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Memoir:

Tumor immunology and immunotherapy: a journey I started from Hangzhou*

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This short article is dedicated to the 90th Anniversary of the School of Life Sciences at Zhejiang University, China. Immunotherapy of cancer is currently a hot topic in the biomedical field, and a research focus of my laboratory is on developing new and effective combinatorial immunotherapeutic strategies for liver cancer. Of note, my interest in immunotherapy of cancer stems from the training as an undergraduate student at Hangzhou University, China, almost 40 years ago.

1 Introduction

It is a great honor to be invited to write a short article at the celebration of the 90th Anniversary of the School of Life Sciences at Zhejiang University, China. The research in my laboratory is currently focused on deciphering mechanisms of liver tumorigenesis (Feng, 2012) and developing novel combinatorial immunotherapeutic strategies of liver cancer (Wen et al., 2019). This stems from the training I received almost 40 years ago.

In March 1978, as a young fellow in China, I was fortunate to be admitted into the Department of Biology at Hangzhou University. This department has now been incorporated into the School of Life Sciences in the new Zhejiang University. Like many others in the entering class of 1977, I had not chosen biology as the subject to study in college. My goal was to become a mathematician, largely inspired by the story of "Jin-run CHEN," who at that time was the most widely known expert in the area of number theory in China. After meeting the admission criteria for Hangzhou University, I was re-assigned to the Biology Department, as a result of my willingness to "obey an assignment," as I had mentioned in the college application form. Retrospectively, this reassignment to the Biology Department transformed my career, for which I have been extremely grateful throughout my life since then.

After completing the core curriculum in college, we were offered a few courses of "elective topics." I enrolled in two important courses, one of which was "Tumor Biology" lectured by Prof. Xi-min JIANG. The main interest of JIANG's research had previously been in the area of comparative physiology. In the early 1970s, his research focus switched to tumor immunology, apparently aimed at tackling a more challenging health issue faced by society. The other course was "Immunology," lectured jointly by several teachers, including Ren-rui DING, Zhi-zhang MA, and Shi-juan ZHE. After taking these two courses, I became deeply interested in tumor immunology, particularly in the immunotherapy of tumors. At that time, it was already widely recognized that either chemo- or radiation-therapy did not only kill tumor cells, but also damaged healthy cells in patients. Immunotherapy was apparently a hope or research direction to pursue, which could allow a specific attack on tumor cells. In my final year at Hangzhou University, I joined the group of teachers MA and

