ผลของ recombinant Oct4 **และ** Sox2 proteins **ต่อการแสดงออกของ** pluripotent genes **ใน** mouse somatic cells

นางสาวสิริลดา สุพรรณคง

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THE EFFECTS OF RECOMBINANT OCT4 AND SOX2 PROTEINS ON PLURIPOTENT GENES EXPRESSION IN MOUSE SOMATIC CELLS

Miss Sirilada Suphankong

A Thesis Submitted in Partial Fulfillment of the Requirements
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อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.นพ.ดร.นิพัญจน์ อิศรเสนา ณ อยุธยา, 50 หน้า.

ในปัจจุบันพบว่าการใช้ไวรัสเป็นตัวนำ transcription factor เพียง 4 ตัว ได้แก่ Oct4, Sox2, Klf4 และ c-Myc เข้าสู่เซลล์ สามารถเหนี่ยวนำให้โซมาติกเซลล์มีคุณสมบัติเข้าใกล้ pluripotent stage ซึ่งเซลล์ใหม่ที่สร้างได้มีคุณสมบัติเหมือนกับเซลล์ต้นกำเนิดตัวอ่อนทุกประการ และเรียกเซลล์ใหม่นี้ว่า “induced pluripotent stem (iPS) cells” การค้นพบในครั้งนี้นำไปสู่ความหวังในการสร้าง patient-specific pluripotent stem cells เพื่อนำไปใช้ในการรักษาและงานวิจัยทางการแพทย์ในอนาคต อย่างไรก็ตามปัญหาสำคัญสำหรับการใช้เทคนิคนี้คือ การที่ไวรัสเข้าไปแทรกในจีโนมของเซลล์เจ้าบ้านอาจก่อให้เกิดการกลายพันธุ์และกลายเป็นมะเร็งได้ ดังนั้นคณะผู้วิจัยจึงสนใจที่จะพัฒนาเทคนิคใหม่ โดยการใช้เปบไทด์สายสั้นๆ ที่เรียกว่า Tat peptide เป็นตัวนำ transcription factor ที่สำคัญดังกล่าวเข้าสู่เซลล์แทนการใช้ตัวพาที่เป็นไวรัส จากผลการทดสอบคุณสมบัติของ recombinant Oct-4 protein พบว่าโปรตีนที่เราสร้างขึ้นเพียงแค่เติมลงใน media ที่ใช้เลี้ยงเซลล์ตามปกติ ก็สามารถผ่าน somatic cell membrane เข้าสู่นิวเคลียสของเซลล์ได้ อีกทั้งยังสามารถกระตุ้นให้เกิดการแสดงออกของ pluripotent gene บางตัวรวมถึงมีผลต่อการเกิด activation ของ Nanog-luciferase reporter อีกด้วย ที่น่าสนใจอีกประการหนึ่งพบว่าเมื่อนำ recombinant Oct4 protein มาใช้ร่วมกับ Sox2/Klf4/c-Myc-retroviral transduction สามารถกระตุ้นให้เกิด iPS colony ได้โดยเปรียบเทียบกับ control ที่ใช้ไวรัสเพียง 3 ตัว (Sox2 , c-Myc และ Klf4) ซึ่งไม่พบ colony เลย จากผลการทดลองนี้ นำไปสู่ความเป็นไปได้ที่จะนำเทคนิค protein transduction มาประยุกต์ใช้ในการเหนี่ยวนำให้เกิดการรีโปรแกรมเซลล์ได้โดยไม่ต้องใช้ไวรัส

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SIRILADA SUPHANKONG: THE EFFECTS OF RECOMBINANT OCT4 AND SOX2 PROTEINS ON PLURIPOTENT GENES EXPRESSION IN MOUSE SOMATIC CELLS. THESIS ADVISOR: ASST. PROF. NIPAN ISRASENA, M.D., Ph.D., 50 pp.

Mouse and human somatic cells can be reprogrammed into induced pluripotent stem (iPS) cells by reretroviral transducing genes encoding four transcription factors: Oct4, Sox2, Klf4 and c-Myc. This provided an opportunity to generate patient-specific pluripotent stem cells for human therapeutic and research application. However, a major limitation of this technology is the viral genome integration. In this study we demonstrated 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. Surprisingly, when using Tat-Oct4 protein combine with three retroviral vectors: Sox2, Klf4 and c-Myc, could be generated iPS colonies from primary mouse fibroblasts. These results suggest that the recombinant Oct-4 protein is active and may be used to substitute viral vector in iPS cell generation.

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LIST OF ABBREVIATIONS

SCs	Stem cells
ADCs	Adult stem cells
HSC	Hematopoietic stem cells
NSC	Neural stem cells
MSC	Mesenchymal stem cells
ESC	Embryonic stem cells
ICM	Inner cell mass
mES	Mouse embryonic stem cells
hES	Human embryonic stem cells
SCNT	Somatic cell nuclear transfer
MEFs	Mouse embryonic fibroblasts
iPS	Induced pluripotent stem cells
Antp	Antennapedia peptide
HIV	Human immunodeficiency virus

PTDs	Protein transduction domains
RA	Retinoic acid
LIF	Leukemia inhibitory factor
HMG	High mobility group
CNS	Central nervous system
P^{SC}	Stem cell-specific promoter
NLS	Nuclear localization signal
SSEA-1	Stage-specific embryonic antigen-1
AP	Alkaline phosphatase
HAT	Histone acetyltransferase complex
NPCs	Neural progenitor cells
HMTase	Histone methyltransferase
HDAC	Histone deacetylase inhibitors
AZA	5-Aza-cytidine
VPA	Valproic acid

CHAPTER I

INTRODUCTION

Background and Rationale

As well known that stem cells (SCs) have been capable of unlimit proliferation, self-renewal and differentiation into all cell types in our body. Stem cells (SCs) are classified according to their differentiation capacity. Pluripotent stem cells are capable to differentiate into somatic three germ layers: mesoderm (muscle, bone, etc.), ectoderm (neuron, skin, etc.) and endoderm (hepatocytes, pancreatic beta cells, etc.) , whereas differentiation potential of multipotent stem cells is more restricted when compare to pluripotent stem cells. Tissue-specific progenitor cells or adult stem cells (ADCs) are believed to be multipotent, the most extensively studies in ADCs are hematopoietic stem cells (HSC), neural stem cells (NSC), and mesenchymal stem cells (MSC). For embryonic stem (ES) cells are the most studied in pluripotent stem cells, which are derived from the inner cell mass (ICM) of the mammalian blastocyst. They have the ability to grow indefinitely while maintaining pluripotency (1,2). In addition, ES cells can also differentiate into a variety of different cell types when cultured in vitro. This property of ES cells, particularly human embryonic stem (hES) cells, holds great promise for cell transplantation therapy, drug screening and tissue engineering (3). However, there are three important obstacles associated with human ES cells transplantation: teratoma formation, immune rejection after transplantation and ethical concerns regarding the use of human embryos. One way to resolve these issues is the generation of pluripotent stem cells directly from the patients' own cells.

Patient-specific pluripotent stem cells may be created by reprogramming somatic cells to a pluripotent state. From previous study reported that a somatic cell can be reprogrammed by somatic cell nuclear transfer (SCNT) and fusion of somatic cells with ES cells. Each method has advantages as well as disadvantages, for example, nuclear transfer can induce nearly complete reprogramming but still concern about ethical issue in requiring oocytes or embryos for generation. However, application of

SCNT has only been achieved in nonhuman primates (4-6). Although cell fusion with ES cells has been achieved with human cells, which results in abnormal tetraploid cells so they are unsuitable source for clinical application (7-9), in addition, tumor formation is a concern for both methods. Breakthrough in 2006, Takahashi and Yamanaka have opened up opportunities, which makes a new approach to produce patient-specific pluripotent stem cells for regenerative medicine (10). They demonstrated that the introduction only four factors: Oct4, Sox2, Klf4 and c-Myc into mouse somatic cells by retroviral vectors were sufficient to reprogram them into pluripotent state, so-called " induced pluripotent stem (iPS) cells " (10). These results were confirmed in mouse cells (11-13) and applied to human cells (14-16). Human somatic cells can also be reprogrammed by the different set of four factors: Oct4, Sox2, Nanog and LIN28 (17). iPS cells were similar to ES cells in morphology, gene expression, in vitro differentiation, DNA methylation pattern and developmental potency. However, the transduction of somatic cells with retroviruses or lentiviruses, which are integrated into the host cell genome causes of mutagenesis and also the reactivation of the transgene encoding c-Myc could lead to tumorigenesis.

To date, iPS cells can be generated by using various genetic approaches, for example using nonintegrating adenovirus to deliver reprogramming genes (18), transient transfection of reprogramming plasmids (19), a *piggyBac* (PB) transposon gene-delivery system (20,21). In addition, the endogenous gene expression in certain cell types also allowed easier reprogramming and/or fewer required exogenous genes (22-24). In order to apply the technology to cell transplantation therapy, it is critical to generate iPS cells without the genetic modification.

One possible way to avoid introducing exogenous genetic modifications to target cells would be to deliver the reprogramming proteins directly into cells. Previous studies have demonstrated that various proteins can be delivered into cells by conjugating them with a short peptide that mediates protein transduction, such as *Drosophila* homeotic transcription protein antennapedia (Antp), the herpes simplex virus structural protein VP22, (11R) poly-arginine peptide and the human immunodeficiency virus (HIV-1) *trans*-activator Tat protein (25-27). Several reports have described the

delivery of peptides or proteins in vivo. From the fact that protein transduction domain (PTDs) can cross the blood-brain barrier may be suitable for neurological application, for example, when expressing as a fusion gene to the Tat transduction domain by direct injection in mice's brain could increase of the distribution of the lysosomal enzyme beta-glucuronidase, which the protein deficient in the mucopolysaccharidosis VII disease (28). Another interesting application of this technology is for cancer gene therapy, it may enable more efficient penetration and delivery at solid tumor sites, than using viral vectors, such an 11R-fused p53 protein (11R-p53) be able to suppress the growth of bladder cancer cells, in addition, safer than virus-mediated gene therapy. The knowledges gained from above studies can be applied with other proteins for regulation in many cell types (29-30).

In the present study, we generate the recombinant Tat-fused Oct4 and/or Sox2 proteins and then study about the effect of these proteins on pluripotent genes expression and Nanog luciferase reactivity in mouse somatic cells. And further aim of this experiment is that protein transduction can be applied and used as a new technique for generating pluripotent stem cells from somatic cells without genetic manipulation including, understanding the molecular mechanisms underlying nuclear reprogramming.

Research Questions

Whether recombinant Oct4 and/or Sox2 proteins effect to pluripotent genes expression and Nanog luciferase reactivity in mouse somatic cells?

Objectives

1. To study the effect of recombinant Oct4 and/or Sox2 proteins by using protein transduction technique on pluripotent genes expression in mouse somatic cells.
2. To develop a new technique for generating pluripotent stem cells from somatic cells without genetic manipulation.
3. To study and understand the reprogramming mechanism.

Hypothesises

Using recombinant Oct4 and/or Sox2 proteins can induce to pluripotent gene expression and activate Nanog-luciferase reporter in mouse somatic cells.

