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Generation of retinal ganglion-like cells from reprogrammed mouse fibroblasts

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Background

Retinal neuron degeneration results in irreversible loss of vision. Neuroprotective strategies are effective for the early stage of degeneration, but cannot prevent the cell death in advanced stage. Stem cell transplantation into injured retina has potential to restore the vision and provide treatment of advanced stages of retinal degeneration.[1,2] Embryonic stem (ES) cells have been identified as a viable source of retinal progenitors. The embryonic retinal stem cells/progenitors are however, not available in sufficient quantity for clinical application. These cells also possess limited self-renewal besides ethical issues because of their embryonic/ fetal origin.

Recently, the direct reprogramming of somatic cells to a pluripotent state attracted the attention of scientists as these could be used for treating degenerative diseases without ethical concerns.[3] Induction of pluripotent stem cells from mouse embryonic or adult fibroblasts was introduced with the help of four factors namely, Oct3/4, Sox2, c-Myc, and Klf4, under ES cell culture conditions.[4–6] The purpose of the study was to determine whether induced pluripotent stem (iPS) cells express retinal progenitor cell (RPC) related genes, and whether iPS cells can directly differentiate into retinal ganglion cells (RGCs)

Study Design

Mouse iPS cells were induced from tail-tip fibroblasts (TTFs) by the four factors (Oct3/4, Sox2, Klf4 or c-Myc) through retrovirus-mediated transient gene transfer. 12 to 14 days after infection, iPS colonies were picked up and transferred onto mitomycin C-inactivated MEF feeder cells. Two iPS cell lines, including iPS clone 6 (iPS-C6) and GFP-iPS clone 5 (GFP-iPS-C5), were used in this study.

Undifferentiated iPS-C6 cells displayed ES-like morphology when cultured on MEF and expressed endogenous *Sox2*. FACS analysis showed that approximately 20% iPS cells were positive for SSEA-1 (surface marker of ES cells). The reprogramming factor *Sox2* participates in eye development and cross-regulates with Pax6 and *Otx2*. Authors first analyzed whether undifferentiated iPS cells express RPC related marker genes. Mouse brain tissue RNA was isolated on E11.5 and served as a positive control. The results of real- time PCR and immunofluorescence revealed that both undifferentiated iPS-C6 and GFP-iPS-C5 cells expressed RPC-related genes, including *Pax6*, *Rx*, *Otx2*, *Lhx2*, and *Nestin*, but not *Six6*, *Chx10*, or *Six3*. All these markers were not observed in TTFs and ES. These results demonstrated that iPS cells (induced by four factors) inherently express RPC-related genes and present potential differentiation into retinal neurons.

To cause iPS cells to differentiate toward RGCs, mouse iPS-C6 cells were expanded on MEF feeders, and EBs were generated by suspension- cultured iPS cells in EB medium for 2 to 3 days. iPS cells were induced to differentiate into RGCs by DL (Dkk1+ Lefty A) or DN (Dkk1+Noggin). Neuronal morphology was displayed by many cells on day 8. Real time PCR demonstrated that RPC-related genes except *Pax6* were decreased after differentiation. DL or DN significantly upregulated the expression of *Pax6* compared with control. Immunofluorescence assay showed the expression of MAP2 (neuron marker) and GFAP (glia marker) in DN induced neuron-like cell. Although *Pax6* acts as a *Math5* upstream gene and directly activates *Math,* the expression of RGC markers, including *Brn3b*, *Islet-1*, and *Thy1.2*, was not detected. These results indicated that DL or DN upregulated *Pax6* but did not sufficiently differentiate iPS toward RGCs.

To directly differentiate iPS cells into RGCs, authors upregulated *Pax6* by DN, overexpressed *Math5*, and further inhibited *Hes1* with DAPT (γ-secretase inhibitor) in iPS cells. After 72 hours of transfection of *Math5*, many iPS-C6 cells displayed RGC morphology with long, straight synapse like structures. Double-immunostaining analysis demonstrated that RG-like cells stained positive for typical markers of RGC precursors, including *Math5, Brn3b, and Islet-1*. 96 hours after transfection approximately 15% of cells stained positive for Math5, 10% of cells expressed Brn3b, and 5% cells were positive for Thy1.2.

RG-like cells derived from GFP-iPS cells were transplanted into the vitreous chamber of normal mice after differentiation by DN + DAPT and *Math5* transfection. After 2 weeks, most GFP

expressing cells were found to be concentrated near the injection site and rarely engrafted into the host retina, because intact normal retinal environment presents a barrier to graft integration. Authors also found teratoma formation in both eyes of one mouse (12 in total mice) after transplantation of the differentiated iPS cells (induced differentiation at passage 9). These results indicated the risk for tumorigenesis after the transplantation of virus-induced iPS cells into the retina.

Implications

Reprogramming somatic cells to pluripotent stem cells and then directly differentiating them into RG-like cells represents a strategy of artificial change of cell fate by transcription factors. iPSCs represent a renewable, reliable, and robust source of retinal progenitors that may be used to formulate stem cell approaches to understand and treat a wide range of retinal degenerative diseases.

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