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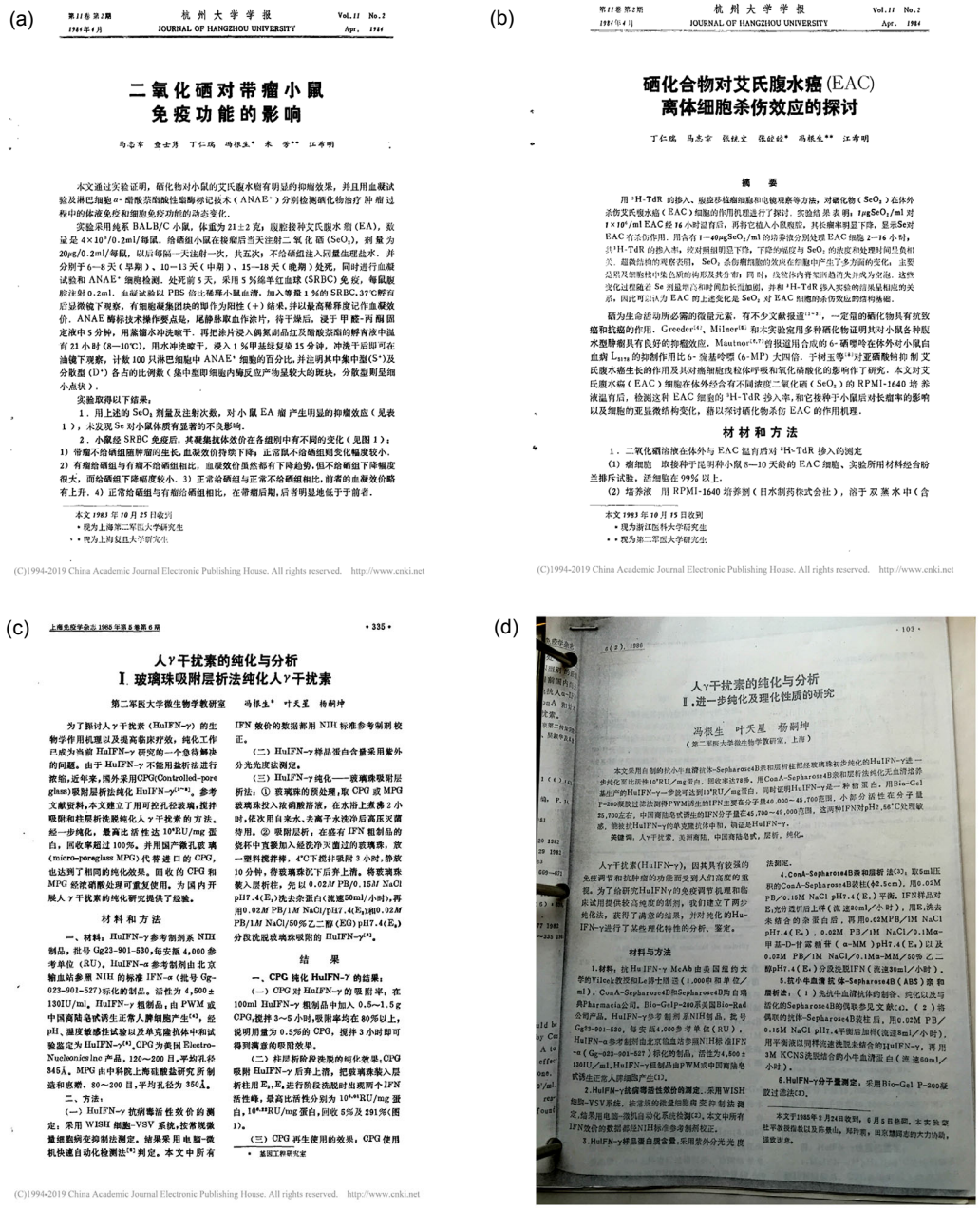
DING to participate in a research project on examining the inhibitory effects of selenium compounds on tumor cell proliferation in mice. In this research, we saw a dramatic extension of mouse survival, as well as an improvement of immune functions in the tumor-bearing mice, following treatment with selenium compounds. However, we also observed the severe liver damage caused by these compounds. The work was published in the *Journal of Hangzhou University* (Natural Science Edition) in 1984 (Figs. 1a and 1b). This was my first experience of biomedical research associated with tumor immunology.


(a)

杭州大学学报 JOURNAL OF HANGZHOU UNIVERSITY

二氧化硒对带瘤小鼠免疫功能的影响

(b)

杭州大学学报 JOURNAL OF HANGZHOU UNIVERSITY

硒化合物对艾氏腹水癌(EAC)离体细胞杀伤效应的探讨

(c)

人γ干扰素的纯化与分析 Ⅰ. 玻璃珠吸附层析法纯化人γ干扰素

(d)

人γ干扰素的纯化与分析 Ⅱ. 进一步纯化及理化性质的研究

Fig. 1 Research papers published in Chinese in the 1980s

(a, b) The first pages of two papers published in the *Journal of Hangzhou University* (Natural Science Edition) (Ding et al., 1984; Ma et al., 1984) on the research work I participated in as an undergraduate student at the Department of Biology, Hangzhou University, China. (c, d) The first pages of two papers published in the *Shanghai Journal of Immunology* (Feng et al., 1985) and *Chinese Journal of Microbiology and Immunology* (Feng et al., 1986), respectively, from my MSc degree research work on interferon-γ purification and characterization

