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A High level of nanog-luciferase activity by recombinant Oct-4 protein : toward developmental tool for iPS cell generation

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Abstract

Induced pluripotent stem cells (iPS) technology which enable a generation of cells with human embryonic stem cells (ES) properties from somatic cells provide the possibility for creating various cell types for autologous transplantation in patient. Nevertheless, the problems associated with using retroviruses and oncogenes for reprogramming process need to be resolved before iPS cells can be considered for human therapy. In this study we demonstrate that recombinant protein Oct-4, one of the four key factors for reprogramming, containing protein transduction domain can pass through somatic cell membrane when added to the culture media. Treating mouse embryonic fibroblast with recombinant Oct-4 protein resulted in an activation of nanog-luciferase reporter. These results suggest that the recombinant Oct4 protein is active and may be used to substitute viral vector in iPS cell generation.

Keywords: induced pluripotent stem cells, recombinant Oct-4 protein, protein transduction domain, nanog luciferase activity.

Introduction

Embryonic stem (ES) cells, possess the remarkable property of pluripotency, the ability to give rise to all cells of the organism. With iPS technology it is now possible to generate es-like pluripotent stem cells from somatic cells. Mouse and human iPS cells could be generated from various types of somatic cells by infected with retroviral encoding four transcription factors; Oct4, Sox2, Klf4 and c-Myc (Takahashi and Yamanaka 2006; Takahashi, Tanabe et al. 2007; Wernig, Meissner et al. 2007). Human iPS cells could also be generated by a different set of four factors: Oct4, Sox2, Nanog, and LIN28 (Yu, Vodyanik et al. 2007). Potential safety concerns in the present reprogramming protocols still hinder human clinical application, especially the use of retroviral vectors (Yamanaka 2009). To solve this issue, we investigated the possibility of replacing viral vector with recombinant proteins. Previous studies have demonstrated that various proteins such as cre-recombinase, GFP protein can be delivered into cells by conjugating them with a short peptide that mediates protein transduction (PTD). The aim of this project is to develop tool for iPS cells generation based on this technology.

Materials & Methods

The open reading frame of mouse Oct4 was amplified and cloned into the expression vector (Invitrogen). Protein expression plasmid was transfected into host cells using FuGENE HD (Roche). Stably protein-expressed cells were selected, and the recombinant protein was extracted using Protein Extraction Reagent (Pierce, Thermo) Based on 6x-Histidine tagged at C-terminal, recombinant mouse Oct4 protein was purified by affinity chromatography using HisTrap HP (GE Healthcare). For identification of recombinant mouse Oct4 protein, western

blot was used. Briefly, purified protein was separated on 8% SDS-PAGE and blotted onto nitrocellulose membrane (Bio-rad). The blot was blocked with TBST (20 mM Tris-HCl, pH7.6, 136 mM NaCl, and 0.1% Tween-20) containing 5% skim milk and then incubated with Oct3/4 (sc-5279; Santa Cruz Biotechnology) antibody solution at 4°C overnight. After washing with TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature. Signal was detected using Supersignal (Pierce, Thermo). Protein transduction of recombinant protein into cells was examined by immunofluorescence. 10 µg of recombinant mouse Oct-4 protein were added into mouse Neural stem cells (NSC) media and cultured for 6 hours. Then cells were fixed and immunostained using Oct3/4 (sc-5279; Santa Cruz Biotechnology) antibody and Alexa 488-conjugated goat anti-mouse IgG_{2b} (Invitrogen). For luciferase reporter assay, Nanog5P reporter plasmid (addgene) was used in combination with pRL-TR (Promega). All plasmids were transfected into mouse embryonic fibroblast (MEF) using FuGENE HD (Roche). Recombinant Oct-4 protein was added at concentration 10 µg/ml. Dual-Luciferase reporter assay system (Promega) were used according to the manufacturer's protocols. Briefly, Two days after transfection, cells were lysed using 1X passive lysis buffer. Lysates were collected and assayed for luciferase activity by using Centro LB 960 detection system (BERTHOLD).

Results

Purified recombinant mouse Oct-4 protein (rmOct-4) was tested for protein identification using western blot analysis. As a result, single band was detected which correlate to Oct4 protein molecular weight (Figure 1). These data showed purity and specificity of isolated protein. Next, we tested protein transduction efficiency of rmOct-4 by adding 10 µg of rmOct-4 into culture media of mouse neural stem cells (mNSCs). Six hours after transduction, mNSCs were fixed and stained for Oct-4 protein. We found that mNSCs treated rmOct-4 were stained with Oct4 protein in nuclease more than untreated control (Figure 2). This data showed that rmOct-4 protein has ability to pass through nuclear membrane.

Oct-4 protein is essential for maintenance of pluripotentcy in Embryonic stem cells (ESCs). By interaction with SOX2, Oct-4 has been reported to regulate pluripotent specific gene expression, such as Nanog. So, we tested whether rmOct-4 has ability to activate Nanog expression *in vitro* using Luciferase assay. rmOct-4 shown to increase luciferase activity more than 4-folds compare to control. Moreover, combination of SOX2 transfection with rmOct-4 protein enhances luciferase activity to 12 folds. These data demonstrated that rmOct-4 protein can activate Nanog luciferase reporter (Figure3).

