Letter to the Editor

Stem Cell INVESTIGATION

Biological and clinical insights offered by chemically induced liver progenitors (CLiPs)

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Recently, we reported on the generation of liver progenitor-like cells, which we named chemically induced liver progenitors (CLiPs), and proposed a new direction for cell transplantation therapy (1). Tanimizu and Mitaka provided an insightful comment on this report (2). Here, we would like to address some of their valuable comments and provide responses and discussion.

Transplantation of hepatocytes or their equivalents has been proposed as an alternative treatment for liver transplantation. However, its clinical feasibility has been questioned because of the lack of appropriate cell sources. Such cell sources should be sufficient in number, replace injured liver tissue and function effectively after transplantation. Moreover, safety issues and ethical issues should be carefully assessed before the clinical application of candidate cell sources. Native mature hepatocytes (MHs) are the only cell source that has been used for cell transplantation therapy (3), but they are not expandable *in vitro*, thereby limiting the availability of this technology. On the other hand, pluripotent stem cell-derived hepatic cells have immature phenotypes as hepatocytes, leading to poor engraftment and hepatic functionality after their transplantation into animal models.

We recently reported that CLiPs can be generated from rat and mouse MHs *in vitro* using three small molecules: Y-27632 (ROCK inhibitor), A-83-01 (ALK inhibitor) and CHIR99021 (GSK3 inhibitor) (this small molecule cocktail was called YAC). CLiPs were bipotential to differentiate into both MHs and biliary epithelial cells (BECs) and were expandable *in vitro* through more than 20 passages. Most importantly, we demonstrated that rat CLiPs extensively repopulated the injured liver of cDNA-uPA/SCID mice (75–90% repopulation efficiency). These results highlight the potential applicability of CLiP technology to cell transplantation therapy in humans.

As noted by Tanimizu and Mitaka, the most important question is whether the CLiP methodology is applicable to humans. In our several attempts, CLiPs have not been generated from commercially available cryopreserved human MHs with the present protocol. We assume that there are several possible reasons for the difference between human and rodent MHs. First, to be reprogrammed into CLiPs, human MHs might require signalling cues distinct from those for rodent MHs. To test this idea, it might be important to explore the differences between human and rodent MHs in signalling pathways that are activated or inactivated during exposure to YAC. If we can find any differently activated/inactivated pathways through such analysis, by adding or subtracting small molecule(s), it might be possible to revise the present protocol for human MH reprogramming so that it mimics the intracellular signalling events in rodent MHs when they are exposed to YAC. Second, in our past attempts, we used frozen human MHs, which are commercially available, whereas we used freshly isolated MHs in our rodent studies. It is important to investigate whether freshly isolated human MHs might be reprogrammed into CLiPs. Nonetheless, possible cellular damage involving freezing-thawing events does not

fully answer our question, because we have confirmed that CLiPs can be generated from frozen rat MHs (personal observation). Third, differences in telomerase activity between humans and rodents might explain the difference in the susceptibility to reprogramming stimuli in MHs. It should be noted that rodent somatic cells, including hepatocytes, have stable telomerase activity, which is, in contrast, tightly silenced in human somatic cells (4,5). Indeed, past studies have demonstrated the requirement of telomerase activity for immortalization or long-term culture of human MHs (6). However, in the absence of YAC, rat MHs did not proliferate, and mouse MHs also stopped proliferation immediately after the first passage. Thus, the proliferative capacity of rodent CLiPs cannot be explained solely by their telomerase activity. In summary, further investigation is required to understand the difference between rodent and human MHs in their proliferative capacity *in vitro* in response to YAC.

Another point raised by Tanimizu and Mitaka is the heterogeneity of MHs (2). Recent studies have provided evidence that MHs consist of phenotypically distinct subpopulations. Heterogeneity of MHs has been conventionally acknowledged as hepatic zonation, namely, the spatial difference in the metabolic characteristics of MHs along the lobule axis (7). In addition to this metabolic heterogeneity of MHs, Font-Burgada *et al.* recently reported that there is a subpopulation of MHs at the periportal region, named "hybrid hepatocytes", which express Sox9, a BEC/liver progenitor cell (LPC) marker. Hybrid hepatocytes undergo extensive proliferation and replenish liver mass after chronic hepatocyte-depleting injuries (8). In contrast, Wang *et al.* reported that Axin2$^+$ proliferative MHs reside at the pericentral region and that this MH subpopulation serves as the reservoir for physiological turnover of hepatocytes (9). Their data also suggest that Axin2$^+$ MHs are frequently diploid, whereas the majority of MHs are polyploid. In our study, inspired by the findings of Wang *et al.*, we investigated the possible association between ploidy status of MHs and their reprogrammability to CLiPs. FACS-based single-cell colony formation analysis clearly demonstrated that in rats, CLiPs originate from diploid MHs. This observation does not simply support the claim by Wang *et al.* that pericentral diploid MHs are the source of hepatocyte turnover, because our microarray analysis showed no significant difference in Axin2 expression levels between diploid and polyploid MHs (personal observation). Thus, whereas it is now clear that reprogrammable MHs are restricted to diploid MHs, it remains unclear whether these reprogrammable MHs reside in the pericentral region. In addition, it also remains unclear whether diploid reprogrammable MHs play any specific roles in physiological and injured liver. Recent studies strongly suggest that MHs can be reprogrammed into proliferative bipotent LPCs in response to chronic liver injury in mice (10-13) and rats (14). Although further investigation is required, our observation implies the possibility that diploid MHs also serve as the origin of such *in vivo* reprogramming of MHs into LPCs. Tanimizu *et al.* recently reported that normal mouse liver contains an Epcam$^-$/Icam-1$^+$ LPC fraction composed of cells that are readily differentiated into MHs *in vitro* and *in vivo* (15). The Icam-1$^+$ LPCs behave like small hepatocytes, resident LPCs which the authors have long investigated in rat studies (16). Taken together with the plasticity of MHs to dedifferentiate into a LPC-like state, it is plausible that hepatocytes maintain their phenotypic equilibrium owing to their heterogeneity, which might contribute to the robust homeostasis in the liver.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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