2 Exploring the anti-tumor effects of cytotoxic cytokines

It was my keen interest in tumor immunology that prompted me to apply for admission into a graduate program in immunology. My choice of mentor was Prof. Tian-xing YE at the Second Military Medical University (SMMU) in Shanghai, China. I had a very high respect for Prof. YE, because of a book entitled "Theory and Practice of Immunology" that he wrote in the late 1970s. That was surely one of the first immunology books written in Chinese; it was printed internally by SMMU and was not available in public bookstores. Prof. DING at Hangzhou University was able to obtain a copy of the book through a relative who was YE's friend and colleague at SMMU. DING was generous enough to let me take the book home and read it during the 1981 Spring Festival holiday. In 1982, I was fortunate to be accepted into SMMU and became a graduate student for a Master of Science (MSc) degree under supervision of Prof. YE (at that time, the Doctor of Philosophy (PhD) system was still at its infancy in China). Aiming at developing immunotherapeutic reagents, my research focused on purification and characterization of interferon-γ (IFN-γ). Part I of the work was published in the *Shanghai Journal of Immunology*, and Part II in the *Chinese Journal of Microbiology and Immunology* (Figs. 1c and 1d). I learned a lot from Prof. YE, who was extremely knowledgeable in many areas of immunology. Indeed, the Immunology Program at SMMU trained and nurtured a large group of immunologists, including Dr. Xue-tao CAO, who was the President of Chinese Immunology Society for many years. Xue-tao is currently the President of Nankai University in Tianjin, China. During the research work, I also received support from Prof. Ping DU, a pioneer in interferon research in China, and Dr. Zhong-tian QI, his former graduate student.

Sometimes things in life seem almost like they were "designed" or arranged by "God." In early 1985, Mrs. Zhi-zhang MA, my college teacher, went to Indiana University in the USA as a visiting scholar, in an exchange program between the two universities. She was working in Dr. Milton TAYLOR's lab in the Department of Biology, on a project related to interferon. Knowing that my MSc thesis work was on IFN-γ purification, MA introduced me to Dr. TAYLOR who was looking for somebody with experience in interferon research. In October 1986, I joined the TAYLOR lab at Indiana University Bloomington and completed my PhD dissertation work there. My first project was to characterize a fusion protein consisting of IFN-γ and tumor necrosis factor-β (TNF-β), based on previously observed synergistic effects between cytotoxic cytokines, in collaboration with a group at Genentech Inc., USA. Indeed, the hybrid protein was shown to have enhanced anti-proliferative activity on ME180 cervical carcinoma cells (Feng et al., 1988). To dissect the molecular mechanisms of the anti-tumor effects of these cytokines, we then isolated mutant ME180 cell lines that were resistant to IFN-γ following somatic cell mutagenesis. Interestingly, we demonstrated that one of the mechanisms of IFN-γ cytotoxicity was induction of indoleamine 2,3-dioxygenase (IDO), which degrades an essential amino acid L-tryptophan, and that the mutant cells failed to produce IDO in response to IFN-γ (Feng and Taylor, 1989).

The PhD training in Indiana University was solid and thorough, and from which I benefited tremendously. The research programs there were especially strong in molecular biology, having several internationally renowned and exceptionally talented scientists including Norman PACE, Barry POLISKY, and Thomas BLUMENTHAL. From the analysis of ME180 mutants that were defective in IDO induction upon treatment with IFN-γ, I learned about the importance of understanding intracellular signaling pathways. Therefore I then decided to receive postdoctoral training in a molecular signaling lab. In 1990, I chose to work with Dr. Bryan WILLIAMS at the Hospital for Sick Children, affiliated with the University of Toronto, Canada. With Bryan's support, I received a three-year postdoctoral fellowship from the Medical Research Council (MRC) of Canada. Shortly before my arrival, Bryan WILLIAMS, in collaboration with Ara HOVANESSIAN in France, cloned the long-sorted human protein kinase (now called PKR) induced by IFN and activated by double-stranded RNA (dsRNA) (Meurs et al., 1990). Because of this work, Bryan and Ara received the Milstein Award from the International Society of Interferon Research (ISIR) in 1990. To continue this project, I cloned the mouse homologue of the human PKR and identified the dsRNA-binding motifs in the N-terminal domain, which was published in *Proceedings of the National*

Academy of Sciences of the United States of America (*PNAS*) (Feng et al., 1992). Soon after joining the lab, Bryan informed me that he had just accepted a job offer as the funding Chair of Cancer Biology at the Cleveland Clinic Foundation, USA; however, I decided to stay on in Toronto instead of moving to Cleveland.

