RGC Ref.: N_HKU712/12 NSFC Ref. :
(please insert ref. above)

The Research Grants Council of Hong Kong
NSFC/RGC Joint Research Scheme
Joint Completion Report

(Please attach a copy of the completion report submitted to the NSFC by the Mainland researcher)

Part A: The Project and Investigator(s)

1. Project Title

Analysis of the role of ATOH8 and HCC cancer stem cell and somatic cell reprogramming

ATOH8 在肝癌幹細胞中的功能研究以及在體細胞重編程過程中的作用

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

	Hong Kong Team	Mainland Team
Name of Principal Investigator *(with title)*	Prof. Xin-Yuan GUAN	Dr. Tao WANG
Post	Professor	Principal Investigator
Unit / Department / Institution	Clinical Oncology The University of Hong Kong	Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences
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Co-investigator(s) *(with title and institution)*		

3. Project Duration

	Original	Revised	Date of RGC/ Institution Approval *(must be quoted*)
Project Start date	01/01/2013		
Project Completion date	31/12/2016		
Duration *(in month)*	48		
Deadline for Submission of Completion Report			

Part B: The Completion Report

5. Project Objectives

5.1 Objectives as per original application

1. To investigate the repressive role of ATOH8 in the regulation of stemness-associated genes;
2. To study whether ATOH8 can increase sensitivity of CSCs to chemotherapeutic agents;
3. To test if ATOH8 silencing can enhance the generation of induced pluripotent stem cells.

5.2 Revised Objectives

Date of approval from the RGC: _____

Reasons for the change: _____

_____

1.
2.
3. ....

6. Research Outcome

Major findings and research outcome
(maximum 1 page; please make reference to Part C where necessary)

In this project, qRT-PCR was applied to compare ATHO8 expression level in 242 pairs of primary HCC cases. Compared with corresponding non-tumor tissues, down-regulation (defined as greater than a 4-fold change) of *ATOH8* was detected in 118/242 (48.8%) of HCC tissues. The average level of *ATOH8* expression in tumor tissues was significantly lower than the level in paired non-tumor tissues (0.34 versus 1.18, $P<0.001$, paired Student's *t* test. Kaplan-Meier survival analysis found that patients with *ATOH8* down-regulation displayed a worse disease-free survival (DFS) (estimated mean=41.4 months) compared to patients without *ATOH8* down-regulation (estimated mean=52.6 months) (log-rank=4.631, $P=0.031$). Clinical association study also found that down-regulation of *ATOH8* was significantly associated with HCC differentiation ($P=0.01$) and AFP expression ($P=0.03$).

Functionally, *ATOH8* plays a tumor suppressive role in HCC. XTT assay showed that *ATOH8* could inhibit tumor cell growth. Soft agar assay found that *ATOH8* could significantly inhibit colon formation in soft agar. Tumor formation in nude mice showed that *ATOH8* could inhibit *in vivo* tumor formation and growth. To study whether *ATOH8* can regulate stemness associated genes (*OCT4*, *NANOG*, *SOX2* and *AFP*) at the transcriptional level, qRT-PCR was used to compare the expression levels of these genes between *ATOH8*- and empty vector-transfected cells. These genes were significantly down-regulated in *ATOH8*-transfected cells compared with empty vector-transfected cells ($P<0.05$), indicating that *ATOH8* bound to the promoters of these genes and as a result led to the repression of their transcription. The repressive effect of *ATOH8* on the transcription of these genes was also further extended in QSG7701 cells following *ATOH8* silencing by two siRNA against *ATOH8* (si22 and si45). Results showed that the expression levels of these genes were significantly increased compared with scrambled control cells when *ATOH8* was repressed ($P<0.05$). Western blot analysis showed that the expression of stemness-associated genes was decreased when *ATOH8* was introduced into cells and increased when *ATOH8* was silenced.

In HCC clinical samples, absent expression of ATOH8 was frequently observed in $CD133^+$ liver CSC subset and their incidence in HCC was significantly associated with poor prognosis ($P=0.020$). Interestingly, knockdown of *ATOH8* could enrich the population of $CD133^+$ cells in HCC cell lines. In QSG7701 and BEL7402 cells, *ATOH8* depletion increased the proportion of $CD133^+$ cells from 0.86 ±0.06% to 10.43 ±3.21% and from 1.12 ±0.33% to 2.13 ±0.43%, respectively. When *ATOH8* was introduced into PLC8024 and Huh7 cells, the subpopulation of $CD133^+$ cells decreased significantly (PLC8024: from 35.37 ±5.66% to 6.32 ±2.11%; Huh7: from 28.94 ±2.54% to 10.72 ±3.36%). In QSG7701 cells, $CD133^+$ cells induced by *ATOH8* depletion have been isolated by flow-sorting. Compared to $CD133^-$ cells, sorted $CD133^+$ cells possessed stronger tumorigenicity, as demonstrated by their increased ability to form foci ($P<0.001$), to form colonies on soft agar ($P=0.002$), as well as to stimulate tumor formation in SCID mice with a fewer number of cells.

