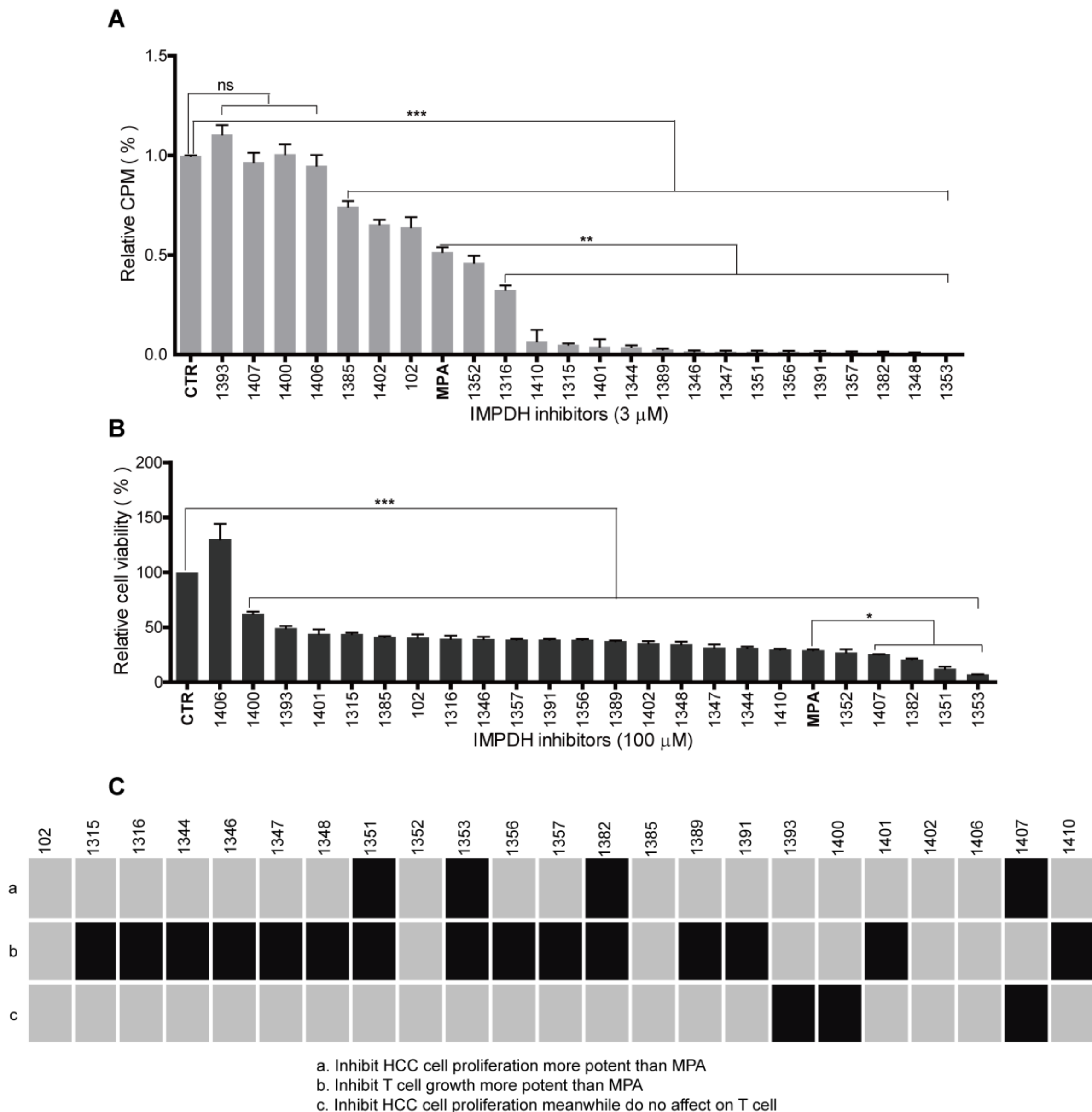


Figure 6. Other IMPDH inhibitors and their immunosuppressive and anti-HCC activity. (A) [3H]-Thymidine assay showed that fifteen compounds were more potent than MPA in inhibiting T cell proliferation (mean ± SEM, n = 3, **P < 0.01); (B) MTT assay showed that four compounds were more potent than MPA in inhibiting HuH6 cells proliferation (mean ± SEM, n = 3, *P < 0.05); (C) Three compounds were verified to be more potent in inhibiting T cells and HuH6 cells than MPA (mean ± SEM, n = 3, **P < 0.01). Three compounds could inhibit HuH6 cells proliferation without effecting T cell proliferation (compounds vs CTR, mean ± SEM, n = 3, ***P < 0.001). Shown is results from at least 3 independent experiments.

Cytoplasmic rods and rings in mycophenolic acid treatment

It has recently been proposed that anti-viral action of ribavarin (RBV) relates to RBV-induced rearrangement of IMPDH to form rods and rings[35]. To obtain further insight whether also the anti-oncogenic action of the IMPDH inhibitor MPA has a similar association to altered IMPDH ultrastructural distribution, we investigated the effects of the drug of subcellular distribution of IMPDH. Interestingly, we observed that MPA exposure can potently induce the cytoplasmic rearrangement of IMPDH to form ring and rod-like structures in human hepatoma cells, and this could not be completely reversed by guanosine supplementation (Figure 7). These observations suggest that the induction of stable ring and rod structures is a common action of IMPDH inhibitors, which correlates with the general clinical effects of these compounds.

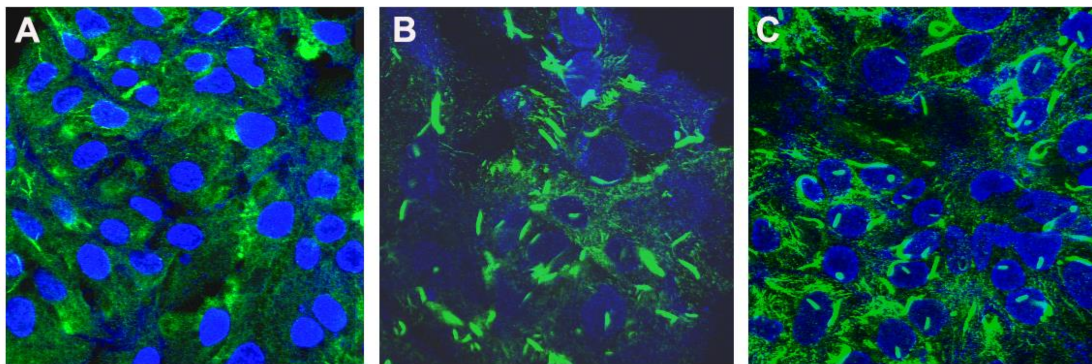


Figure 7. MPA treatment induces RR structure in the human hepatoma Huh7 cells. (A) The IMPDH protein shows a dispersed distribution in the cytoplasm of Huh7 cells; (B) After MPA treatment at the concentration of 3 µM for 24 hrs, IMPDH was aggregated into RR structure; (C) Supplementation of guanosine (25 µM) was unable to reverse MPA-induced IMPDH aggregations. Blue: DAPI nuclear staining. Green: antibody against Human IMPDH2.

Discussion

Although it is suspected that immunosuppressive medication following LT facilitates HCC recurrence, the issue of how specific immunosuppressive drugs affect the disease process is poorly understood[36]. Obviously, a regimen that can perform its immunosuppressive function which is necessary for preventing graft rejection but that concomitantly exerts anti-tumor effects should be the preferential clinical choice in this particular setting. In this aspect, mTOR inhibitors attract attention. However, only approximately 50% of all HCC patients exhibit activation of mTOR downstream signaling elements in their tumors[10, 37]. Indeed, both experimental and clinical evidence suggest that tumors bearing different genetic mutations can respond differentially to mTOR inhibitors[38, 39]. Given the heterogeneity of HCC, other immunosuppressive regimens also deserve careful attention. Several studies have reported that MPA could inhibit cancer cell proliferation across different types of cancer cell lines[40-43] as well as potentially supportive evidence from patients[44, 45].

In this study, we have demonstrated an anti-cancer effect of MPA in experimental HCC models including human HCC cell lines and mouse primary liver tumor organoids. Culture of primary liver cancer cells from either human or mouse has been proven to be very difficult. The organoid technology (culturing “mini-organ” in 3D) has endowed the possibility of establishing stable cultures from primary tumors, including for liver tumors[27, 28, 34]. Our data support that MPA has potent inhibitory effects on HCC growth *in vitro*. More importantly, clear inhibition of mouse liver tumor organoids initiation and growth were also observed after MPA treatment. We further provided clinical evidence that the use of MMF, the prodrug that metabolizes into MPA after administration, is associated with reduced disease recurrence and improved survival in HCC-related liver transplant patients. These results indicated an anti-tumor action of MPA occurring.

Although the anti-tumor effects of MPA have been substantially established, it is still unclear how this drug exerts the anti-tumor activity. Several molecular pathways appear to play a pivotal role in MPA-induced apoptosis[46]. Two p53 induced genes (TP53I3 and TP53INP1), as well as the p53 protein, are known to be up-regulated by MPA[46]. The increase of p53 level provides a mechanism for rapid growth arrest or apoptosis in the event of DNA damage during S phase of cell cycle[47]. In our study, the induction of S phase arrest in HCC cells by MPA is in agreement with these known findings. We surprisingly found that

supplementation of exogenous guanosine counteracts only to a minor extent to the inhibitory effect of MPA in HCC cells. Although depletion of guanine nucleotide pools by inhibiting IMPDHs is the predominate mechanism in inhibiting lymphocyte proliferation, this however only partially explain the mechanism-of-action in anti-HCC by MPA.

Although the exact mechanism by which MPA acts remains unclear, Covini *et al.* has proposed a scenario in which enzymatic activity of IMPDH is shuttled down as a consequence of ring and rod formation, which in turn provokes IMPDH to become autoantigenic, and hence the production of specific autoantibodies[35]. This was also found in our study during MPA treatment, which induce the ring and rod formation. Thus, targeting IMPDH is expected to inhibit cancer by simultaneously blocking nucleotide synthesis and provoking immune response through RR structure induced autoantibodies. We think this is particularly relevant to therapeutic targeting IMPDH in cancer treatment. The IMPDH2 isoform is upregulated in a wide range of cancer tissues, associated with disease aggressiveness, and related to poor patient survival[6]. Of note, a general feature of many IMPDH inhibitors (e.g. MPA) is immunosuppressive. Therefore, the development of new inhibitors retaining the potent antiviral and anti-cancer effects but avoiding immunosuppressive activity represents as a new direction to move forward.

Excitingly, after performing a retrospective analysis in our LT cohort, we found an association between MMF use and reduced HCC recurrence and improved patient survival. Importantly, there are no significant differences regarding patient and tumor characteristics[29] between these two groups. It must be said that our observations may also be related to a potential inferior immunosuppressive effect of MMF containing treatment regimens. Because of the small sample size, the single center setting, and the retrospective nature of these findings, further clinical evaluation is warranted preferentially in randomized studies to confirm our findings. Moreover, three out of twenty-three other IMPDH inhibitors were found to possess both stronger immunosuppressive and anti-tumor activity than MPA and may therefore be considered as potential alternatives for MMF in the LT set.

In summary, this study has demonstrated that clinically relevant concentrations of MPA are capable of constraining HCC cell growth in experimental models. We further provided clinical evidence that MMF is associated with reduced HCC recurrence and improved survival in liver transplant patients. Confirming these experimental findings and

retrospective clinical observations by prospective randomized trials could lead to better management of immunosuppressive medication for HCC patients after LT.

Supplementary Information

Supplementary Table S1. Clinical information of patients using MMF

No	Age (yrs)	LTx date	Start date MMF	End date MMF	MMF Period (weeks)	Recurrence date	Death date
1	58	22-May-1992	26-Jan-1998	21-Feb-2008	525	-	-
2	50	18-Jan-1998	26-Sep-2002	04-Feb-2011	436	-	-
3	55	18-May-2006	24-May-2006	04-Jul-2013	371	-	-
4	53	28-Jul-2007	13-Aug-2007	12-Jun-2013	304	-	-
5	60	21-Dec-2005	25-Jan-2006	07-May-2009	171	-	-
6	69	21-May-2000	19-Dec-2005	22-Sep-2008	144	-	-
7	63	05-Sep-2007	11-Sep-2007	15-Jun-2010	144	-	-
8	65	20-Nov-2004	15-Sep-2005	17-Mar-2008	130	19-Jun-2007	18-Mar-2009
9	58	01-Jan-2007	29-Jan-2007	21-Jan-2010	155	-	-
10	24	09-Feb-2005	02-Mar-2005	20-Apr-2006	59	-	-
11	65	23-Aug-2007	23-Aug-2007	21-Sep-2007	4	-	-
12	56	22-Jan-2007	26-Jan-2007	15-Oct-2007	37	-	-
13	52	27-Mar-2007	-	-	-	-	-
14	50	07-Nov-1997	-	-	-	-	18-Jan-2001
15	52	19-Jul-1997	-	-	-	-	09-Dec-2003
16	43	02-Mar-1998	-	-	-	-	12-Feb-2000
17	55	10-Sep-2004	-	-	-	-	23-Aug-2005
18	60	16-Feb-2000	-	-	-	-	-
19	54	22-Mar-2002	-	-	-	-	11-Jan-2004
20	63	25-Jan-1994	-	-	-	-	09-Jun-1996
21	67	26-Jul-1998	-	-	-	-	-
22	56	25-Apr-2005	-	-	-	-	31-Mar-2007
23	42	31-Mar-2005	-	-	-	-	23-Feb-2006
24	61	10-Oct-2006	-	-	-	-	-
25	69	05-Apr-2000	-	-	-	-	23-Feb-2001
26	55	04-Jul-1995	-	-	-	-	13-Sep-1998
27	58	24-May-2006	-	-	-	-	-
28	24	04-Oct-1989	-	-	-	-	02-Dec-1990
29	67	04-Mar-2007	-	-	-	-	-
30	64	17-Sep-2007	-	-	-	-	-
31	53	15-Feb-2001	-	-	-	-	21-May-2002
32	50	03-May-2000	-	-	-	-	-
33	60	17-Oct-1999	-	-	-	-	05-Dec-2008
34	66	06-Aug-2001	-	-	-	-	22-Jul-2004
35	48	08-Jul-2004	-	-	-	-	-
36	53	05-Jan-1990	-	-	-	-	26-Jul-1992
37	46	14-Apr-2004	-	-	-	-	17-Sep-2005
38	44	01-May-1996	-	-	-	-	30-Aug-1996
39	61	10-Oct-1999	-	-	-	-	-
40	58	16-Nov-2002	-	-	-	-	-

41	57	29-Nov-2003	-	-	-	-	-
42	57	17-Oct-1996	-	-	-	-	10-Jul-2000
43	57	12-Jun-2002	-	-	-	-	-
44	66	15-Mar-2007	-	-	-	-	-

Note: - no recurrence/no death/no MMF treatment

Supplementary Figure 1

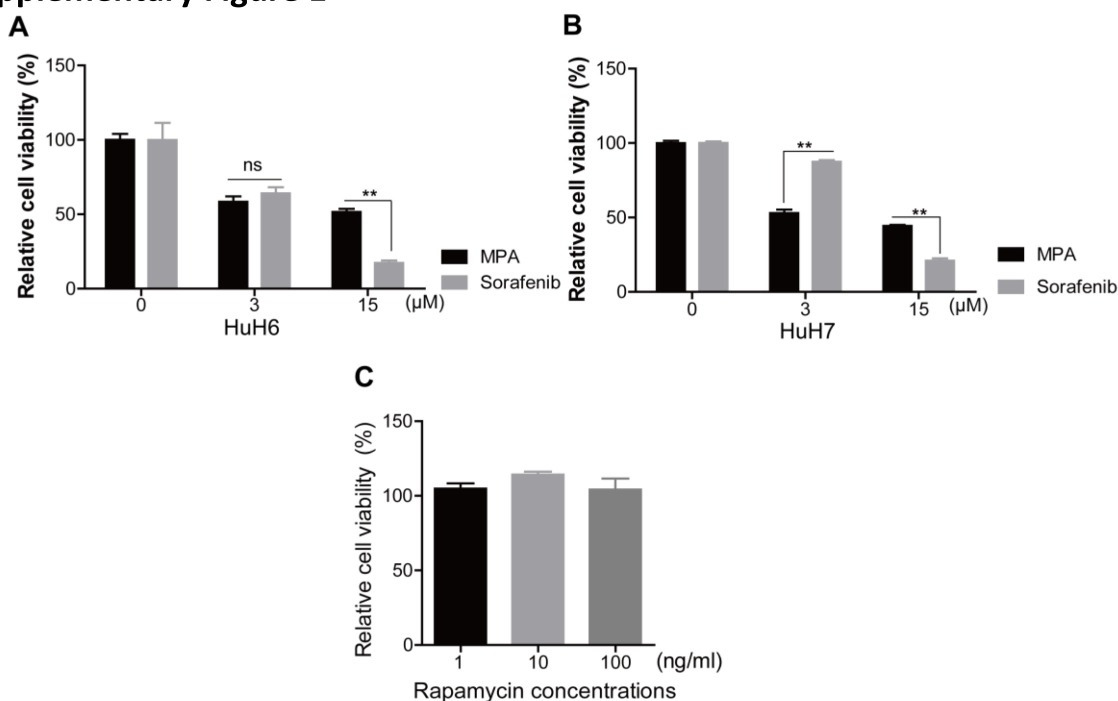


Figure S1. The effects of MPA, Sorafenib and Rapamycin on HCC cell lines. (A) At the low concentration of 3 μM, the inhibitory effects of MPA and Sorafenib have no significant difference, while at the concentration of 15 μM, Sorafenib* showed more potent effect in HuH6 cell line; (B) At the low concentration of 3 μM, MPA has stronger inhibitory effect, while at the concentration of 15 μM, Sorafenib showed more potent inhibition in HuH7 cell line; (C) The effects of Rapamycin (at the concentrations of 1ng/ml, 10ng/ml and 100ng/ml, respectively) were not significant in HuH6 cell line, determined by MTT assay. (mean ± SEM, n = 3, respectively, **P < 0.01, ***P < 0.001).