CHAPTER II

LITERATURE REVIEWS

Embryonic stem (ES) cells

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of the mammalian blastocyst. They are able to self-renewing cell division under specific cell culture conditions while maintaining pluripotency. In addition, ES cells can also differentiate into a variety of different cell types when cultured in vitro. They are capable of giving rise to cells of the three germ layers: mesoderm (muscle, bone, etc.), ectoderm (neurons, skin, etc.) and endoderm (hepatocytes, pancreatic beta cells, etc.), so they are pluripotent cells (Figure 1). This property of ES cells, particularly for human embryonic stem (hES) cells, holds great promise for regenerative medicine, such as Parkinson's disease, spinal cord injury, and diabetes (3). However, there are ethical difficulties regarding to the use of human embryos, as well as the problem of tissue rejection after transplantation in patients. One possible way to avoid these issues is by generating the pluripotent cells from the patients' own cells. The first step, we should be studied and understood about the molecular basic mechanism and key regulators of pluripotency.

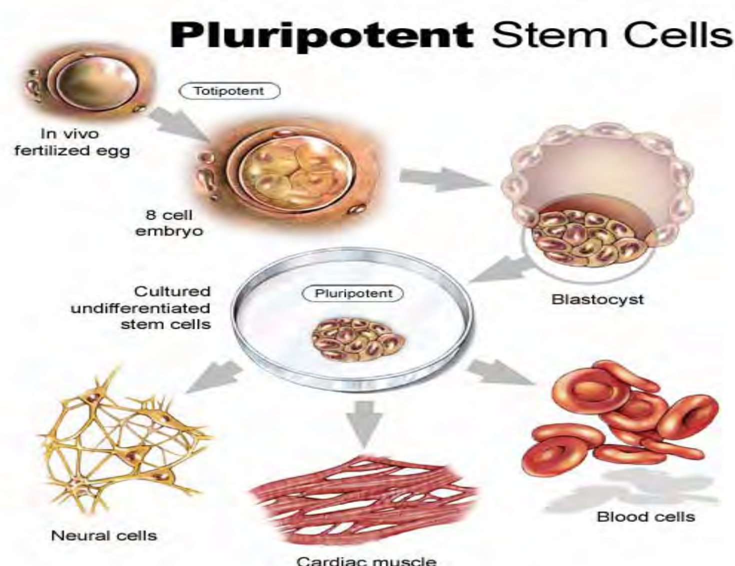


Figure 1: Pluripotency of embryonic stem cells (31).

Understanding the molecular basic mechanism of ES cells may a molecular tool to examine the pluripotency of each induced pluripotent cells line, especially before these cells are used for cell transplantation therapy.

1. Key transcriptional regulators of ES cells

So far known that the core transcription factors; Oct-4, Sox2 and Nanog have been shown function in the maintenance of pluripotency in both early embryos and ES cells. And also several genes that are frequently up-regulated in tumors, such as *Klf4* and *c-Myc*, have been shown to contribute to the long-term maintenance of the ES cell phenotype and the rapid proliferation of ES cells in culture.

1.1 Oct-4 (also known as Oct3 and encoded by *Pou5f1*)

The transcription factor Oct-4, a POU (Pit, Oct, and Unc) family, in early embryo, is essential to establish pluripotency. In addition, it is highly expressed in undifferentiated ES cells and germ cells (32), and in vitro its down-regulated when these cells are induced to differentiate by retinoic acid (RA) treatment or the removal of leukemia inhibitory factor (LIF) (33,34).

In 2000, Niwa's group found that the quantitative expression of *Oct-4* defines differentiation, dedifferentiation or self-renewal of ES cells. A less than twofold increase in expression causes differentiation into primitive endoderm and mesoderm. In contrast to, repression of *Oct-4* induces loss of pluripotency and dedifferentiation to trophectoderm. Thus a critical amount of Oct-4 is required to maintain self-renewal (35).

Oct-4 null embryos die during the pre-implantation stage of development. Although these embryos are able to reach the blastocyst stage, in vitro culture of the ICM of homologous mutant blastocysts produces only trophoblast lineages. ES cells can not be derived from Oct-4 null blastocyst. Suppression of Oct-4 resulted in spontaneous differentiation into the trophoblast lineages in both mES and hES cells. These data demonstrate the essential roles of Oct-4 in maintenance of pluripotency and also plays important roles in promoting differentiation.

From genome-wide studies in human and mouse revealed a large group of target genes with Oct-regulatory elements, and these include *Fgf4, Utf1, Opn, Rex1/Zfp42, Fbx15, and Sox2* (36-40).

1.2 Sox2

The transcription factor Sox2 is a member of the Sox (SRY-related High Mobility Group box) gene family that encodes transcription factors with a single HMG DNA-binding domain. Consistent with this role, Sox2 is necessary for embryonal development and to prevent ES cells differentiation, so that like Oct4. However, unlike Oct4, Sox2 also marks stem/precursor cell populations within the central nervous system (CNS) (41,42).

Sox2 null embryos die during implantation due to a failure of epiblast development (43). Homologous mutant blastocysts appear morphologically normal, but undifferentiated cells fail to proliferate, and only trophectoderm and primitive endoderm-like cells are produced. Consistant with down-regulation of Sox2 in ES cells by RNA interference promotes trophectoderm differentiation. Therefore, Oct-4 and Sox2 are essential for the maintenance of pluripotency (44,45).

In the previous study reported that Sox2 forms a heterodimer with Oct-4 on the enhancer DNA sequences of *Fgf4*, however, is not a unique target of the Sox2-Oct4 complex. At least three other genes, *Utf1, Fbx15,* and *Sox2* itself, are regulated by the Sox2-Oct4 complex (46,47). It is possible that other ES-cell associated genes are regulated by combination of Sox2 with different transcription factors as in the case of *Rex1* which is mainly activated by a combination of Sox2 and Nanog.

1.3 Nanog

The transcription factor Nanog is a divergent homeobox transcription factor especially expressed in early mouse embryo and ES cells. In 2003, Mitsui's group reported the two important properties of Nanog –the fundamental role in pluripotency of both ICM and ES cells, and the ability to maintain ES cell self-renewal without LIF (48). Over-expression of nanog in mES cells enables them to maintain ES cells self-renewal in the absence of LIF. In contrast with, the deletion of *nanog* resulted in loss of pluripotency in both ICM and ES cells. Nanog-deficient ICM failed to generate epiblast and only produced partial endoderm-like cells and nanog-deficient ES cells lost pluripotency and differentiate into extra-embryonic endoderm lineage (49,50).

The transcription factors Oct-4, Sox2, and Nanog have essential roles in early development of embryos and been shown to function as core transcription factors in maintaining pluripotency (51-53). These three factors collaborate to form regulatory circuitry consisting of auto-regulatory and feed-forward loops. Oct-4, Sox2 and Nanog contribute to pluripotency and self-renewal by activating their own genes encoding components of key signaling pathways and by repressing genes that a key to developmental processes.

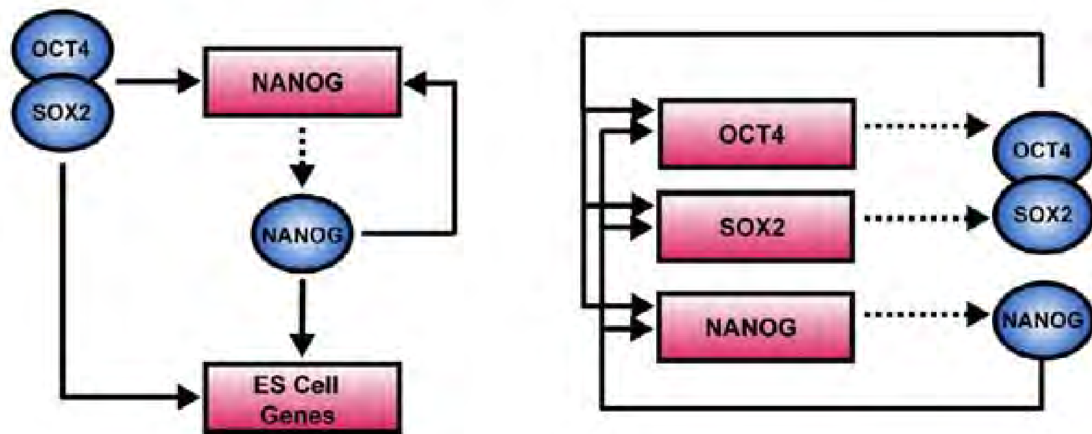


Figure 2: auto-regulatory and feed-forward loops of core transcription factor in ES cells. (left) An example of feed-forward transcriptional regulatory circuitry in ES cells. Regulators are represented by blue circles; gene promoters are represented by red rectangles. Binding of a regulator to a promoter is indicated by a solid arrow. Genes encoding regulators are linked to their respective regulators by dashed arrows. (right) The interconnected auto-regulatory loop formed by Oct-4, Sox2, and Nanog. (51)

1.4 Proto-oncogene c-Myc

The helix-loop-helix/leucine zipper transcription factor Myc is associated with a number of cellular functions including cell growth, differentiation and proliferation but also with oncogenic transformation. c-Myc has been proposed as a major downstream target for two pathways that support maintenance of pluripotency: the LIF/STAT3 and the Wnt signaling cascades. Its potential in stem cell renewal has been demonstrated by ability of ES cells expressing a stable c-Myc mutant, to contribute to chimeric mice even when cultured in absence of LIF (54).

The first pathway, LIF signaling, LIF triggers by binding to a hetero-dimeric LIF-receptor a signaling cascade that results in activation and nuclear translocation of transcription factor STAT3. STAT3, when over-expressed, is sufficient for the continued self-renewal of mES cells even in absence of LIF. The c-Myc transcriptional activation was found to be one of the downstream targets of STAT3 in ES cells. Wnt signaling can maintain mouse and human stem cell populations, independently of the LIF/STAT3 pathway. The Wnt signal is thought to act via inhibition of GSK3β, a GSK3β-inhibitor, can support self-renewal of ES cells in the absence of Wnt-ligand (56). In addition, c-Myc may open up chromatin by binding to numerous sites in the genome and by recruiting histone acetylase complexes (57).

In c-Myc -/- mouse embryos die between 9.5 and 10.5 days of gestation. Pathologic abnormalities include the heart, pericardium and neural tube. In addition, the c-Myc -/- ES cells are defective in vascular differentiation. Furthermore, over-expression of c-Myc, either in vitro or in vivo, leads to malignant transformation, suggesting that the accumulation of c-Myc mRNA and protein in numerous and diverse human tumors plays a role in the pathogenesis of these neoplasms.

1.5 Gut-enriched Kruppel-like factor (GKlf, Klf4)

The Klf4 is a Kruppel-like zinc finger transcription factor. It can act as an oncogene but also as a tumor suppressor protein. Klf4 is, like c-Myc, a downstream target of activated STAT3 in LIF-induced ES cells. Its over-expression leads to sustained expression of Oct-4 and inhibition of differentiation in ES cells (58). Similar to Sox2, Klf4 can also act as a co-factor for Oct4-mediated regulation of gene transcription.

Klf4 may be indirectly involved in the up-regulation of Nanog protein by repressing p53, a negative regulator of Nanog (59). Klf4 also works in conjunction with RAS oncogenic signal transduction protein to stimulate cellular proliferation which lead to cell immortalization. Rowland's group showed that ectopic expression of Klf4 suppresses cell proliferation. In p21 null cells, Klf4 promotes cell proliferation by down-regulating p53 (60,61). The inactivation of STAT3 in mES cells markedly decreases Klf4 expression, and forced expression of Klf4 enables LIF-dependent self-renewal.

In addition, Klf4 cooperates with Oct-4 and Sox2 to activate the *Lefty1* core promoter in mES cells (62).

