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發展正電性奈米粒子作為 Sox2, Klf4, Oct4 及 c-Myc 蛋白質載體製造人類誘導多能幹細胞之研究

Development of cationic nanoparticles with Sox2, Klf4, Oct4 and c-Myc proteins in induced pluripotent stem cells' generation

夏瑞景

Jui-Ching Hsia

指導教授：陳彥榮 博士

Advisor：Yen-Rong Chen, Ph.D.

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本論文係 夏瑞景 君（學號 R01B22020）在國立臺灣大學生化科技學系完成之碩（博）士學位論文，於民國 103 年 7 月 4 日承下列考試委員審查通過及口試及格，特此證明

口試委員：

陳彥華 （簽名）

（指導教授）

黃義侑

江宏仁

系主任、所長 黃慶璨 （簽名）

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中文摘要

人類誘導多能性幹細胞 (induced pluripotent stem cells, iPS cells) 是一種將體細胞再程序化後產生的人造幹細胞，其特點在於將已分化的細胞退分化回具有分化能力的幹細胞，改變了過往認為分化只能是單行道的概念。目前最主要的人類誘導多能性幹細胞製造方法是山中伸彌博士於 2006 年所發展，利用帶有四因子 (Klf4, Sox2, Oct4 以及 c-Myc) 的反轉錄病毒感染體細胞再程序化而成。自該研究之後，許多藉由 DNA 嵌入宿主基因體來表現四因子，進一步將體細胞再程序化成為人類誘導多能性幹細胞的方式陸陸續續被發展出來，這些以 DNA 作為因子運送載體的人類誘導多能性幹細胞也被稱為第一代人類誘導多能性幹細胞。

然而，雖然以 DNA 做為四因子運送載體其再程序化的效率比其他方法來的高，此種方法的最大缺點在於載體所攜帶的四因子是隨機嵌入細胞染色體中，宿主基因突變的疑慮導致了第一代人類誘導多能性幹細胞在醫療上的發展大受限制。為了解決基因嵌入的問題，利用非 DNA 的載體來製造人類誘導多能性幹細胞成為近期研究重心。這些非 DNA 的載體包括蛋白質、信使 RNA 以及微小 RNA (MicroRNA) 等，其共通特點在於再程序化的過程中不需對宿主基因體做任何更動，其誘導出來的人類誘導多能性幹細胞具有更佳的醫療應用前景。

基於前述理由，在此研究中我希望利用蛋白質作為四因子的載體送入人類包皮纖維母細胞 (HS68) 中來誘導人類誘導多能性幹細胞的產生。蛋白質具有易於大量表現、純化以及儲存的優點，其作為載體的主要障礙在於蛋白質無法主動穿過細胞膜，需藉由奈米粒子或穿膜短肽的幫助才能順利進入細胞內；因此，我利用醫工所開發的 gelatin-polyethyleneimaine (gelatin-PEI) 奈米粒子來包裹綠色螢光蛋白 (作為模式蛋白) 以及四因子,藉此將這些蛋白質送入人類纖維母細胞中，並在吞吃後藉由奈米粒子上的一級氨與二級氨作為質子海綿 (proton sponge) 協助蛋白質逃脫核內體 (endosome)。

在奈米粒子的吞吃效率測試中，可見當濃度提升到 50ug/ml 時，HS68 能夠吞吃將近九成的奈米粒子；而在細胞毒性測試中，HS68 雖會在投藥後減少約三成的細胞，卻能在三天的培養後回復到原有數量，顯示未來再程序化時反覆投藥是可行的。

而在蛋白質表現的結果中可見，不論是四因子或是綠色螢光蛋白都能成功的在大腸桿菌表現系統中表現，同時此五種蛋白質也都能利用鎳親合管柱來進行純化。在純化後的綠色螢光蛋白接受 gelatin-PEI 奈米粒子包裹後，我將兩者的混合物加入 HS68 的培養基中，發現綠色螢光蛋白以及奈米粒子能順利被細胞吞吃，且綠色螢光蛋白依舊能產生螢光。顯示利用這樣的奈米粒子去包裹蛋白質來送入細胞是可行的，對於蛋白質的損傷也是可接受的。

以上研究結果顯示未來利用 gelatin-PEI 奈米粒子來包裹四因子蛋白，藉此進行 體細胞再程序化製造人類誘導多能性幹細胞是值得嘗試的,未來這樣的方法可能成為更安全的細胞再程序化方式。

Abstract

Induced pluripotent stem cells (iPS cells) are artificial stem cells, generated by somatic cell reprogramming. In 2006, Shinya Yamanaka and Kazutoshi Takahashi investigated 24 candidates that are specifically expressed in embryonic stem cells (ES cells) as pluripotent-correlated genes, they finally found out that Klf4, Sox2, Oct4 and c-Myc, which are known as Yamanaka factors, are able to derive iPS cells from adult fibroblasts. Since then many DNA-dependent reprogramming methods have been developed, and these methods have the same problem, which hinder the clinical application of this type of iPS cells. The problem of DNA-dependent methods is uncontrollable genome integration during reprogramming process, so the following researcher focused on DNA-free reprogramming vectors, such as proteins, microRNA and mRNA. Those DNA-free methods won't modify host genome and therefore those methods are more promising in regenerative medicine area.

Based on these reasons, I used proteins as DNA-free reprogramming vectors to generate iPS cells. Proteins are easy to overexpress, purify and store up, but without specific peptide sequence or protein carrier, most proteins are unable to cross cell membrane, and hence I cooperated with Dr. Yi-You Huang and Dr. Ming-Ju Chou using their gelatin–polyethyleneimine (gelatin-PEI) nanoparticles as my protein carrier. On

the other hand, gelatin-PEI nanoparticles have many primary and secondary amines work as proton sponge, which provide high efficiency of endosomal escape.