To test whether *ATOH8* can increase the chemo-sensitivity of HCC cells, Huh-*ATOH8* and -Vec cells were treated with 5-fluorouracil (5-FU) and CDDP at various concentrations. After 48 hours, the cell viability was detected by an XTT assay, and results showed that the cell viability was significantly decreased in Huh7-*ATOH8* cells. The IC_{50} of 5-FU was decreased from 8.54 to 5.50, and the IC_{50} of CDDP was decreased from 5.22 to 4.28 compared with Huh7-Vec cells. Because *ATOH8* represses expression of many stemness-associated genes, we further tested whether *ATOH8* depletion could increase the efficiency of iPSC generation. To test whether *ATOH8* depletion can enhance the efficiency of reprogramming of fibroblasts into iPSCs induced by SKOM, fibroblasts (SF002) at passage 8 were infected by lentiviruses producing SKOM with or without shRNA targeting *ATOH8*. The frequency of AP-positive colony was significantly increased in fibroblasts treated with *ATOH8* depletion (sh4: 4.18±0.19‰; sh7: 2.37±0.15‰) compared with fibroblasts treated without *ATOH8* depletion (0.46±0.1‰, $P<0.001$), suggesting that *ATOH8* depletion could enhance the efficiency of iPSC generation.

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

Cancer stem cells (CSCs) play critical roles in cancer development and progression. Currently, no specific therapy can effectively target CSCs. In this work, we find that ATOH8 plays an important suppressive role in cancer stemness, which open a novel insight to understand how a non-CSC to reprogram into a CSC under a given genetic alteration. In addition, ATOH8 can be used as a stemness suppressor for cancer treatment.

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 stem cells (CSCs) is a small group of cells in cancer that play critical role in cancer development and progression. In this study, we demonstrate, the first time in the world, that non-CSCs can be reprogrammed into CSCs under some genetic change, such as the downregulation of ATOH8 gene in human liver cancer. We find that *ATOH8* is frequently down-regulated in liver cancer, which is significantly associated with poor outcome. Further study find *ATOH8* to efficiently repress transcription activity of many stemness-associated genes. Knockdown of *ATOH8* can induce $CD133^-$ cells into $CD133^+$ cells, which possessed CSC properties including the abilities to self-renew, differentiate and resist chemotherapy. Taken together, our data provides solid evidence to show that non-CSCs ($CD133^-$) can be reprogrammed into $CD133^+$ CSCs through *ATOH8* depletion. Therapeutically, re-introduction of *ATOH8* into HCC cells can increase the chemo-sensitivity of cancer cells, which has immense potential to increase sensitivity to chemotherapies.

Part C: Research Output

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

(Please attach a copy of each 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)	Title and Journal/ Book	Submitted to RGC	Attached to this report	Acknowledged the support of this Joint Research Scheme	Accessible from the institutional repository
Year of publication	Year of Acceptance *(For paper accepted but not yet published)*	Under Review	Under Preparation *(optional)*	***(bold** the authors belonging to the project teams and denote the corresponding author with an asterisk*)*	*(with the volume, pages and other necessary publishing details specified)*	*(indicate the year ending of the relevant progress report)*	*(Yes or No)*	*(Yes or No)*	*(Yes or No)*
2015				Song Y, Pan G, Chen L, Ma S, Zeng T, Chan TH, Li L, Lian Q, Chow R, Cai X, Li Y, Li Y, Liu M, Li Y, Zhu Y, Wong N, Yuan YF, Pei D, Guan XY	Loss of ATOH8 Increases Stem Cell Features of Hepatocellular Carcinoma Cells. ***Gastroenterology*** 149(4):1068-81.	No	Yes	Yes	

2015				Jiang L, Kwong DL, Li Y, Liu M, Yuan YF, Li Y, Fu L, Guan XY	HBP21, a chaperone of heat shock protein 70, functions as a tumor suppressor in hepatocellular carcinoma. ***Carcinogenesis*** 36:1111-20	No	Yes	Yes	
2017				Cao TT, Lin SH, Fu L, Tang Z, Che CM, Zhang LY, Ming XY, Liu TF, Tang XM, Tan BB, Xiang D, Li F, Chan OY, Xie D, Cai Z, Guan XY	Eukaryotic translation initiation factor 5A2 promotes metabolic reprogramming in hepatocellular carcinoma cells. ***Carcinogenesis*** 38:94-104.	No	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 delivered paper. All listed papers must acknowledge RGC's funding support by quoting the specific grant reference.)*

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 this Joint Research Scheme *(Yes or No)*	Accessible from the institutional repository *(Yes or No)*
April 5-9, 2014, San Diego, USA	ATOH8 depletion can reprogram non cancer stem cells into cancer stem cells	AACR Annual Meeting 2014	No	No	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
Miss Yang-Yang SONG	Ph.D.	September 2010	August 2014

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