Figure 1 Western blot analysis of rmOct-4 protein using Oct3/4 antibody and horseradish peroxidase (HRP)-conjugated secondary antibody.

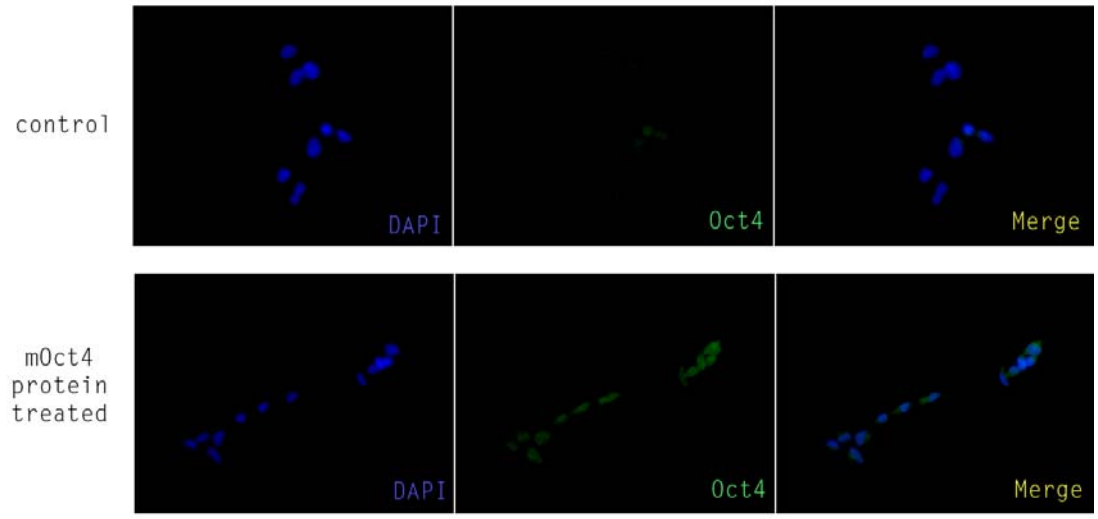

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Figure 2 Protein transduction of rmOct-4 protein into mNSC cells was examined by immunofluorescence. Oct4 (GREEN), DAPI (BLUE) and the images were merged. Magnification: 40X

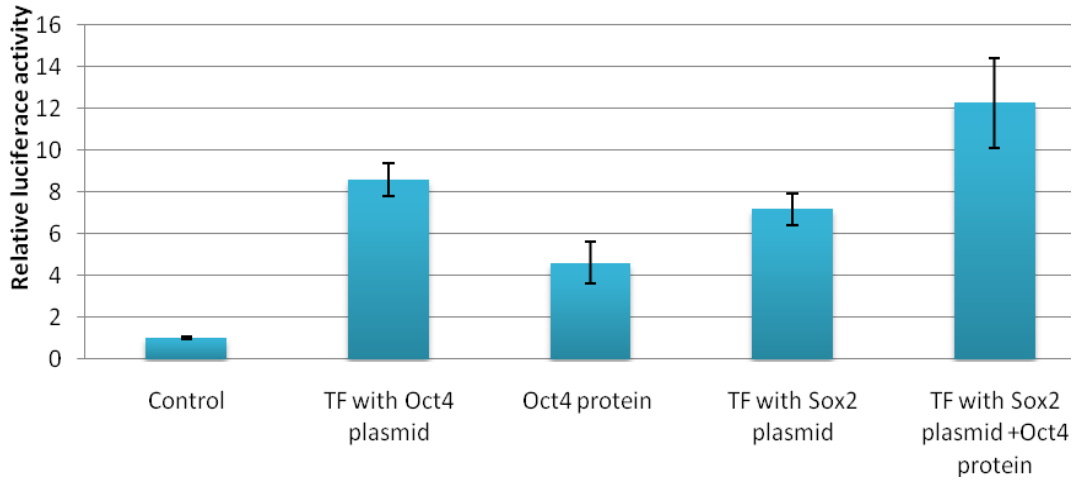

Figure 3 Effect of rmOct-4 protein on luciferase activity in MEF.

Discussion & Conclusion

In this study we show that we have generated a transducible versions of Oct4, a key transcription factor required for pluripotency. Immunofluorescence staining indicated that Oct4-PTD is internalized by cells and can translocate into the nucleus. This recombinant protein, when added to culture media, is not toxic to the cells within the concentration range used in our experiments (10 µg/ml) and can function to activate Oct-4 target genes as shown by nanog reporter assay. Our results suggest that Oct4-PTD may become a useful tool for iPS cells generation. It would provide advantage over those based on gene transfer because it would allow pluripotency genes control without interfering with host cell genome and the protein administration can be stopped when its function is no longer required.

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