In my research, the uptake efficiency experiment performed by flow cytometry showed that HS68 cells are able to uptake almost 90% of gelatin-PEI nanoparticle when particle concentration reach 50ug/ml. Besides, cell viability assay showed that although HS68 population will decrease after 24 hours particle application, HS68 cells will repopulate after three days, indicating that repeating protein delivery is possible.

On the other hand, I also showed that both enhanced green fluorescent proteins (eGFPs) and Yamanaka four factors can be overexpressed in *Escherichia coli* expression system, and these proteins were able to be purified by Ni-NTA affinity columns. Furthermore, after I mixed gelatin-PEI nanoparticles and eGFPs with HS68 cells, these particles are able to transport our model protein eGFPs into HS68 cell line, and transfection process won't cause severe cell death.

Overall, these data showed that gelatin-PEI nanoparticles are able to carry our model proteins, eGFPs, into human foreskin fibroblasts. I suppose that we can combine proteins and nanoparticles as reprogramming vectors, and this might be a new method to generate iPS cells

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Chapter one Introduction

1.1 Induced pluripotent stem cells: History, characteristics and further applications

Stem cells are cells that are able to renew themselves through mitotic cell division and differentiate into a diverse range of specialized cell types. Therefore, stem cells can be used in developmental research, therapies and regenerative medicine, such as cardiac cell therapy (*1*) and islet-beta cell generation (*2*). Also, stem cells are classified into several classes, including embryonic stem cells, fetal stem cells, perinatal stem cells, adult stem cells and induced pluripotent stem cells based on their origin (*3*). The self-maintenance and differentiation of stem cells are controlled not only by some specific genes but also affected by "stem cell niche", the microenvironment surrounding stem cells *in vivo* (*4*).

Before reprogramming techniques were available, it was believed that adult somatic cells would not switch fates automatically after committed to specific cell type. In 1952, Briggs and King (*5*) generated tadpoles from blastocyst nucleus with enucleated oocyte by somatic cell nuclei transfer (SCNT) technique. Later on, Gurdon (*6*) combined mature somatic cell's nucleus with enucleated oocyte by using same technique to generate adult frog. With SCNT, reprogramming is even possible for fetal

and adult mammalian cells (*7*).

On the other hand, cell-cell fusion is another method to generate dedifferentiated somatic cell before induced pluripotent stem cells were available. In 1976, Miller and Ruddle (*8*) mixed pluripotent PCC4aza1 embryonal carcinoma cells with thymocytes from young adult mice, and these mixed-up cells were able to generate tumors which contained differentiated tissues after transplanted into nude mice. After Miller and Ruddels' research, both Tada M *et al.* (*9*) and Cowan CA *et al.* (*10*) have successfully reprogrammed somatic cells with embryonic stem cells using cell-cell fusion technique. These experiments together indicated that unfertilized eggs and ES cells contain factors that can confer totipotency or pluripotency to somatic cells (*11*).

Based on this idea, Yamanaka and Takahashi have scanned 24 factors which play pivotal roles in the maintenance of ES cell identity (*11*). Eventually, they found out that by delivering some specific factors, such as Sox2, Klf4, Oct4 and c-Myc, murine fibroblasts were able to be reprogrammed back into embryonic-stem-cell-like state, and these cells are what we called iPS cells today. Compared with human embryonic stem (hES) cells, iPS cells possessed some advantages, such as ethical-issues-free, easier to obtain, and immunologically compatible when applying to disease modeling (*12*), cell therapy (*13*) and organ reconstruction (*14*).

Considering pluripotency and the ability of differentiation, ES cells are the best choice of all. But due to the ethical problem, ES cells are not convenient to obtain. From previous researches, iPS cells' genome-wide expression patterns and histone modifications have high similarity with ES cells, and they have similar differentiation behavior (*15*). In addition, the problem of epigenetic memory due to reprogramming of iPS cells generation is not as serious as SCNT and cell-cell fusion techniques (*16*).

Although iPS cells generation is a well-known techniques these days, the mechanism behind those transcription factors (that is to say, Sox2, Klf4, Oct4 and c-Myc) is still a mystery. In the experiments executed by Soufi A *et al.*, they found out that c-Myc plays an important role in OSK chromatin engagement, and low reprogramming efficiency of iPS cells might due to massive H3K9me3 in many genes required for pluripotency (*17*). Besides, p53 (*18*) and Mbd3 (*19*) were both correlated to iPS cells' reprogramming process, p53 knockout or Mbd3 knockout cells can both achieved higher reprogramming efficiency. Moreover, the process of reprogramming can be further divided into different stages (*20*), and either epithelial-to-mesenchymal transition (EMT) or mesenchymal-to-epithelial transition (MET) is required for reprogramming (*21*). These experiments indicated that reprogramming might not be a one-step process, but a series of epigenetic modification, gene activation and

inactivation processes.

In addition to the mechanism of iPS cells' reprogramming, the efficiency of factor delivery and the elimination of iPS cells' tumor-genic potential are also important during iPS cells generation, and these problems are associated with the methods used to generate iPS cells.

1.2 Factor delivery: First step in iPS cells generation

The factor delivery methods can be generally classified into two groups: DNA-dependent and DNA-free methods. With former can be further subdivided into two major categories: Integrating and non-integrating vectors (*11*, *22*). From lentiviral vectors (*11*) to retroviral vectors (*23*), non-specific genome mutation caused by these integrating vectors is the major problem during reprogramming process. Although using integrating vector is a prevalent way during the iPS cell generation, but it is still not proper when applying to clinical use (*24*).

For non-integrating vectors like piggyBac transposition (*25*) or episomal vectors (*22*), the efficiencies of iPS cells generation by both methods are much more higher than general DNA-free methods, but whether any piece of vector sequence will remain in the host genome or not is still a possible problem. Besides, transposon needs a

specific integration site, but it is still unclear whether this method would induce nonspecific genome alteration in iPS cells (*25*). As a result, the DNA-free method might be a safer way to generate iPS cells.