3 Discovery of an SH2-containing tyrosine phosphatase Shp2 (Syp)

After spending a year in the WILLIAMS lab, I carried my MRC fellowship and joined the late Tony PAWSON's group in the Samuel Lunenfeld Research Institute of Mt. Sinai Hospital in Toronto, Canada. The PAWSON lab was known for discovering the Src-homology 2 (SH2) domain in mediating protein–protein interaction in orchestrating intracellular signaling events (Koch et al., 1991). In discussing potential projects, I expressed interest in searching for a tyrosine kinase involved in interferon signaling. At that time, i.e. in 1991, no protein tyrosine kinase (PTK) was found to act in this pathway yet. Soon after, tyrosine kinase 2 (Tyk2) was identified as a critical signaling component in interferon response, using a genetic complementation assay (Velazquez et al., 1992). Through collaborative efforts of many labs, the Janus kinase/signal transducers and activators of transcription (Jak-STAT) pathway was discovered for interferon and cytokine signaling (Darnell et al., 1994). Tony told me very nicely, "as you have come to join us with your own MRC fellowship, you are certainly welcome to do whatever you like in cell signaling"; however, he went on, "it would be great if you could make your story related to the SH2 domain, which is my baby and is also the center of research in the lab." Along this line, I then expressed an interest in searching for tyrosine phosphatase with SH2 domain by polymerase chain reaction (PCR). Tony thought that this was a cool idea to try. Working towards this goal, I designed a PCR strategy that used one primer based on a conserved motif in SH2 domains and another primer corresponding to a motif shared in catalytic domains of known protein-tyrosine phosphatases (PTPs). During my PhD program, I learned the principles of PCR but had never actually touched a PCR machine. Therefore, I carefully read the PCR manual and a few technical papers; this reading allowed me to acquire several critical points important for the experimental design. First, it was important to include "limited degeneracy" in the design and synthesis of the degenerative primers. Located in the center of the conserved motif of SH2 domains was an arginine (Arg, R) residue involved in direct contact with a phospho-tyrosine (p-Tyr) residue in ligands. Examining the known SH2 domains revealed that not all six genetic codons were used for arginine at this position. Thus, I only included two codon possibilities in the primer design, which dramatically reduced degeneracy and therefore improved specificity. Also important was the requirement to use the "touchdown" PCR program and variable magnesium concentrations in the buffer. Careful design and execution of a PCR strategy resulted in production of a single specific DNA product of approximately 500 bp under all conditions, in addition to many other bands, observed in agarose gel analysis. Cloning and sequencing of this 500-bp PCR product revealed that it indeed contained DNA sequences coding for a typical but novel SH2 domain. Since the other primer was based on a conserved motif within catalytic domains of PTPs, we were very positive about identifying a novel SH2-containing tyrosine phosphatase at that time. The data were obtained shortly before Christmas day in 1991, which allowed me to have a relaxing and happy Christmas vacation in Ann Arbor, Michigan, USA, with two close friends and former roommates from Indiana University, Jin-song LIU and Wei-dong HUANG.

After this initial breakthrough, it was quite straightforward to obtain overlapping complementary DNA (cDNA) fragments by screening a randomly primed cDNA library using that PCR product as a probe. Recombination of these partially overlapping fragments generated a longer cDNA product of approximately 2000 bp. Sequencing of the 2-kb fragment indicated that it encoded a protein containing two SH2 domains at the N-terminus and a single catalytic domain of tyrosine phosphatase. Meanwhile, cloning the SH2 domains into a bacterial expression vector generated a glutathione *S*-transferase (GST)-fusion protein, which was injected into rabbits for the production of a specific antiserum used for biochemical analysis in cell signaling. The whole project continued quickly and smoothly. In September 1992, we submitted a manuscript to *Science*, which was published in March 1993 (Feng et al., 1993). We named the enzyme Syp, standing for an SH2-containing

tyrosine phosphatase. Almost at the same time, several other groups cloned this enzyme from human cells, which was published under different names (Feng and Pawson, 1994).