*Clinical use of sorafenib is 400 mg twice daily, and the C_{trough} sorafenib average concentration in patients treated with the dose of 400 mg is 8.78 ± 4.82 μg/ml (equivalent to 13.78 ± 7.57 μM)

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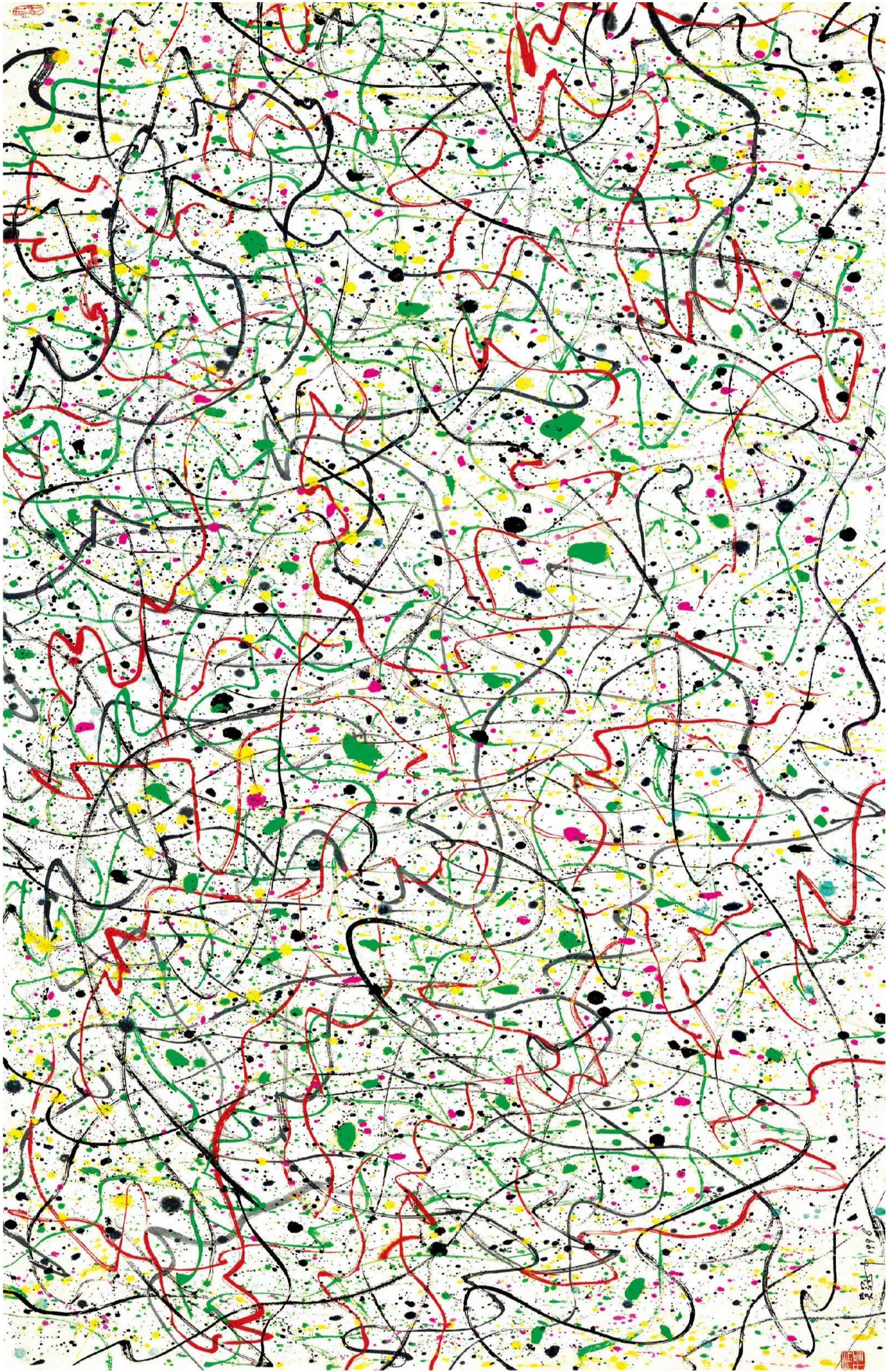

Chapter 4

Dichotomal functions of phosphorylated and unphosphorylated STAT1 in hepatocellular carcinoma

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J Mol Med (Berl). 2019 Jan; 97(1):77-88.

Abstract

Interferons (IFNs) with antiviral and immune-stimulatory functions have been widely used in prevention and treatment of hepatocellular carcinoma (HCC). Signal transducer and activator of transcription 1 (STAT1) is a key element of the IFN signaling, and the function of STAT1 is critically determined by its phosphorylation state. This study aims to understand the dynamics and functions of phosphorylated (p-) and unphosphorylated (u-) STAT1 in HCC. We found that u-STAT1 is significantly elevated in patient HCC tumor tissues and predominantly expressed in cytoplasm; while p-STAT1 is absent. Loss of u-STAT1 potently arrested cell cycle and inhibited cell growth in HCC cells. Induction of p-STAT1 by IFN-α treatment effectively triggers the expression of interferon-stimulated genes (ISGs), but has moderate effect on HCC cell growth. Interestingly, both u-STAT1 and p-STAT1 are induced by IFN-α, though with distinct dynamics. Importantly, artificial blocking the induction of u-STAT1, but not p-STAT1, sensitizes HCC cells to IFN-α treatment. Therefore, p-STAT1 and u-STAT1 exert opposite functions and coordinately regulate the responsiveness to IFN treatment in HCC.

Keywords: Hepatocellular carcinoma (HCC), Signal transducer and activator of transcription 1 (STAT1), Interferon (IFN) signaling, Immune response

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors[1] and the second leading cause of cancer related-death worldwide[2]. As a major etiology, chronic infection with hepatitis B or C virus (HBV or HCV) triggers liver fibrosis, cirrhosis, and eventually the development of HCC[3]. To prevent from or treat for viral hepatitis-related HCC, interferons (IFNs) have been explored in clinic[4, 5]. In context of tumors, IFNs can be produced by various cell types, including immune cells, as well as tumor cells. They elicit antitumor effects by directly controlling tumor cells or indirectly by regulating immune response[6]. However, the exact mechanisms remain poorly understood due to their multitude functions in respect to both intra-tumoral and micro-environmental determinants[7]. Although benefits of reducing cancer risk have been observed in clinical studies[5], IFN treatment for the management of HCC is still controversial and no clear recommendations have been proposed[7].

Signal transducer and activator of transcription 1 (STAT1), an important upstream regulator of the IFN signaling, functions as the core transcription factor to drive the transcription of a subset of IFN-regulated genes (IRGs)[8]. Upon IFN stimulation, phospho-STAT1 (p-STAT1) acts as a key element for STAT1 homodimerization (STAT1-STAT1) or heterodimerization (STAT1-STAT2-IRF9 complex, ISGF3). These complexes translocate to the nuclear with subsequent binding to Interferon-Stimulated Response Elements (ISRE) and Interferon-Gamma Activated Sequences (GAS), and then stimulate the transcription of IRGs to regulate host immune response and cell growth[9]. Although STAT1 has been found to be deregulated in a variety of cancers, the exact role of STAT1 in cancer, especially in different types of cells, remains controversial. On one hand, STAT1 is recognized as a tumor suppressor which can inhibit tumor growth through regulating cell proliferation, differentiation and death[10-13]. On the other hand, STAT1 can also be a tumor promoter as it can promote tumor cell growth, therapy resistance, and immune suppression[14, 15]. In addition, expression of STAT1 has been found to correlate with both good or poor prognosis in different types of cancers[16]. Although STAT1 was reported to be a potential suppressor in HCC[17], the findings are based on limited numbers of patients and a modest effect on HCC cell growth.

Upon IFN stimulation, p-STAT1 and unphospho-STAT1 (u-STAT1) act as two forms of STAT1 to perform its function[18]. Although p-STAT1 is recognized as the key activator of IFN signaling, u-STAT1 can also regulate gene transcription in the absence of IFN stimulation[19]. Thus, p-STAT1 and u-STAT1 stimulate transcription of different subsets of genes, which have distinct functions in immune responses of tumors to IFN-related therapy[7]. ISGs selectively controlled by u-STAT1, denoted as IFN-related DNA damage resistance signature (IRDS), have been identified in patients resistant to radio- and chemotherapy. Therefore, p-STAT1 and u-STAT1 were thought to have distinct functions and have been used as independent prognostic markers in predicting disease outcomes in cancer[20].

In this study, we investigated the expression and functions of p-STAT1 and u-STAT1 in HCC. Remarkably, we found that STAT1 was predominantly present as u-STAT1 form and was highly expressed in the cytoplasm of tumor cells from HCC patients. Although p-STAT1 induced by IFN-α treatment robustly stimulated ISG expression by activating the IFN signaling pathway and inhibited HCC growth, its function was quickly blocked by intrinsic or induced u-STAT1. Thus, the tumor suppressive or promoting role of STAT1 largely depends on its phosphorylation status. The dynamic induction of p-STAT1 and u-STAT1 by IFN treatment coordinately regulates the growth of tumor cells.

Material and Methods

Tissue microarray (TMA)

Archived formalin fixed paraffin-embedded tissue samples from 133 patients who underwent hepatic resection for HCC at Erasmus MC-University Medical Center between 2004 and 2014, were used for this study. Clinical data of this HCC cohort have been published previously[21]. The use of patient materials was approved by the medical ethical committee of Erasmus MC. TMA slides contained three or four 0.6 mm cores from the tumorous area and two 0.6 mm cores from the paired tumor-free liver (TFL) area of these patients.

Immunohistochemistry

Paraffin-embedded TMA slides were deparaffinized with xylene and rehydrated in graded alcohols (100%, 95%, 70%) for further immunohistochemistry staining. Slides were then washed with Phosphate Buffered Saline with Tween 20 (PBST) and boiled in citric acid buffer

(pH6.0) for 20 min for antigen retrieval. Peroxidase was blocked by adding 3% H_2O_2 for 10 min at room temperature. The slides were incubated overnight with the primary antibody against STAT1 (rabbit polyclonal; sc-592) (1:300) and p-STAT1 (58D6; rabbit monoclonal; #9176) (1:150) at 4°C. After being rinsed in PBST, slides were incubated with second anti-rabbit IgG antibody conjugated with HRP for 1 h at room temperature. DAB solution (0.05% DAB, 0.0017% H_2O_2) was then prepared and added to the slides to visualize antibody binding. The reaction was stopped by washing with distilled water. Subsequently, hematoxylin were employed for background staining of tissue. Negative control staining was carried out by omitting the primary antibody.

Cytoplasmic and nuclear staining were scored separately. Percentages of cells with cytoplasmic or nuclear expression were scored as follows: low for 0-30%; moderate for 30-70%; high for > 70%. Scoring of expression intensity was performed as: grade 1 for weak; grade 2 for moderate; grade 3 for strong. A final immune-reactivity score (IRS) was obtained for each case by multiplying the percentage and the intensity values, ranging from low, moderate and high. The scorings were done by two investigators.

Colony formation assay

Cells were trypsinized, harvested and suspended in culture medium. After quantified through counting, 2 × 103 cells were seeded into 6-well plates and the medium was refreshed for every four days. After two weeks culture, formed colonies were washed with PBS and fixed by 70% ethanol. Followed by counterstaining with crystal violet and washed with PBS, colony sizes were measured microscopically through digital image analysis.

MTT assay

Cells were trypsinized and seeded in a 96-well plate at a concentration of 1×10^3 cells/well. After overnight incubation, cells were treated with IFN-α (1000, 5000, 10000 IU/ml) for one week and the medium was refreshed for one time. Cell viability was analyzed by incubating cells with 0.5 mg/ml MTT (Sigma-Aldrich) for 4 h. After discarding the cell supernatant, 150 µl DMSO was added followed 10 min shaking. The absorbance was determined using enzyme mark instrument at the wavelength of 490 nm.

Cell cycle analysis

Cells (5 × 10^5/well) were plated in six-well plates and allowed to attach overnight. When the cell confluence reached 60% to 80%, cells were trypsinized and washed with PBS for two times and then fixed in cold 70% ethanol overnight at 4°C. The cells were then washed twice with PBS and incubated with 50 µl RNase (100 µg/ml) at 37°C for 30 min, and then 250 µl propidium iodide (PI) (50 µg/ml) was added and cells were incubated at room temperature for 5 min. The samples were analyzed immediately by FACS. Cell cycle was analyzed by FlowJo software.

Cell apoptosis analysis

Cell apoptosis analysis was performed by staining cells with annexin V-FITC (BD Pharmingen) and PI. Cells (5 × 10^5/well) were seeded into six-well plates and incubated at 37°C in 5% CO2 overnight, then cells were treated with IFN-α (Thermo Scientific, the Netherlands) (1000 IU/ml), TNF-α (Peprotech, USA) (20 ng/ml) or the combination. After 72 h, all of the cells were trypsinized and resuspended in annexin-binding buffer (BD Pharmingen) and stained with Alexa Fluor 488 Annexin V and PI at room temperature for 15 min. Detection of apoptosis was performed by FACS and the results were analyzed by FlowJo software.

Statistical analysis

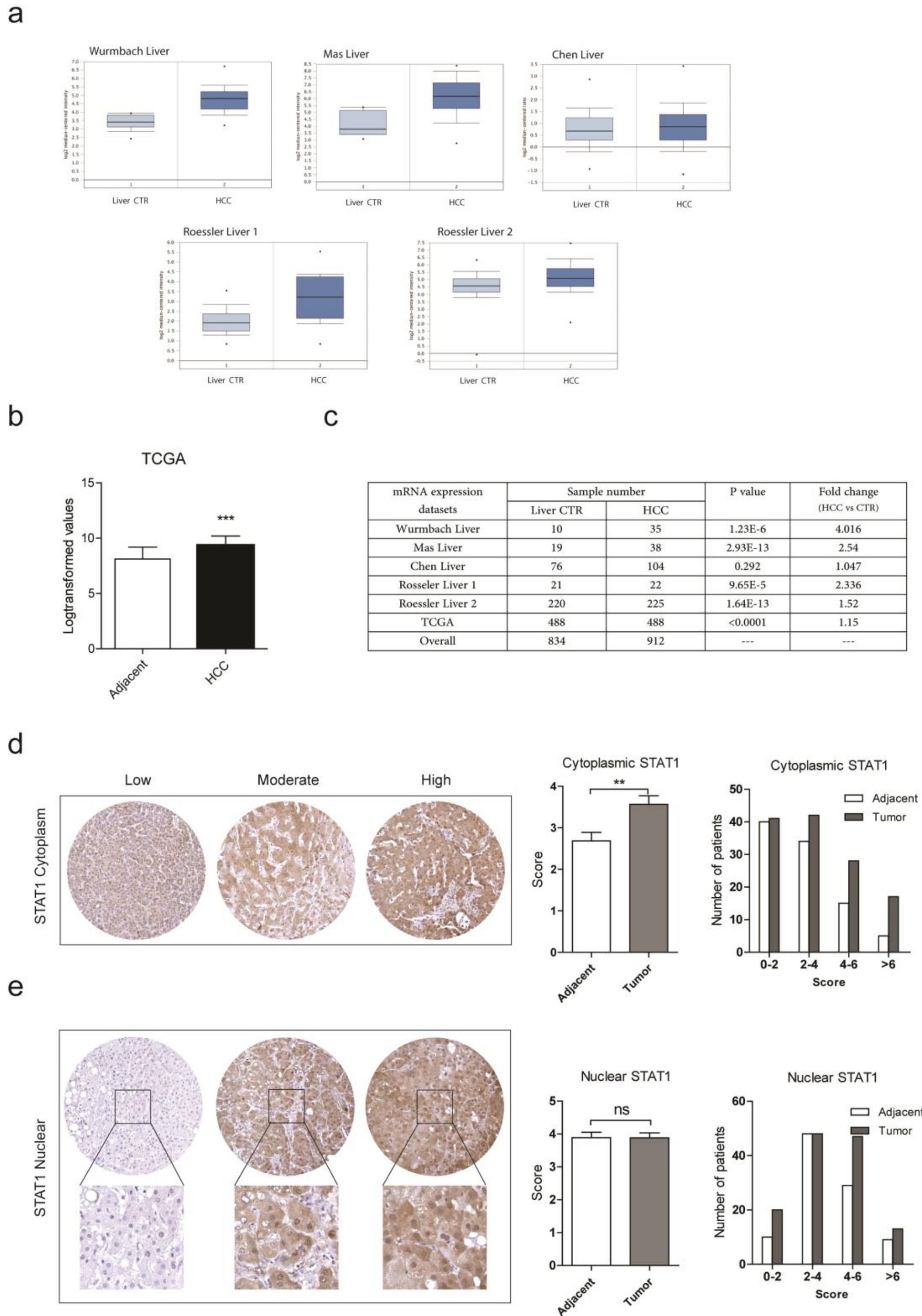
Statistical analysis was performed by using the nonparametric Mann–Whitney test for paired or non-paired data, or the paired t test using GraphPad InStat software as appropriate. Crude (non-adjustment) survival analysis (Kaplan-Meier curve) was first used to display the overall survival difference. Hazard ratio (HRs) and 95% Cls were calculated to evaluate the prognostic power of variables of patients. P-value < 0.05 was considered statistically significant.