Since 2006, DNA-free methods including microRNA (*26*), mRNA (*24*), small molecules (*27*) and proteins (*28*) were developed rapidly. Messenger RNA or microRNA were both able to avoid genome mutation and gene insertion during reprogramming process. Nevertheless, the complex technique and the instability of RNA may limit its universalization and reprogramming efficiency. Also, even though iPS cells generated by small molecules provide promising reprogramming efficiency (about 0.2%), but the interaction between different small molecules and the reprogrammed cells is still unclear, and the reprogramming process is much more complicated than usual methods. Therefore, proteins were chosen to be the reprogramming vector in this research.

In previous researches, Hongyan Zhou *et al.* combined Yamanaka factors with poly-arginine, which was also known as cell-penetrating peptide, to deliver bare protein into the cells. These recombinant proteins were able to transform human fibroblast into ES cells-like cells (*28*). Compared with other delivering means, its efficiency is particularly low even with the addition of deacetylase (HDAC) inhibitor valproic acid

(VPA) (*28*). On the other hand, Dohoon Kim *et al.* also combined Yamanaka factors with poly-arginine tag, and successfully deliver these proteins into human newborn fibroblast (HNF). The iPS cells which them generated from these recombinant proteins were able to maintain for more than 35 passages and exhibit morphology similar to that of hES cells (*29*). However, the reprogramming efficiency of both experiment were extremely low, this phenomena indicates that there is still room for improvement, especially for protein delivery and protein protection.

Overall, DNA-free methods are able to substitute for viral vectors and become the new delivering way in the future. Nevertheless, the obstacles, such as the reprogramming efficiency, of the iPS cell generation process still need to be eliminated.

1.3 Yamanaka factors: Sox2, Klf4, Oct4 and c-Myc

SRY-related HMG box (Sox) 2 is a member of the *Sox* gene family with a single HMG DNA-binding domain. In 2000, Zappone *et al.* found out that Sox2 expression is correlated with uncommitted dividing stem and precursor cells of the developing central nervous system (*30*). Also, Avilion *et al.* showed that Sox2 marks the pluripotent lineage of the early mouse embryo. In contrast, Sox2 was down-regulated in development committed cells (*31*). These results indicated that Sox2 might play an important role in pluripotency maintenance.

Krüppel-like factors (Klfs) are transcription factors that bind to specific DNA sequences and regulate gene transcription. Klf4 is highly expressed in epithelial tissues, especially in postmitotic epithelial cells of the intestinal mucosa. This characteristic suggests that klf4 may be link to cell grow arrest (*32*). During iPS cells generation, klf4 binds to the Oct3/4-Sox2 complex to co-regulate the expression of Nanog, which is the pluripotency-defining protein (*33*), with homeobox protein PBX1.

OCT4, a POU domain protein also known as Oct3, is expressed in blastomeres, pluripotent early embryo cells and later in germ cells (*34*). In 1998, Jennifer Nichols *et al.* discovered that Oct4-deficient embryos developed to the blastocyst stage, but the inner cell mass cells were not pluripotent. Furthermore, the trophoblastic proliferation of Oct4 knockout embryos was restricted (*35*). That is to say, Oct4 plays a vital role in embryonic development and pluipotency maintenance.

c-Myc, a cellular homolog of the retroviral v-Myc oncogene (*36*), belongs to a family of helix-loop-helix/leucine zipper transcription factors. In several animal and human tumors, c-Myc proto-oncogene was found to be activated (*37*). c-Myc mutation is lethal in homozygotes between 9.5 and 10.5 days of gestation, and the embryos are smaller and retarded in development than normal ones (*38*). In 2004, Peter Cartwright *et al.* reveled that Myc expression alone can render self-renewal and maintenance of

pluripotency, even in the absence of LIF (*39*).

Overall, these Yamanaka factors are either ES cells proliferation correlated or pluripotent correlated, and participated in several different early developmental stages. These facts may be the clues that why iPS cell can be generated by applying four Yamanaka factors to adult somatic cells.

1.5 Gelatin- Polyethyleneimaine (Gelatin-PEI) nanoparticles

Biomaterials have been use in several bioengineering areas, including tissue engineering, tissue regeneration and drug delivery. By using biocompatible materials as bio-macromolecules carriers, the tolerability and bioavailability of bio-macromolecules can be significantly elevated. Those biocompatible carriers including nanoparticles, nanocapsules, micellar systems, and conjugates (*40*). The nanoparticle-based carriers possessed several advantages, such as high uptake efficiency due to sub-micron particle size, longer retention time, good surface-to-volume ratio and acceptable drug-release control (*41*). In fact, those advantages are the most important factors which can be manipulated by specific process during nanoparticles construction, including size, encapsulation efficiency, surface charge (zeta potential) and release characteristics.

In past decades, the most widely-used polymers were poly lactic acid (PLA), poly

glycolic acid (PGA), and poly lactide-co-glycolide (PLGA). The polymers described above are known for their biocompatibility and plasticity, and these polymers were all approved by Food and Drug Administration (FDA). On the other hand, natural polymers such as gelatin (*42*), chitosan (*43*), collagen (*44*) and silk fibroin (*45*) were also possible to be the ingredients of nanoparticles. These natural polymers have two major advantages, one is the various functional groups on natural polymers makes then easy to be modified depending on the needs. The second is the natural derivatives after polymer degradation are mostly amino acids and saccharides, which can be easily cleared after medical applications.