Reprogramming into induced pluripotent stem (iPS) cells

Patient-specific pluripotent stem cells may be created by reprogramming somatic cells to a pluripotent state. A somatic cell can be reprogrammed by somatic cell nuclear transfer (SCNT) and by fusion of somatic cells with ES cells (4-9). In 2002, Jaenisch's group (63) showed that ES cells derived from SCNT could be differentiated into hematopoietic progenitor cells and rescue immune deficiency in Rag2 knockout mice. This paper demonstrated proof of principle that SCNT might be used to create patient-specific stem cells. However, positive results of application of SCNT have only been achieved in nonhuman primates (64). In 1997, Surani's group (65) developed a cell fusion method to reprogram mouse somatic cells into ES cells. This technique was later applied to humans, however, these experiments generated abnormal tetraploid cells (66). From successful reprogramming of somatic cells by cell fusion with ES cells indicates that ES cells have some important factors that are able to induce pluripotency.

2.1 Reprogramming somatic cells towards pluripotency by defined factors

Base on this hypothesis, the first study, Takahashi and Yamanaka selected 24 candidate genes and screened them in mouse somatic cells. A neomycin-resistant gene was inserted into the mouse *Fbx15* locus by homologous recombination, and the 24 candidate genes were transduced into mouse fibroblasts using retroviral vector. By screening combinations of these 24 genes using G418-resistant ESC-like clones, they identified key transcription factors; *Oct-4, Sox2, Klf4* and *c-Myc* as being sufficient to reprogram mouse fibroblasts into ES-like cells that were called "induced pluripotent stem (iPS) cells" (10). The *Fbx15*-selected iPS cells were similar to mES cells in morphology, proliferation, the gene expression and the teratoma formation. However, *Fbx15*-iPS cells had a different global gene expression pattern from ES cells and failed to produce adult chimeric mice (40).

In 2007, three groups demonstrated a notable improvement in quality of iPS cells. They chose either *Nanog* or *Oct-4* expression in place of *Fbx15* expression for selection (67-69). *Nanog*- and *Oct-4*-iPS cells share with ES cells abivalent pattern of histone trimethylation on histone 3 lysine 4 (H3K4), associated with transcribed genes, and on histone 3 lysine 27 (H3K27) thatis associated with silenced genes. (70). Functionally, *Nanog*- and *Oct-4*-iPS cells contributed in teratomas to tissues derived from all three germ layers. Both types of iPS cells, in contrast to *Fbx15*-iPS cells, also contributed to live chimeric mice and demonstrated potential for germ-line transmission.

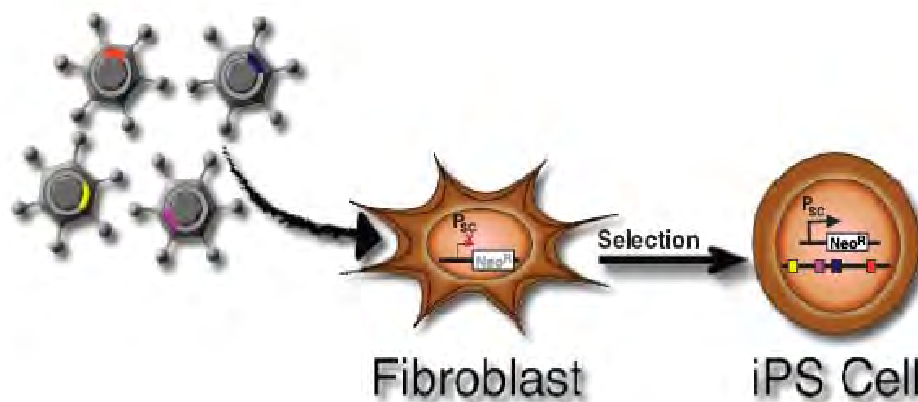


Figure 3: Reprogramming the differentiated stage by viral transduction. The approach initially outlined by Takahashi and Yamanaka (2006) involves the transduction of fibroblast with four pluripotent stem cell factors *Klf4, Oct-4, Sox2 and c-Myc*. Transduced cells are then placed under neomycin selection for reactivation of a stem cell-specific promoter (P^{SC}), such as *Fbx15*, *Oct4 or Nanog*. (71)

More recently, human iPS cells could be generated from human fibroblast by retroviral transduction encoding the same factors or a different set of four factors: *Oct4, Sox2, Nanog*, and *LIN28* (72-75). In addition, mouse and human iPS cells were generated in the absence of a transduced c-Myc gene, but with a lower efficiency (76,77). However the major concern is the viral integration which has been to an elevated frequency of mutagenesis and tumor formation in iPS cell-derived chimeric mice

2.2 The developmental protocols for generating iPS cells

From the viral integration concern, there are three groups show that:

(i) mouse pluripotent stem cells could generate from adult mouse liver cells, which have previously been shown to require fewer sites of viral integration (78).

(ii) mouse pluripotent stem cells could generate from fibroblasts and liver cells by using adenoviral system that does not integrate into genome (79).

(iii) mouse pluripotent stem cells could generate from MEFs by plasmid or mRNA transient transfection. They used 2A self-cleavage sequences to express Oct-4, Sox2 and Klf4 in a single expression vector. The repeated transfection with this plasmid and c-Myc cDNA enabled generation of iPS cells, resulting in without evidence of plasmid integration in iPS cells (80).

Moreover, iPS cells have been generated by genomic integration of the four reprogramming factors using lentiviruses (81), or transposons (82), followed by transgene removal using Cre-mediated excision. However the efficiency of iPS cell induction using adenoviruses or plasmid transfection protocol is lower than that with the retroviral method.

Recently, there are many groups reported that endogenous expression of Sox2 plus exogenous expression of one or two factors including Oct-4, and/or combining with small-molecule compound, is sufficient to induce the generation of iPS cells from adult NSC (83-86).

2.3 Application of mouse and human iPS cells

Proof of principle for iPS cell-based therapy was the first demonstrated in mice by Jaenisch’s group. iPS cells were generated from tail-tip fibroblast of humanized sickle cell anemia mice. After the sickle cell gene mutation in the generated iPS cells was corrected via homologous recombination, the cells were directed to differentiate into hematopoietic progenitor cells were transplanted back into donor mice, which were rescued from sickle cell anemia without immune rejection (87). For successful application, it is necessary to demonstrate the differentiation competency of iPS cells.

It has been shown that mouse iPS cell-derived neurons could functionally integrate into donor brains, and that symptoms of Parkinson's disease in donor mice were relieved after transplantation of iPS cell-derived dopaminergic neurons (88).

Human iPS cells could be generated from patients with specific diseases. These cells can be used to study the mechanisms of diseases or drug screening for individual patients. In 2008, the first iPS cell line containing genetic disorder, a human sickle cell anemia iPS cell line, was successfully established (89-92).

Although iPS cells have been effective for autologous transplantation in animal models without immune rejection including may be a promising tool to study the mechanisms of diseases and the effects of drugs. Before iPS cells can be used in diagnostic and therapeutic application, several criteria have to be fulfilled.

Protein transduction

3.1 Protein transduction technology

Recent developments in protein transduction, delivery of protein into cells, suggest that is now a realistic approach for compensation to viral transduction in delivery the reprogramming factors into the somatic cells. Protein transduction is an emerging technology with potential application of proteins into the cells from the external environment. Previous studies have demonstrated that various proteins can be delivered into cells by conjugating them with a short peptide that mediates protein transduction, such as *Drosophila* homeotic transcription protein antennapedia (Antp), the herpes simplex virus structural protein VP22, (11R) poly-arginine peptide and the human immunodeficiency virus (HIV-1) *trans*-activator Tat protein (93-96). Although the Antp protein transduction domain (PTD) can transduce cells when associated with short peptides, the efficiency decreases when incorporated into larger proteins. In addition, VP22 fusion proteins have been reported to be internalized, but little data about the efficiency of this protein delivery mode is available. Next study shown that fusions created with the Tat (49-57) PTD show markedly better cellular uptake than other short basic peptide sequences.

In 1988, Green (97) and Frankel (98) independently discovered that human immunodeficiency virus type 1 (HIV-1)-encoded *trans-activator* Tat protein, an 101-amino acids protein, which encoded by two exons, and is essential for the transcription of viral genes and viral replication.

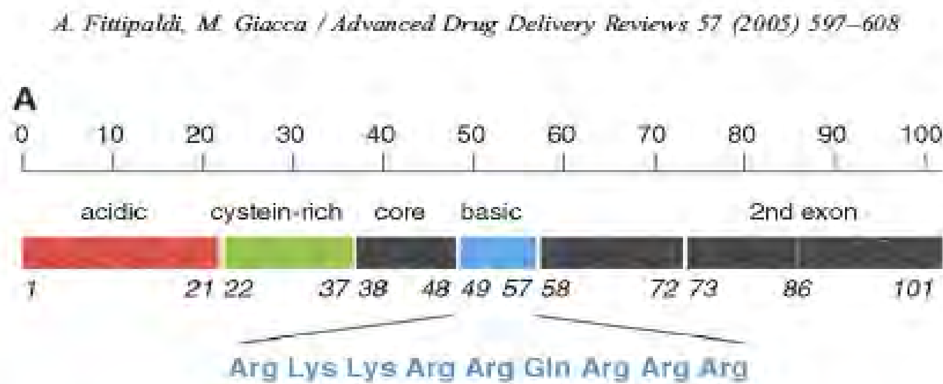


Figure 4: Domain of HIV-1 Tat protein. The amino acid sequence of the basic domain of the protein, which is required for transcellular protein transduction, is indicated. (99)

The first exon (aa 1-72)	:	an N-terminal acidic domain (aa 1-21)
	:	a domain containing 7 cysteins (aa 22-37)
	:	a core region (aa 38-48)
	:	a basic domain enriched in arginine and lysine amino acids (HIV-1 Tat) (aa 49-57)
The second axon (aa 73-101)	:	a relatively more variable sequence

Previous studies have shown that the 11-amino acids Tat peptide YG**RKKRR**Q**RRR** (recidues 47-57), rich in basic amino acids (highlighted in bold), is sufficient for intracellular transduction and subcellular localization (100). It is believed that this short Tat motif can functionally be dissected into two parts: **GRKKR** can act as a potential nuclear localization signal (NLS), whereas, **RRR** appears to be very critical for protein transduction (101). Not only the protein enters the cells, but also that it is transported to the nucleus in a transcriptionally active form. And found that, after 6 hrs, more than 80% of the internalized Tat was detectable in the nuclear compartment.

Most of these applications are based on the fusion of the protein transduction domain of Tat to the protein of interest, either at the N-terminus or at the C-terminus, followed by the addition of the recombinant fusion protein to the culture medium of the cells of interest. Most notably, fusion or conjugation to Tat has been shown not only to mediate cell internalization of heterologous proteins (102), but also of larger molecules or particles, including magnetic nanoparticles (103), phages and retroviral vectors (104,105), liposomes (106) and plasmid DNA (107), with variable efficiency. Another potentially interesting application of the delivery of genes encoding Tat fusion proteins is for cancer gene therapy.

