論文審査の結果の要旨

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Restoration of therapeutic transgene expression by administering hematopoietic stem cell gene therapy (SCGT) is largely thought to be beneficial for treatmenting primary immunodeficiencies (PIDs). However X-linked chronic granulomatous disease (XCGD), which is characterized by impaired neutrophil functionality, is unique in that the persistence of gene marked cells following SCGT is transient only including the most recent clinical trial carried out in Japan last year. During the defense of his thesis, Mr. Lin Huan-Ting described the following results:

1. An efficient protocol was developed for differentiating neutrophils first from embryonic stem cells (ESCs) and then induced pluripotent stem cells (iPSCs). Differentiated neutrophils exhibited the characteristic features of peripheral blood neutrophil thus underlining the validity of the protocol to model a disease affecting neutrophil functionality.

2. During the characterization of neutrophils, Mr. Lin discovered that the combination of CD64 and CD15 allowed him to accurately distinguish between developing and mature neutrophils within the same culture. This was used to optimize a multi-colored method of analysis by FACS. Therefore specific differentiating cell populations (developing or mature) could be targeted alongside concomitant analyses of these cells.

3. Patient autologous iPSCs could be successfully generated from an XCGD patient that possessed all characteristic features of pluripotent stem cells. The same point mutation that had been identified in the patient was retained in these XCGD iPSCs. Indeed, mature neutrophils differentiated from untransduced XCGD iPSCs showed functional impairment in generating ROS thus indicating that iPSCs could successfully recapitulate the disease phenotype.

4. In order to accurately correlate transgene expression with functional recovery, Mr. Lin described the use of an emerging technology called droplet digital PCR (ddPCR) to accurately and reliably estimate the vector copy number (VCN) in transduced cells. Using purified gDNA as template, the concentration of the transgene and that of a reference control was quantified per unit volume of input. The VCN value was calculated as the ratio between the two concentrations.

5. To mimic the clinical scenario, integrating alpharetroviral vectors were used to transduce XCGD iPSCs. After differentiating into neutrophils, it was found that despite transduced cells carrying multiple provirus insertions, cellular recovery in terms of gp91phox, the deficient protein, and ROS production in mature neutrophils was considerably less compared with the healthy wild type control. Silencing alone would not fully explain these observations because puromycin selection was maintained throughout such that only transgene positive cells were analyzed.

6. By analyzing the expression of the intracellular components of NAPDH oxidase (p47 and p67phox) there was a negative correlation with transgene-derived gp91phox in mature neutrophils.

7. Central to his study, Mr. Lin demonstrated the expression of "ectopic" gp91phox only in the developing fraction of neutrophils differentiated from transduced XCGD iPSCs. No expression was detected in the corresponding fraction from healthy controls.

8. It was found that developing neutrophils ectopically expressing gp91phox showed raised levels of certain ER stress markers. Although this may not be the main mechanism, it has been reported that sustained ER stress can lead to cell apoptosis, which may be the ultimate fate of these cells.

9. To determine the hierarchical transition in maturation of *in vitro* cultured neutrophils, FACS sorting was performed to obtain only developing neutrophils differentiated from both XCGD and healthy control iPSCs. Following a further two days of differentiation, mature neutrophils appeared in the culture suggesting that they originated from this developing fraction.

10. During the time-course assessment of gp91phox expression, mature neutrophils differentiated from healthy iPSCs showed peak expression after 7 days. In gp91phox-transduced cells, expression levels were comparable with healthy controls up to 5 days after differentiation. However, it was abruptly lost and by day 7, gp91phox expression became almost undetectable.

11. In this study, whether the control (no gp91phox) or therapeutic (gp91phox-conatining) vector was used, transduced cells can be identified by NGFR expression. Time-course assessment revealed that expression was sustained through 7 days of differentiation in control vector-transduced cells but in the therapeutic group, NGFR expression was abruptly lost after 5 days. Since both vector constructs are very similar, neither would be considered to be more susceptible to silencing than the other. Therefore silencing alone is unlikely to fully explain the disappearance of transgene-derived gp91phox.

12. By analyzing cell viability over time, on day 3, there was no significant levels of cell death detected in the developing fraction in any of the experimental groups. However on day 5, cells ectopically expressing gp91phox showed an increased susceptibility for undergoing cell death. By day 7, the same group of therapeutic vector-transduced cells showed normal cells viability, which is consistent with the previous observation that ectopic gp91phox expression was lost by this time point.

In summary, Mr. Lin found that ectopic gp91phox expression may compromise the viability of developing neutrophils, which may at least partially explain why their transient persistence in the peripheral blood. He was successful in demonstrating the practical utility of iPSC-based disease modeling systems. By intensively repeating experiment, he used it to optimize multi-color FACS analysis permitting concomitant surface staining and florescence detection-based cell assays. In collaborating with BioRad, the ddPCR system may eventually be used to estimate VCN states in a

variety of research and clinical settings. Indeed, a second manuscript based on the ddPCR work has been submitted for publication and is currently under review. Most importantly the core findings from his work may well have a significant clinical impact. In this study, the potentially detrimental effects of non-physiologically regulated transgene expression is clearly shown despite conventional thinking that it is largely beneficial. More specific to XCGD, there is valuable insight into the underlying pathophysiological mechanisms and for the first time, there is some experimental evidence to support a reason that could explain why there has been limited success from clinical trials. Taken together, given the number of noticeable discoveries and achievements made over the course of this study, it is my recommendation that Mr. Lin should be conferred with his Ph.D. degree by the University of Tokyo.