Cloning of this important signaling molecule provided a fresh view on the dynamic interactions between PTKs and PTPs in cell signaling. Nowadays, it is clear that there are two SH2-containing tyrosine phosphatases in mammals: Shp1 is predominantly expressed in hematopoietic and lymphocytic cells, and Shp2 (which we previously called Syp) is widely expressed in all cell types. Interestingly, Shp2, acting in signaling events immediately downstream of receptor tyrosine kinases (RTKs) (Fig. 2), has been found to promote RTK signaling to the Ras-extracellular signal-regulated kinase (Ras-ERK) pathway (Fig. 3). Consistent with this positive effect in RTK signaling, Shp2/Ptpn11 was identified as the first oncogenic tyrosine phosphatase (Chan and Feng, 2007). With the *Science* paper in press, I received several job offers in Canada and USA, and decided to go back to Indiana, by accepting an assistant professor position in the Walther Oncology Center and the Department of Biochemistry and Molecular Biology in Indiana University Medical School, Indianapolis.

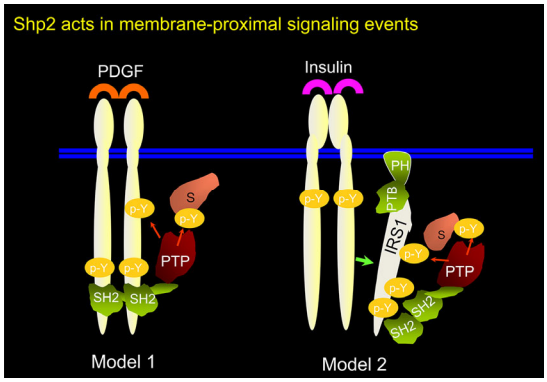


Fig. 2 Structure and function of protein-tyrosine phosphatase Shp2

Shp2 is a PTP that contains two SH2 domains. It can dock on a p-Tyr residue on RTK through its two SH2 domains, such as PDGF-R, and dephosphorylate another p-Tyr site on the RTK (Model 1). However, Shp2 can also dock on a substrate of RTK in the insulin system, and dephosphorylate another site (Model 2). In either Model 1 or 2, the phosphatase was presumed to act as a negative regulator in RTK signaling. PTP: protein-tyrosine phosphatase; p-Tyr: phospho-tyrosine; RTK: receptor tyrosine kinase; PDGF-R: platelet-derived growth factor receptor; PTB: phosphotyrosine binding; PH: pleckstrin homology; IRS1: insulin receptor substrate 1

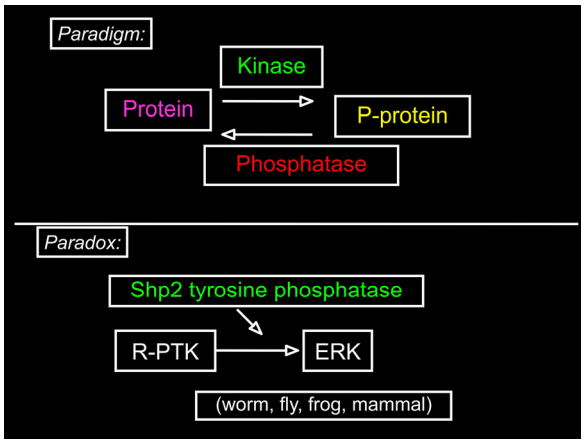


Fig. 3 A positive role of Shp2 in promoting RTK-to-ERK signaling

Working in the paradigm in which a phosphatase acts to reverse a kinase activity in cell signaling, we found, however, that Shp2 acts to promote signaling from kinase to kinase (RTK-to-ERK). The underlying mechanism is still not fully understood. PTK: protein tyrosine kinase; RTK: receptor tyrosine kinase; ERK: extracellular signal-regulated kinase

4 Dissecting mechanisms of intracellular signaling events

After setting up my own lab in Indianapolis in 1994, the focus of our research was naturally on dissecting mechanisms of intracellular signaling pathways centered around Shp2. The first project was on functional analysis of Shp2 in the control of hematopoietic cell differentiation from mouse embryonic stem (ES) cells, carried out by postdoctoral fellow Cheng-kui QU, who came to the lab with a strong background in hematology and was extremely hard-working. By establishing an in vitro ES cell differentiation assay and chimeric animal analysis in vivo, he was able to demonstrate a positive requirement of Shp2 for the development of all blood cell lineages in mammals (Qu et al., 1997, 1998, 2001). QU is now a full professor at Emory University in Atlanta, USA, and is doing very well.