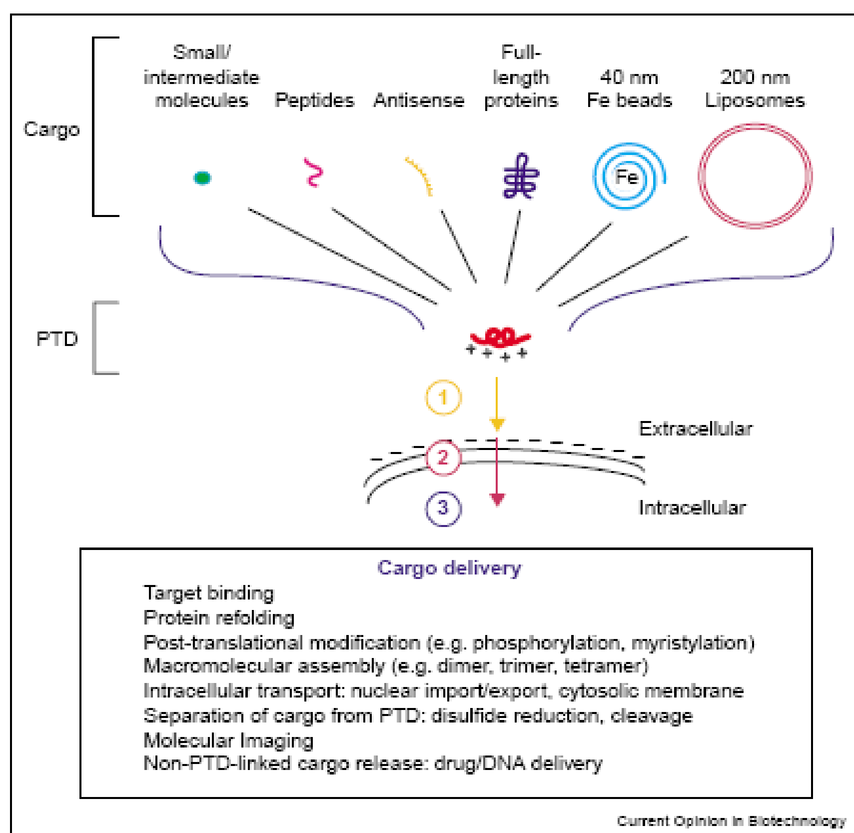


Figure 5: Utility of protein transduction technology. A wide variety of cargo has been covalently linked to PTDs. Currently, most molecules can be transduced into cells when linked to a PTD. (108)

3.2 Protein transduction: an alternative to generate iPS cells

The protein transduction technique has several advantages for generating of iPS cells that suitable for therapeutic application. First, it can reduce any risk of genetic manipulation and offers a method for generating safer iPS cells. Second, it can be used for directly control the expression of interested factors by controlling the concentration and time-duration of protein. And third, TAT-fused proteins only have a limited active half-life, so their effect are only transient that is benefit to silence the expression of reprogramming factor after the endogenous gene reactivation. Previous study, have been shown that intraperitoneal injection of the 120 kDa β-galactosidase protein, fused to the TAT PTD, results in delivery of the biologically active fusion protein to all tissues in mice, including the brain. These results open new possibilities for direct delivery of reprogramming factors into patient's cells toward pluripotency. However, the limitations of this technology in this field are ability of proteins that may be only guided cells into partial reprogramming and toxicity of them.

CHAPTER III

MATERIALS AND METHODS

1. Cell cultures

J1 ES cells were maintained on feeder layers of mitomycin C-treated MEFs (www.stemcells.atcc.org). ES cells were passaged every 3 days, maintained in DMEM/High Glucose containing 15% fetal bovine serum (ES qualified), 2.0 mM L-Alanyl-L-Glutamine, 0.1 mM non-essential Amino acids, 0.1 mM 2-Mercaptoethanol, 1% Penicillin/Streptomycin and 10^3 U/ml mouse leukemia inhibitory factor (LIF)

For MEFs isolation, uteri isolated from 13.5-day-pregnant mice (10) were maintained in DMEM/High Glucose containing 10% fetal bovine serum (USA grade), 20mM L-Alanyl-L-Glutamine and 1% Penicillin/Streptomycin. In this study, we used MEFs within four passages to avoid replicative senescence.

2. Protein transduction technology

2.1 Insect cells culture

High Five™ cell lines were maintained in Express Five SFM medium containing 10% L-Alanyl-L-Glutamine and 1% Penicillin/Streptomycin and incubated at 27°C or room temperature (in dark room).

2.2 Plasmid constructions

We designed and amplified the TAT sequence by PCR (40 bp) and cloned into *KpnI/BamHI* site of pIZ/V5-His vector (Invitrogen). Then transformation into *E. Coli* strain like TOP10, and selecing transformants on Low salt LB agar plates containing 25-50 ug/ml Zeocin™ (Invitrogen). Next, we checked the completed ligation by sequencing.

2.3 Interested genes

In the interested genes should contain a Kozak translation initiation sequence and ATG start codon for proper initiation of translation.

Note that the G or A at position -3, and the G at position +4 are the most critical for function (shown in bold). The ATG start codon is shown underlined.

(G/A) NN<u>ATG</u> **G**

The open reading frame of mouse Oct4 was amplified by PCR and cloned into *EcoRI/*XhoI site of TAT-pIZ/V5-His expression vector. Next, we checked the completed ligation by enzymatic cut check and sequencing, respectively.

2.4 Transient expression in Insect cell lines and selecting stable cell lines

The cells will be needed log-phase cells with > 95% vaiability to perform a successful transfection. Review page 9-20 of the InsectSelect™ System manual protocol, download at www.invitrogen.com. Briefly, 1ug/ul in TE of plasmid DNA was added into transfection mixture and incubated at room temperature about 15 minutes. Next, we added them into insect cells on 60 mm dish and incubated at room temperature for 4-8 hours. Only insect cells that could grow in 500 ug/ml zeocin condition medium would be selected to make stable clones for protein extraction.

2.5 Purification proteins

For the first step of purification, the recombinant protein was extracted Using I-PER® Insect Cell Protein Extraction Reagent (Pierce).

- Procedure for protein extraction from monolayer-culture Insect cells
  - A. Wash the Insect cells
    1. Aspirate the media from the plate.
    2. Gently add a volume of PBS to the plate that is equal to the culture volume. Aspirate the PBS from the plate. Repeat this step.
  - B. Lyse the Insect cells using I-PER® reagent

    Note: Add protease inhibitors to the I-PER® reagent immediately before use.
    1. Add an appropriate volume of I-PER® reagent 1 ml / 100 mm dish.

2. Incubate cells for 10 minutes at 4 ^{O}C. Incubate plates on shaker platform with vigorous shaking.
3. Use a pipette to transfer the cells and debris to 1.5 ml eppendorf tube.
4. Centrifuge tube at 15,000 x g for 15 minutes at 4 ^{O}C.
5. Use a pipette to carefully transfer the supernatant containing soluble proteins to a new tube. Store at -80 ^{O}C.

The second step, 6x-Histidine tagged protein was purified again by ion-exchange chromatography using HisTrap HP (GE Healthcare) according to the manufacturer's instructions.

3. Retroviral production

pMXs-based retroviral vectors encoding the human complementary DNAs of Oct4, Sox2, Klf4 and c-Myc, were transfected into Plate-E cells using FuGENE HD transfection reagents (Roche). The mixture of plasmids for the three factors was transfected into a single dish of Plate-E cells. Twenty-four hours after transfection, the medium was replaced. Twenty-four hours later, virus-containing supernatant was used for retroviral infection.

4. Generation of induced pluripotent stem (iPS) cells

The first step, we seeded at a density of $5X10^{5}$ MEFs per 100 mm dish and incubated with virus-containing supernatant for the three factors (1:1:1). Next, 24 hours after infection, we changed media to ESC completed medium supplement with 10^{3} U/ml mLIF and 10 ug/ml rmOct-4 or rmSox2 protein for 1 week (change media everyday). Seven days after that the cells were transfered onto feeder layers of mitomycin C-treated MEFs. These cells were incubated in ESC completed medium supplement with 10^{3} U/ml mLIF and 10 ug/ml rmOct-4 or rmSox2 protein for 2 up to 4 weeks (change media every 2 day). During cultures the cells were observed the morphological changes by inverted-microscope every day until colonies emerged around day 14-21. Next, the colonies were selected for expansion and characterization.

5. Immunofluorescence staining

The cells were washed with PBS at least four to five times to avoid possible aggregation of proteins and then fixed with PBS containing 4% paraformaldehyde for 10 min at room temperature. After washing with PBS, the cells were treated with PBS containing 5% normal goat serum (Invitrogen), 1% bovine serum albumin (BSA, Sigma), and 0.1% Triton X-100 for 45 min at room temperature. Next, the treated cells were stained with Oct-3/4 (Santa Cruz Biotechnology), Oct-4 (Abcam) or Sox2 (Abcam). Secondary antibodies used were Alexa 488-conjugated goat anti-mouse IgG2b (Invitrogen) and Alexa 546-conjugated goat anti-rabbit IgG (Invitrogen. Nuclei were counterstained with 1 ug/ml DAPI (Invitrogen).

6. Western blot analysis

The purified protein (10 ug) was separated by electrophoresis on 8% SDS-PAGE and transferred to a Nitrocellulose membrane (Biorad). 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 primary antibody solution at $4^{O}C$ 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 with Supersignal (Pierce) and X-film (Pierce). Antibodies used for western blotting were anti-Oct-4 (Abcam), anti-Sox2 (Abcam) and anti-rabbit IgG-HRP (Biorad).

7. RNA extraction and Gene expression analysis

Total RNA was purified with Trizol reagent (Pacific science). One microgram of total RNA was used for synthesis of single-strand cDNA by First strand cDNA synthesis kits (Fermentus), according to the manufacturer's instructions. Quanlitative PCR was performed with Taq DNA polymerase recombinant (Fermentus). For quantitative PCR was performed with Maxima SYBR Green qPCR master mix (2X) (Fermentus) and reported by the ABI 7500$^{@}$ software analysed by relative quantification method (comparative Ct method).

8. Nanog-luciferase reporter assay

Condition I : Transfect the reporter plasmids (1 ug) containing the firefly luciferase gene into MEFs with 50 ng of pRL-TR (Promega)

Condition II : Transfect the pMXs-mOct4 plasmid (1 ug) into MEFs

Condition III : TAT-mOct4 protein treatment (10 ug/ml)

Condition IV : Transfect the pMXs-mSox2 plasmid (1 ug) into MEFs

Condition V : Transfect the pMXs-mSox2 plasmid (1 ug) into MEFs combined with TAT-mOct4 protein treatment (10 ug/ml)

Forty-eight hours after transfection, the cells were lysed with 1X passive lysis buffer (Promega) and incubated for 15 min at room temperature. Luciferase activities were measured with a Dual-Luciferase reporter assay system (Promega) and Centro LB 960 detection system (BERTHOLD), according to the manufacturer's protocols.

9. Toxicity test

Vary concentration of Tat-fusion protein and add into ES medium every day. Range of concentration 2 ug/ml to 10 ug/ml and then selected the most suitable concentration of recombinant protein that is not toxic to mouse somatic cells.

CHAPTER IV

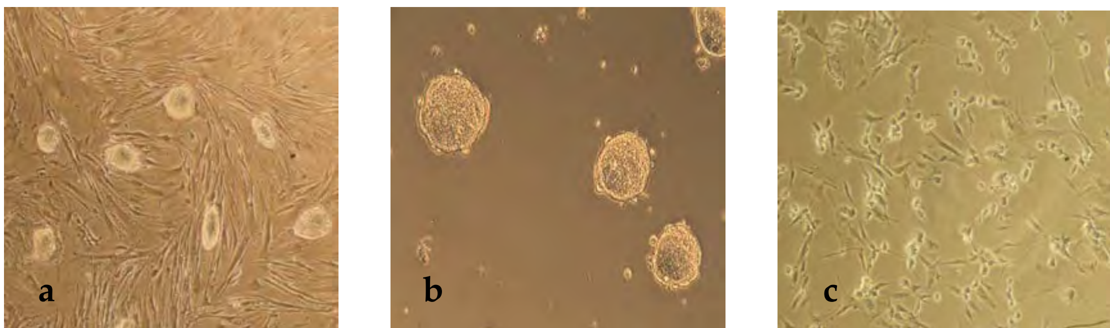
RESULTS

1. J1 mouse embryonic stem (ES) cells and mouse fibroblasts culture.

Mouse embryonic fibroblasts (MEFs) were isolated from 13.5-day-pregnant mice by standard protocol and cultured in DMEM/High Glucose supplement with 10% fetal bovine serum (USA grade). Note that, we used MEFs within four passages to avoid replicative senescence. The ES cells were cocultured on feeder layers of mitomycin C-treated MEFs or on gelatin coated plate and maintained in ESC medium supplement with 10^3 U/ml mLIF.