Results

STAT1 expression is elevated in tumor tissues of HCC patients

In order to investigate STAT1 expression in HCC patients, we first searched the online datasets from Oncomine and TCGA, including six cohorts of 912 HCC tumor tissues with 834 paired tumor-free liver tissues from the same patients. To our surprise, STAT1 mRNA expression was significantly upregulated in tumors of five of the six cohorts (Figure. 1A-C).

To further confirm these results, TMA slides including tumor tissues and paired tumor-free liver tissues of 133 HCC patients were stained for STAT1. Positive staining of STAT1 in both nuclear and cytoplasm was found in most of the patients. Nuclear STAT1 is often recognized



mRNA expression datasets	Sample number		P value	Fold change (HCC vs CTR)
	Liver CTR	HCC		
Wurmbach Liver	10	35	1.23E-6	4.016
Mas Liver	19	38	2.93E-13	2.54
Chen Liver	76	104	0.292	1.047
Rosseler Liver 1	21	22	9.65E-5	2.336
Roessler Liver 2	220	225	1.64E-13	1.52
TCGA	488	488	<0.0001	1.15
Overall	834	912	---	---

Figure 1. STAT1 expression is upregulated in tumors of HCC patients. a The Oncomine microarray database (https://www.oncomine.org) was searched to analyze mRNA expression of STAT1 in HCC

patients. In total, five cohorts of 424 HCC tumor tissues compared with 346 paired tumor-free tissues from the same patients were identified. STAT1 mRNA was significantly upregulated in tumors tissues compared with tumor-free tissues in four of the five cohorts, P<0.001. b STAT1 expression profile across 488 HCC tumors and paired tumor-free liver tissues derived from TCGA (https://cancergenome.nih.gov/). STAT1 mRNA expression was significantly higher in tumor tissues comparing with tumor-free tissues (mean ± SD, n=488, ***P<0.001). c Landscape of all the online cohorts. d Cytoplasmic STAT1 was significantly upregulated in HCC tumors. The cytoplasmic STAT1 protein immune-reactivity scores (IRS), obtained by multiplying the scores for proportions of stained cells and the scores for expression intensity, range from low (score: 0-3), moderate (score: 3-6), high (score: 6-9) (mean ± SEM, n=133, **P<0.01). d No significant difference was found in nuclear STAT1 expression IRS scores (mean ± SEM, n=133, ns, no significant).

as p-STAT1, while cytoplasmic STAT1 is referred as u-STAT1[22]. Therefore, we scored the nuclear and cytoplasm expression of STAT1 separately. Consistent with the RNA expression data derived from the online datasets, cytoplasmic STAT1 protein expression in tumor tissues was significantly higher than that in tumor-free tissues (Figure. 1D), but no difference in nuclear STAT1 expression between tumors and tumor-free tissues was found (Figure. 1E).

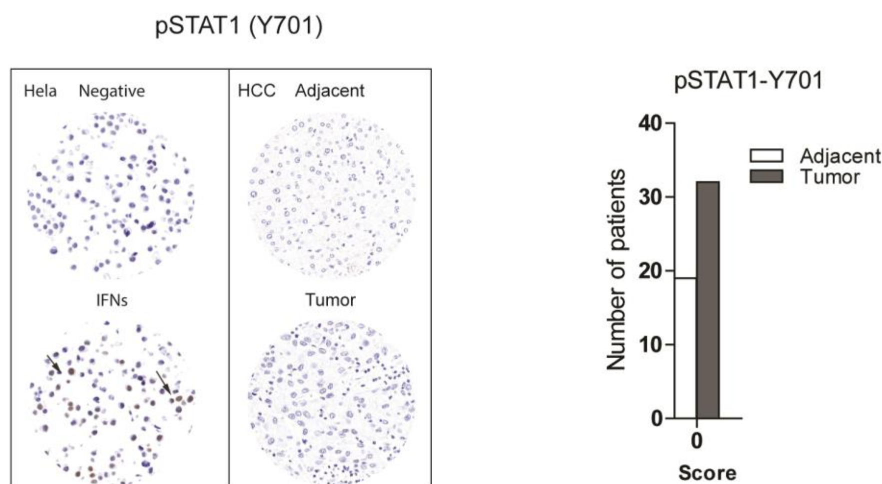
Among all the clinical factors, alpha-fetoprotein (AFP) serum level and tumor differentiation were significantly associated with higher patient mortality (Table. S2). This result is consistent with the general consensus that serum AFP is an independent indicator for HCC prognosis[23]. Correlation of STAT1 expression with clinical behavior were further analyzed. High cytoplasmic STAT1 was not significantly associated with the analyzed factors (Table. S3). However, high nuclear STAT1 expression was significantly associated with patient age (Table. S4). Furthermore, no significant correlation was observed between STAT1 expression and patient survival outcome (Figure. S1). Collectively, we found that cytoplasmic STAT1 expression in tumor tissues appears higher compared to tumor-free tissues.

P-STAT1 is absent in tumor tissues of HCC patients and human hepatoma cell lines

As the key component of JAK-STAT signaling, STAT1 is phosphorylated after activation and then translocates to the nucleus. Although u-STAT1 has been generally recognized as present in cytoplasm, emerging evidence has indicated its translocation to nuclei and its function as a transcription factor[24].

To clarify the phosphorylation status and localization of STAT1, we stained TMA slides with tissues of 32 patients with a specific antibodies against phosphorylated STAT1. Hela cells treated with IFNs were used as a positive control. Surprisingly, we did not observe positive staining for p-STAT1 in both tumor or tumor-free tissues (Figure. 2A). Consistently,

p-STAT1 was absent in all HCC cell lines, whereas u-STAT1 was highly expressed (Figure. 2B). Thus, we have demonstrated that STAT1 is predominantly present in unphosphorylated state in HCC tissues and human hepatoma cell lines.



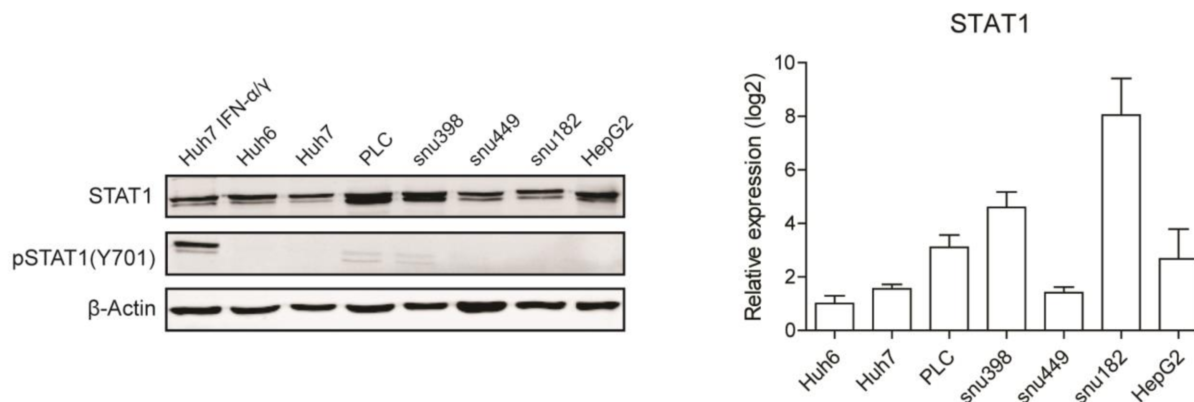


Figure 2. P-STAT1 is absent in both HCC tumors and cell lines. a No expression of p-STAT1 in HCC tumors and tumor-free tissues. Tumors (n=32) and tumor-free tissues (n=19) were stained for p-STAT1 (Y701). Paraffin-embedded Hela cells treated with IFNs were used as a positive control. b Absence of p-STAT1 in HCC cell lines. Cell lysates were collected for Western blot, and qRT-PCR was used to measure the mRNA levels of STAT1 (mean ± SD, n=3 independent experiments, two biological repeats for each).

Knockout of u-STAT1 impairs HCC cell growth

To determine the functions of u-STAT1, we generated u-STAT1 knockout cells by Lenti-CRISPR/Cas9 system in Huh7 and Huh6 HCC cell lines. Complete loss of STAT1 was demonstrated at protein level by western blot analysis (Figure. 3A). Finally, three wild-type and three knockout clones of both cell lines were selected for subsequent experimentation.

The colony formation unit (CFU) assay measures the ability of single cells to form clones. Strikingly, we observed that knockout of u-STAT1 inhibited CFU formation of HCC cells (Figure 3B), in contrast to previous findings that STAT1 served as a tumor suppressor[17, 20]. Cell cycle analysis revealed that loss of u-STAT1 significantly increased the proportion of Huh7 and Huh6 cells in the G1 phase and concomitantly decreased the proportion of cells in S-phase (Figure. 3C). These data suggest that u-STAT1 sustains HCC cell growth by promoting cell cycling.

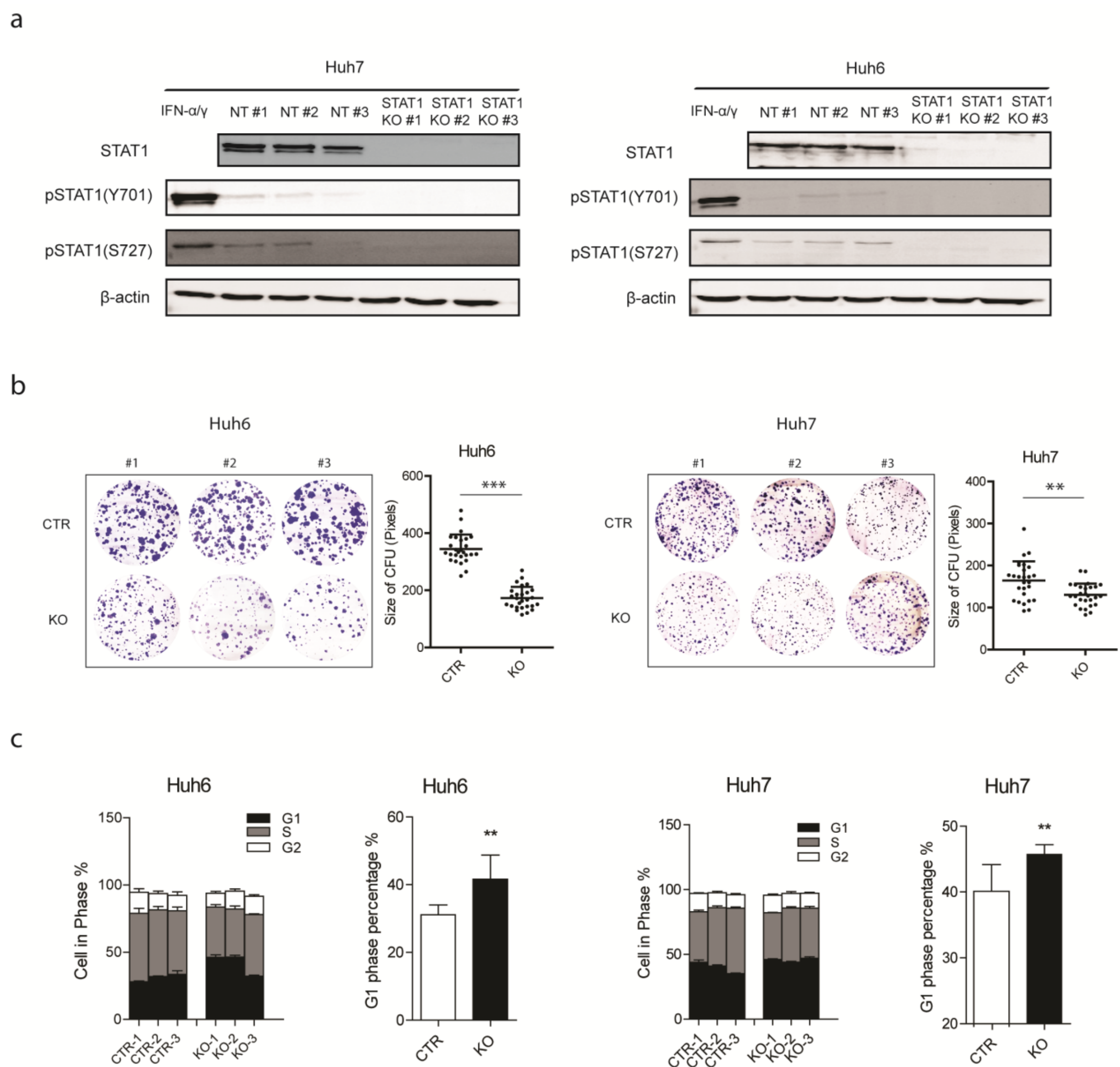
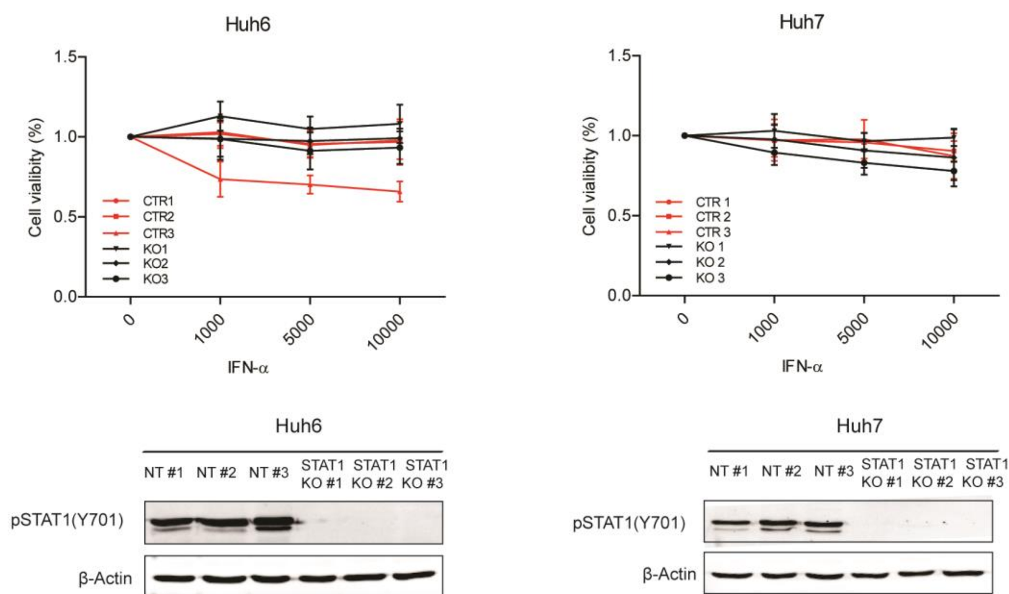


Figure 3. Knockout of u-STAT1 inhibits HCC cell growth. a Knockout of STAT1 in HCC cell lines. Cell lysates from Huh7 and Huh6 clones transduced with LentiCRISPR/Cas9 vector were collected for western blot. β-actin served as loading control. b U-STAT1 knockout significant inhibited the colony formation of Huh7 and Huh6 cell lines, as measured by clone size (mean ± SD, n=27, ***P<0.001, **P<0.01). c U-STAT1 knockout arrested cell cycling. U-STAT1 knockout arrested Huh7 and Huh6 cells in G1 phase determined by flow cytometric analysis (mean ± SD, n=9. **P<0.01).

