CRF 8G (Revised Dec 08)

RGC Reference HKU1/CRF/10
please insert ref. above

The Research Grants Council of Hong Kong
Collaborative Research Fund Group Research Projects
Completion Report

(*for completed projects only*)

Part A: The Project and Investigator(s)

1. Project Title

Programming the Second Generation Tumor-targeting Bacteria

2. Investigator(s) and Academic Department/Units Involved

Research Team	Name/Post	Unit/Department/Institution
Project Coordinator	Prof. Jiandong Huang Professor	Biochemistry/HKU
Co-investigator(s)	Prof. Xiaodong Cui Professor	Physics/HKU
	Prof. Terence Hwa Professor	Biophysics/UC San Diego/HKU
	Prof. Kwan Man Professor	Surgery/HKU
	Prof. Lei-Han Tang Professor	Physics/HK Baptist University
Others Collaborator:	Prof. Guanhua Chen Professor	Chemistry/HKU
	Dr. Wei Huang Research Assistant Prof.	Physics/Biochemistry/HKU
	Prof. Erwei Song Professor	Surgery/Zhongshan University
Adviser:	Dr. Neal Copeland Dean of Cancer Biology	The Methodist Academy
	Dr. Antoine Danchin CEO	AMAbiotics SAS SEM Genopole

3. Project Duration

	Original	Revised	Date of RGC Approval *(must be quoted*)
Project Start Date	February 1, 2011	March 1, 2011	January 31, 2011
Project Completion Date	January 31, 2014	February 28, 2014	
Duration (*in month*)	36	36	
Deadline for Submission of Completion Report	October 31, 2014	November 30, 2014	

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Part B: The Final Report

5. Project Objectives

5.1 Objectives as per original application

Objective 1. Enhancement of preferential gene expression in tumors
Objective 2. Evaluation of the synthetic bacteria using tumor models
Objective 3. Characterizing the tumor microenvironment for the improvement of synthetic bacteria rational design
Objective 4 Bacterial-cancer population dynamics

5.2 Revised objectives

Date of approval from the RGC: February, 2012

Reasons for the change: The funded budget is only 68% of the requested amount.

Objective 1. Enhancement of preferential gene expression in tumors
Objective 2. Evaluation of the synthetic bacteria using tumor models
Objective 3. Characterizing the tumor microenvironment for the improvement of synthetic bacteria rational design
Objective 4 Bacterial-cancer population dynamics

6. Research Outcome

6.1 Major findings and research outcome

Despite advances in treatments, cancer remains a leading cause of death worldwide. The main causes of mortality are metastasis, relapse, and resistance to chemo- and radiotherapy. This has prompted the development of new approaches to treat cancer. One approach uses viral and non-viral vectors to deliver anti-cancer genes to the tumors. However, this approach has inadequate tumor specificity and the vectors cannot normally replicate and are often short-lived. Furthermore, these vectors are poorly distributed throughout the tumor mass. We need novel delivery systems that can specifically target both primary and metastatic tumors.

We previously engineered a 1st-generation *Salmonella* strain that preferentially targeted tumor tissues. Injecting this strain into tumor-bearing host animals resulted in tumor growth retardation without causing lethality, whereas using a typical *Salmonella* strain at similar doses would have significantly reduce the survival rate of the host due to infection. In this project, through the efforts of our multidisciplinary team, we further engineered a 2nd-generation anti-cancer *Salmonella* strain that used a newly designed inter-kingdom delivery and expression system (IKDE) to improve the tumor-suppression capability.

The 2nd-generation anti-tumor *Salmonella* strain, ST1, was engineered from our original YB1 *Salmonella* strain through a series of genetic manipulations coupled with the incorporation of an IKDE system. The IKDE system has several key features: (i) an in vivo plasmid maintenance mechanism, (ii) a T7 RNA polymerase positive feedback expression cassette that can amplify the T7 polymerases only after cytoplasmic entry, (iii) the IKDE expresses both protein and RNA as therapeutics, and (iv) the IKDE-expressed protein or RNA could spread to more tumor regions more extensively than the bacteria itself, likely due to exosome-mediated long distance delivery. Treatment with ST1 armed with IKDE-controlled therapeutic genes in immunocompromised and immunocompetent mouse models bearing multidrug-resistant tumors resulted in potent therapeutic effects in both models, although ST1 was confined to the tumor hypoxic regions.