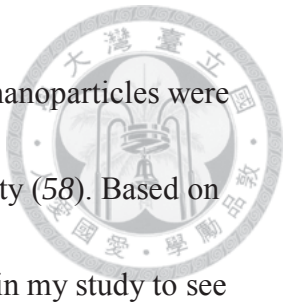
Gelatin is a natural, biocompatible (*46*) and biodegradable (*47*) polymer, which derived from animal skin white connective tissue, bones and collagen . It is wildly used in pharmaceutical products, medical products and, most important of all, as a carrier matrix due to its low cell toxicity (*48*) and capability of preserving the bioactivity of encapsulated agent in vivo (*49*).

Gelatin is obtained from partial hydrolysis of collagen, and the charge it possesses depends on the methods that were used in collagen pre-treatment. Due to the proportion of the carbonyl groups in gelatin, the polymer is positively charged in acidic solutions and negatively charged in alkaline solutions (*50*). Also, the isoeletric point (IEP) is

ranging from 4.8 to 9.4 depends on the pH value of the pre-treatment (*51*). By adjusting the charge and the IEP value, gelatin polymer will be able to attract either positively or negatively charged proteins due to ionic strength. In fact, gelatin and the albumin were the first nanoparticles for pharmaceutical applications (*52, 53*).

Polyethyleneimaine (PEI) is a synthetic cationic polymer, which is regarded as the most effective DNA carrier since 1995 (*54*). The numerous amine groups within PEI provide PEI ability to interact with negatively charged drugs and biomolecules, including DNA and proteins (*55*). Besides, these amine groups provide several reactive sites, and therefore a wide range of chemical modifications are possible. During biomolecules delivery, amine groups were believed to act as proton sponge which can help biomolecules escape from endosomes, and cationic polymer may induce nanoscale holes on cell membrane which increase the permeability of the membrane (*56*). The amount of primary and secondary amines was indicated to be the most important factor for transfection efficiency and cytotoxicity (*57*), so it is important to find the balance between these two.

In this research, I cooperated with Dr. Yi-You Huang and his lab by using their gelatin-PEI nanoparticles as protein carriers. In 2010, Wei-Ti Kuo *et al.* developed a gelatin (1.8kDa)-PEI nanocarries with high positive ζ potential and buffering effect.



And with specific nanoparticles-to-proteins ratio (30:1), gelatin-PEI nanoparticles were able to deliver 2.12×10^4 RLU/μg protein with acceptable cell viability (*58*). Based on this research, gelatin-PEI nanoparticles were used as protein carriers in my study to see whether it can deliver Yamanaka factors into human fibroblasts or not, and if so, target cells might be able to be reprogrammed into iPS cells.

1.6 Research aims

The first generation of iPS cells were generated by using constitutively active viral vectors that can integrated into the host genome, but gene insertion might cause genome instability, which limited the possibility of iPS cells in clinical usage. Also, iPS cells were able to be derived via transposon, transient plasmid, episomal or adenovirus, but the problem of insertion mutagenesis and the complex operation, such as sequential selection and vector construction during generation process remains to be solved. (*25, 59-62*)

To solve these problems, I try to deliver proteins of four Yamanaka factors (Sox2, Klf4, Oct4 and c-Myc) into somatic cells with gelatin-PEI nanoparticles which generated by Dr. Yi-You Huang and his group. Dr. Yi-You Huang and his research group mainly focused on tissue reconstruction and drug delivery with several different

nanoparticles, such as liposome and polymersome in the past. I wanted to combine their techniques in biomolecule delivery with iPS cells generation, hoping to overcome the disadvantage of DNA-dependent methods we mentioned above. It is possible that by using proteins with nanoparticles as delivery vectors, the genome integrity and cell uptake efficiency would be improved largely compared with DNA-dependent methods. And, application of this technique to tissue reconstruction might provide a possible therapeutic use in the future.

Chapter two Materials and methods

2.1 Cell culture

2.1.1 Cell line

- HS68 (ATCC® CRL-1635™)

  HS68 cell line is a kind of human foreskin fibroblast cell line, and HS68 cell line's donor was possibly suffered Canavan disease, a deficiency in aspartoacyclase. In this research, HS68 cell line was bought from Bioresource Collection and Research Center, and this cell line is going to be reprogrammed by Yamanaka factors.

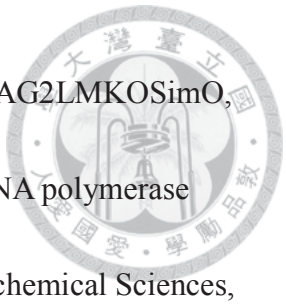
2.1.2 Cell culture condition

HS68 cells were cultured and transfected in Dulbecco's modified eagle medium (Life technologe) with 10% fetal bovine serum (Hyclone) and 3.7 g/l Sodium hydrogen carbonate. HS68 cells were culture in 10 cm cell dish (Thermo) at 37℃.

2.2 Plasmid construction

2.2.1 Plasmid

The following plasmids were used in protein overexpression in *E.coli* strain BL21: pETDUET-1-Sox2, pETDUET-1-Klf4, pETDUET-1-Oct4, pETDUET-1-c-Myc and pETDUET-1-eGFP. Due to frame shift, adaptors were design for Sox2, Klf4, Oct4 and c-Myc as Table.1.



Sox2, Klf4, Oct4 and c-Myc sequences were amplified from pCAG2LMKOSimO, and eGFP was amplified from pEGFP-C1 (Clontech) by LA taq^{TM} DNA polymerase (Takara). pETDUET-1 (Novagen) was obtained from Institute of Biochemical Sciences, National Taiwan University. Cloning primers were designed as Table.1. Sox2, Klf4, Oct4 and c-Myc were inserted by restriction site EcoRI (C-terminal) and NotI (N-terminal). eGFP was inserted by restriction site NotI on both ends.