Activation of STAT1 phosphorylation by IFN-α treatment hardly inhibits HCC cell growth

As the active form of STAT1, p-STAT1 has been widely recognized as the functional form in inhibiting tumor growth through inducing cell apoptosis and arresting cell cycle. Because p-STAT1 is absent in HCC cells, IFN-α was employed to activate STAT1 phosphorylation. Upon



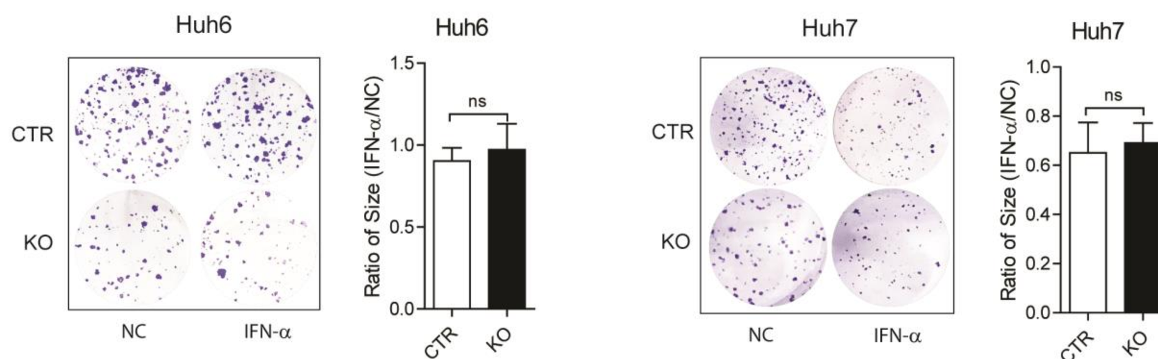


Figure 4. IFN-α exerts modest inhibition on HCC cells independent of p-STAT1. a IFN-α treatment did not or only modestly inhibit HCC cell growth independent of p-STAT1. Huh7 and Huh6 cells were treated by IFN-α (1000, 5000 and 10000 IU/ml) for 7 days and cell growth was determined by MTT assay (mean ± SD, n=4). P-STAT1 was measured by western blot and was strongly stimulated by treatment of IFN-α (1000 IU/ml) for 30 min. b IFN-α (1000 IU/ml) modestly inhibited the colony formation. Clone size of IFN-α untreated HCC cells were normalized to treated cells and data were present as STAT1 KO cells comparing with controls (CTR) (mean ± SD, n=3, ns, no significant).

IFN-α treatment, p-STAT1 was strongly induced in Huh7 and Huh6 cells, but not in STAT1 knockout cells (Figure. 4A). Huh7 and Huh6 with or without STAT1 were treated with differnt

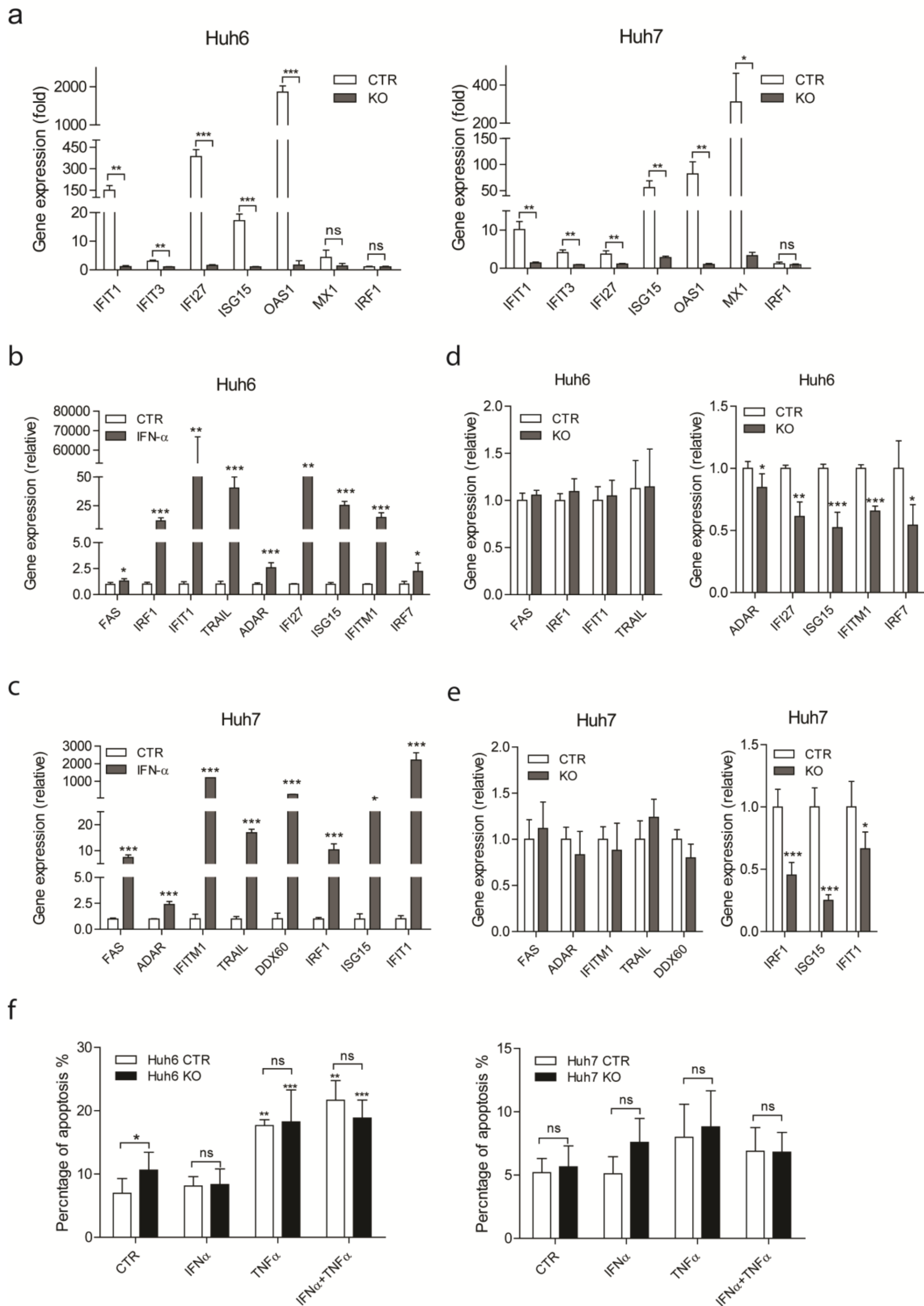


Figure 5. STAT1 is the key component for IFN-α induced ISG expression but not for cell apoptosis. a STAT1 knockout abolished the induction of ISGs by IFN-α. Huh6 KO and Huh7 KO cells were treated with IFN-α (1000 IU/ml) for 24 h. ISG expression was determined by qRT-PCR (mean ± SD, n=3, two

biological replicates for each independent experiment, *P<0.05, **P<0.01, ***P<0.001). b-c p-STAT1 strongly induced the expression of different ISGs. Huh6 and huh7 cells were treated with IFN-α for 4h. ISGs were quantified by qRT-PCR (mean ± SD, n=4, *P<0.05, **P<0.01, ***P<0.001). c-d U-STAT1 regulates IRDS genes but not pro-apoptotic ISGs. The expression of ISGs was compared between control and knockout cells in huh6 and huh7 by qRT-PCR (mean ± SD, n=4, *P<0.05, **P<0.01, ***P<0.001). f HCC cell lines are resistant to apoptosis induction by treatment of IFN-α. Huh6 KO and Huh7 KO cells were treated with IFN-α (1000 IU/ml), TNF-α (20 ng/ml) or the combination for 72 h. Cells were collected and stained with Anexin V/PI, and subsequently analyzed by FACS (mean ± SD, n≥4, *P<0.05, ns, no significant).

concentrations of IFN-α. Surprisingly, both cells lines were resistant to IFN-α treatment on cell proliferation, although Huh7 cells showed modest growth inhibition. Furthermore, no significant difference of cell growth between STAT1 knockout cells and WT controls was observed (Figure. 4A). Consistent to the MTT results, only Huh7 cells showed a slight inhibition on colony formation and no difference was found between knockout and WT cells in both cell lines (Figure. 4B).

Induction of ISGs is the hallmark of STAT1 activation[25]. They are thought to be the anti-tumor effectors of IFN-α treatment[26]. As expected, a subset of ISGs were strongly induced by IFN-α treatment; while the stimulation was abolished in STAT1 knockout cells (Figure. 5A). Besides, p-STAT1 has also been reported as an apoptosis inducer. However, IFN-α failed to induce apoptosis in HCC cells, while TNF-α did in Huh6 cells (Figure 5B). In addition, IFN-α did not further enhance apoptosis in the presence of TNF-α. These results suggest that HCC cells are resistant to growth regulation by IFN-α treatment, although p-STAT1 and ISGs are robustly activated.

U-STAT1 serves as a feedback loop to block the inhibitory effect of p-STAT1 on HCC cell growth

To understand why HCC cells are insensitive to IFN-α treatment, we profiled the dynamics of p-STAT1 and u-STAT1 expression. In fact, STAT1 is one of the most important ISGs. Both p-STAT1 and u-STAT1 were strongly induced by IFN-α. P-STAT1 peaked at 0.5 hour after IFN-α treatment and thereafter decreased gradually; whereas u-STAT1 started to gradually increase eight hours post-treatment (Figure. 6A). The expression of JAK1 was not changed, which has been demonstrated to be inhibited by u-STAT1[18]. We hypothesize that the distinct dynamics of these two forms may antagonize each other, and eventually deters the response to IFN-α treatment.

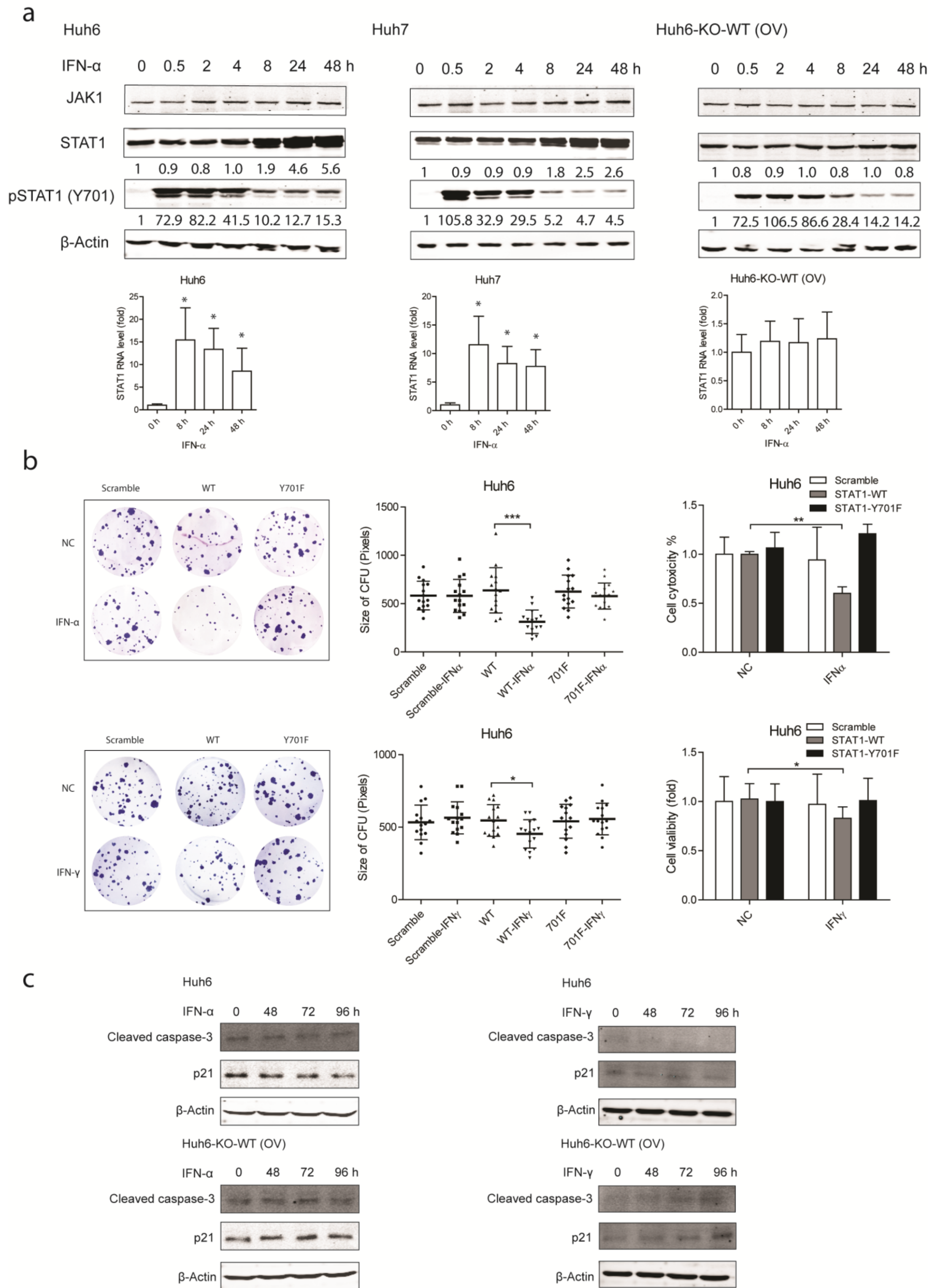


Figure 6. U-STAT1 works as a feedback loop in blocking p-STAT1 function. a IFN-α treatment induced u-STAT1 and p-STAT1 expression. Expression of u-STAT1 and p-STAT1 were both induced in Huh7 and Huh6 cells by IFN-α but not in Huh6-KO-WT determined by western blot and qRT-PCR

(mean ± SD, n=4, *P<0.05). b Attenuating u-STAT1 expression sensitized Huh6 cell to IFNs treatment. Huh6-KO-WT and Huh6-KO-Y701F cells were treated with IFN-α (1000 IU/ml) or IFN-γ (1000 ng/ml). Decreased colony formation efficiency was found in Huh6-KO-WT and also cell growth inhibition but not in Huh6-KO-Y701F cells determined by MTT assay (mean ± SD, n=3, **P<0.01). c Blocking IFN-α induced u-STAT1 expression sensitized HCC cells to apoptosis and cell cycle arrest. Cell lysates of Huh6 cells and Huh6-KO-WT treated with IFN-α (1000 IU/ml) or IFN-γ (1000 ng/ml) were collected for western blot analysis. β-actin served as loading control.

To dissect these complicated interactions, we artificially control STAT1 expression by genome modification. We exogenously expressed CMV promotor controlled WT (Huh6-KO-WT) or mutant (Y701F) (Huh6-KO-Y701F) STAT1 in STAT1 knockout Huh6 cells. Thus, STAT1 mRNA is constitutively expressed driven by the exogenous CMV promoter and therefore no longer be induced by IFN-α. Treatment of IFN-α activates p-STAT1, but the expression of u-STAT1 was not affected in these cells (Figure 6A). As expected, ISG expression was strongly induced by IFN-α, although no major effect on the basal expression of ISGs (Figure. S3). Importantly, blocking the induction of u-STAT1 expression greatly sensitized Huh6-KO-WT cells to IFN-α treatment. In contrast, this effect was not observed in Huh6-KO-Y701F, indicating the requirement of p-STAT1 activation (Figure. 6B and 6C). Furthermore, exogenous expression of u-STAT1 did not exerted major effect on HCC growth (Figure 6D). Cleaved caspase-3 and p21 are the key components of apoptosis and cell cycling. We found that both cleaved caspase-3 and p21 expression were stimulated in Huh6-KO-WT cells, but decreased in Huh6 cells (Figure. 6E).These results suggest that the induction of u-STAT1 as a feedback loop antagonizes the function of p-STAT1 and protects HCC cells from IFN-α treatment.

Discussion

As the key component of IFN signaling, STAT1 have been reported with both pro- and anti-tumor functions during cancer development from clinical studies in cancer patients[16]. Deregulated expression of STAT1 has been observed in a variety of cancer types[27-30]. It is closely correlated to clinical behaviors of patients, either good or poor prognosis[14, 29]. In HCC, the expression of STAT1 has been reported to be lower in tumor tissues and is negatively associated with the histological grade[17]. However, we found that expression of STAT1 is higher in HCC tumor tissues in both our patients and other cohorts from online datasets, consisting of a large number of patients. Compared to the tumor-free tissues, we found higher levels of STAT1 is in the cytoplasm of HCC cells; whereas the levels in nuclear are comparable. The exact reasons accounting for the discrepancy between our results and the previous studies remain to be further investigated[17].

The phosphorylation status is essential for the functions of STAT1. In general, p-STAT1 is supposed to locate in nuclear; while u-STAT1 is considered predominately present in cytoplasm[22]. Surprisingly, we found that p-STAT1 is completely absent in our HCC tumor tissues and HCC cell lines, indicating that u-STAT1 is the dominant form located in both nuclear and cytoplasm. This is consistent with previous finding that u-STAT1 can shuttle between cytoplasm and nuclear, and reinforces host defense against viral infection[24]. However, the expression levels of STAT1 in either nuclear or cytoplasm are not significantly related to survival in our patients.

Experimental studies in STAT1 knockout mouse have demonstrated a tumor suppressor function mainly through tumor intrinsic and extrinsic mechanisms[15, 31]. Cell cycle regulator, apoptosis inducers and genes of immune system have been recognized as downstream targets of STAT1. However, several oncogenes have been reported to be regulated by STAT1, which are involved in promotion of tumor growth and invasiveness, suppression of immune surveillance and induction of therapy resistance[16]. Thus, STAT1 plays multifaceted roles in cancer development. In HCC, we found that silencing u-STAT1 inhibits cell growth and arrests cell cycle, indicating u-STAT1 sustains the growth of HCC. These results are partially consistent with previous finding that u-STAT1 can protect tumor cells from apoptosis stimuli, radio- and chemotherapy[22, 30, 32, 33].

