主論文の要旨

Establishment of induced pluripotent stem cells from aged mice using bone marrow-derived myeloid cells

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Introduction

The generation of induced pluripotent stem (iPS) cells from murine and human somatic cells through forced expression of defined transcription factors constitutes a major breakthrough in regenerative biology. This technology opens the possibility of personalized cell therapies for treating human disease and/or repairing the damaged tissues of elderly patients. To use iPS cells to treat damaged tissues or repair aged organs, it will be necessary to establish iPS cells from elderly patients. Since bone marrow (BM) cells are relatively easy to obtain by BM aspiration, we asked if it was possible to establish iPS cells from BM cells of aged mice. Murine iPS cells have been established from mouse embryonic fibroblast (MEF) and adult fibroblast cells. Although the efficiency of producing iPS cells from MEF is low, the efficiency of producing iPS cells from other cells is even lower. For example, T cells are difficult to convert to iPS cells without p53 deficiency. Here, we established iPS cells from aged BM-derived myeloid (BM-M) cells. We present data that show the efficiency of reprogramming aged BM-M cells and the pluripotency of the BM-M-iPS cells in generating teratoma with three germ layers. Further, we differentiated BM-M-iPS and MEF-iPS back to myeloid cells and compared the histological morphology, pluripotent gene expression, myeloid gene expression and myeloid cell surface marker expression of these cells. Finally, we compared the state of gene promoter methylation of undifferentiated BM-M-iPS and differentiated cells.

Object and Methods

BM cells from aged C57BL/6 mice carrying the green fluorescence protein (GFP) gene were cultured with granulocyte macrophage-colony stimulating factor (GM-CSF) for 4 days. Four factors (Oct3/4, Sox2, Klf4 and c-Myc) were introduced into the BM-derived myeloid (BM-M) cells. The efficiency of generating iPS cells from aged BM cultured in GM-CSF was compared with MEF and 2 month BM-M cells from C57BL/6 mice by counting the positive colonies. The pluripotency of BM-M-iPS was evaluated by pluripotent marks expression, teratoma formation, and in vitro differentiation to 3 germ layers. Both BM-M-iPS and MEF-iPS were differentiated back to myeloid cells and were compared the histological morphology by HE staining, pluripotent gene expression, myeloid gene expression and myeloid cell surface marker expression by PCR. Finally, the state of gene promoter methylation of undifferentiated BM-M-iPS and differentiated cells were compared by ChIP assay.

Results

1. Establishment of iPS cells from BM-M cells from aged mice

First, the efficiency of generating iPS cells from aged BM cultured in GM-CSF was compared with MEF and 2 months BM-M cells from C57BL/6 mice. The colony number was less than those obtained from MEF cells or 2-month-old BM-M cells. Then, We tried to establish iPS

clones by using BM of 21-month-old EGFP-C57BL/6 mice. The morphology of the cells was similar to that seen with established iPS. Pluripotent makers SSEA-1 and Pou5f1 were both positive in BM-M-iPS cells and MEF-iPS cells. Strong AP activity was observed in both BM-M-iPS 1 and MEF-iPS cells (Fig 1).

2. Teratoma formation by 21-month-old BM-M-iPS cells

BM-M-iPS cells were transplanted to the dorsal flank of syngeneic C57BL/6 mice to evaluate the pluripotency. We found that aged BM-M-iPS cells produced teratoma 21 days after transplantation. The teratoma contained various tissues belonging to three germ layers. All the teratoma tissues expressed GFP protein. Tissues of teratoma were positively stained with alpha-smooth muscle actin (mesoderm), a-fetoprotein (endoderm) and neurofilament H (ectoderm), respectively (Fig 2).

3. In vitro differentiation of 21-month-old BM-M-iPS cells

The BM-M-iPS cells were hanging drop cultured and differentiated to 3 germ layers in vitro. Differentiated BM-M-iPS cells expressed GFP and alpha-smooth muscle actin, a-fetoprotein and neurofilament H. Same as MEF-iPS, the BM-M-iPS were also can be differentiated back the myeloid cells (Fig 3).