We also found that *Salmonella* treatment induced reduction of metastasis. Particular, the YB1 treated liver cancer model showed over 90% reduction of metastasis. In this model, a luciferase-labeled MHCC97L human liver cancer cell line were subcutaneously implanted in nude mice and a tumor was transplanted into the liver by surgery. YB1 was then administered and the luciferase signal was recorded. After 3 weeks post YB1 treatment, we found metastasis in the lung was significantly less in the treated group compared to the control group. The effect of *Salmonella* on metastasis was confirmed by an allograft metastatic breast cancer model using the multidrug-resistant mouse breast cancer 4T1 cell line that mimics human late stage breast cancer. We implanted 4T1 in the fat pad of immunocompetent BALB/c mice, and then treated them with YB1 or ST1. H&E staining of lung tissues demonstrated reduction of metastasis.

We constructed an intravital animal model and live imaging system. The dorsal skinfold Window Chamber is a sophisticated animal model, which allows live observation of dynamic changes in mouse tissue. This chronic model provides a repeatable analysis of tumor progression, treatment, and angiogenesis during 2-3 weeks after tumor implantation. The observations were performed using a Nikon inverted microscope eclipse Ti-s/L 100 or an auto-focusing imaging microscope system. Using these two imaging systems, we were able to track the growth of cancer cells and the movement of bacteria in vivo.

By using the Window Chamber model and intravital imaging, we observed solid tumor formation using tdTomato-labeled 4T1 breast cancer cells that were implanted in the Window Chamber model. After 3 days, tumor growth was seen in the chamber. Between day 5 and 12, the tumor growth was rapid and large amounts of tumor-associated blood vessels were generated. After day 14, large hypoxic areas started to appear in the center of tumor as the tumor-associated blood vessels could not supply enough nutrients and oxygen.

With the intravital live imaging system, we observed the *Salmonella*-tumor interaction. We made an unexpected finding that *Salmonella* induced occlusive microvascular thromboses in tumor-associated blood vessels but not in normal tissues. The blockage of these blood vessels led to cancer cell death and tumor shrinkage. This discovery may lead to new directions in engineering anti-tumor bacteria.

6.2 Potential for further development of the research and the proposed course of action *(maximum half a page)*

Intravital live imaging studies of bacterial-tumor interaction suggested that the bacteria could eliminate small-sized tumors by interacting with the host blood vessels. The progress made in our CRF project not only provides us with useful theoretical and experimental tools for further investigation of cancer bacteriotherapy, but also raises some important questions. Why is the *Salmonella*-induced occlusive microvascular thrombosis specific to the tumor region? What is the underlying mechanism? Is the *Salmonella*-induced occlusive microvascular thrombosis important to bacterial colonization of tumors? What further engineering improvements can be made to achieve complete cancer eradication?

In the future, we plan to study the mechanisms underlying *Salmonella*-mediated bacterial cancer therapy and to provide mechanistic insight for further engineering of bacteria targeting the tumor microenvironment to trigger complete cancer eradication. This is based on our exciting observation that *Salmonella* had a profound impact on the tumor-associated vascular system, and was able to retard tumor growth and metastasis in both immune deficient and competent mice models. Elucidation of key mechanisms of *Salmonella*-induced blockage of tumor-associated blood vessel may reveal previously unknown aspects of tumor biology.

We plan to characterize *Salmonella*-blood vessel interaction in vitro with a recapitulated vascular microenvironment using microfluidic platforms, intravital live imaging, Optical Coherence Tomography (OCT) and theoretical analysis. Genetic analysis will be used to dissect the pathways in *Salmonella* that contribute to the bacterial effect on the tumor vascular system.

Possible future studies could also yield detailed and specific knowledge on the properties of blood vessels and angiogenesis, subjects of great interest in other fields of biomedical research. The collaborative efforts among biologists, physicists and engineers will provide innovative approaches for cancer therapy. The project can bring together a truly interdisciplinary team of researchers consisting of biologists, physicists and engineers from Hong Kong and worldwide, reinforcing the international status of Hong Kong in synthetic and quantitative biology. It will also provide excellent training opportunities for students and other young researchers.

Finally and most importantly, future clinical trials in large animals such as dogs and pigs as well as human are warranted since the engineered bacteria have both very potent therapeutic effect on primary tumors and metastasis.

6.3 Research collaboration achieved *(please give details on the achievement and its relevant impact)*

Extensive collaboration has been carried out during the project.