2.2.2 Polymerase chain reaction (PCR) condition

Polymerase chain reaction was performed with following condition: LA Taq^{TM} DNA Polymerase (Takara, 1%) was mixed with 10x LA PCR buffer (10%), dNTP mix (16%), Mg^{2+} (10%), template DNA (4%), each primer (2%) and ddH_2O up to total value as 50µl.

2.2.3 Gel filtration

After PCR, DNA product was analyzed by 1% TAE agarose gel. DNA was further purified with Gel/PCR DNA Fragments Extraction Kit (Geneaid) following by kit's protocol. In brief, 500 µl DF buffer was added into each tubes which contained gel less than 300 mg, then incubated at 60°C for 15 minutes. Then DF column was placed in a new collection tube. The sample was transferred to the DF column 800µl each time, then centrifuged at 15000 rpm for 30 seconds. Discard the solution in the collection

tube. Added 400 μl W1 buffer into DF column and centrifuged with same condition again. Discard the solution in the collection tube then add 600 μl wash buffer. The DF column was centrifuged at same condition and discarded the solution in the collection tube again. The empty column was centrifuged at 15000 rpm for 3 minutes. The DF column was moved to a new tube, then added 30 μl ddH2O and stood at room temperature for 2 minutes. And the tube was centrifuged at 15000 rpm for 2 minutes. The DNA sample was preserved at 4°C.

2.2.4 Restriction enzyme digestion

Purified DNA inserts and pETDUET-1 were cleavage with EcoRI-HF® (New England Biolabs) and NotI-HF® (New England Biolabs) for 2 hours at 37°C. Reaction mixture contained NEBuffer (10%), restriction enzyme (1%), template (up to 5 μg) and ddH_2O. After restriction process, the reaction mixture was placed in dry bath at 65°C for 30 minutes to eliminate enzyme activity.

2.2.5 Competent cell preparation

For plasmid preparation and protein overexpression, DH5α and BL21 competent cells were prepared according to the Molecular Cloning (Sambrook, 2001). The E. coli cultured from a single colony were incubated at 18°C for about 40 hours to reach absorbance of 0.4-0.8 at O.D. 600 nm. Let the culture medium stand on ice for 10

minutes. Medium was centrifuged at 3600 rpm under 4°C for 10 minutes. Added 1/3 total volume transformation buffer (10mM PIPES, 15mM $CaCl_2 \cdot 2H_2O$, 250mM KCl, 55mM $MnCl_2 \cdot 4H_2O$, pH 6.7) then let the tube stand on ice for another 10 minutes. Centrifuged at 3600 rpm under 4°C for 10 minutes. Added transformation buffer (8% total medium volume) and DMSO (Bioman, 7 % transformation buffer volume) into the pellet and resuspended. Then let liquid nitrogen freeze the sample and stored in -80°C.

2.2.6 Ligation and transformation

The insert and pETDUET-1 were mixed with 2 μl ligation high ver.2 (Toyobo) at 16°C for 30 minutes, eGFP and pETDUET should both be treated with shrimp alkaline phosphatase (sAP) first to prevent self-ligation. After ligation, I added 50 μl DH5α competent cell into the ligation solution and put the mixture on ice stood for 10 minutes. Then the tube was incubated at 37°C for 3 minutes. Let it stood on ice for another 2 minutes. 50 μl SOB (2% bacto tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5mM KCl, 10mM Mg^{2+} added after autoclaved) was added into the tube and the transformed cells were recovered for 30 minutes at 37°C. Transformed cells were further spread out on the LB plate with ampicillin (5% tryptone, 2.5% yeast extract, 5% NaCl, 2% 1N NaOH, 1.5% agarose, 100 μg/ml ampicillin) and cultured at 37°C for 14~16 hours.

2.3 Plasmid mini-preparation

The proper colony was picked up and cultured in up to 2 ml Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract and 5 g sodium chloride in 1 liter dH_2O) with ampicillin at 37°C for 14~16 hours. Then the medium and the cells were centrifuged at 15000 rpm for 5 minutes. After the supernatant was discarded, 150 μl MP I solution (25 mM Tris-HCl, 10 mM EDTA, pH 8.0, 50 mM glucose, RNase μg/ml) was added to resuspend cell pellet. Then 150 μl MP II (0.2N NaOH, 1% SDS, freshly prepared) solution was added to lysis pellet and immediately followed by adding 150 μl MP III (3M potassium acetate solution, pH 5.2) solution III to neutralize the solution. The mixture was centrifuged at 15000 rpm for 5 minutes and transferred the supernatant to the new tube twice. Isopropanol (KANTO KAGAKU, 0.7x total volume) was added then the mixture was centrifuged at 15000 rpm for 15 minutes. The supernatant was discarded then 250 μl 80% alcohol was added to wash the DNA pellet. The mixture was centrifuged at 15000 rpm for 5 minutes and the supernatant was discarded again. 250 μl 80% alcohol was added to wash again and the mixture was centrifuged again. The DNA sample was dissolved in 20 μl ddH_2O and stored at -20°C.

2.4 Plasmid midi preparation

The proper colony was picked up and cultured in 50 ml LB broth with ampicillin at

37°C for 14~16 hours. The cultured medium was transferred into the 50 ml centrifuge tube and centrifuged at 3000 rpm for 10 minutes under 4°C, then the supernatant was discarded. Preparation procedures were referred to HiPure Plasmid Midiprep Kit's protocol (Invitrogen). The sample was then added Suspension Buffer (RNase added), Lysis Buffer, and chilled Neutralization Buffer, 4 ml each. The flow-through was then transferred into the column which was equilibrated by 10 ml Equilibrium Buffer. Discard flow-through and washed the column by 10 ml Wash Buffer twice.