Both of them (MEFs and ES cells) from passage 2 were observed the morphology and then extracted RNA for real-time PCR analysis. The results show that ES cells exhibited a round shape, large nuclei and scant cytoplasm (Figure 6A; a-b), whereas the MEFs morphology were spindle shape (Figure 6A;c).

A



Quantitative real-time PCR analysis using primers specific for Oct4, Sox2 and Nanog, PCR primers are listed in Table1. We detected that in ES cells showed a significant high level expression of ES marker genes when compared to MEFs (Figure 6B). As the results, confirmed that these cells could be used to test for our experiments.

B

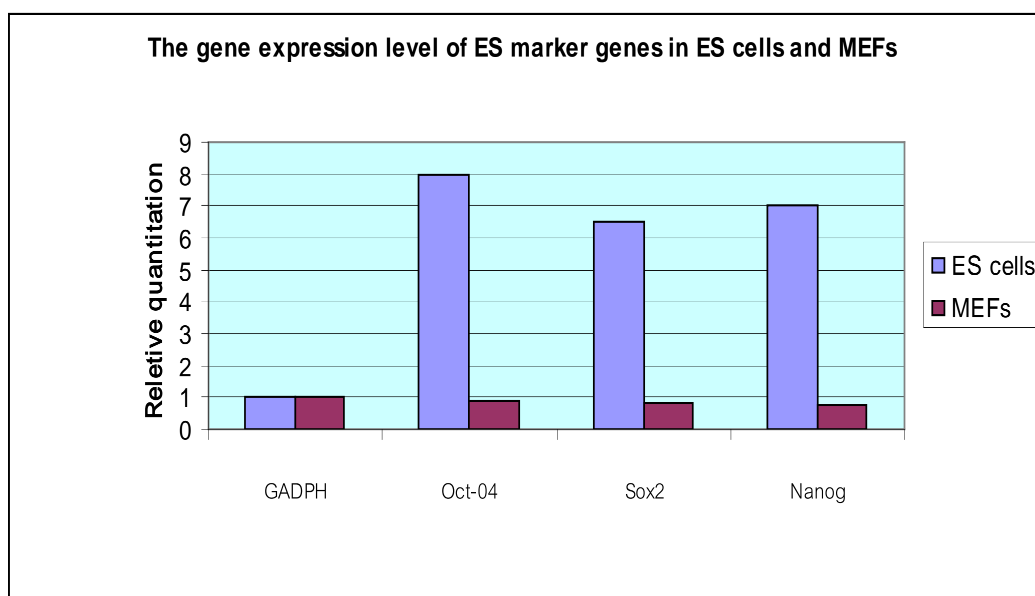


Figure 6: Characterization the properties of ES cells and MEFs.

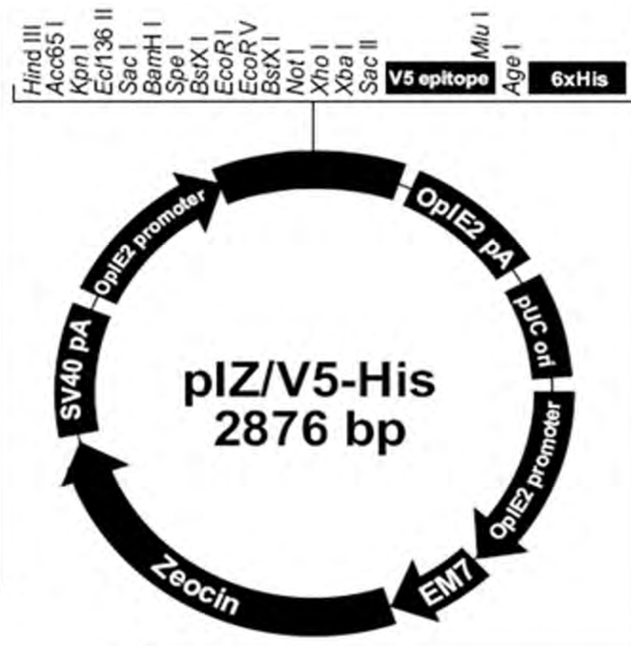
(A) Morphology of ESC passage 2 on feeder layers of mitomycin C-treated MEFs (a), ESC passage 2 on gelatin coated plate (b) and MEFs passage 1 (c). Magnification: 20X

(B) Real-time PCR analysis of ES marker gene expression in ES cells and MEFs. GAPDH was used as a loading control.

2. Protein transduction technology

To generate recombinant proteins that can penetrate across the plasma membrane of mouse somatic cells, we designed and fused TAT *trans*-activator domain into C-terminal of pIZ/V5-His expression vector (Figure 7A). Next, the open reading frame of interested genes (*mOct-4* and *mSox2*) was amplified and cloned into the TAT-fused expression vector (Figure 7B). After that the protein expression plasmid was transfected into insect cells using Cellfectin® transfection reagent (Invitrogen). Only insect cells that could grow in 500 ug/ml zeocin condition medium would be selected to make stable clones for protein extraction.

A



B

Figure 7: Plasmid constructions and characterization of recombinant reprogramming protein.
(A) pIZ/V5-His Map and Features. (B) Schematic of the protein expression vector.

The recombinant mOct-4 and mSox2 proteins were expressed in Insect cell lines (Figure 8) and then extracted using I-PER® extraction reagent. The whole protein identities were confirmed by western blot analysis. The results show corrected band of rTAT-Oct4 protein from treated cells (left), was similar to protein extract from ESC (middle). In contrast, no band in protein extracts from untreated cells (Right).

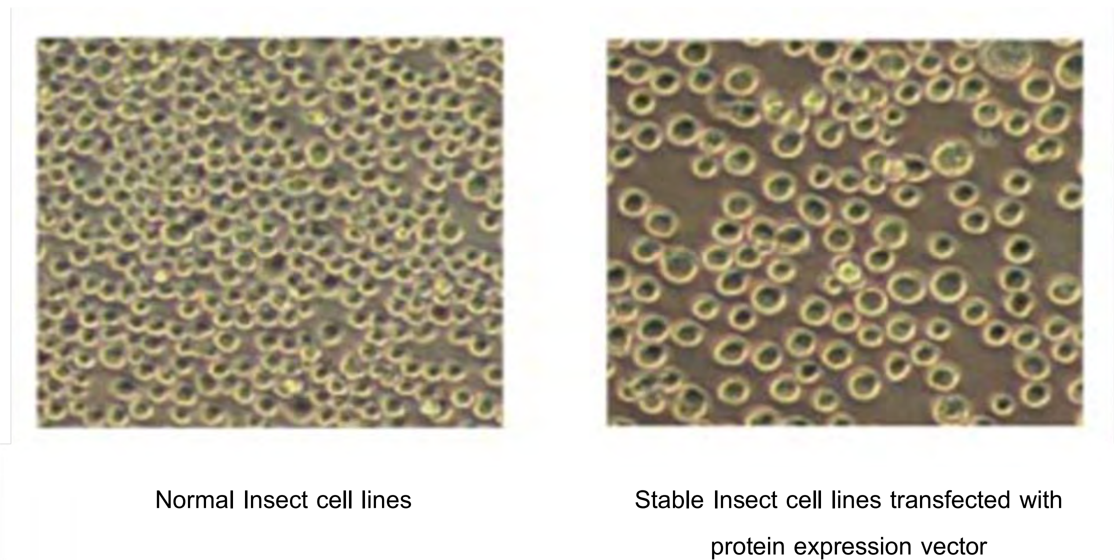


Figure 8: The different morphology between normal Insect cell line and stable Insect cell lines. After transfection the Insect cells with TAT-fused Oct4 or Sox2 vector (Right), the size of cells were larger than normal cells (Left) and slowly growth rate.

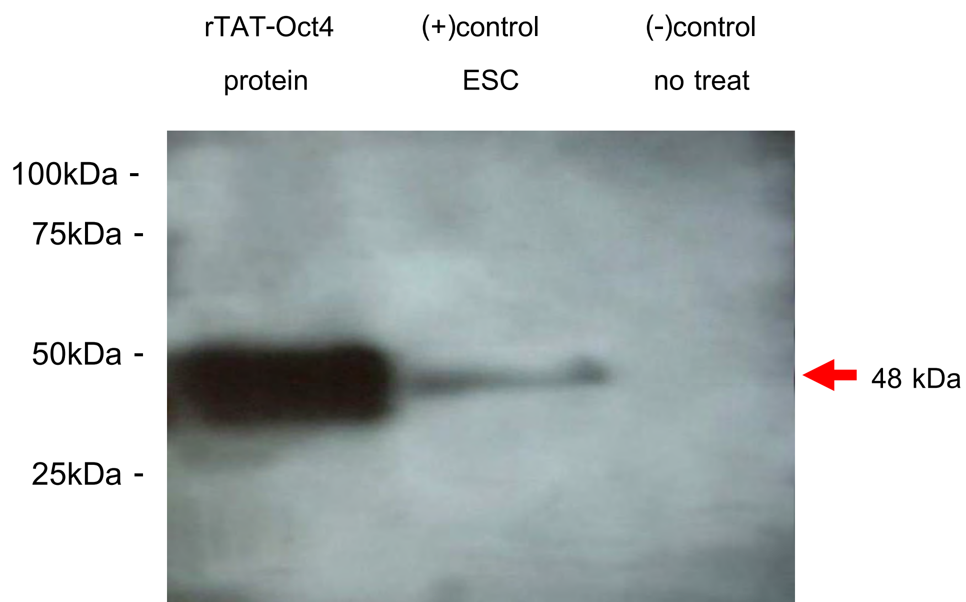


Figure 9: Western blot analysis of whole extraction of Tat-fused Oct4 protein. The 10 ug/ml of whole protein extract from treated cells, ESC and untreated cells, respectively, were detected by western blot analysis using mOct-4 antibody and horseradish peroxidase (HRP)-conjugated secondary antibody.

3. Purification of recombinant TAT-fused proteins

From previous western blot results, the whole extracted protein showed a single band, not all of them are only recombinant desired protein. Based on 6x-Histidine tagged at C-terminal, recombinant mOct-4 and mSox2 protein were purified again by ion-exchange chromatography using HisTrap HP and identified by western blot analysis. The result showed a sharp single band was detected which correlated to molecular weight of each protein (Figure 10A,10B).

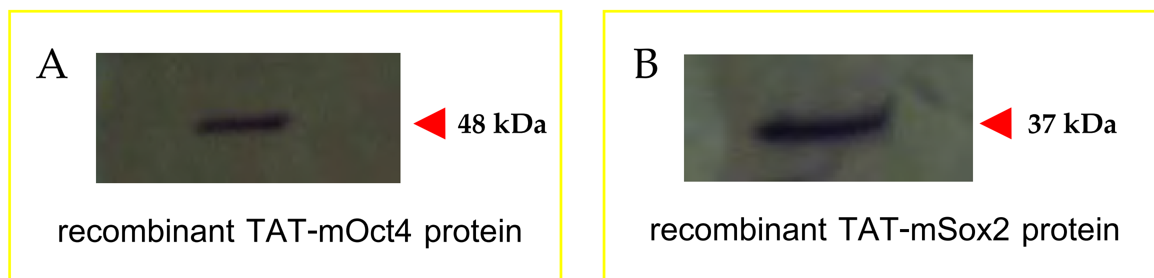


Figure 10: The specific band of purified recombinant proteins. Western blot analysis of recombinant mOct-4 protein using mOct-4 antibody and horseradish peroxidase (HRP)-conjugated secondary antibody (A) and recombinant mSox2 protein using mSox2 antibody and horseradish peroxidase (HRP)-conjugated secondary antibody (B).