4. Gene expression, ChIP assays and cell surface markers of BM-M-iPS cells and differentiated BM-M-iPS cells

By co-culturing with OP9, Both the BM-M-iPS and MEF-iPS cells can be differentiated to the myeloid lineage, which lost the expression of pluripotent genes and gain the expression of myeloid linage genes and cell surface markers. The differentiated BM-M-iPS lost expression of Nanog and Pou5f1, at least in part, due to methylation of their promoters. On the contrary, TNF-α and IL1β gene expression was upregulated and their promoters were hypomethylated (Fig 4).

Discussion

For optimized clinical use of iPS cells for personalized cell therapies, it is important to select a relatively simple and safe way to obtain tissue sources. Several sources such as adult fibroblasts, hepatocytes, gastric epithelial or immune cells, including B and T cells have been used to establish iPS cells. For regenerative medicine, it is not ideal to use B cells or T cells to establish iPS cells because of gene rearrangement. Both Okabe et al. and Kunisato et al. (2010) reported the generation of iPS cells from adult murine BM cells respectively. However, in those experiments, the sources of iPS cells from BM contained B cells in which genes had already been rearranged. In order to avoid gene rearrangement, we cultured BM cells for 4 days with GM-CSF, which induces myeloid lineage cells.

In the case of elderly patients, the use of iPS cells for personalized clinical cell therapy requires the establishment of iPS cells from relatively old autologous tissue. Here, we demonstrated that it was possible to establish iPS cells from 21-month-old C57BL/6 mice. It has been shown

previously that fibroblasts from aged mice or highly differentiated hematopoietic cells have reduced capacities to produce iPS cells. Our experiments showed the efficiency of iPS cell generation in aged mice is indeed lower than that of young mice. Another problem is whether iPS cells from aged mice can be differentiated to somatic cells. It has been shown that human iPS cells established from adults are capable of differentiating into hemangioblast cells, but with dramatically decreased efficiency and early senescence. In our present experiments, aged BM-M-iPS cells could be differentiated into myeloid lineage cells and the differentiated cells proliferated well in the presence of GM-CSF.

We found that the gene expression pattern of the pluripotent markers of aged BM-M-iPS cells was similar to that of MEF-iPS cells. BM-M-iPS cells can be differentiated back to myeloid cells by co-culturing with OP9 in the presence of GM-CSF. The differentiated cells proliferated in the presence of GM-CSF and expressed myeloid cell surface markers. Thus, differentiated cells have markers similar to those of monocytes or macrophages.

It has been shown that gene expression can be regulated by histone methylation. Histone methylation causes gene silencing and heterochromatin formation, which is an essential mechanism of epigenetic regulation. Expression of Nanog and Pou5f1 is important for maintaining the pluripotency of both embryonic stem cells and iPS cells. Using chromatin immuno-precipitation assays, we investigated histone H3 methylation of both Nanog and Pou5f1 in undifferentiated BM-M-iPS cells and differentiated BM-M-iPS cells. The results of ChIP assays indicated that both Nanog and Pou5f1 were hypomethylated in undifferentiated BM-M-iPS cells and hypermethylated in differentiated BM-M-iPS cells, which is consistent with previous reports of the epigenetic regulation of Nanog and Pou5f1 genes in embryonic stem cells. In contrast, both TNF-α and IL1β were hypermethylated in undifferentiated BM-M-iPS cells and hypomethylated in differentiated cells, which explains the gene expression patterns obtained with RT-PCR. The results of ChIP assays support the idea that changes in histone methylation may play an important role in both the reprogramming required to achieve pluripotency and the directed differentiation of pluripotent stem cells, which affects the expression of genes associated with pluripotency and those required for myeloid differentiation.

Conclusion

We successfully established iPS cells from aged mice using BM-derived myeloid cells. Our experiments demonstrated three important findings. First, iPS cells can be generated from BM-derived myeloid cells. Second, iPS cells can be derived from aged mice. Finally, the iPS cells derived from C57BL/6 mice expressed GFP, thus these iPS cells can be used for regenerative medicine experiments in vivo, allowing us to follow their fate.