Then the column was put onto a new 15 ml centrifuge tube after washing. 5 ml elution Buffer was added into the column to elute the plasmid, and the plasmid was precipitated by adding 3.5 ml isopropanol into the centrifuge tube. The tube was then centrifuged at 9000 rpm for 30 minutes under 4°C, and the supernatant was further discarded. DNA pellet was washed by 1 ml chilled 70% ethanol, then the sample was centrifuged at 12000 rpm for 10 minutes under 4°C.The sample was later transferred into a new microcentrifuge tube and the washing process should be repeated at least two times. Let the plasmid pellet stood for 30 minutes before dissolved by 30 μl TE-8.0 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

2.5 Total protein extraction.

Transformed BL21 and Rosetta™ competent cells (Novagen) were cultured in 50

ml LB broth with ampicillin under 37°C until O.D. 600 reached 0.4~0.6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was later added into the culture to a final concentration of 1mM and cells were cultured at 27°C for several hours (depends on the experiment). The medium was later transferred into a 15ml centrifuge tube and spun at 6000 rpm for 10 minutes under 4°C. Then we discarded the supernatant and added 5 ml lysis buffer (50 mM $Na_3PO_4 \cdot 12H_2O$, pH 7.0, 0.1 M NaCl, 0.1 mM EDTA, 0.2% Triton X-100, added and 20 μg/ml Roche cOmplete protease inhibitor cocktail before use) to resuspend the pellet. Cells were destructed by sonication (Misonix Sonicator 3000). During the whole sonication process, the sample should be placed on ice. The lysate was later transferred into microcentrifuge tubes and spun at 12000 rpm for 20 minutes under 4°C. Collect both supernatant and pellet separately, then the supernatant should be filtrated with 0.45 μm PVDF filter (Millipore) before SDS-PAGE and affinity chromatography.

2.6 SDS-PAGE and CBR staining.

The protein samples were mixed with 5x sample buffer (250 mM Tris-HCl, pH 6.8, 10% SDS, 0.5% (w/v) bromophenol, 50% glycerol, 5% β-mercaptoethanol) and placed on dry bath incubator (Major Science) under 100°C for 10 minutes. The SDS-polyacrylamide gel (10% separation gel and 4% stacking gel) was placed in the tank

filled with TGS buffer (50 mM Tris-HCl, pH 8.3, 380 mM Glycine, 0.1% SDS). The gel was further stained by CBR solution (1 g coomassie brilliant blue R-250 [Sigma B-0149] dissolved in 10% glacial acetic acid and 20% methanol) for 30 minutes and the stained gel was destained by destain buffer (10% glacial acetic acid, 20% methanol).

2.7 Western analysis

PVDF membrane (Roche) and filter papers (Blot Absorbent Filter Paper [extra thick], Bio-Rad) were rinsed with western transfer buffer (25 mM Tris-HCl, 192 mM Glycine, pH 8.3). The membranes and gel should be placed on platinum anode of the cell (Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell, Bio-Rad) in following arrangement: filter paper, PVDF membrane, SDS-PAGE gel, and filter paper. The current should be set at 0.06~0.18 A (3 mA/cm^2, depends on the membrane size) during the transferring and the membrane should be transferred for 1 hour.

After protein transfer, TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) was used to wash the membrane twice. The membrane would later be blocked with blocking buffer (5% steam milk dissolved in TBST) for 1 hour. The membrane should be washed with TBST thrice (10 minutes each) after blocking, then the membrane was soaked in 1^{st} antibody solution, which would be anti-His mouse monoclonal antibody (Santa Cruz) or anti-eGFP rabbit polyclonal antibody (Bioman), at

room temperature for an hours. Membrane was washed with TBST three times after 1st antibody application, and the membrane was soaked in 2nd antibody solution (Anti-mouse goat antibody conjugated with horseradish peroxidase [Jackson 115-035-075] or anti-rabbit goat antibody conjugated with horseradish peroxidase [Jackson 111-035-003]) for an hour. The membrane should be washed with TBST three times after 2nd antibody application and then rinsed with HRP substrate (Millipore WBLUC0500) for 1 minute. Use ddH_2O to wash the membrane and then the membrane was imaged by BioSpectrum 2D Imaging System (UVP BioSpectrum 800).

2.8 Affinity chromatography.

Ni-NTA spin column (Qiagen 31314) and His-Trap gravity column (GE) were used to purify 6x His fusion proteins, and the experiments were performed according to each column's protocol. For Ni-NTA spin columns, the Ni-NTA spin column was equilibrated with 600 μl NPI-10 buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl, 10 mM imidazole, pH = 8.0). Centrifuge for 2 minutes at 890 g. Load up to 600 μl of the cleared sample onto the pre-equilibrated Ni-NTA spin column. Centrifuge for 5 minutes at 270 g, and collect the flow-through. Wash the Ni-NTA spin column twice with 600 μl NPI-20 buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl, 20 mM imidazole, pH = 8.0). Centrifuge for 2 minutes at 890 g. Elute the protein twice with 300 μl buffer NPI-500 (50 mM $Na_2H_2PO_4$, 300 mM

NaCl, 500 mM imidazole, pH = 8.0). Centrifuge for 2 minutes at 890 g, and collect the flow through.

For Ni-NTA gravity columns, the His-Trap gravity columns were equilibrated with 10 ml binding buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl, 10 mM imidazole, pH = 8.0). Load up to 3 ml of the cleared sample into the pre-equilibrated column and collect the flow-through. Wash the Ni-NTA spin column with 10 ml binding buffer and collect the flow through. Elute the protein with 5 ml elution buffer (50 mM Na2H2PO4, 300 mM NaCl, 250 mM imidazole, pH = 8.0) and collect the flow through.

2.9 Protein concentration determination

Different concentration of bovine serum standards (100, 200, 300, 400 and 500 μg/ml) were prepared. Standards and protein samples were added into 96-well microtiter plate in duplicate. The Dye-Reagent (Bio-Rad 500-0006, diluted 5 folds with dH_2O) was later added to each samples and standards. Samples and dye were mixed and placed at room temperature for 3 minutes before measured the O.D. 595 nm (Thermo Multiskan FC).