4. The nuclear localization of recombinant protein in mouse fibroblasts and mNSC

Next, we examined protein transduction efficiency of recombinant mOct-4 and mSox2 protein by adding 10 ug/ml of them into culture media of MEFs and mNSC. Twenty-four hours after transduction, treated cells were fixed and immunostained with specific antibody. We found that rmOct4 protein was mainly observed in the nucleus more than untreated-cell control (Figure 11A-D), while rmSox2 protein was mainly observed in the cytoplasm (Figure 11E-F). These results suggested that rmOct-4 and rmSox2 proteins have ability to pass through somatic cell membrane when added to the culture media. Note that protein transduction efficiency of rmOct-4 protein is better than that of rmSox2 protein.

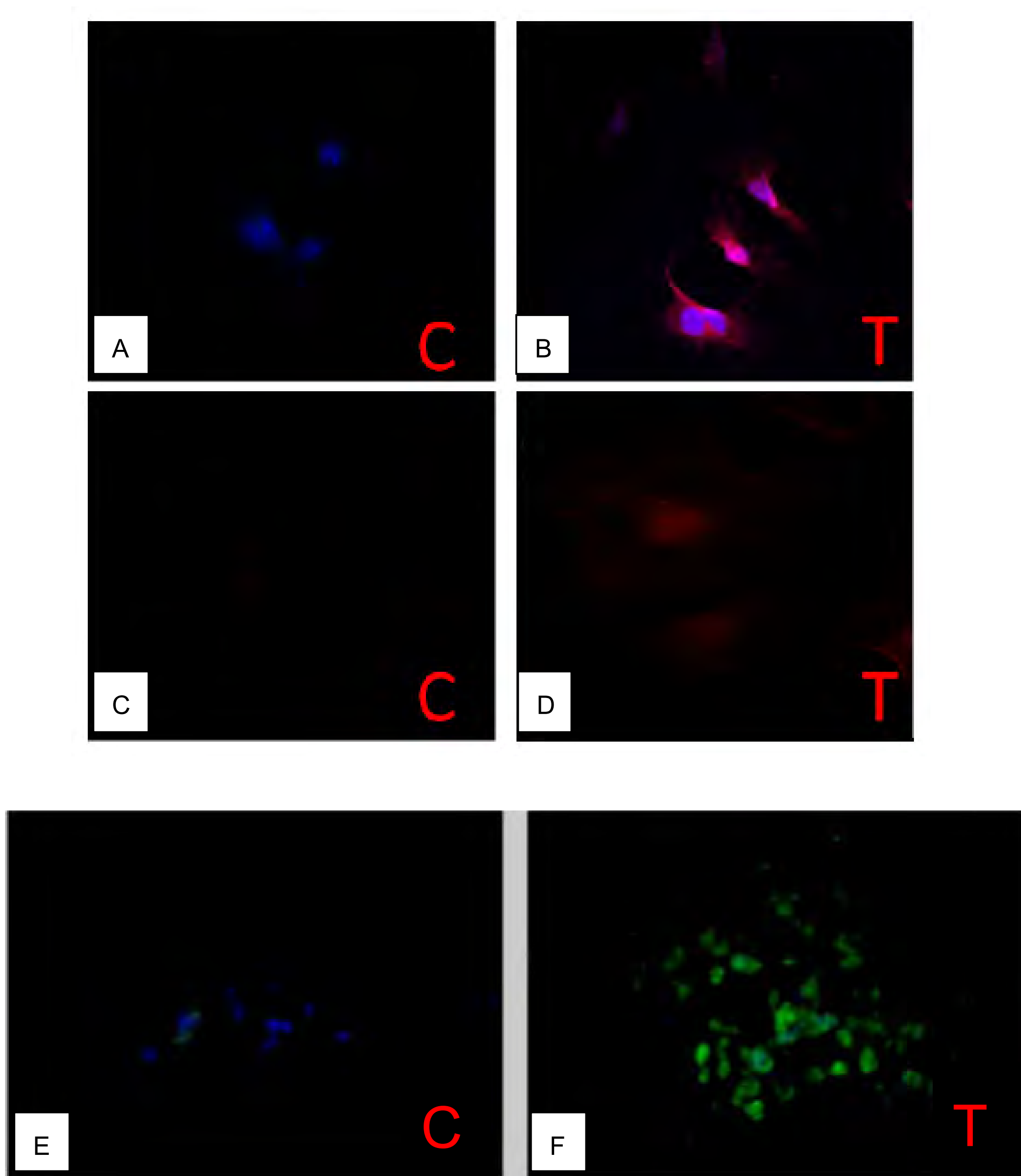


Figure 11: Protein transduction of recombinant protein into MEFs and mNSC cells was examined by imunofluorescence staining.

(A-B) MEFs no treat recombinant protein (left) and MEFs treated with rmOct4 protein 10 ug/ml for 24 hours (right). mOct-4 (RED), DAPI (BLUE) and magnification : 40X

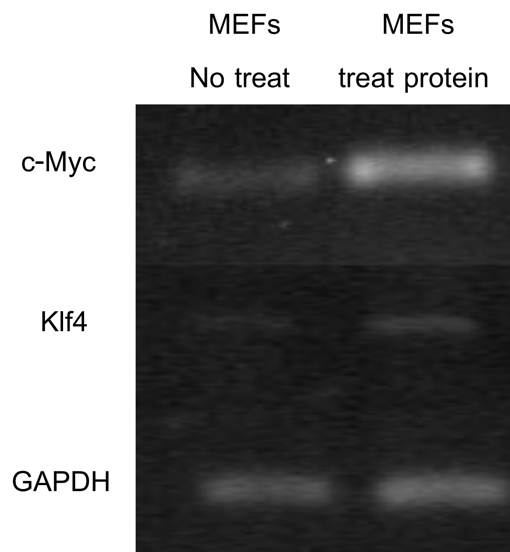
(C-D) MEFs no treat recombinant protein (left) and MEFs treated with rmSox2 protein 10 ug/ml for 24 hours (right). mSox2 (RED), DAPI (BLUE) and magnification : 40X

(E-F) mNSC no treat recombinant protein (left) and mNSC treated with rmOct4 protein 10 ug/ml for 24ours (right). mOct-4 (GREEN), DAPI (BLUE) and magnification : 40X

6. Gene expression of another genes by PCR and quantitative Real-time PCR analysis

To test the effects of rmOct-4 protein on pluripotent gene expression, we treated MEFs with 10 ug/ml rmOct-4 protein for 7 days and changed media every 24 hours. In this experiment, we chose another pluripotent factors such as, *c-Myc* and *Klf4* gene and examined the level of gene expression using quanlitative PCR and quantitative real-time PCR, compared between MEFs with and without rmOct-4 protein. We detected significantly up-regulation of *c-Myc*, but slightly up-regulation of *Klf4* in MEFs that were treated rmOct-4 protein as shown in Figure 12A.

A



In the same way, we confirmed the previous result by quantitative real-time PCR. In treated MEFs showed *c-Myc* was 6 folds up-regulated and *Klf4* was 2 folds up-regulated, respectively. As shown in Figure 12B. These results suggested that recombinant mOct-4 protein can induce some pluripotent gene expression when adding them to the cell culture medium in the suitable concentration.

B

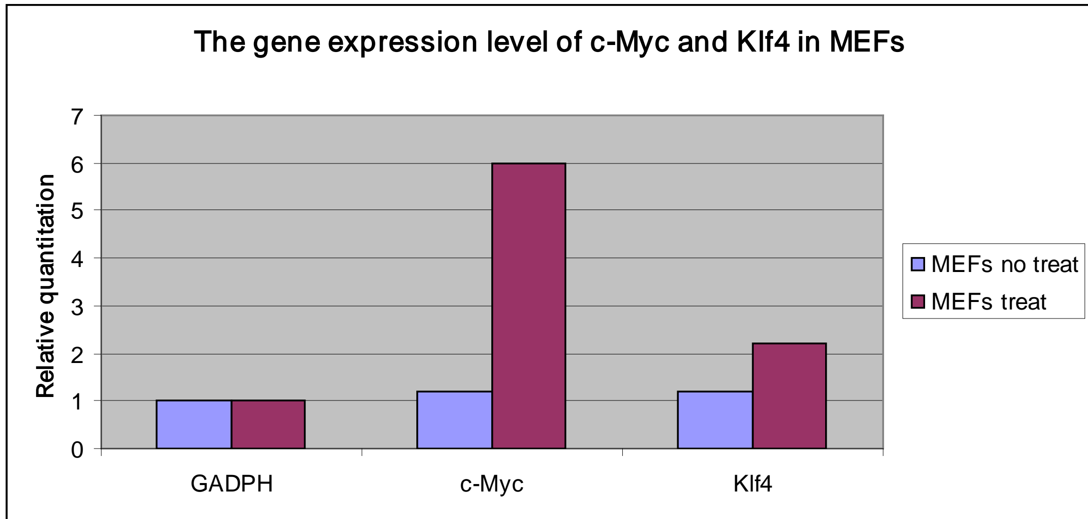


Figure 12: The expression level of c-Myc and Klf4 compared between MEFs with and without rmOct-4 protein. The c-Myc expression level was highly up-regulated in MEFs that culture in media with rmOct-4 protein whereas Klf4 was not different in both conditions. Then we confirmed these results by quantitative Real-time PCR (Figure 12A-B).

7. Nanog-luciferase reporter assay

From the previous studies, Oct-4 protein is essential for maintenance of pluripotency in embryonic stem cells (ESC). By interaction with Sox2, Oct-4 has been reported to regulate pluripotent specific gene expression, such as Nanog. So we tested whether recombinant mOct-4 has ability to activate Nanog expression *in vitro* using Luciferase reporter assay. We determined the level of Nanog promoter reactivation in 5 condition cultures :

Condition I : Transfect the reporter plasmids (1 ug) containing the firefly luciferase gene into MEFs with 50 ng of pRL-TR (Promega) in Figure 13A.

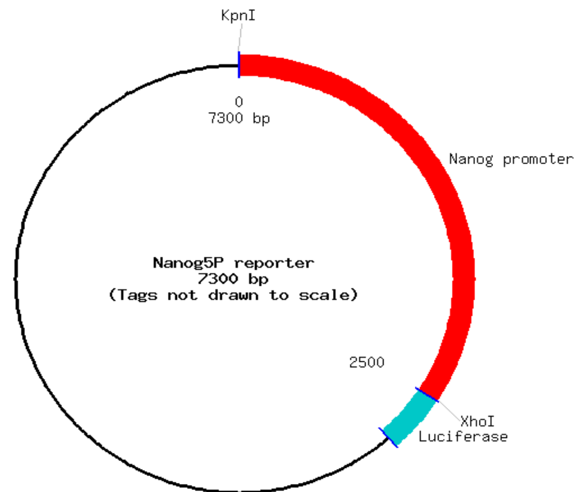
Condition II : Transfect the pMXs-mOct4 plasmid (1 ug) into MEFs

Condition III : Recombinant mOct-4 protein treatment (10 ug/ml)

Condition IV : Transfect the pMXs-mSox2 plasmid (1 ug) into MEFs

Condition V : Transfect the pMXs-mSox2 plasmid (1 ug) into MEFs combined with Recombinant mOct-4 protein treatment (10 ug/ml)

A.