First, our theorist collaborators established integrative models for quantitative evaluation of bacteriotherapy. Together, we developed continuum models for the growth of a solid tumor, whereby the proliferation of cancer cells was limited by nutrient and/or oxygen supply via diffusive transport. Analytic expressions were derived for the tumor expansion velocity vF under fairly generic situations. Suppression of cell proliferation by bacteria was modeled via nutrient competition and induction of apoptosis by direct bacterial entry into cancer cells or secretion of anti-cancer drugs diffused from the vector. The typical layered structure of a solid tumor is modeled. We found that the tumor expansion rate vF was essentially set by the width of the viable layer and the cell proliferation rate. The mathematical relationship we obtained compared favorably with the experimental observations under realistic choices of the model parameters. More importantly, the study allowed us to examine and compare the effectiveness of different therapeutic strategies, and to sort out the interdependencies of various components of the system brought about by the complex microenvironment. We have drafted a manuscript to report our findings.

Second, our physicist collaborators established the intravital imaging system that is critical for the observation of bacterial tumor interactions. This enabled us to discover that *Salmonella* induced occlusive microvascular thromboses in tumor-associated blood vessels and the blockage of these blood vessels led to cancer cell death and tumor shrinkage. This finding points to a new direction of our future research.

Third, the collaborations among biologists and physicists allowed us to explore beyond bacterial cancer therapy. For example, we studied the bacterial movement and was able to publish our collaborative work in *Science*. We also studied the role of microvesicles secreted by macrophages. We found that the microvesicles shuttle invasion-potentiating microRNAs into breast cancer cells. This result has been published in *Molecular Cancer*.

Fourth, the CRF project allow us to promote collaboration outside Hong Kong. Since the start of the CRF project, we are able to establish further collaboration with French CNRS on studies of bacteria by using theoretical

physics and synthetic biology approaches; with Shenzhen 2nd Hospital and Shenzhen Institutes of Advanced Technology, CAS on synthetic biology studies of bladder cancer; with University of Strathclyde, UK on delivery and expression of shRNA targeting SK1 and SK2 by *Salmonella* to tumours. We are also exploring possible collaborations with Saarland University, Germany and The Beijing Computational Science Research Center (CSRC) on sophisticated modeling of tumor vascular systems.

7. The Layman's Summary

(describe in layman's language the nature, significance and value of the research project, in no more than 200 words)

Cancer remains a leading cause of death with current therapeutic methods. Novel therapies are therefore in urgent needs. One potential method is to use bacteria as cancer therapeutic agent. Since bacteria can sense their environment, distinguish between cell types, synthesize and deliver drugs into cancer cells, attempts are made to program bacteria to attack tumors. Current advances in synthetic biology technology offer great opportunities in refining this approach. In this project, we have established a stepwise approach to program a 2nd generation of bacterial strains that are able to detect tumor microenvironment, effective in killing cancer cells and safe to normal tissues, so that they can ultimately be used clinically to treat cancer patients. Our results show that our 2nd generation of bacteria, equipped with a newly designed system that is capable express and deliver therapeutic proteins and RNAs, have potent therapeutic effects on the primary tumors in both immunocompromised and immunocompetent mice. More importantly, metastasis was greatly reduced when mice were treated with the engineered bacteria. These results encourage us to seek clinical trials in the future.

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Part C: Research Output

8. Peer-reviewed journal publication(s) arising directly from this research project

(Please attach a copy of the publication and/or the letter of acceptance if not yet submitted in the previous progress report(s). All listed publications must acknowledge RGC's funding support by quoting the specific grant reference.)

The Latest Status of Publications				Author(s) *(denote the corresponding author with an asterisk*)*	Title and Journal/Book *(with the volume, pages and other necessary publishing details specified)*	Submitted to RGC *(indicate the year ending of the relevant progress report)*	Attached to this report *(Yes or No)*	Acknowledged the support of RGC *(Yes or No)*
Year of publication	Year of Acceptance (For paper accepted but not yet published)	Under Review	Under Preparation (optional)					
2011				Bin Yu, Mei Yang, Ho Yin Bosco Wong, Rory M. Watt, **Erwei Song,** Bo-Jian Zheng, Kwok-yung Yuen, **Jian-Dong Huang***	A method to generate recombinant *Salmonella typhi* Ty21a strains expressing multiple heterologous genes using an improved recombineering strategy. **Applied Microbiology and Biotechnology** V91(1), P177-188	9/2012	No	No
2011				Ye Jin and **Jian-Dong Huang***	Engineering a portable riboswitch-LacP hybrid device for two-way gene regulation. **Nucleic Acids Research** V39(19):e131	9/2012	No	Yes
2011				Mei Yang, Jingqi Chen, Fang Su, Bin Yu, Ling Lin, Yujie Liu, **Jian-Dong Huang*** and **Erwei Song***	Microvesicles secreted by macrophages shuttle invasion-potentiating microRNAs into breast cancer cells.	9/2012	No	Yes