2.10 Cell viability analysis

Cell viability was measured by MTT (Sigma M5655) assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a water soluble tetrazolium

salt (yellow), and the concentration of MTT stock solution was 5 mg/ml . According to MTT product applications, after MTT was cleft by active mitochondrial dehydrogenases of living cells, the soluble yellow dye will be converted to the insoluble purple formazan. The amount of formazan can be measured by dissolving formazan in 0.08 N HCl in isopropanol, and the absorbance of converted dye was measured at a wavelength of 570 nm by Microplate Reader (Beckman DTX 880).

In this experiment, 3 x 10^4 HS68 cells were seeded on 96 well plates (BD) 12 hours before adding different amount of gelatin-PEI nanoparticles (Obtained from Dr. Yi-You Huang and Dr. Min-Ju Chou). Serum-free DMEM should be used during gelatin-PEI nanoparticles application. Wash with PBS three times after 2 hours nanoparticles application. HS68 cells were further cultured in 10% FBS DMEM with 24, 48 or 72 hours. Each well was treated with 10 μl MTT stock solution for 3 hours. Remove the medium and 0.08 N HCl in isopropanol was added. Measure the wavelength of 570 nm by Microplate Reader (Beckman DTX 880).

2.11 Cellular uptake analysis

Gelatin-PEI nanoparticles (conjugated with rhodamine isothiocyanate, RITC) uptake efficiency was determined by the RITC-positive cells detected by flow cytometry (BD FACSCanto II). In brief, HS68 cells were cultured in 6 well plate

(Corning) for 12 hours. Gelatin-PEI nanoparticles were added into each well with different concentration (0, 5, 10 and 50 μg/ml). Serum-free DMEM should be used during gelatin-PEI nanoparticles application. Wash with PBS three times after 2 hours nanoparticles application. HS68 cells were further cultured in 10% FBS DMEM with 22 hours. Use 0.05% trypsin to collect HS68 cells and fix them with 4 % paraformaldehyde (Sigma P6148). Wash the cells two times with PBS between previous steps. Cells were filtrated with .40 nm mesh filter (BD) before flow cytometry analysis. Flow data was analyzed by FlowJo 7.6.1.

2.10 Intracellular protein delivery of gelatin-PEI nanoparticles

Purified eGFP and gelatin-PEI nanoparticles were mixed with weight ratio as 1:2 (final eGFP concentration = 10 μg/ml). The mixed solution was kept in dark and stood at room temperature for an hours. HS68 cells were cultured in 24 well plate (Corning) with cover glass (Deckgläser 18 mm) for 12 hours. Gelatin-PEI nanoparticles and eGFP mixture were added into each well with different concentration (0 and 10 μg/ml eGFP). Serum-free DMEM should be used during gelatin-PEI nanoparticles application. Wash with PBS three times after 2 hours nanoparticles application. HS68 cells were further cultured in 10% FBS DMEM with 4 hours. Discard culture medium and fix HS68 cells with 4 % paraformaldehyde (Sigma P6148). Fix cover glass with mounting medium on

the micro slide glass (MATSUNAMI pro-01). Red fluorescence and green fluorescence were observed and recorded by confocal microscopy (Zeiss LSM 780 Confocal).

Chapter three Result

3.1 Identification of His-eGFP and His-Yamanaka factors overexpressed by BL21 and Rosetta™ competent cells

3.1.1 Total protein analysis by coomassie brilliant blue and Western blotting

After IPTG induction, total protein extract from empty pETDUET-1 and target genes were collected after sonication and centrifugation. Protein concentration after extraction was determined by Bradford method.

In total protein SDS-PAGE, total proteins were stained by coomassie brilliant blue-R for 5 minutes. Total protein analysis (figure 1-1) showed that eGFP was overexpressed only in eGFP-pETDUET-1 sample, but not in empty pETDUET control group. Western blot analysis also showed the same result obviously (figure 1-2). Due to high expression level of his-eGFP, only BL21 was used in eGFP overexpression experiments.

For Yamanaka four factors, all factors were first overexpressed by BL21, but the expression level were not obvious. So Rosetta™ competent cells were used to improve His-Yamanaka factors' expression level.

Total protein analysis (figure 1-3) showed that only Oct4 in BL21, Sox2 in both BL21 and Rosetta™ and c-Myc in Rosetta™ had clear overexpression pattern, but other

groups had no significant change. In contrast, Western blot analysis (figure 1-4) showed that all four factors were successfully overexpressed after IPTG induction, including Klf4 and Oct4, indicated that overexpression level or the solubility are quite different between all four factors and different competent cells.

Overall, His-eGFP and all Yamanaka factors were successfully overexpressed by these two competent cells, and except for His-eGFP, all other factors needs further condition modification to elevate the expression amount of these fusion proteins.

3.1.2 Induction time optimization of His-eGFP and His-SKOM

In order to optimize the best IPTG induction time for His-eGFP fusion proteins, the inducted cells (three replicates, Group A, B and C) was collected every 3 hours after IPTG application. After only 3 hours IPTG induction, His-eGFP was able to be overexpressed by BL21 (figure 2-1), and after 9 to 15 hours IPTG induction, expression level of His-eGFP would reach the highest point, than gradually decreased according to Western blot quantification by ImageJ (figure 2-2).

These results indicated that His-eGFP should be inducted between 9~15 hours to reach the highest expression level, and longer induction would not be suggested.