The classically active form of STAT1, p-STAT1, is strongly induced during immune response and rapidly regulates downstream gene expression. It has been demonstrated that p-STAT1 remarkably arrests tumor cell growth[34-36]. In line with this, we found that p-STAT1 inhibits HCC cell growth by arresting cell cycle and inducing cell apoptosis. However, p-STAT1 is quickly dephosphorylated within only a few hours. u-STAT1, which is transcribed by p-STAT1, subsequently substitutes p-STAT1 expression and lasts for several days. Consequently, the anti-tumor effect of p-STAT1 is attenuated by the pro-tumor effect of u-STAT1. Thus, the function of STAT1 is highly dependent on its phosphorylation state, and p-STAT1 and u-STAT1 exert opposing functions.

IFNs have been widely explored for treating various malignancies[6]. However, IFN monotherapy has limited efficacy, although combination of IFNs with other tumoricidal therapies have been proven effective[37]. Systemic thermotherapy with IFNs for HCC has limited benefit on patient survival and in some instances is accompanied with significant toxicity[38], although antiviral therapy with IFNs might reduce the risk of virus infection in cancer patients[4]. Reasons for the clinical failure of IFNs likely include inherent biological mechanisms, changes in cell population, and institution of counter-regulatory pathways[39]. IFN signaling is generally considered to stimulate immune response, but it has also been reported to induce immunosuppression[7]. Different forms of STAT1, p-STAT1 and u-STAT1, have shown different transcription properties that contribute to the complexity of IFN signaling[7]. In our study, we have demonstrated that p-STAT1 and u-STAT1 have opposing functions in HCC during IFN-α treatment. These results may explain the possible mechanisms of the ambiguous effects of IFNs in cancer treatment.

In summary, STAT1 is dominantly present as the form of u-STAT1 in HCC cells. The phosphorylation state deters the functions of STAT1 that u-STAT1 sustains but p-STAT1 inhibits HCC growth. Upon IFN treatment, the expression, phosphorylation and localization of STAT1 are dynamically regulated and coordinately control the responsiveness to IFN treatment. Thus, these findings provide mechanistic insight on the role of STAT1 in HCC, and provide scenario for future optimization of IFN treatment.

Supplementary Materials

Bioinformatics analysis of online datasets

To analyze mRNA expression of STAT1 in HCC, the Oncomine microarray database (https://www.oncomine.org) was analyzed using the online tool. In the Gene Expression Omnibus (GEO) database , datasets of HCC gene expression were searched and analyzed (accession codes GSE14520). STAT1 mRNA expression was analyzed in identified cohorts by comparing expression levels in HCC tumors with tumor-free tissues. Moreover, survival data of 360 HCC cases in The Cancer Genome Atlas (TCGA) were available (https://portal.gdc.cancer.gov/projects/TCGA-LIHC).

Immunohistochemistry

Paraffin-embedded TMA slides were deparaffinized with xylene and rehydrated in graded alcohols (100%, 95%, 70%) for further immunohistochemistry staining. Slides were then washed with Phosphate Buffered Saline with Tween 20 (PBST) and boiled in citric acid buffer (pH6.0) for 20 min for antigen retrieval. Peroxidase was blocked by adding 3% H_2O_2 for 10 min at room temperature. The slides were incubated overnight with the primary antibody against STAT1 (rabbit polyclonal; sc-592) (1:300) and p-STAT1 (58D6; rabbit monoclonal; #9176) (1:150) at 4°C. After being rinsed in PBST, slides were incubated with second anti-rabbit IgG antibody conjugated with HRP for 1 h at room temperature. DAB solution (0.05% DAB, 0.0017% H_2O_2) was then prepared and added to the slides to visualize antibody binding. The reaction was stopped by washing with distilled water. Subsequently, hematoxylin were employed for background staining of tissue. Negative control staining was carried out by omitting the primary antibody.

Cytoplasmic and nuclear staining were scored separately. Percentages of cells with cytoplasmic or nuclear expression were scored as follows: low for 0-30%; moderate for 30-70%; high for > 70%. Scoring of expression intensity was performed as: grade 1 for weak; grade 2 for moderate; grade 3 for strong. A final immune-reactivity score (IRS) was obtained for each case by multiplying the percentage and the intensity values, ranging from low, moderate and high. The scorings were done by two investigators.

Cell culture and reagents

Seven different human hepatoma cell lines (Huh7, Huh6, PLC, snu398, snu449, snu182, HepG2) were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza). Media were supplemented with 10% (v/v), fetal bovine serum (FBS) (Hyclone Technologies), 100 units/mL of penicillin and 100 µg/mL of streptomycin. All the cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. All the cell lines were a kind gift from Dr. Ron Smits (department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center) [1] and confirmed mycoplasma-free and their STR genotyping was analyzed at the Department of Pathology, Erasmus Medical Center Rotterdam.

Generation of STAT1 knockout cells using LentiCRISPR/Cas9 system

The sgRNA (TCCCATTACAGGCTCAGTCG) targeting *STAT1* was designed by online tool "MIT CRISPR Design" (http://crispr.mit.edu/) and cloned into the lentiviral backbone vector lentiCRISPR v2 (Addgene). To produce lentivirus, HEK293T cells were transfected with 0.6 µg of pMD.2G, 1.5 µg of psPAX2, and 2 µg of lentiCRISPR v2 in a 6-well plate. Lentivirus-containing culture supernatants were collected and filtered through a 0.45 µm filter. Cells were then infected with lentivirus for two days and selected using 3.0 µg/ml puromycin (Sigma-Aldrich). From stably transduced cell lines, single cells were sorted by FACS, and genomic DNA was isolated using Wizard® Genomic DNA Purification Kit (Promega) to identify the introduced mutations (Figure. S2). To further validate the knockout effect, western blot was performed to detect the expression of STAT1 at protein level.

Colony formation assay

Cells were trypsinized, harvested and suspended in culture medium. After quantified through counting, 2×10^3 cells were seeded into 6-well plates and the medium was refreshed for every four days. After two weeks culture, formed colonies were washed with PBS and fixed by 70% ethanol. Followed by counterstaining with crystal violet and washed with PBS, colony sizes were measured microscopically through digital image analysis.

MTT assay

Cells were trypsinized and seeded in a 96-well plate at a concentration of 1×10^3 cells/well. After overnight incubation, cells were treated with IFN-α (1000, 5000, 10000 IU/ml) for one week and the medium was refreshed for one time. Cells were then incubated with 0.5

mg/ml MTT (Sigma-Aldrich) for 4 h. After discarding the cell supernatant, 150 µl DMSO was added followed 10 min shaking. The absorbance was determined using enzyme mark instrument at the wavelength of 490 nm. The formula: (Absorbance $_{\text{treated cells}}$-Absorbance $_{\text{DMSO}}$)/(Absorbance $_{\text{negative control}}$-Absorbance $_{\text{DMSO}}$) was used to analyze the cell viability.

Cell cycle analysis

Cells (5×10^5/well) were plated in six-well plates and allowed to attach overnight. When the cell confluence reached 60% to 80%, cells were trypsinized and washed with PBS for two times and then fixed in cold 70% ethanol overnight at 4°C. The cells were then washed twice with PBS and incubated with 50 µl RNase (100 µg/ml) at 37°C for 30 min, and then 250 µl propidium iodide (PI) (50 µg/ml) was added and cells were incubated at room temperature for 5 min. The samples were analyzed immediately by FACS. Cell cycle was analyzed by FlowJo software.

Cell apoptosis analysis

Cell apoptosis analysis was performed by staining cells with annexin V-FITC (BD Pharmingen) and PI. Cells (5×10^5/well) were seeded into six-well plates and incubated at 37°C in 5% CO_2 overnight, then cells were treated with IFN-α (Thermo Scientific, the Netherlands) (1000 IU/ml), TNF-α (Peprotech, USA) (20 ng/ml) or the combination. After 72 h, all of the cells were trypsinized and resuspended in annexin-binding buffer (BD Pharmingen) and stained with Alexa Fluor 488 Annexin V and PI at room temperature for 15 min. Detection of apoptosis was performed by FACS and the results were analyzed by FlowJo software.

Western blot assay

Laemmli sample buffer containing 0.1 M DTT (freshly made) was used to lyse the cells. Then, cell lysates were denaturalized by heating 5-10 min at 95°C followed by loading onto a 10-15% sodium dodecyl sulfate-polyacrylamide SDS gel and separated by electrophoresis (SDS-PAGE). After 90 min running in 120 V, proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen) for 1.5 h with an electric current of 250 mA. Subsequently, the membrane was blocked with blocking buffer (Li-COR, Lincoln, USA) mixed with PBST in ratio of 1:1. And then followed by overnight incubation with rabbit anti-STAT1, anti-p-STAT1 (Y701) (1:1000) antibody at 4°C. Membrane was washed 3 times with PBST, which was followed by incubation for 1 h with anti-rabbit or anti-mouse IRDye-

conjugated secondary antibodies (Li-COR, Lincoln, USA) (1:5000) at room temperature. Blots were assayed for actin content as standardization of sample loading, and scanned and quantified by odyssey infrared imaging (Li-COR, Lincoln, USA). The results were analyzed with Odyssey 3.0 software.

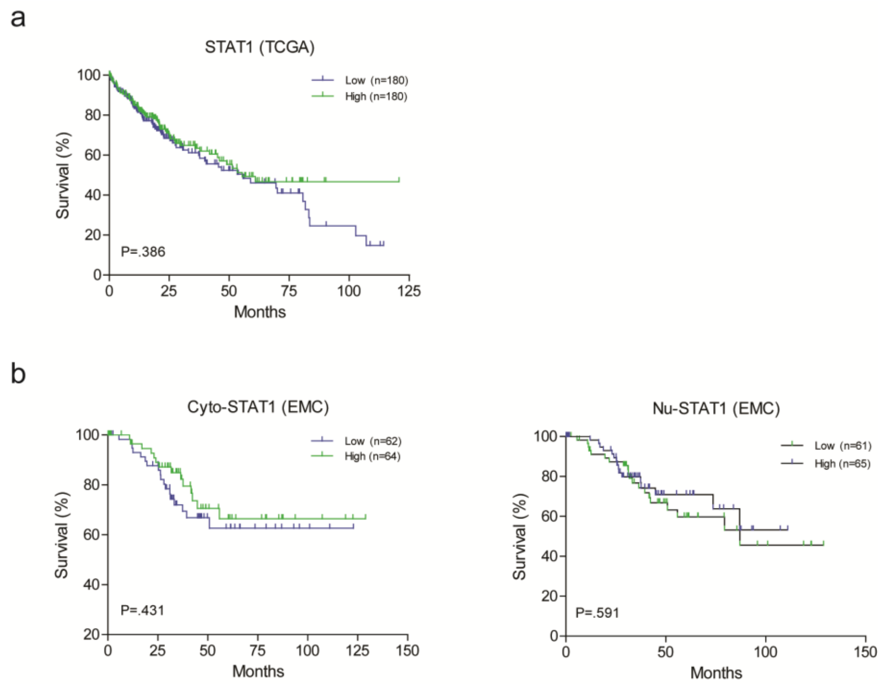


Figure S1. Survival analysis of HCC patients. a Kaplan Meier analysis of HCC patients from the TCGA cohort. Expression of STAT1 is not strongly associated with patient outcome (n=360, p=0.386). b Kaplan Meier analysis of the EMC patient cohort. Both cytoplasm (n=126, p=0.431) and nuclear expression (n=126, p=0.591) were analyzed for patient survival outcome. No significant correlation with patient outcome was found.

STAT1 locus ATAATCATGCCCTTCCCATTACAGGCTCAGTCGGGGAATATTCAGAGCACAGTG

Huh7

#1
ATAATCATGCCCTTCCCATTACAGGCTCAGAGTCGGGGAATATTCAGAGCACAGTG +2
ATAATCATGCCCTTCCCATTACAGGCTCAGAGTCGGGGAATATTCAGAGCACAGTG +2

#2
ATAATCATGCCCTTCCCATTACAGGCTCAAGTCGGGGAATATTCAGAGCACAGTG +1
ATAATCATGCCCTTCCCATTACAGGCTCAAGTCGGGGAATATTCAGAGCACAGTG +1

#3
ATAATCATGCCCTTCCCATTACAGGC----------TCGGGGAATATTCAGAGCACAGTG -4
ATAATCATGCCCTTCCCATTACAGGC---------------GGGAATATTCAGAGCACAGTG -8

Huh6

#1
ATAATCATGCCCTTCCCATTACAGGCTCAGAGTCGGGGAATATTCAGAGCACAGTG +2
ATAATCATGCCCTTCCCATTACAGGCTCAG--------GGGGAATATTCAGAGCACAGTG -4

#2
ATAATCATGCCCTTCCCATTACAGGCTCAG-----CGGGGAATATTCAGAGCACAGTG -1
ATAATCATGCCCTTCCCATTACAGGCTCAGAGTCGGGGAATATTCAGAGCACAGTG +2

#3
ATAATCATGCCCTTCCCATTACAGGCTCAAGTCGGGGAATATTCAGAGCACAGTG +1
ATAATCATGCCCTTCCCATTACAGG--------------------GAATATTCAGAGCACAGTG -10

Figure S2. Genome sequencing of STAT1 knockout cell clones. The sequence locus of STAT1, red labeled as the sgRNA targeting site and green labeled as the PAM sequence. All the clones show frameshift mutation with nucleotides deletion (dash line) or insertion (red).

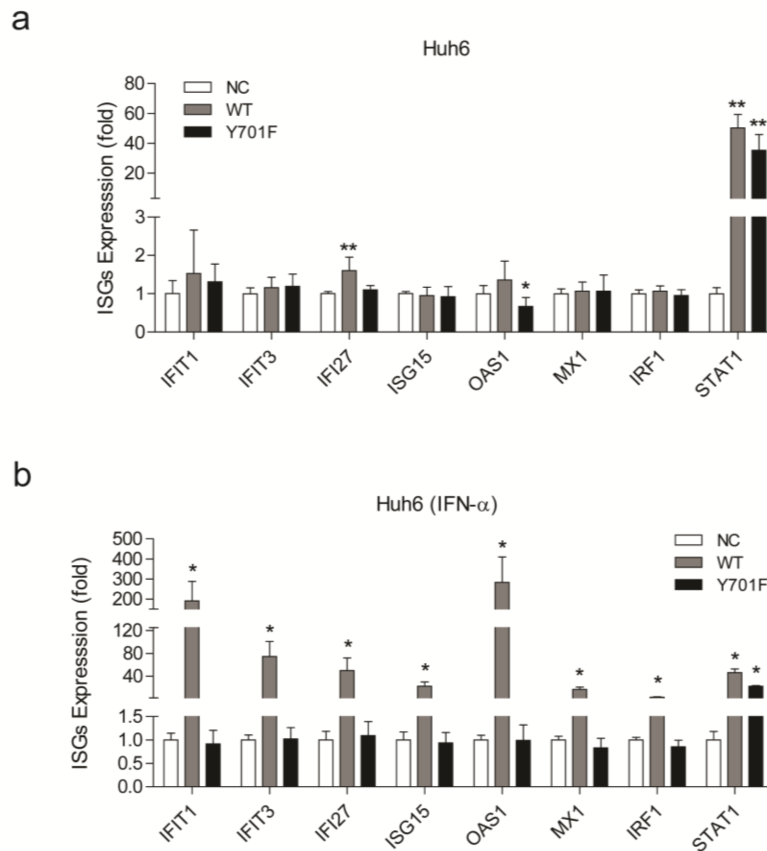


Figure S3. Expression of ISGs are restored by STAT1 overexpression. a STAT1 re-expression does not further induce the expression of ISGs (mean ± SD, n=3, two replicates *p<0.05). b STAT1 expression restores the function of IFN-α in inducing ISG expression. Huh6 cells transduced with lentiviral vector expressing STAT1 were treated with IFN-α for 24 h (mean ± SD, n=3, two replicates *p<0.05), NC=negative control (Huh6 transfected with empty lenti-vector)

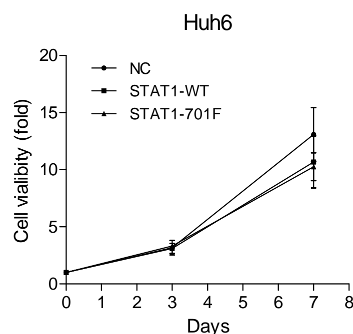


Figure S4. STAT1 did not promote HCC cell growth Huh6-KO-WT and Huh6-KO-Y701F cells were grown for one week. Cell growth was determined by MTT assay (mean ± SD, n=3, two biological replicates for each independent experiment).

Table S1. Human qPCR primer sequences

	5' FORWARD	3' REVERSE
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
IFIT1	GCCTTGCTGAAGTGTGGAGGAA	ATCCAGGCGATAGGCAGAGATC
IFIT3	CCTGGAATGCTTACGGCAAGCT	GAGCATCTGAGAGTCTGCCCAA
IFI27	CGTCCTCCATAGCAGCCAAGAT	ACCCAATGGAGCCCAGGATGAA
ISG15	CTCTGAGCATCCTGGTGAGGAA	AAGGTCAGCCAGAACAGGTCGT
OAS1	AGGAAAGGTGCTTCCGAGGTAG	GGACTGAGGAAGACAACCAGGT
MX1	GGCTGTTTACCAGACTCCGACA	CACAAAGCCTGGCAGCTCTCTA
IRF1	GAGGAGGTGAAAGACCAGAGCA	TAGCATCTCGGCTGGACTTCGA
STAT1	ATGGCAGTCTGGCGGCTGAATT	CCAAACCAGGCTGGCACAATTG

Table S2. Patient characteristics according to HCC specific mortality.