To interrogate specific functions of Shp2 in various cell types in health and diseases, we decided to generate a conditional mutant allele of Shp2 using the Cre-loxP system. Er-quang (Eric) ZHANG, a PhD student who came from Fudan University, Shanghai, China, took on this challenging job and successfully generated a conditional Shp2 knockout allele in mice. We have now distributed this mutant mouse line to many labs around the world. Eric is now doing very

well in research on circadian rhythm as a principal investigator at the National Institute of Biological Sciences (NIBS) in Beijing, China. Also worthy of mention, Yue-hai KE was another successful post-doctoral fellow in the lab. Yue-hai also got his Bachelor of Science (BSc) degree from the Department of Biology, Hangzhou University and completed his PhD thesis work with Dr. Jin LI at Fudan University focused on human genetics. Yue-hai is now Associate Dean of the Medical School and also Associate Dean of the Sci-Tech Academy at Zhejiang University, Hangzhou, China. Helen (He) ZHU was a very talented PhD student who defined a positive role of Shp2 in adult hematopoiesis in mice. Furthermore, she was able to demonstrate directly opposing effects of Shp2 and Pten in control of myeloproliferation and leukemogenesis as well as cooperative functions of the two signaling molecules in mammalian erythropoiesis (Zhu et al., 2015). Helen is now a full professor in the Research Institute of Renji Hospital affiliated with Shanghai Jiao Tong University, China.

5 Deciphering the anti-oncogenic effects of pro-oncogenic molecules in the liver

Emilie BARD-CHAPEAU was a visiting graduate student from the University of Lyon, France. She came to the lab with little research experience. For her PhD thesis, we wanted to set up a partial hepatectomy and liver regeneration system, to determine if Shp2 is indeed a positive regulator of cell proliferation in response to proliferative signals in mammals in vivo. With no previous experience and no help from others in the lab, Emilie was able to independently set up the mouse liver surgery procedure. She demonstrated that loss of Shp2 indeed impaired hepatocyte proliferation following a conventional two-third removal of liver mass in mice (Bard-Chapeau et al., 2006). Consequently, it was truly a big surprise when we observed that tumor nodules developed in the livers wherein was Shp2 deleted in the hepatocytes in some aged mice. These old mice were not used in the partial hepatectomy experiment, which was performed on young mice at age of 2–3 months. Fortuitously, Emilie examined the liver phenotype of these old "useless" mice before disposal. To confirm and extend the observation, we treated the mice with chemical carcinogen diethylnitrosamine (DEN), and found that Shp2 deficiency in hepatocytes did aggravate development of hepatocellular carcinoma (HCC) induced by DEN in mice, thus pointing Shp2 as a tumor suppressor in the liver (Bard-Chapeau et al., 2011). This observation was unanticipated, given the positive role of Shp2 in enhancing Ras-ERK signaling and in hepatocyte proliferation in the regenerating liver.

Importantly, consistent with our data on Shp2 in the liver, a number of groups also reported similar "paradoxical" results; for example, liver tumorigenesis was ironically enhanced by deleting pro-oncogenic molecules in hepatocytes, including c-MET, β-catenin, IκB kinase β (IKKβ), and Jnk (Feng, 2012). Realizing that these observations may have disclosed some previously unappreciated but important mechanisms of liver tumorigenesis, the main focus of our lab research has shifted to elucidating the anti-oncogenic effects of pro-oncogenic molecules in HCC development, after joining the faculty at UCSD in 2009.