B.

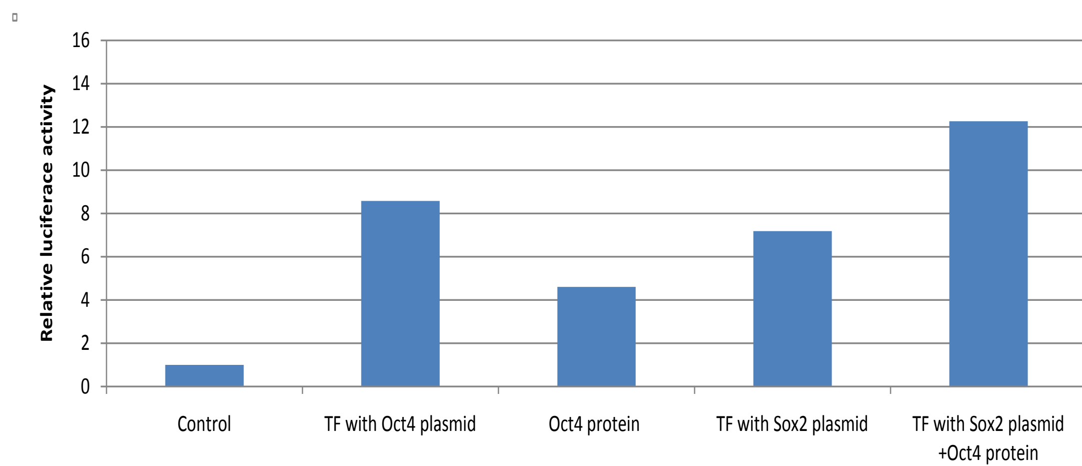


Figure 13: The level of Nanog promoter reactivation in MEFs.

(A) Nanog promoter-LUC Map and Features.

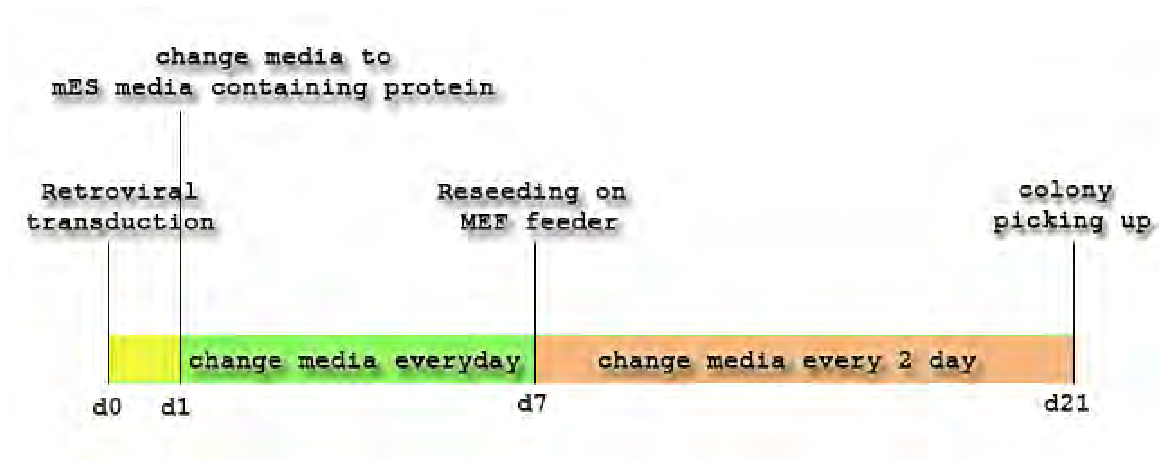
(B) Effect of recombinant mOct-4 protein on luciferase activity in MEFs.

We found that recombinant mOct-4 protein shown to increase luciferase activity more than 4-folds compare to control. Moreover, combination of Sox2 transfection with recombinant mOct-4 protein enhances luciferase activity to 12-folds. These results suggested that recombinant mOct-4 protein can activate Nanog luciferase reporter (Figure 13B).

8. Generation of induced pluripotent stem (iPS) cells

To tested ability of recombinant protein when combined with three retroviral transduction to generate iPS colony. In an initial series of experiments, we seeded at a density of $5X10^5$ MEFs per 100 mm dish and incubated with virus-containing supernatant for the three factors (1:1:1). Next, 24 hours after infection, we changed media to ESC completed medium supplement with 10^3 U/ml mLIF and 10 ug/ml rmOct-4 or rmSox2 protein for 1 week (change media everyday). Seven days after that the cells were transfered onto feeder layers of mitomycin C-treated MEFs. These cells were incubated in ESC completed medium supplement with 10^3 U/ml mLIF and 10 ug/ml rmOct-4 or rmSox2 protein for 2 up to 4 weeks (change media every 2 day). During cultures the cells were observed the morphological changes by inverted-microscope every day until colonies emerged around day 14-21. Next, the colonies were selected for expansion and characterization as shown in Figure 14A.

A.



We found that using recombinant mOct-4 protein combined with retrovirus Sox2, c-Myc and Klf4 transduction could be able to generate iPS colony at day 14 after reseeding on MEF feeder, while another condition (no treat rmOct4 protein at day 7 after infection) not appear colony. In the same way, recombinant mSox2 protein combined with retrovirus Oct-4, c-Myc and Klf4 transduction could be able to generate iPS colony but the morphology quite different from mES colony.

B.

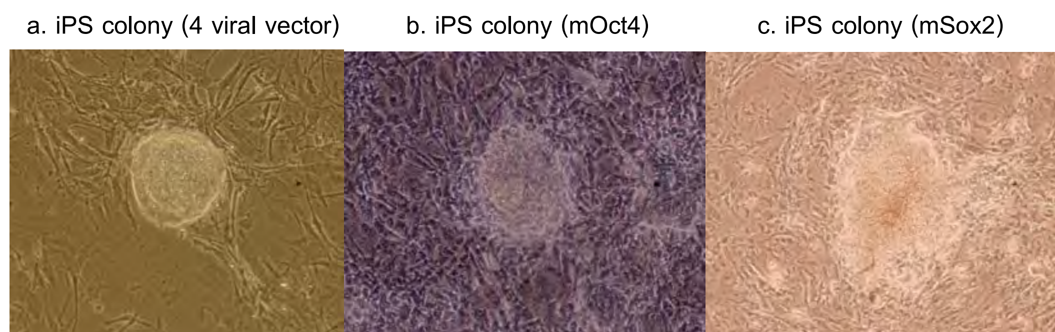


Figure 14: Generation of iPS cells from unmodified MEFs cultures by direct delivery reprogramming protein fused with TAT trans-activator domain. (A) The schematic protocol and timeline for generating iPS cells from unmodified MEFs. (B) Morphology of ES-like colonies on day 14 after reseeding on MEF-feeder. We found that the morphology of iPS colonies derived from MEFs that treated with rmOct4 protein (b) were similar to ESC colonies (a) but the iPS colonies derived from MEFs that treated with rmSox2 protein exhibited flat and rough shape (c).

Table 1: Primers Used in This Study.

Primer	Sequence	Purpose
cTAT_F cTAT_R	CATGTACGGTCGTAAAAAACGTCGTCAGCGTCGTCGTGGTG GATCCACCACGACGACGCTGACGACGTTTTTTACGACCGTACATGGTAC	RT-PCR for cloning *TAT*
pTAT_F pTAT_R	TACGGTCGTAAAAAACGTCGT TCCACCACGACGACGCTGACG	RT-PCR for *TAT*
cOct4_F cOct4_R	GCCG**GAATTC**CACC**ATG**GTCCCTAGGTGAGCCGTC GCCG**CTCGAG**CGGTTTGAATGCATGGGAGA	RT-PCR for cloning *Oct3/4*
pOct4_F pOct4_R	TCT TTC CAC CAG GCC CCC GGC TC TGC GGG CGG ACA TGG GGA GAT CC	RT-PCR for endogenous *Oct-4*
cSox2_F cSox2_R	GCCG**GAATTC**CACC**ATG**GCCCGCATGTATAACATG GCCG**CTCGAG**CGGGGCAGTGTGCCGTTAAT	RT-PCR for cloning *Sox2*
pSox2_F pSox2_R	TCA CAT GTG CGA CAG GGG CAG TAG AGC TAG ACT CCG GGC GAT GA	RT-PCR for endogenous *Sox2*
pNanog_F pNanog_R	CAG GTG TTT GAG GGT AGC TC CGG TTC ATC ATG GTA CAG TC	RT-PCR for endogenous *Nanog*
mGAP_F mGAP_R	CCAAGGAGTAAGAAACCCTGGA CGAGTTGGGATAGGGCCTCT	RT-PCR for *GAPDH*
pKlf4_F pKlf4_R	GCG AAC TCA CAC AGG CGA GAA ACC TCG CTT CCT CTT CCT CCG ACA CA	RT-PCR for endogenous *Klf4*
pMyc_F pMyc_R	CAG AGG AGG AAC GAG CTG AAG CGC TTA TGC ACC AGA GTT TCG AAG CTG TTC G	RT-PCR for endogenous *c-Myc*

CHAPTER V

DISCUSSION AND CONCLUSION

In our study, we demonstrate the generation of recombinant mOct-4 and mSox2 protein, containing TAT-transduction domain that can pass through somatic cell membrane when added to the culture media within the suitable concentration range (10 ug/ml). We demonstrated that treating MEFs with recombinant mOct-4 protein can function to induce pluripotent gene expression (*c-Myc* and *Klf4*) as shown by RT-PCR and quantitative real-time PCR analysis. Moreover, treating MEFs with recombinant mOct-4 protein can function to activate Oct-4 target gene as shown by Nanog reporter assay. Surprisingly, we found that using recombinant mOct-4 protein combined with retrovirus Sox2 , c-Myc and Klf4 cold be able to generate iPS colony, while another condition (no treat rmOct-4 protein at day 7 after infection) not appear colony. In the same way, recombinant mSox2 protein combined with retrovirus Oct-4, c-Myc and Klf4 transduction could be able to generate iPS colony but morphology quite different from mES colony. These results suggest that the recombinant mOct-4 protein is active and may be used to substitute viral vector for iPS cell generation. However, the efficiency of iPS generation is lower using this protein transduction protocol, compared to virus-base protocol.

Notably, the concentration of the purified-protein used in the present study was stable (10 ug/ml) throughout experiments, thus suggesting that purified reprogramming protein may be easily controlled to study the reprogramming process and how to work of each reprogramming factors, while Kim's group (109) used the whole-protein extracts. Recently, Ding's group (110) reported the generation of mouse iPS by combining the use of recombinant reprogramming proteins and small molecule valproic acid. In this study, we generated mouse iPS with direct delivery of reprogramming protein in the absence of any chemical treatment. In addition, we used reprogramming proteins expressed in mammalian system, while Ding's group used refolded proteins after expression in *E.coli*. The benefits and limitations of three Protein-based iPS cells studies as shown in Table 2.