Variable	**HR(95%CL)**	***P***
Age	0,735 (0,287-1,884)	0,522
AFP (>200)***	6,808 (2,207-16,797)	0,000
Tumor Size	3,298 (0,967-11,244)	0,057
Fibrosis	1,341 (0,392-4,582)	0,640
Cirrhosis	1,238 (0,436-3,516)	0,688
Vascular invasion	1,524 (0,644-3,604)	0,338
HBV positive	0,003 (0,000-1,659E+63)	0,941
HCV positive	0,006 (0,000-3,011E+63)	0,947
Viral hepatitis	305,841 (0,000-1,505E+68)	0,941
Differentiation*	0,242 (0,070-0,831)	0,024
Gender	1,468 (0,590-3,651)	0,409

Table S3. Patient characteristics according to cytoplasmic STAT1 expression

Variable	**HR(95%CL)**	***P***
Age	0,981 (0,466-2,068)	0,960
AFP (>200)	0,959 (0,314-3,006)	0,959
Tumor Size	1,707 (0,632-4,607)	0,291
Fibrosis	1,878 (0,543-6,494)	0,319
Cirrhosis	0,887 (0,371-2,122)	0,788
Vascular invasion	1,261 (0,576-2,762)	0,561
HBV positive	0,855 (0,361-2,026)	0,721
HCV positive	0,422 (0,153-1,167)	0,097
Differentiation	0,541 (0,191-1,533)	0,248
Gender	0,850 (0,390-1,852)	0,683

Table S4. Patient characteristics according to nuclear STAT1 expression

Variable	**HR(95%CL)**	***P***
Age**	0,372 (0,178-0,778)	0,009
AFP (>200)	2,401 (0,967-5,964)	0,059
Tumor Size	0,976 (0,468-2,032)	0,947
Fibrosis	0,606 (0,202-1,814)	0,371
Cirrhosis	1,235 (0,510-2,991)	0,640
Vascular invasion	0,632 (0,303-1,319)	0,222
HBV positive	0,308 (0,025-3,749)	0,356
HCV positive	0,602 (0,059-6,142)	0,668
Viral hepatitis	1,994 (0,156-25,487)	0,595
Differentiation	2,662 (0,960-7,369)	0,060
Gender	0,717 (0,363-1,415)	0,338

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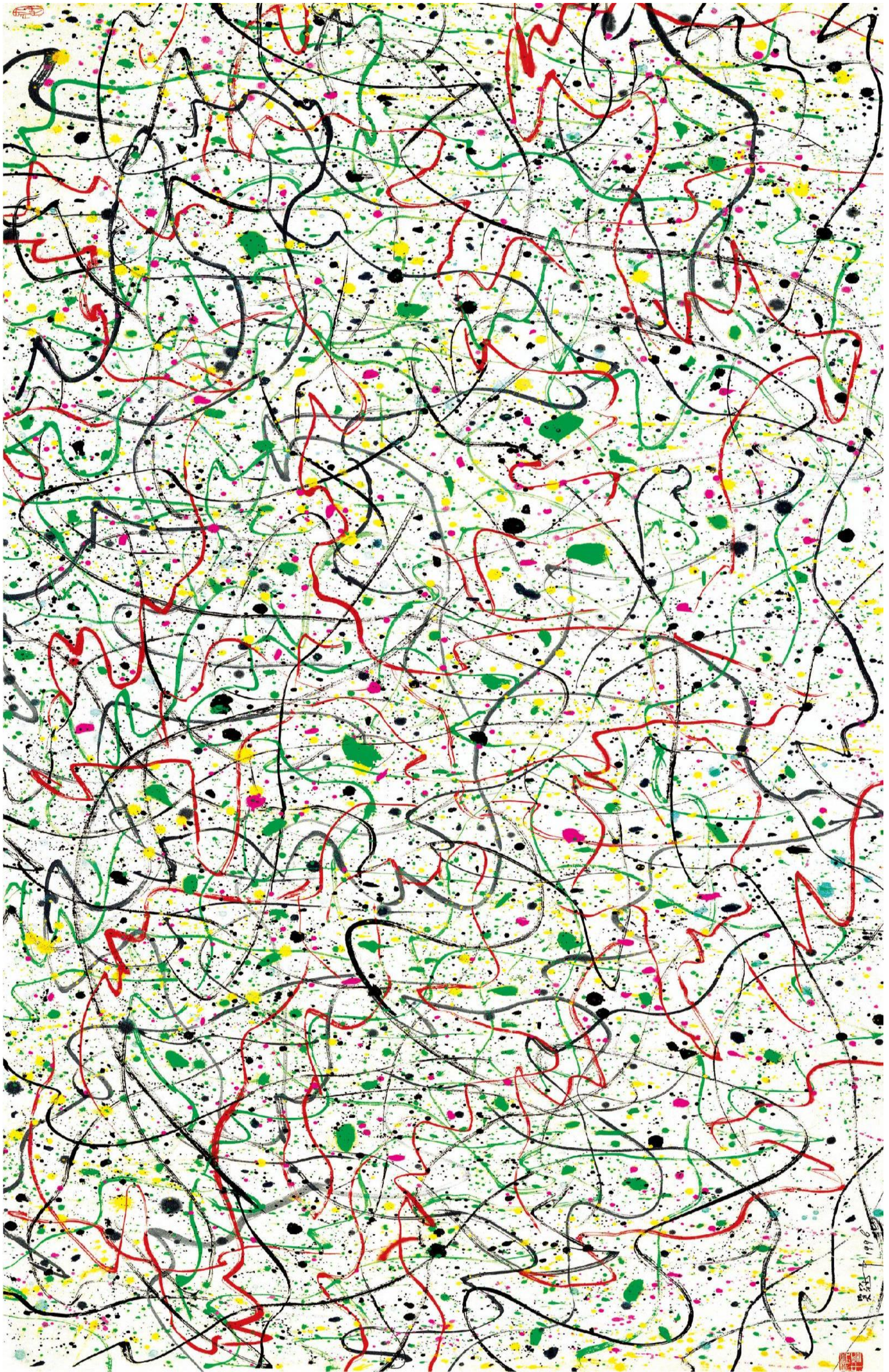

Chapter 6

Genetically engineered bacteria for treating human disease

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Trends in Pharmacological Sciences. 2017 Sep; 38(9):763-764

Abstract

Bacteria have now been harnessed to combat human diseases, especially to meet the challenge of antimicrobial resistance. Modulating the microbiome, particularly by genetically engineering the bacteria, has provided proof-of-concept as potential pharmacotherapy, but this field should engage in discussion as how to move forward.

Over the ages, the rapid and invisible transmission of infectious diseases has inspired shock and awe to human society. With the emergence and advent antibiotic medication, fear for at least bacterial infection has been substantially subdued. However, rapidly spreading antibiotic resistance because of inappropriate use of this class of medications has provided new urgency to the quest of developing alternative anti-microbial strategies[1]. Intriguingly, these efforts have led to realization that living organisms can be used therapeutically to combat infectious diseases, defining a novel group of therapeutic entities which in turn are also used for the treatment of non-infectious diseases. DNA recombinant technology, through which bacteria are manipulated to express biomedical molecules ectopically, can greatly add to the power of living organisms as therapeutic agents. There is, however, very little standardization with respect to mode of delivery, consensus as to measure pharmacokinetics of genetically engineered bacteria and the required precautions with respect of contamination of ecosystems with genetically modified organisms or the transmission of harmful organisms to patients. Thus, the field should engage in discussion as how to move forward in these respects.

Such discussion is especially called for in view of the rapid development in the field. Consider for instance, the recent study of Hwang *et al.* published in Nature Communications in which a genetically engineered *Escherichia coli* Nissle, for preventing and treating intestinal *Pseudomanas asruginosa infection* was described[2]. In this elegant study, an engineered microbe was used that can specifically detect *P. aeruginosa*-secreted autoinducer N-acyl homoserine lactone and subsequently responds to this pathogen by inducing its own lysis, thus releasing an anti-*P. aeruginosa* toxin and an anti-biofilm enzyme. The bacteria establish chronic colonization of the intestine, hence preventing further colonization of the pathogen and providing prophylactic activity. These findings open a new era in the treatment of antibiotic-resistant infection, especially as it is highly specific to a defined pathogen associated with both prevention and treatment to the specific microorganism. Concomitantly, new questions emerge with respect how to define, dose and measure amounts of this therapeutic organism, and how to assess potential ecological risks of such bacteria, following excretion of modified organisms out of the body.

Some guidance in this respect may be obtained from the experience with probiotics and prebiotics. Probiotics are bacterial which naturally occur in fermented foods and drinks and are associated with a variety of health benefits, These include protection against

pathogenic infection through niche occupation, reduced intestinal inflammation and increased Ca^{++} uptake in the gut. They are used as preventive or supportive medication in a variety of conditions such as irritable bowel syndrome, inflammatory bowel disease, infectious and antibiotic-related diarrhea, allergy and eczema, oral health as well as certain urinary and vaginal health-related conditions[3]. Prebiotics are dietary fibers that allow intestinal expansion of probiotic bacteria[4]. However, despite the regular use of probiotics and prebiotics as medication and functional food, there is still little in guidance as to how assess pharmacokinetics of such preparations. This partly relates as to doubts as to their clinical efficacy. Although some evidence of clinical effectiveness of probiotics has been provided for certain diseases, such as antibiotic-associated diarrhea, *Clostridium difficile*-associated colitis, irritable bowel syndrome and inflammatory bowel disease [3], much of the data presented in contemporary body of medical literature is inconsistent. Furthermore, doubts have been raised with respect to safety of probiotic preparations. The PROPATRIA trial (which assessed the clinical efficacy of probiotics in pancreatitis) showed excessive mortality to be associated with such treatment[5]. Given the fact that almost no standard quality or content of probiotics has been established, it is imperative that the field develops guidelines in this respect.

A similar situation holds true for fecal microbiota transplantation (FMT). FMT has seen an almost stellar increase in popularity for treatment of a variety of gastrointestinal and non-gastrointestinal disorders, in particular *Clostridium difficile*-associated colitis[6] but also inflammatory bowel disease and metabolic syndrome. In this strategy, feces of healthy individuals are used for reestablishing the homeostasis in a dysbiotic human gut, usually by delivery through endoscopy[7]. For *Clostridium difficile*-associated colitis, an impressive efficacy in resolution of infection was observed in over 90% of FMT-treated as compared to 31% and 23% in conventional antibiotic treatment. Nevertheless, the complex compositions of fecal might unavoidably expose patients to undetected organisms or even harmful pathogens[8]; whereas the importance of using living organisms for this treatment has also been challenged[9]. Nevertheless, the approach remains superior relative to the use defined consortia of bacteria[10] and its popularity raises important question as how to define dosing and other pharmacokinetic parameters and prompt further development in this area.

It is important to point that this field is developing quickly driven by the advances in molecular biology. The production of therapeutic proteins through genetically modified

organisms at mucosal surfaces has important advantages, in particular the possibility to achieve local delivery which increases efficacy and at the same time reduces side effects. The successful Phase I clinical trial with interleukin 10-producing *Lactococcus lactis* for the treatment of Crohn's disease illustrates the promise of such strategies[11]. Bacteria can be manipulated to express a plethora of potentially biomedically interesting molecules, such as hormones, interleukins and antibodies. In this way, they can circumvent barriers associated with conventional strategies in delivery and production of these proteins and help lowering the side effects as well as productions cost[12]. The field, however, needs to anticipate such developments and should pro-actively address questions (Box 1) as to how to define pharmacokinetic parameters of therapeutic preparations consisting of living bacteria.

Box 1. Development and challenges in harnessing living organisms as pharmaceutical modality.

- The use of bacteria for disease prevention or treatment has become clinical reality and benefits specificpatients. However, some of the clinical effects appear modest and need to be further mechanistically clarified, also in view of that almost no standard for quality control of the bacteria has been established.
- Risks have been raised in treating certain groups of patient. For instance, sepsis caused by probiotics (neonates, immunosuppression, pancreatitis) remains a feared complication Furthermore, FMT may unavoidably cause the transmission of undetected or unknown pathogens.
- Different strains of bacteria and the aspecific composition of bacteria preparations may vary dramatically in their therapeutic efficacy. To define the underlining mechanisms will help to provide guidance in treating with particular strains for specific diseases.
- Genetically modified bacteria have shown their advantages in combating diseases in some clinical trials, but may hold the risks of contamination of ecosystems and transmission between the human beings. Developing biocontainment bacteria with resistant to evolutionary escape will greatly meet the future demand in considering of bacteria biosafety.

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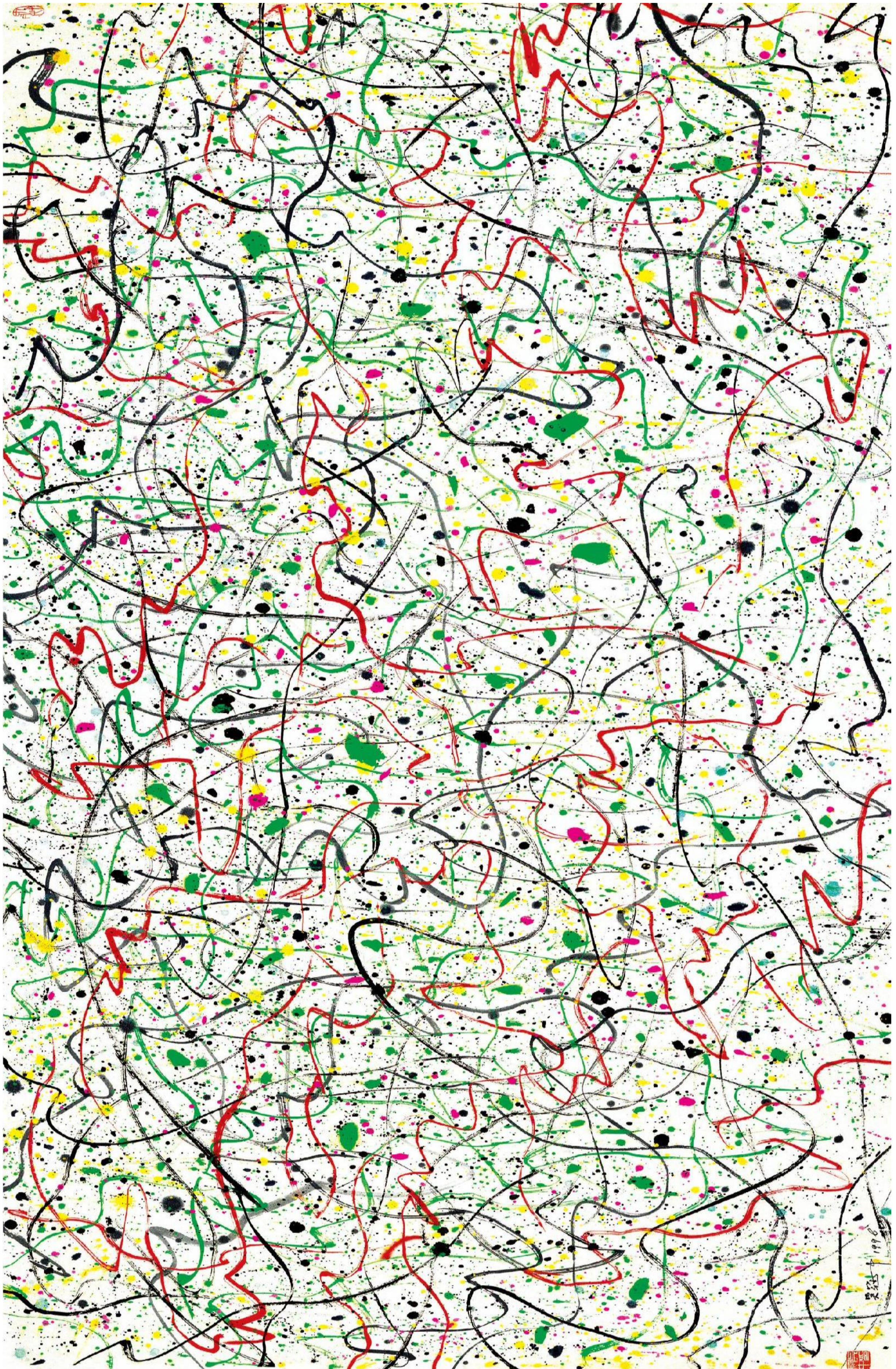

Chapter 7

Summary and discussion

Exploring molecular alterations for HCC therapy

Cancer has now been recognized a specific type of pathology for at all millennia and during all this time efforts at treatment have proven frustrating disappointing. For much of the time, cancer treatment was hampered by a lack of understanding of the underlying driving forces. The advent of nucleotide sequencing technology, however, has allowed analysis of the cancer process in unprecedented detail. Overwhelming evidence now shows oncogenesis to be cause of genetic mutation and to involve both of gain function changes in oncogenes and concomitant loss of function in tumor suppressor genes[1]. These changes are in the vast majority of cases sequential in which succession of the alterations in the genome transforms normal cells to cancer cells, while diversity in the gene mutation patterns provokes cancer cell heterogeneity, a phenomenon that substantially hampers successful treatment[2]. Efforts to categorize the cancer process and also its diversity in presentation have led to of the definition of eight hallmarks of cancer, that in conjunction should provide a logical framework describing the disease on a mechanistic level and which I used during my thesis research to study the liver cancer problem (see also introduction). Let us here summarize as what has been achieved.