					Molecular Cancer V10(1):117			
2011				Chenli Liu, Xiongfei Fu, Lizhong Liu, Xiaojing Ren, Carlos K.L. Chau, Sihong Li, Lu Xiang, Hualing Zeng, **Guanhua Chen**, L**ei-Han Tang**, Peter Lenz, **Xiaodong Cui**, **Wei Huang***, **Terence Hwa***, **Jian-Dong Huang***	Sequential establishment of stripe patterns in an expanding cell population. **Science** V334(6053), P238-241	9/2012	No	Yes
2012				Xiongfei Fu, **Lei-Han Tang**, Chenli Liu, **Jian-Dong Huang**, **Terence Hwa**, and Peter Lenz*	Stripe formation in bacterial systems with density-suppressed motility. **Physical Review Letters**. V108, 198102	9/2012	No	Yes
2012				Bin Yu, Mei Yang, Lei Shi, Yandan Yao, Qinqin Jiang, Xuefei Li, **Lei-Han Tang**, Bo-Jian Zheng, Kwok-yung Yuen, David K. Smith, **Erwei Song***, **Jian-Dong Huang***	Explicit hypoxia targeting with tumor suppression by creating an "obligate" anaerobic *Salmonella* Typhimurium strain. **Scientific Reports** V2:436	9/2012	No	Yes
2013				Ye Jin, Jianting Wu, Yannan Li, Zhiming Cai and **Jian-Dong Huang**	Modification of the RpoS network with an artificial small RNA. **Nucleic Acids Research**, V41(17):8332-8340		Yes	Yes
2012				Chang Xian Li, Yan Shao, Kevin T. P. Ng, Xiao	FTY720 Suppresses Liver Tumor	9/2012	No	Yes

				Bing Liu, Chang Chun Ling, Yuen Yuen Ma, Wei Geng, Sheung Tat Fan, Chung Mau Lo, **Kwan Man***	Metastasis by Reducing The Population of Circulating Endothelial Progenitor Cells. PLoS ONE, 7（2） e32380			
2014				Chang Xian Li, Yan Shao, Kevin T. P. Ng, Xiao Bing Liu, Chang Chun Ling, Yuen Yuen Ma, Wei Geng, Sheung Tat Fan, Chung Mau Lo, **Kwan Man***	Regulatory B cells accelerate hepatocellular carcinoma progression via CD40/CD154 signaling pathway. **Cancer Letters**, : S0304-3835(14)00553-9	10/2014	No	Yes
		√		Bo-Tao Ning, Bin Yu, Shing Chan, Jian-liang Chan, Jian-Dong Huang *, Godfrey Chi-Fung Chan *	Treatment of Neuroblastom a with an engineered "Obligate" anaerobic Salmonella typhimurium strain YB1, Journal of Translational Medicine (2014) (under review)	9/2014	Yes	Yes
		√		Lei Shi, Bin Yu, Chunhui Cai, Wei Huang, Bo-Jian Zheng, David K. Smith, Kwok-Yung Yuen, Jian-Dong Huang	Combined prokaryotic-eu karyotic (inter-kingdom) delivery of therapeutic expression vectors to solid tumours **Nature Biotech**. (2014) (under review).	11/2014	Yes	Yes
		√		Changxian Li, Bin Yu , Lei Shi, Mei Yang, Jian-Dong Huang *, Man Kwan *	Treatment of Liver cancer with an engineered "Obligate" anaerobic Salmonella typhimurium strain YB1 (2014), **BMC Cancer** (under	11/2014	Yes	Yes

					review).			
			√	Xue-Fei Li, Lei-Han Tang, Bin Yu, Lei Shi, Qiu-Bin Lin, and Jian-Dong Huang, Mei Yang, Terence Hwa	Quantitative evaluation of bacteria-based cancer therapy (2014), (in preparation).	11/2014	Yes	Yes

9. Recognized international conference(s) in which paper(s) related to this research project was/were delivered *(Please attach a copy of each conference abstract)*

Month/Year/ Place	Title	Conference Name	Submitted to RGC *(indicate the year ending of the relevant progress report)*	Attached to this report *(Yes or No)*	Acknowledged the support of RGC *(Yes or No)*
May 26-29, 2013, Jeju, Korea	"Synthetic Patterns: Sequential Establishment of Stripe Patterns in an Expanding Cell Population"	the 8th symposium of Asian Biophysics Association		Yes	Yes
11/2012 Guangzhou, China	Engineering Bacteria to Reveal Biological Principles and to Target Tumors	International Symposium on Genetic Regulation and Targeted Therapy of Cancer		Yes	Yes
10/2012 Suzhou, China	Synthetic Patterns—Sequential establishment of stripe patterns in an expanding cell population	Cold Spring Harbor Asia Conference on High Throughput Biology		Yes	Yes
10/2012 Taipei, Taiwan	Autonomous Formation of Sequential Periodic Stripes from Density-dependent Motility	2012 Asia Pacific Developmental Biology Conference		yes	Yes
8/2012 Tianjin, China	Autonomous Formation of Sequential Periodic Stripes from Density-dependent Motility	2012 Sino-USA Chinese Collaborative Workshop-Opportunities and Challenges in Synthetic Biology		yes	Yes