6 Development of combination immunotherapy of liver cancer

In dissecting a putative role of Shp2 in mediating cell–cell communication between hepatocytes and Kupffer cells in the liver, we generated two different mutant mouse lines: one with Shp2 ablated in hepatocytes using albumin-Cre and the other with Shp2 removed from the hepatocytes and non-parenchymal cells (NPCs), including Kupffer cells, using Mx1-Cre. Of note, the expression of Mx1-Cre is induced by interferon or synthetic dsRNA, polyIC, an interferon inducer. This is actually the first established inducible gene deletion approach in mice. This strategy was previously used by several groups to dissect signaling molecules with bidirectional roles in HCC development. However, based on my previous experience with polyIC and interferons, I knew that an injection of polyIC would not simply induce Mx1-Cre expression, but actually trigger the production of numerous inflammatory cytokines. Given the tight association of liver tumorigenesis with inflammation, we set up stringent controls to rule out the possible effect of polyIC itself in the process. With that in mind, we injected polyIC into controls and albumin-Cre mice in addition to the Mx1-Cre line. Interestingly, we detected

tumor-inhibitory effects of polyIC in all three lines of mice. This careful design of experiments led us to find a robust liver tumor-inhibitory effect of the synthetic dsRNA. Our subsequent experiments focused on this intriguing inhibitory effect of polyIC on HCC. Interestingly, polyIC potently prevented HCC initiation, if given at the pre-cancer stage, but had no effect on suppressing tumor progression (Lee et al., 2017).

RNA-sequencing analysis demonstrated that polyIC treatment potently induced programmed cell death ligand 1 (PD-L1) expression in liver sinusoid endothelial cells (LSECs). It was the data that prompted us to test a combination of polyIC and anti-PD-L1 antibody in HCC treatment. Indeed, our most recent data clearly demonstrated that this combinatorial treatment effectively suppressed HCC progression and significantly extended mouse survival (Wen et al., 2019). The key point is to simultaneously boost innate and adaptive immunity, to turn the "cold" liver tumors to be "hot" tumors, and responsive to immunotherapy (Fig. 4). In the past few years, three visiting students from the First and Second Affiliated Hospitals of Zhejiang University, Zhuan-hui PENG, Liang WEN, and Panyisha WU, have worked on this project. Based on the exciting data of polyIC and PD-L1 blockade, our current efforts are concentrated on deciphering novel mechanisms of immunosuppression in liver tumors, to develop more effective therapeutic protocols. Success of this project may be transformative for changing the therapeutic landscape of HCC, which is a most malignant disease.

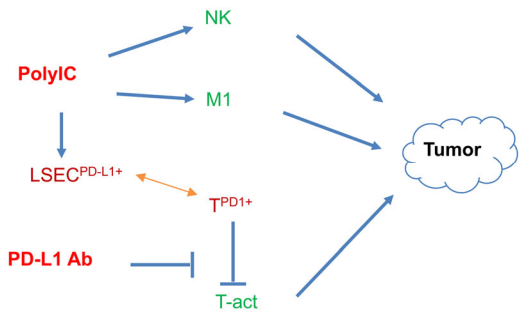


Fig. 4 Rationale of combination immunotherapy of liver cancer

The most recent data from our lab demonstrate that coordinated activation of innate and adaptive immune functions by polyIC and anti-PD-L1 antibody may be an effective strategy for immunotherapy of liver cancer. NK: natural killer; PD-L1: programmed cell death ligand 1; LSEC: liver sinusoid endothelial cell

7 Concluding remarks

Looking back at my career path in the past 40 years, I see a cycle of starting from the naïve interest in tumor immunology as a college student, to developing combination immunotherapy for HCC as the focus of current lab research, with better understanding of the complexity of tumor immunology. The outset of this journey, which is actively ongoing, was no doubt made at the Department of Biology, Hangzhou University, China. At this special occasion of the 90th birthday of the Department, now the School of Life Science at Zhejiang University, I write this article to express my deep and sincere gratitude.

Compliance with ethics guidelines

Gen-sheng FENG declares that he has no conflict of interest.

This article does not contain any studies with human or animal subjects performed by the author.

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中文概要

题　目：肿瘤免疫学和免疫治疗：我的旅程从杭州出发

概　要：为庆祝浙江大学生命科学学院九十华诞，谨写此文。作者冯根生是原杭州大学生物学系七七级学生；在杭大求学期间，选修了免疫学和肿瘤生物学，深受启发。冯现在是加州大学圣地亚哥校区医学院病理系和生命科学学院的教授。实验室目前的研究重点是肝癌发病机理和肝癌免疫治疗。这得益于当年在杭大的启蒙教育。

关键词：肝癌免疫治疗；肿瘤免疫学；浙大生科院九十华诞