Treatment of HCC is only curative if the primary cancer is physically removed and no metastases remain in the body. To achieve this, medical professionals often have to resort to liver transplantation. This is obviously a demanding procedure and its consequence if life-long use of immunosuppressive medicine to prevent rejection of the liver graft. Intriguingly this entails possibilities. Graft rejection and cancer share a need for cell division, the former to expand the lymphocyte compartment, the latter to expand the tumor compartment. As a consequence immunosuppressive medication sometimes shares characteristics with anti-cancer medication and if liver cancer is particularly sensitive to specific immunosuppressive medication, such medication may actually prevent recurrence. This notion is explored in Chapter 2, which is the synthesis of two of our separately studies, in which we show that IMPDH inhibition not only protects liver grafts but also counteracts the liver cancer process. Mechanistically, I could link this process to altered subcellular distribution of the enzyme involved. As other medication, e.g. ribavirin, also provokes altered subcellular distribution of IMPDH, it should prove interesting to investigate its effects on HCC as well and I feel that further studies investigating this possibility are called for.

In Chapter 3 and Chapter 4, we aim to investigate the function of STAT1 and IMPDHs in HCC progression, which are related to immune response and metabolism of cancer.

In Chapter 3, we demonstrated that two isoforms of the IMPDH enzyme exert distinct functions in HCC. The expression of IMPDH2 is downregulated in HCC tumors and positively associated with prognosis of HCC patients. This is unexpected in view of that elevated expression of IMPDH2 is associated with aggression of cancer. Intriguingly, in the subpopulation of patients in which nuclear localization of IMPDH2 was detected, it was significantly associated with longer survival. In contrast, expression of IMPDH1 is significantly up-regulated in the tumor tissue, and higher expression is related to better cumulative survival. The *in vivo* and *in vitro* experiment confirmed that IMPDH1 sustains but IMPDH2 inhibits the initiation and growth of HCC tumors.

Elevation of the expression of IMPDH2 across a wide spectrum of cancer types suggests that it is an excellent target for anticancer therapy[6]. However, even if these inhibitors exert potent effects on cancer cell growth, their efficacy remains obscure for now[7, 8]. With the exploring distinct function of two IMPDH isoforms, I hope I help understanding the complexity of the tumor cell in response to IMPDH2 inhibitors. Nevertheless, the complexity observed highlights the problems in devising and defining better therapy and stress the need for further research.

IMPDH inhibitors, such as mycophenolic acid (MPA) and its prodrug MMF, have been widely used in the clinic for the prevention of allograft rejection in organ recipients. Organ transplantation patients are generally at higher risk of developing malignancy[9]. Prevention of tumor recurrence is the major challenge of achieving this goal. With the finding of IMPDH in the HCC progression, it would be interesting to evaluate the effect of MMF/MPA treatment on HCC recurrence in liver transplantation patients.

In Chapter 4, I found that expression of STAT1 was higher in tumor tissues and mainly presented as u-STAT1, maybe to be expected in the absence of IFN treatment. It is known that high p-STAT1 levels following IFN treatment are transient and only last for several hours, after which STAT1 mainly is mainly manifest as u-STAT1. Intriguingly, I demonstrated the existence of both a nuclear and cytoplasmic u-STAT1 fraction, maybe consistent with a previous finding that u-STAT1 can shuttle between the cytoplasm and nucleus. By translocating to the nucleus, u-STAT1 can exert a transcription factor function and regulate

the expression of a subset of ISGs to aid host defense against viral infection and conceivably cancer. However, the spectrum of ISGs apparently regulated by p-STAT1 and u-STAT1 substantially differes. Some ISGs were found only to be regulated by u-STAT1 but not p-STAT1. Importantly, previous studies have demonstrated that p-STAT1 can protect tumor cells from apoptosis stimuli, radio- and chemotherapy. An implication of my result thus is that changing the balance between p-STAT1 and u-STAT1 (in favor of the latter) would in increased propensity of the cancer to respond to treatment while simultaneously substantial anti-viral activity of the transcription factor would remain. With advent of medication like tofacitinib clinically achieving such an effect looks feasible and I feel this possibility should be actively investigated.

Type I IFNs have attracted substantial interest for the treatment of various malignancies[3]. However, most studies show only limited efficacy with respect to tumor suppression and much of these effect mainly being related to prevention of virus-related cancers, suggesting such effects mainly relate to direct combat of viral infection[4, 5]. Reasons for the clinical failure of IFNs in cancer medicine likely include inherent biological mechanisms, changes in cell population, and institution of counter-regulatory pathways. IFN signaling is generally considered to stimulate immune response, but it has also been reported to induce immunosuppression under specific conditions[4]. As the key transducer of signaling by IFNs, phosphorylation of STAT1 following IFN stimulation is close linked to expression of IRGs as also explained above. I speculate that production of designer IFNs and their delivery to precancerous lesions in the tract, for instance by genetically modified bacteria (see later), would be capable of eliminating these not yet full-blown cancer cells, also by altering the balance between u-STAT1 and pSTAT1. Testing such an idea in practice, however, is still quite far away.

Developing novel strategies for anticancer therapy

With remarkable progress in research on cancer pathogenesis, a myriad of plausible therapies has become developed or even introduced into the clinic. Disappointingly, however, cancer, however, still ranks as the second leading cause of death worldwide to date. Surgery, radiotherapy and chemotherapy remain the main adequate and effective modes of treatment and thus curative rates largely dependent on early detection. Limited efficacy and unavoidable side effects toward normal cells make the development of new

targeting strategies and drugs urgently needed. In Chapter 5 and Chapter 6, I aim to evaluate the safety of telomerase targeted anticancer strategy and discuss the application of microorganisms for disease treatment.

Targeting telomerase appears an attractive approach bar the potential side effects on stem cell populations which thus may negatively affect medium term health of the patient. In Chapter 5 and given that the intestine and liver are at forefront of drug absorption and metabolism, it found it essential to understand how adult stem cells in these tissues/organs cope with these telomerase-targeted agents. I found that telomerase-targeted agents strongly inhibited the HCC and CRC cell growth, while intestinal and liver stem cell were relatively tolerant to these agents. Sensitivity of stem cells to telomerase-targeted agents also correlated with telomerase activity: intestinal stem cells but not liver stem cell have telomerase activity. Adult stem cells are known to be well-equipped to cope with DNA damage and also to maintain genetic stability. I found that Wnt signaling, which is important for stem cell renewal and tissue regeneration, is also important for tolerance of stem cells to telomerase length challenging stimuli. This function was dependent on the regulation of different DNA repair genes including TERT. Telomerase is normally absent in quiescent stem cells and will be activated during cell division. It seems that Wnt signaling, telomerase activity, cell proliferation are concurrent events, and the cooperation of these factors will balance the sensitivity and resistance of stem cells to telomerase targeted agents.

In Chapter 6**,** I discussed how to use the genetic modified bacteria for disease treatment. Despite the advancement in cancer gene therapy, specific and efficient gene delivery systems are still lacking. Incomplete tumor targeting, inadequate tissue penetration and limited toxicity are three main reasons responsible for the limitations of the cancer therapy[10, 11]. Alternative, genetically engineered bacteria have attracted more attentions and being developed as delivery vector for gene therapy. With advanced engineering technology, bacteria can be modified into a tiny *robot factories* with the function of targeting tumors, producing cytotoxic molecules, self-propel, response to triggering signaling, sensing local environment and producing external detectable signals[12].

Recently, numerous experiments have shown that bacterial therapies can successfully regress tumor size and promote cancer survival in mice. Challenges, however, remain with respect to limited drug production, intrinsic bacterial toxicity, targeting efficiency, genetic instability and combination with other therapies[13]. Furthermore, there is

little standardization, hampering use in the clinic, including in that of the mode of delivery. There is also little consensus as hoe to measure pharmacokinetics of genetically engineered bacteria, and the required precautions with respect to contamination of ecosystems with genetically modified organisms or the transmission of harmful organisms to patients. Nevertheless, in view of incremental improvements reached by alternative approaches, also due to complexity of the biological systems involved, a radical departure of existing approaches is called for, and the use of genetically-modified organisms may offer this.

Conclusions and future perspectives

- Commonly, gain-of-function mutation in oncogenes and loss-of-function mutations in tumor suppressor genes are the essential genetic alterations in cancer development. Different post-translational manifestations of gene products, however, will also remarkably influence cellular phenotype. This thesis provides important examples of this: the phosphorylation state of STAT1 and the different isoforms and subcellular localization of IMPDH were associated with distinct or even opposite functionality in HCC development. As STAT1 and IMPDH are the targets of clinically approved drugs (*e.g.* IFNs and MPA), this observation will help developing and optimizing drug treatment. As a genetic disease, exploring functions of different genes and key signaling pathways will largely improve the knowledge of molecular pathogenesis of cancer and contribute to the development of new anticancer therapies.
- Human adult stem cells, such as intestinal and liver stem cells, are remarkably tolerant for telomerase-targeting agents. Our study bears important implications for advancing the development of telomerase-targeted anticancer therapy, and has provided an important example for using human organoid models in the evaluation of drug safety, an observation also important in the quest for reduced use of experimental animals. Wnt signaling is identified as a signaling module that helps coping with DNA damage and is a regulator of TERT expression. In combination with the fact that telomerase and Wnt signaling are both needed for the proliferation of the stem cells, we speculate that all these factors are concurrent to balance sensitiveness and resistance of stem cells to drug treatment.
- With the various advantages associated with harnessing engineered bacteria for disease treatment, the successful use of engineered bacteria for cancer therapy may be just over the horizon. Challenges and opportunities are coexist regarding the advance in synthetic biology and understanding the host-bacteria interaction, however, lacking of the comprehensive test in human and bacteria strains with high efficiency, safety and biocontainment.

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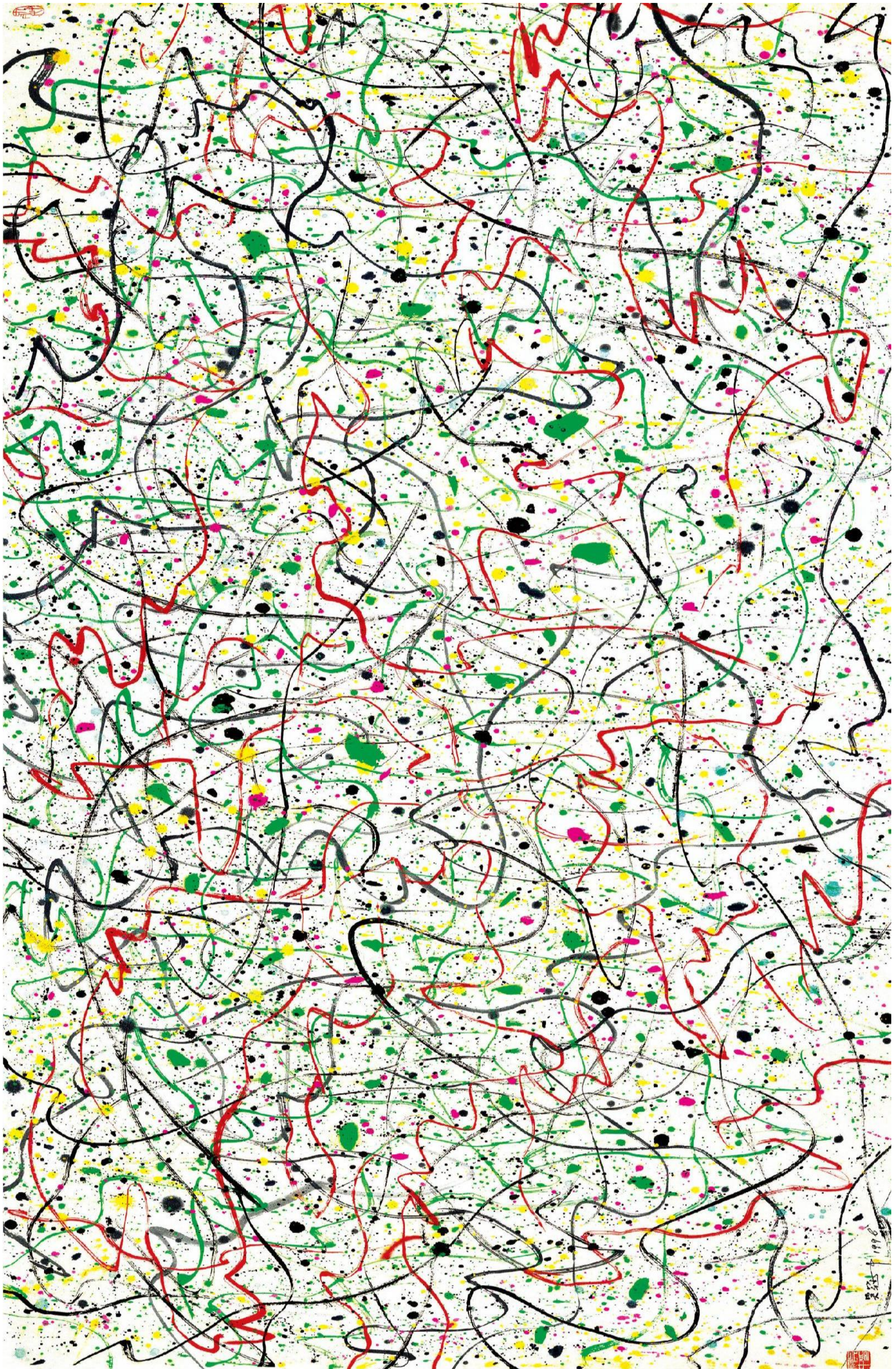

Chapter 8

Nederlandse samenvatting

Dutch summary

Het paraplubegrip kanker is afgeleid van het Latijnse woord "*cancer*" wat op haar beurt een leenvertaling is van het Griekse woord 'karkínos', dat oorspronkelijk "krab" betekende, maar door Hippocrates ook werd gebruikt voor gezwellen in het lichaam. Een lichaamsgezwel deed Hippocrates denken aan een in het zand ingegraven krab. Woekerende cellen, die aanleiding geven tot tumoren en kanker vormen één van de meest hardnekkige gezondheidsproblemen en voorzaken onnoemelijk menselijk leed. In dit proefschrift probeer ik bij te dragen aan strijd der mensheid tegen deze ziekte. Ik concentreer mij hierbij op leverkanker en dikke darmkanker.

Leverkanker, en met name het hepatocellulair carcinoom (HCC) als oook dikke darmkanker zijn kwaadaardige wildgroeisels die ontstaan na genetische beschadigingen van de darmcellen dan wel de levercellen (hepatocyten). Op mondiaal niveau zijn beide vormen van zeer veel voorkomend, waaraan jaarlijks vele miljoenen mensen komen te verscheiden. In Nederland is HCC relatief zeldzaam, maar is het wel de meest voorkomende vorm van primaire kwaadaardige tumoren in de lever. Dit promotieonderzoek is uitgevoerd aan het Erasmus Medisch Centrum van de Erasmus Universiteit van Rotterdam, en dit centrum heeft van alle Academische Centra in ons land in absolute termen de meeste leverkankerpatiënten en het is dus een logische locatie om deze ziekte te gaan onderzoeken. Een uitvoerige motivatie en achtergrond voor de gestelde doelen met betrekking ott het in dit proefschrift beschreven onderzoek wordt in Hoofdstuk 1 door mij gegeven.

In Hoofdstuk 2 presenteer ik de synthese van twee gepubliceerde studies waaraan ik een belangrijke heb geleverd (Chen K, Sheng J, Ma B, Cao W, Hernanda PY, Liu J, Boor PPC, Tjon ASW, Felczak K, Sprengers D, Pankiewicz KW, Metselaar HJ, Ma Z, Kwekkeboom J, Peppelenbosch MP, Pan Q. Suppression of Hepatocellular Carcinoma by Mycophenolic Acid in Experimental Models and in Patients. **Transplantation** 2019 May;103(5):929-937 en Chen K, Ma B, Peppelenbosch MP, Pan Q. Cytoplasmic rods and rings in mycophenolic acid treatment. **Liver Int.** 2017 Nov;37(11):1742-1743). In dit hoofdstuk presenteer ik experimenten dia laat zien dat de immuunsuppressieve medicatie mycofenolzuur de groei van HCC remt zowel in proefdieren als in weefselkweken van HCC. Een belangrijke observatie hierbij was dat HCC patiënten die via transplantatie een nieuwe lever kregen en ook werden behandeling met het mycofenolzuur kregen, zowel langer niet dood gaan en ook minder vaak terugval van de ziekte vertonen wanneer deze werden vergeleken met HCC patiënten welke een levertransplantatie ondergingen en therapie kregen met alternatieve

immunosuppressiva. Dit effect ging gepaard met specifieke veranderingen in het celskelet. Mijn studies wijzen sterk in de richting van dat mycofenolzuur gebruik na transplantatie van de lever bij HCC patiënten een goed idee met betrekking tot behandeling is.

