Abstract

Hematopoietic stem cells (HSCs) originating from aorta-gonad-mesonephros (AGM) could self-renew and develop into various immune cells, such as T cells, neutrophil and natural killer (NK) cells, rendering them as a promising cell source for immunotherapy. NK cells are one of the innate lymphoid cells, and NK cell-based immunotherapy serves as a curative therapy for several cancers including leukemia. Neutrophils are one of the granulocytes, and clinical applications of neutrophils are as a therapeutic target in cancer. Due to the lack of reliable sources for the amounts of HSCs and immune cells required for clinical infusions (~10^9 cells/patient), it remains as a major challenge to realize their full potential in targeted cell and immunotherapy. While substantial efforts have been made to generate native cell-like HSCs and immune cells from human pluripotent stem cells (hPSCs), detailed molecular mechanisms regulating the differentiation of HSCs and immune cells remain elusive, preventing the development of robust strategies for HSC and immune cell productions.

In this study, we first demonstrated that temporal manipulation of Wnt signaling is both sufficient and essential to induce AGM-like hematopoiesis from 11 hPSC lines. TGFβ inhibition at the stage of aorta-like $SOX17^+CD235a^-$ hemogenic endothelium, through downregulation of Wnt signaling, yielded a chemically-defined, feeder-free culture platform for robust generation of AGM-like hematopoietic cells. Furthermore, we investigated how hypoxia affects the *in vitro* hPSC differentiation into HSCs, which resulted in a hypoxia-enhanced HSC differentiation platform.

Next, the temporal roles of transcription factors (TFs), including *NFIL3*, *ID2*, and *SPI1*, in regulating and promoting NK cell differentiation from hPSCs are determined. *NFIL3* and *SPI1* have been reported to influence the early stages of NK cell development, while *ID2* has an impact

on the generation of NK cells throughout the early and intermediate stage. We genetically modified hPSCs with doxycycline-inducible expression of *NFIL3*, *ID2*, and *SPI1*, and investigated their roles in NK cell induction from hPSCs. Among these three TFs, forced expression of *ID2* yielded the highest percentage of NK cells under a chemically-defined, feeder-free monolayer culture condition, demonstrating that forced expression of NK-specific TFs improves the efficiency of NK cell differentiation from hPSCs.

Chimeric antigen receptor (CAR) is an artificial cell receptor expressed on immune T or NK cells that has been engineered to allow T or NK cells to re-target cancer cells by exclusively binding to a cancer-specific protein. CAR engineering has significantly improved the anti-tumor efficacy of NK cell therapy, resulting in 6 FDA-approved CAR-T therapies and many other ongoing clinical trials. Recently, a chlorotoxin (CLTX)-based CAR was developed and shown to specifically bind to a variety of heterogenous glioblastoma (GBM) cell lines. To test whether CLTX-CAR could improve the anti-tumor cytotoxicity of hPSC-derived NK cells, hPSCs were engineered with CLTX-CAR for stable and homogenous CAR expression via Cas9-mediated homologous recombination. The expression of CLTX-CAR did not affect the pluripotency and NK cell differentiation potential of hPSCs, and CLTX-CAR significantly improved the cytotoxicity of hPSC-derived NK cells against GBM.

Finally, we implemented a GBM-on-a-chip microfluidic model to interrogate the tumor microenvironment (TME). Microfluidics are an emerging device for investigating cancer biology with spatiotemporal control over signaling modulators by using a small volume. The interaction between hPSC-drived neutrophils and GBM was explored in this microfluidic device. GBM TME is very complex and involves many cell types, including neurons, microglia, immune T and NK

cells. In the future, microfluidic models with isogenic cell components will be designed and implemented to better model GBM TME.

In summary, these findings provide evidence that Wnt signaling plays critical roles in directing hPSCs towards hematopoietic cell lineages and *ID2* enhances NK cell differentiation from hPSC-derived hematopoietic progenitor cells. CAR engineering further improves anti-tumor activities of hPSC-derived NK cells. Microfluidic models are also used to interrogate GBM TME.