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海 外 特 別 研 究 員 最 終 報 告 書

独立行政法人日本学術振興会 理事長　殿

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（氏名は必ず自署すること）

海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

1. 用務地（派遣先国名）用務地：　ケンブリッジ　（国名：イギリス　）

2. 研究課題名（和文）※研究課題名は申請時のものと違わないように記載すること。
ヒト初期胚の多能性状態を制御する細胞周期機構の解明

3. 派遣期間：平成 31 年 1 月 14 日　～　令和 3 年 4 月 13 日

4. 受入機関名及び部局名
受入機関名： University of Cambridge
部局名：Department of Surgery

5. 所期の目的の遂行状況及び成果…書式任意　**書式任意（A4 判相当 3 ページ以上、英語で記入も可）**
（研究・調査実施状況及びその成果の発表・関係学会への参加状況等）
（注）「6. 研究発表」以降については様式 10－別紙 1～4 に記入の上、併せて提出すること。

5. 所期の目的の遂行状況及び成果 **書式任意（A4 判相当 3 ページ以上、英語での記入も可）**
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The aim of this research is to obtain deeper understandings of cell cycle genes regulating pluripotency and differentiation on the pre- and post-implantation stages of human development. We hypothesized that some of the cell cycle regulators become functional and influence cell fate choice during the transition through the naive and primed pluripotent states towards differentiated cell states. To examine this hypothesis, we use human embryonic stem cells (hESCs) as an in vitro model of human embryos and focus on retinoblastoma family genes (RB, RBL1 and RBL2) as a key cell cycle regulator involved in the fate decision over this period of development.

We have successfully established the RB/RBL1/RBL2 triple-disturbance system to bypass the functional redundancy of the gene family of interest. First, we generated RBL1 and RBL2 double knock out hESC lines by targeting their protein coding regions using CRISPR/Cas9 genome editing technology. The induced non-sense mutations and the removal of the target proteins were confirmed by the genotyping assay and the western blot analysis, respectively. We also kept the cells carrying intact alleles of RBL1 and RBL2 after going through the same gene editing processes, as the wild type controls for the double knockout cells. Subsequently, we transfected the double knockout cells and the targeted wild type cells with the optimized induced knockdown (OPTiKD) gene cassettes to express shRNA against RB, which allows the tetracycline-inducible knockdown of RB. The optimal shRNA sequence was selected based on the knockdown efficiency measured by quantitative RT-PCR and the efficient elimination of the RB proteins was confirmed by the western blot analysis.

Using the established RB/RBL1/RBL2 triple-depletion system, we first analyzed the functions of RB genes in human primed pluripotent state. To our surprise, the RB/RBL1/RBL2 triple depletion did not cause clear morphological changes or downregulation of pluripotency marker genes in

the pluripotency maintenance condition. However, the double knockout of RBL1/2 together with the induced knockdown of RB exhibited shorter G1 phase and longer S phase. These results suggest that RB family genes have central roles in regulating the cell cycle of primed state human embryonic pluripotent cells although they are dispensable for the maintenance of human primed pluripotency. In order to study the functions of RB family genes in human ground pluripotent state, we tried to reprogram the hESCs to the naive pluripotent state. However, the establishment and maintenance of reprogrammed naive state hESCs from the conventional hESCs could not be successful.

In order to study the functions of RB family genes during the differentiation from the primed pluripotent state, we also examined the effects of RB/RBL1/RBL2 triple depletion in directed differentiation into the definitive endoderm and the foregut lineage. In the early stages of definitive endoderm formation, the morphology of cells and the expression levels of selected marker genes were not affected by the knockdown of RB in either of the targeted wild type cells or the RBL1/RBL2 double knockout cells, suggesting that the RB family genes are not important in the exit from primed pluripotency. Interestingly, on the day 6 of endoderm differentiation (foregut stage), the knockdown of RB lead to a significant downregulation of an endoderm marker, *SOX17*, in the double knockout cells but not in the targeted wild type cells. These cells progressively deteriorated in the later stages of the differentiation. These phenotypes were also confirmed with the other combination of targeted wild type and double knockout cells generated with different guide RNAs. Interestingly, removal of the RB family genes did not exert any significant influences on the cell cycle profile while the differentiation itself dynamically rearranged the length of phases. Transcriptome analysis utilizing high throughput sequencing on day 3 and 6 of differentiation revealed that the depletion of all the RB family genes resulted in ectopic upregulation of the genes related to mesenchymal and neural crest cell fates. These results suggest that RBs are not critical in regulating the G1/S-phase transition during definitive endoderm and foregut formation while the disturbance of all the family genes impair the later

stage of foregut formation.

As Postdoctral Fellowship for Research Abroad finishes, we are preparing a paper to report our findings described above. The RB cell lines that we established in this project provide useful *in vitro* model to study the molecular functions of RBs in various human cell types accessible from human pluripotent stem cells. Although we had to redirect the project because of the results not supportive to our initial hypotheses about pluripotent states, we obtained interesting insights in the roles of RBs during the formation of endodermal lineages. The results that disturbed RBs drive the endoderm cells to wrong lineages indicates their unknown functions in this lineage commitment.