Table 2. The benefits and limitations of three Protein-based iPS cells studies

Sheng Ding and et.al Cell Stem Cell (2009)	Dohoon Kim and et.al. Cell (2009)	In our study ……
➢ Bacterial system : Used refolded proteins after expression in *E*.coli	➢ Mammalian system : Used recombinant proteins expressed in HEK293 cell lines	➢ Mammalian system : Used recombinant proteins expressed in Insect cell lines
➢ Poly-arginine 11R domain	➢ TAT *trans*-activator domain	➢ TAT *trans*-activator domian
➢ In the present of VPA	➢ In the absence of small molecules	➢ In the absence of small molecules
➢ Not study in human cells	➢ Both study in mouse and human cells	➢ Not proof in mouse cells
➢ Purified extract proteins : Used combining the 4 factors with VPA	➢ Cruded extract protins : Used combining the 4 factors without small molecule	➢ Purified extract protein Used only one factor with 3 retroviral transduction

Recently, some investigators have attempted to use chemicals as substitutes for reprogramming factors, in order to eliminate the integration of viral vector into genome. Now two groups of scientists have identified small molecules that enhance reprogramming efficiency and replace at least one of the reprogramming factors. The first group, Sheng Ding and Hans Scholer (111), worked on neural progenitor cells (NPCs) that express high levels of Sox2. These cells could be reprogrammed with the addition of only two genes, *Oct4 and Klf4* but very low rate. They also found that the MEK inhibitor PD0325901 could inhibit growth of nonreprogrammed cells while boosting growth of reprogrammed cells.

Another groups, Dong Melton and co-worker, reported that the 5-aza-cytidine (AZA), DNA methyltransferase inhibitor could induce a rapid and stable transition to a fully reprogramming (112-115). The valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, enables reprogramming of primary human fibroblasts with just two transcription factors, Oct4 and Sox2.

All of above knowledges and our results suggest that it may be possible to reduce the number of retroviral vector generated mouse and human iPS cells by combining the used of recombinant reprogramming proteins and small molecule(s), in order to making therapeutic use of reprogrammed cells safer and more practical and lead to a better understanding of nuclear reprogramming resulting that it will provide great benefits to many patients.

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APPENDIX

APPENDIX

REFERENCE SEQUENCES OF MOUSE OCT4

LOCUS NM_013633 1346 bp mRNA linear ROD 20-JAN-2008

DEFINITION Mus musculus POU domain, class 5, transcription factor 1 (Pou5f1), mRNA.

ACCESSION NM_013633

VERSION NM_013633.2 GI:125490391

```
   1 aaccgtccct aggtgagccg tctttccacc aggcccccgg ctcggggtgc ccaccttccc
  61 catggctgga cacctggctt cagacttcgc cttctcaccc ccaccaggtg ggggtgatgg
 121 gtcagcaggg ctggagccgg gctgggtgga tcctcgaacc tggctaagct tccaagggcc
 181 tccaggtggg cctggaatcg gaccaggctc agaggtattg ggatctcccc catgtccgcc
 241 cgcatacgag ttctgcggag ggatggcata ctgtggacct caggttggac tgggcctagt
 301 cccccaagtt ggcgtggaga ctttgcagcc tgagggccag gcaggagcac gagtggaaag
 361 caactcagag ggaacctcct ctgagccctg tgccgaccgc cccaatgccg tgaagttgga
 421 gaaggtggaa ccaactcccg aggagtccca ggacatgaaa gccctgcaga aggagctaga
 481 acagtttgcc aagctgctga agcagaagag gatcaccttg gggtacaccc aggccgacgt
 541 ggggctcacc ctgggcgttc tctttggaaa ggtgttcagc cagaccacca tctgtcgctt
 601 cgaggccttg cagctcagcc ttaagaacat gtgtaagctg cggcccctgc tggagaagtg
 661 ggtggaggaa gccgacaaca atgagaacct tcaggagata tgcaaatcgg agaccctggt
 721 gcaggcccgg aagagaaagc gaactagcat tgagaaccgt gtgaggtgga gtctggagac
 781 catgtttctg aagtgcccga agccctccct acagcagatc actcacatcg ccaatcagct
 841 tgggctagag aaggatgtgg ttcgagtatg gttctgtaac cggcgccaga agggcaaaag
 901 atcaagtatt gagtattccc aacgagaaga gtatgaggct acagggacac ctttcccagg
 961 gggggctgta tcctttcctc tgcccccagg tccccacttt ggcaccccag gctatggaag
1021 cccccacttc accacactct actcagtccc ttttcctgag ggcgaggcct ttccctctgt
1081 tcccgtcact gctctgggct ctcccatgca ttcaaactga ggcaccagcc ctccctgggg
1141 atgctgtgag ccaaggcaag ggaggtagac aagagaacct ggagctttgg ggttaaattc
1201 ttttactgag gagggattaa aagcacaaca ggggtggggg gtgggatggg gaaagaagct
1261 cagtgatgct gttgatcagg agcctggcct gtctgtcact catcattttg ttcttaaata
1321 aagactggga cacacagtag atagct
```

REFERENCE SEQUENCES OF MOUSE SOX2

LOCUS NM_011443 2457 bp mRNA linear ROD 27-JAN-2008

DEFINITION Mus musculus SRY-box containing gene 2 (Sox2), mRNA.

ACCESSION NM_011443

VERSION NM_011443.3 GI:127140985

```
   1 ctattaactt gttcaaaaaa gtatcaggag ttgtcaaggc agagaagaga gtgtttgcaa
  61 aaagggaaaa gtactttgct gcctctttaa gactagggct gggagaaaga agaggagaga
 121 gaaagaaagg agagaagttt ggagcccgag gcttaagcct ttccaaaaac taatcacaac
 181 aatcgcggcg gcccgaggag gagagcgcct gtttttttcat cccaattgca cttcgcccgt
 241 ctcgagctcc gcttcccccc aactattctc cgccagatct ccgcgcaggg ccgtgcacgc
 301 cgaggccccc gcccgcggcc cctgcatccc ggcccccgag cgcggccccc acagtcccgg
 361 ccgggccgag ggttggcggc cgccggcggg ccgcgcccgc ccagcgcccg catgtataac
 421 atgatggaga cggagctgaa gccgccgggc ccgcagcaag cttcgggggg cggcggcgga
 481 ggaggcaacg ccacggcggc ggcgaccggc ggcaaccaga agaacagccc ggaccgcgtc
 541 aagaggccca tgaacgcctt catggtatgg tcccgggggc agcggcgtaa gatggcccag
 601 gagaacccca agatgcacaa ctcggagatc agcaagcgcc tgggcgcgga gtggaaactt
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 721 atgaaggagc acccggatta taaataccgg ccgcggcgga aaaccaagac gctcatgaag
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 841 ggggttgggg tgggcgccgg cctgggtgcg ggcgtgaacc agcgcatgga cagctacgcg
 901 cacatgaacg gctggagcaa cggcagctac agcatgatgc aggagcagct gggctacccg
 961 cagcacccgg gcctcaacgc tcacggcgcg gcacagatgc aaccgatgca ccgctacgac
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1081 acctacagca tgtcctactc gcagcagggc acccccggta tggcgctggg ctccatgggc
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1441 aaagaggaga gtaggaaaaa tctgataatg ctcaaaagga aaaaaaatct ccgcagcgaa
1501 acgacagctg cggaaaaaaa ccaccaatcc catccaaatt aacgcaaaaa ccgtgatgcc
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2101 aaaggcaaaa aaaaaatgtc catgcaggtt gatatcgttg gtaatttata atagcttttg
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2221 ttttcttatg gtttgtaata tttctgtaaa ttgtgatatt ttaaggtttt tccccccttt
2281 tattttccgt agttgtattt taaaagattc ggctctgtta ttggaatcag gctgccgaga
2341 atccatgtat atatttgaac taataccatc cttataacag ctacattttc aacttaagtt
2401 tttactccat tatgcacagt ttgagataaa taaattttg aaatatggac actgaaa
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REFERENCE SEQUENCES OF MOUSE KLF4

LOCUS NM_010637 2905 bp mRNA linear ROD 18-NOV-2007

DEFINITION Mus musculus Kruppel-like factor 4 (gut) (Klf4), mRNA.

ACCESSION NM_010637

VERSION NM_010637.2 GI:142369926

1 gccaagagag cgagcgcggc tccgggcgcg cggggagcag aggcggtggc gggcggcggg
61 ggcacccgga gccgccgagt gcccctcccc gcccctccag ccccccaccc aggaacccgc
121 ccgtgacccg cgcccatggc cgcgcgcacc cggtacagtc cccaggactc cgcaccccgc
181 gccaccgtcc agctcgcagt tccgcgccac cgcggccatt ctcacctggc ggcgccgccc
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301 ggggacactg ctgagtccaa gagcgtgcag cctggccatc ggacctactt atctgccttg
361 ctgattgtct atttttataa gagtttacaa cttttctaag aatttttgta tacaaaggaa
421 cttttttaa agacatcgcc ggtttatatt gaatccaaag aagaaggatc tcgggcaatc
481 tgggggtttt ggtttgaggt tttgtttcta aagtttttaa tcttcgttga ctttggggct
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601 gcagccacct ggcgagtctg ac**atg**gctgt cagcgacgct ctgctcccgt ccttctccac
661 gttcgcgtcc ggcccggcgg gaagggagaa gacactgcgt ccagcaggtg ccccgactaa
721 ccgttggcgt gaggaactct ctcacatgaa gcgacttccc ccacttcccg gccgccccta
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901 cctagacttt atcctttcca actcgctaac ccaccaggaa tcggtggccg ccaccgtgac
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2221 tccaagagac aaaacagaaa tactaaaaac aaacaaacaa aaaaacaaac aaaaaaacca
2281 agaaaaaaaa atcacagaac agatggggtc tgatactgga tggatcttct atcattccaa
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2581 atgttctaac agcctaaatg atggtgcttg gtgagttgtg gtcctaaagg taccaaacgg
2641 gggagccaaa gttctccaac tgctgcatac ttttgacaag gaaaatctag ttttgtcttc
2701 cgatctacat tgatgaccta agccaggtaa ataagcctgg tttatttctg taacattttt
2761 atgcagacag tctgttatgc actgtggttt cagatgtgca ataatttgta caatggttta
2821 ttcccaagta tgcctttaag cagaacaaat gtgtttttct atatagttcc ttgccttaat
2881 aaatatgtaa tataaattta accca

REFERENCE SEQUENCES OF MOUSE MYC

LOCUS NM_010849 2399 bp mRNA linear ROD 20-JAN-2008

DEFINITION Mus musculus myelocytomatosis oncogene (Myc), mRNA.

ACCESSION NM_010849

VERSION NM_010849.4 GI:100913213

1 cccgcccacc cgccctttat attccggggg tctgcgcggc cgaggacccc tgggctgcgc
61 tgctctcagc tgccgggtcc gactcgcctc actcagctcc cctcctgcct cctgaagggc
121 agggcttcgc cgacgcttgg cgggaaaaag aagggagggg agggatcctg agtcgcagta
181 taaaagaagc ttttcgggcg tttttttctg actcgctgta gtaattccag cgagagacag
241 agggagtgag cggacggttg gaagagccgt gtgtgcagag ccgcgctccg gggcgaccta
301 agaaggcagc tctggagtga gaggggcttt gcctccgagc ctgccgccca ctctccccaa
361 ccctgcgact gacccaacat cagcggccgc aaccctcgcc gccgctggga aactttgccc
421 attgcagcgg gcagacactt ctcactggaa cttacaatct gcgagccagg acaggactcc
481 ccaggctccg gggagggaat ttttgtctat ttggggacag tgttctctgc ctctgcccgc
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601 tggaaacccc gcagacagcc acgacgatgc ccctcaacgt gaacttcacc aacaggaact
661 atgacctcga ctacgactcc gtacagccct atttcatctg cgacgaggaa gagaatttct
721 atcaccagca acagcagagc gagctgcagc cgcccgcgcc cagtgaggat atctggaaga
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1621 tcctgaagca gatcagcaac aaccgcaagt gctccagccc caggtcctca gacacggagg
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2281 cagttacaaa agattttaag acatgtacca taattttttt tatttaaaga cattttcatt
2341 tttaaagttg atttttttct attgttttta gaaaaaaata aaataattgg aaaaaatac

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