Een vraag die daarna opborrelde uit de bovengenoemde resultaten, was hoe dan het mycofenolzuur een dergelijk effect zou kunnen bewerkstelligen. Het Mycofenolzuur interfereert met de werking van twee enzymen welke betrokken zijn bij de zogenaamde nucleotidesynthese, namelijk het inosine monophosphate dehydrogenase 1 en het inosine monophosphate dehydrogenase 2 (IMPDH1 en IPDMH2). Het is een logische gedachte dat dat dan deze twee enzymen ook een rol zouden hebben zijn bij de anti-kanker werking van mycofenolzuur. De eerste aanleiding dat deze gedachte inderdaad juist is, kan worden gevonden in Hoofdstuk 3. In dit hoofdstuk presenteer ik data die aantonen dat het specifiek blokkeren van IMPDH2 het delen van tumorcellen afremt, wederom zowel in proefdieren alsmede in kankerkweekjes. Vooral het snel delende compartiment van HCC is gevoelig voor het remmen van IMPDH2, waar het langzaam-groeiende compartiment minder gevoelig hiervoor is. Hoewel deze observaties doen veronderstellen dat HCC niet compleet verdwijnt na IMPDH2 remming, vormt het tegengaan van snelle HCC groei an sich, al vaak een belangrijk doel bij de behandeling van deze ziekte. Daarnaast kan ik mij voorstellen dat door het verminderen van de groei van HCC, het immuun-systeem van de HCC patiënt meer tijd gegeven wordt om een effectief antwoord tegen het kankerproces te formuleren. Vooral omdat, zoals boven reeds gesteld na transplantatie van de lever immuun-suppressie per se noodzakelijk blijkt tijdens de behandeling en als gevolg daarvan de patiënten dus een in verhouding zwak immuun-systeem hebben, is dit punt niet onbelangrijk.

In een vierde studie (Hoofdstuk 4), inmiddels reeds gepubliceerd (Ma B, Chen K, Liu P, Li M, Liu J, Sideras K, Sprengers D, Biermann K, Wang W, IJzermans JNM, Cao W, Kwekkeboom J, Peppelenbosch MP, Pan Q. Dichotomal functions of phosphorylated and unphosphorylated STAT1 in hepatocellular carcinoma. **J Mol Med** (Berl). 2019 Jan;97(1):77-88.) kijk ik naar de rol van de zogenaamde STAT eiwitten. Recentelijk zijn middelen die STAT eiwitten kunnen remmen op de markt gekomen voor menselijk gebruik. Omgekeerd, kan medicatie als interferon juist STAT eiwitten activeren. STATs lenen zich dus goed voor therapeutische interventie. Ik kon inderdaad aantonen dat HCC gekarakteriseerd werd door de aanwezigheid van meer STAT en geactiveerd en niet geactiveerd verschillende rollen

hebben in de lever (kanker) cel. Met behulp van deze informatie is rationeel gebruik van STAT modulatoren bij de behandeling van leverkanker dichterbij gekomen.

Voor mij het belangrijkste hoofdstuk van dit proefschrift is Hoofdstuk 5. In dit hoofdstuk ga ik in op telomerase, was ook wel de Achilleshiel van kanker wordt genoemd. Telomerase is een enzym dat voorkomt dat het DNA tijdens de celdeling steeds korter wordt. Bij elke celdeling wordt in elk chromosoomeinde zo'n 100 nucleotiden korter na zo'n 50 delingen is de DNA lengte niet meer verenigbaar met celdeling en stop de proliferatie. Zowel kankercellen alsook stamcellen hebben echter het enzym telomerase dat het DNA weer verlengd tot de oorspronkelijke lengte. Het ligt dus voor de hand om het telomerase enzym te remmen bij de behandeling van kanker. Echter, de angst bestaat dat dit ook het eind zou betekenen van gezonde stamcelcompartimenten in het lichaam, iets wat uiteindelijk onacceptabele bijwerkingen zou opleveren. Ik heb nu gevonden dat stamcellen, althans in de lever en de darm, relatief goed telomeraseremming kunnen weerstaan en ook de onderliggende moleculaire details opgehelderd, wat de weg opent naar het klinisch testen van zulke inhibitoren.

De laatste studie in dit proefschrift (Hoofdstuk 6) betreft een literatuurstudie naar een radicale nieuwe manier om therapeutische eiwitten in het menselijk lichaam te krjgen en zelfs louter lokaal hun werk te laten doen zonder systemische (lichaamsbrede) bijwerkingen. Het gaat hier om genetisch gemodificeerde bacteriën. Bacteriën zijn goedkoop te kweken en vele voelen zich van nature prettig thuis in de darm. Waar therapeutische eiwitten normaliter in de darm worden afgebroken, geldt dit niet voor eiwitten die door darmbacteriën worden gemaakt. Door bacteriën genetisch te modificeren kunnen deze therapeutische eiwitten gaan maken. Mogelijkheden zijn ontstekingsremmende eiwitten maar ook eiwitten die preventief zijn met betrekking tot kanker. De haalbaarheid en mogelijkheden worden geëxploreerd in dit hoofdstuk, wat ook gepubliceerd werd in een toonaangevende tijdschrift (Ma B, Pan Q, Peppelenbosch MP. Genetically Engineered Bacteria for Treating Human Disease. **Trends Pharmacol Sci.** 2017 Sep;38(9):763-764).

In het laatste hoofdstuk (Hoofdstuk 7) vat ik de kennis samen die ik elders in dit proefschrift heb vergaard met betrekking tot lever- en darmcellen en hun relatie tot het kankerproces. Ook probeer ik een meer helder beeld te schetsen hoe mogelijke nieuwe therapie met als doel het behandelen en zelfs het genezen van HCC, er mogelijk uit zou

kunnen komen te zien. Alles tezamen hoop ik met dit proefschrift een aanzet te hebben kunnen geven voor zulke nieuwe therapie.

Appendix

Acknowledgements

Publications

PhD Portfolio

Curriculum Vitae

Acknowledgements （致谢）

It is my great honor to have the precious opportunity provided by China Scholarship, which support me to continue my study as a PhD in Netherlands. Four years study is colorful and memorable and I would like to give my greatest gratitude to all the people who helped with my PhD study and my daily life. I would never possible to achieve and finish the work presented in this thesis without all of your dedications.

Prof. Maikel P.Peppelenbosch, thank you for the guidance and help for my research. As an leading experimental gastroenterologist, you have tremendous amount of knowledge in this filed and always give eye-open suggestions to my project. Working with you improves me to be an eligible PhD student and to have scientific feelings. Working the “forum” article together with you open a window of my PhD career, which gives me feelings of how to do scientific work. Thank you for all your supports to my PhD study and being my supervisor.

Dr. Qiuwei (Abdullah) Pan, thank you for giving me the opportunity to be your PhD student and supervising me during my four years study. You encouraged me a lot when I was struggling with my projects. You are such a brilliant person with sharp sight in research area. You always encouraged me to be confident during presentation and to be more positive in regular project discussion.

Prof. Herold Metselaar, Prof. Robbert de Man and Prof. James Hardwick, I am honored to have you as the inner committee for my promotion and thanks for your valuable time to evaluate and revise my thesis. Thanks you!

Dr. Ron Smits, you are such a knowledgeable, kindness, and patient person. You do give me a lot of useful suggestions and also a lot of “food” to digest after discussion. Also, thanks for your help during my postdoc application. I really enjoy the time with you. Dr. Jaap Kwekkeboom, thank you for all the suggestions during the GIO meeting and the support with kindly providing patient materials to my project Thank you for all the collaboration and help. Dr Dave Sprengers, thanks for giving suggestions during our weekly meeting. Your clinical perception helps me a lot. Prof. Luc J W van der Laan,

thanks for your help in liver organoid culture and suggestions on GIO meeting. You do help me a lot.

Dr. Kan Chen, you are my bachelor's teacher and the only person I familiar with before I come here. You guided me with the lab work and also my daily life in my first year. I appreciate all the help from you and enjoy the times with you. Dr. Pengyu Liu, we used to be a teammate, the "transcription factor" team. You are always hardworking and have different research ideas. We ever worked together to figure out the technical issues. I enjoy the time with you and wish you a bright further. Dr. Shan Li, you are a rigorous person and always strict with your research. we share four years in the some office with discussion, eating, playing. You give me a lot of help and support to my project. I wish you and your family a bright further. Dr. Wen, you are the happy "bean" of our lab. Thanks for organizing so many wonderful activities during my PhD and also the help and suggestions for my project.

Dr. Wenshi Wang, Dr. Lei Xu and Dr. Yuebang Yin, you three laid the foundation of the virus research in our lab. All of you worked really hard and also took care of all other colleague members in our lab. You three set the PhD models of all of us. The time we shared together are memorable and all of you will have a bright future. Dr. Wenhui Wang, you like a "old sister" to us, thanks for your help to my daily life and my study. Dr. Yijin wang, you are always confident and follow your heart and many thanks for your help in my first year.

To Manzhi, you are a sincere girl and always thanks for your numerous support of my daily life and my research work. We share some memorable time in our first year. To Changbo and Meng, we come here in the same year, we have memorable time in our first year. Thanks for your help and I wish you best in the future. Dr. Sunrui Chen and Jiaye Liu, you two are smart, sunshine and fashion guys in our lab. You always known the information of shopping and travelling discount. I wish you all success in your PhD research.

To Wanlu and Guoying, you two are the excellent researchers in the lab. Xumin and Qin, you two are both creative, excutive and full of ideas. Ruyi, its good to know you and thank you for your contribution to my work. Zhijiang, you are hospital to everyone,

thanks for preparing so many delicious food for us. Yang, you work hard and follow your own step in research. Peifa, you are smart and have the feelings of how to do research. Yunlong, you are a kind and little bit shy boy. Ling, you are such a kind and hard-working girl. Zhouzhou, you are smart and hard-working, thanks for helping analyze the online data. Pengfei, you are the expert of modeling HEV-organoids infection. Shihao and shaoshi, you two are very energetic in our lab and always following the advances of science.

To Marla and Natasha, you both are very kind and thank you for all your help and technical supports to my project. To my current ‘office members’, Lucia, Patrick, Zhouhong Ge, talking with you improves my knowledge of Flow Cytometry and also thanks for your suggestions during my postdoc application. To my former ‘office members’, Monique, Aafke, Janine, Gulce, Ishaki et al. Thanks for the great time we had together. To all MDL members, Andre, Hugo, Andrea, Auke, Raymond, Leonie, Marcel, Monique, Thomas, Henk, Pauline, Adriaan, Jan, Paula, Patrick, Sonja, Anthonie, Lauke, Gertine, Kim, Shanta, Petra et al., everyone in our lab are so great! I want to thank all of you for any help and support during my study here.

感谢浙江理工大学生命科学学院刘新元院士和王毅刚副研究员在本科和硕士期间对我的科研指导以及对生活的关心，出国学习离不开你们的支持和鼓励！感谢新元研究所所有老师和师兄师姐在硕士期间对我的帮助和鼓励！

感谢父母，姐姐至始至终对我学业的支持和理解，以及对我无微不至的关爱，你们永远是我坚强的后盾！感谢所有亲戚和朋友对我从小到大一路走来的支持，帮助和鼓励！让我能够最终走到这一步！希望你们能永远健康快乐！

Publications

International (refereed) journals

1. **Ma B**, Pan Q, Peppelenbosch MP*. Genetically Engineered Bacteria for Treating Human Disease. Trends Pharmacol Sci 2017;38:763-764.
2. **Ma B**, Chen K, Liu P, Li M, Liu J, Sideras K, Sprengers D, Biermann K, Wang W, JNM IJ, Cao W, Kwekkeboom J, Peppelenbosch MP, Pan Q*. Dichotomal functions of phosphorylated and unphosphorylated STAT1 in hepatocellular carcinoma. J Mol Med (Berl) 2019;97:77-88.
3. **Ma B**, Zhang R, Liu J, Li S, Liu P, Verstegen MMA, Li M, Wang Y, Chen K, van der Laan LJW, Cao W, Smits R, Peppelenbosch MP, Pan Q*. Human intestinal and liver stem cells counteract telomerase-targeted anticancer therapy. Submitted.
4. Chen K[#], **Ma B**[#], Cheng Y, Sideras K, Liu Y, Cao W, Ma X, Sprengers D, Feng C, Jonge Jd, Ye X, Ma J, Polak WG, Verheij J, Gulik Tv, Biermann K, Ijzermans JNM, Ma Z, Kwekkeboom J, Peppelenbosch MP, Pan Q*. The two isoforms of IMPDH distinctively associate with patient outcome and exert dichotomic functions in hepatocellular carcinoma. Submitted.
5. Chen K, Sheng J, **Ma B**, Cao W, Hernanda PY, Liu J, Boor PPC, Tjon ASW, Felczak K, Sprengers D, Pankiewicz KW, Metselaar HJ, Ma Z, Kwekkeboom J, Peppelenbosch MP, Pan Q*. Suppression of hepatocellular carcinoma by mycophenolic acid in experimental models and in patients. Transplantation 2019.
6. Liu P, Cao W, **Ma B**, Li M, Chen K, Sideras K, Duitman JW, Sprengers D, Khe Tran TC, Ijzermans JNM, Biermann K, Verheij J, Arnold Spek C, Kwekkeboom J, Pan Q, Peppelenbosch MP*. Action and clinical significance of CCAAT/enhancer-binding protein delta in hepatocellular carcinoma. Carcinogenesis 2019;40:155-163.
7. Liu J, **Ma B**, Cao W, Li M, Bramer WM, Peppelenbosch MP, Pan Q*. Direct-acting antiviral agents for liver transplant recipients with recurrent genotype 1 hepatitis C virus infection: Systematic review and meta-analysis. Transpl Infect Dis 2019;21:e13047.
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9. Dang W, Xu L, **Ma B**, Chen S, Yin Y, Chang KO, Peppelenbosch MP, Pan Q*. Nitazoxanide Inhibits Human Norovirus Replication and Synergizes with Ribavirin by Activation of Cellular Antiviral Response. Antimicrob Agents Chemother 2018;62.
10. Chen K, **Ma B**, Peppelenbosch MP, Pan Q*. Cytoplasmic rods and rings in mycophenolic acid treatment. Liver Int 2017;37:1742-1743.
11. Wang W, Wang Y, Qu C, Wang S, Zhou J, Cao W, Xu L, **Ma B**, Hakim MS, Yin Y, Li T, Peppelenbosch MP, Zhao J, Pan Q*. The RNA genome of hepatitis E virus robustly triggers an antiviral interferon response. Hepatology 2018;67:2096-2112.

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PhD Portfolio

Name PhD Student	Buyun Ma
Erasmus MC Department	Gastroenterology and Hepatology
PhD Period	October 2015 - September 2019
Promotor	Prof. Dr. Maikel P. Peppelenbosch
Copromotor	Dr. Qiuwei (Abdullah) Pan

PhD training

Seminars

- 2015-2019, Weekly MDL seminar program in experimental gastroenterology and hepatology (attending); (42 weeks/year;@1.5h) (ECTS, 9.0).
- 2015-2019, Weekly MDL seminar program in experimental gastroenterology and hepatology (presenting); (preparation time 16h; 2 times/year) (ECTS, 4.6).
- 2015-2019, Biweekly research group education (attending); (20 times/year; @1.5h) (ECTS, 4.3).
- 2015-2019, Biweekly research group education (presenting); (preparation time 8h; 4 times/year) (ECTS, 4.6).

General Courses and workshops

- 2015, the course biomedical research techniques XIV (ECTS, 1.5)
- 2016, the workshop ingenuity pathway analysis (IPA) (ECTS, 0.5)
- 2016, the microscopic image analysis: from theory to practice (ECTS, 0.8)
- 2016, the course on gene expression data analysis using R (ECTS,2.0)
- 2016, the workshop on NCBI & other open source software (ECTS, 1.0)
- 2016, the galaxy for NGS (ECTS, 0.6)
- 2017, the Erasmus MC Cancer Institute Research Day (ECTS, 0.3)
- 2018, the course on biomedical English writing course for MSc and PhD-students (ECTS,2.0)

National and International Conferences

- 2018, 53th The International Liver Congress™ (EASL, European Association for the Study of the Liver), Paris, France (Poster presentation)

- 2018, Annual Day of the Molecular Medicine Postgraduated School, Rotterdam, the Netherlans. (Poster presentation)

Academic Awards

Scientific Awards and Grants

- 2015, China Scholarship Council (CSC) Scholarship (File No. 201508330291)

Curriculum Vitae

Buyun Ma was born in October 6, 1990, in Dongyang, Zhejiang, China. He attended primary, middle and high school in Dongyang.

In 2008, he graduated from high school and move to Hangzhou to strat his Bachelor study in Zhejiang Sci-Tech University. He graduated in 2012, and continue his Master study in Zhejiang Sci-Tech University. His master research mainly focus on harnessing adenovirus armed with suicide genes for hepatocellular carcinoma (HCC) treatment.

In 2015, with the support of China Scholarship Coouncil, he moved to the department of Gastroenterology and Hepatology, Erasmus Medical Center Rotterdam, the Netherlands, to carry out his PhD research. Under supervision of Prof. Maikel P. Peppelenbosch and Dr. Qiuwei (Abdullah) Pan, he focused on molecular pathogenesis and treatment of HCC. He devoted to understand how gene network contribute to HCC development and develop new anticancer strageties.