4/2012 Beijing, China	用合成生物学的方法来编码细胞的时空分布	The 1st Joint Symposium in Translational Medicine Chinese Academy of Medical Sciences (CAMS) & Li Ka Shing Faculty of Medicine, the University of Hong Kong		Yes	Yes
4/2012 Beijing, China	Programming Cell Distribution in Time and Space: A Case Study for Innovative Genetic Circuit Design at the University of Hong Kong	the First International Sc2.0 Meeting		Yes	Yes
12/2011 Physikzentrum Bad Honnef, Germany	Autonomous Formation of Sequential Periodic Stripes from Density-dependent Motility	484th Heraeus Seminar: Physics of Biological Function – Multicellular Systems		Yes	Yes
11/2011 Suzhou, China	An Engineered Tumor Targeting Salmonella	Cold Spring Harbor Asia Conferences - 2011 Symposium: Design & Synthesis of Biological Systems		Yes	Yes

10. ***Student(s) trained*** (please attach a copy of the title page of the thesis)

Name	Degree registered for	Date of registration	Date of thesis submission/ graduation
Dr. Bin Yu	PhD	12/1/2007	11/30/2011
Dr. Chenli Liu	PhD	3/1/2008	8/31/2011
Dr. Songyue Zheng	PhD	9/1/2009	8/31/2012
Dr Lei Shi	PhD	8/1/2010	8/31/2012
Mr. Qiubin Lin	PhD	9/1/2011	8/31/2015
Dr. Xuefei Li	PhD	8/1/2008	8/1/2012
Mr. ChangXian Li	PhD	5/1/2012	4/30/2016
Miss Chen Xu	PhD	9/1/2014	8/31/2017
Dr. Ming-Chun Lee	Postdoctoral fellow	7/15/2011	
Dr. Chenli Liu	Postdoctoral fellow	8/1/2011	
Dr. Ye Jin	Postdoctoral fellow		

11. Other impact *(e.g. award of patents or prizes, collaboration with other research institutions, technology transfer, etc.)*

Patents obtained/Pending

(1) Two-Way, Portable Riboswitch Mediated Gene Expression Control Device
U.S. Non-Provisional Application No. 61/503,453 filed on June 30, 2011.
U.S. Application No. 13/534,774 filed on June 27, 2012
Inventor(s): Jian-Dong Huang and Ye Jin

(2) Modified Bacteria and their Uses thereof for the Treatment of Cancer
U.S. Non-Provisional Application No. 61/687,975 filed on May 4, 2012.
Inventor(s): Jian-Dong Huang, Bin Yu, Mei Yang, and Lei Shi

(3) Therapeutic Delivery and Expression System, Methods and Uses Thereof
U.S. Provisional Application No. 14/019,058 filed on September 5, 2013.
Inventor(s): Jian-Dong Huang, Bin Yu, and Lei Shi

Collaborations established with other research institutions

1. Shenzhen 2nd Hospital and Shenzhen Institutes of Advanced Technology, CAS, Professor Zhiming Cai and Dr. Chenli Liu on synthetic biology studies of bladder cancer;
2. University of Strathclyde, Professor Nigel Pyne on delivery and expression of shRNA targeting SK1 and SK2 by *Salmonella* to tumours;
3. French CNRS, Dr. Julien Tailleur on studies of bacteria by using theoretical physics and synthetic biology approaches.

Promotions and appointments since inauguration of the project

Since the inauguration of the project, Dr. Jiandong Huang, Dr. Xiaodong Cui and Dr. Kwan Man have been promoted to full professor at the University of Hong Kong. Dr. Wei Huang has been appointed as an associate professor at the South University of Science and Technology (SUSTC). Dr. Chenli Liu has been appointed as an investigator (full professor equivalent) at Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences. Dr. Ye Jin has been appointed as a research assistant professor at the Chinese University of Hong Kong. Dr. Yu Bin and Dr. Xuefei Li have been appointed as postdoctoral fellows at the University of Hong Kong and Hong Kong Baptist University, respectively.