On the other hand, all His-Yamanaka factors were able to be detected after 4 hours

IPTG induction (figure 2-3), but no specific overexpression pattern could be observed in His-Sox2 group, and 4 hour IPTG induction is enough for all His-Sox2 groups (figure 2-4). For His-Klf4 and His-Oct4, highest expression level appeared after 4 hour IPTG induction, but no significant expression could be seen after 12 hours (figure 2-4). Besides, the highest expression level of His-Myc appeared after 16 hour IPTG induction, but 8 hour induction might be enough (figure 2-4). These result indicated that except for c-Myc, 4 hours IPTG induction is enough to obtain sufficient amount of His-SKO, longer induction culture is unnecessary.

3.2 Purification of His-eGFP and His-Yamanaka factors by affinity columns

After protein extraction, His-eGFP and His-Yamanaka factors were purified by Ni-NTA spin columns and HisTrap gravity columns. Total protein analysis of His-eGFP (figure 3-1) and Western blot analysis (figure 3-2) both showed that most of the His-eGFP could be purified by both spin and gravity columns, and purified extracts contained only a small portion of proteins other than His-eGFP, especially in spin column group. According to this data, His-SKOM were further purified by spin columns only to obtain purer samples.

For His-Yamanaka factors, total protein analysis (figure 3-3) showed that purified

extracts contained less proteins that were not His-Yamanaka factors, but His-Yamanaka factors had no significant change. Western blot analysis (figure 3-4) showed that after purification, all four fusion proteins could be successfully purified even though protein amount of all factors were less than total extracts.

3.3 Cellular uptake of gelatin-PEI nanoparticles in HS68 cell line

To evaluate the uptake efficiency of gelatin-PEI nanoparticles in HS68 cell line, gelatin-PEI nanoparticles were added into culture medium with different concentration (0,5,10,50 μg/ml separately) and analyzed by flow cytometry.

Histogram analysis by FlowJo 7.6.1 (figure 4) showed that when nanoparticle's concentration was below 50 μg/ml, no significant peak shift could be observed, but almost 90 % cells were RITC-positive when nanoparticle's concentration reach 50 μg/ml. Further experiments are needed to determine uptake efficiency when concentration over 50 μg/ml.

3.4 Cytotoxicity analysis of gelatin-PEI nanoparticles in HS68 cell line

Nanoparticle cytotoxicity at different concentration (10, 20, 30, 40 and 50 μg/ml) was measured by MTT assay. The result of MTT assay (figure 5) indicated that after

nanoparticle application, cell viability would decrease to about 70% for all groups except 0 μg/ml control after 24 hours culture. On the other hand, cells would recover to more than 90% of original population after 72 hours culture compared with control group.

To successfully generate iPS cells with proteins, nanoparticles and proteins should be apply to cells several times. Therefore, according to the result of MTT assay, it is possible to use HS68 cell line as reprogramming target due to low cytotoxicity of the nanoparticles and good recovery rate of HS68 cells.

3.5 His-eGFP delivery by gelatin-PEI nanoparticles in HS68 cell line

Gelatin-PEI and His-eGFP were applied to HS68 cells after 2 hours mix with 2:1 weight ratio (30 μg/ml: 15 μg/ml), and the delivery results were taken by confocal microscopy. Green fluorescence (His-eGFP) and red fluorescence (RITC tag on nanoparticles) were both detectable after 24 hours application, and co-localization was observed (figure 6). Even though there were a lot of nanoparticles that were not successfully entering the cells can be observed, some of them truly penetrated into the cells and remain in the cells can be detected by Z-stack analysis by confocal microscopy.

Overall, gelatin-PEI nanoparticles were able to deliver His-eGFP into HS68 cells without destroy eGFP's function. This result indicated that His-Yamanaka factor may be transported into the cells with same method, but further tests are still needed.

Chapter four　Discussion

iPS cells are considered to be the future star in regenerative medicine due to its ethical- and transplant rejection-free characteristics. But the reprogramming vectors of iPS cells, especially viral systems, limit the possibility of iPS cells in clinical use. In order to solve this problem, several DNA-free methods have been developed. However, almost every method has its limitation and advantages, and these methods are not as user-friendly as traditional viral vectors. Therefore, in this research proteins were combined with nanoparticles as a new reprogramming vectors, hope to find out a balance between potential risk of genome instability and transfection efficiency.

In this study, although no primary reprogramming colony has been observed yet, proteins have been shown to be a possible reprogramming vectors in previous research with extremely low reprogramming efficiency (*28*) (*29*). In 2013, Majad Khan ***et al.*** developed a cationic bolaamphiphile combine with purchased Yamanaka factors as reprogramming vectors (*63*). These researches indicated that using proteins as reprogramming vectors is possible, but none of them can avoid co-treatment with specific chemical compounds or even culture transformed cells on Matrigels during reprogramming process. It suggested that during protein transduction process, the amount of proteins might be not enough to sustain the reprogramming condition.

Protein loss during reprogramming and delivery may due to several reasons, including encapsulation waste, endosome escaping failure and protein degradation within the host cells. By using cationic nanoparticles, the protein escaping percentage may evaluate due to massive proton acceptors in the polymers, and the encapsulation waste may be reduced due to free of organic solvent during protein encapsulation.

Besides, with different cationic polymers and target proteins, the protein-nanoparticle binding efficiency and surface conformation after binding would be different, so it is important to find out a proper cationic polymer which can match the need in different delivery aims. Although Majad Khan *et al.* successfully generate iPS cells by this method, more cationic nanoparticles need to be test in order to increase the protein binding efficiency, and further increase the amount of proteins that can be successfully delivered into the host cells.

In addition to cationic polymer optimization, there are several important factors need to be examined in future experiments. One is the cytotoxicity of protein samples, especially overexpressed by *E.coli*. The endotoxins generated by bacteria should be further removed before reprogramming experiments, and test whether endotoxin removal can enhance the cell viability or not. Another problem is the proper amount of proteins that need to be loaded during every reprogramming cycle, and find out the

balance between reprogramming efficiency and the toxicity of nanoparticles. Also, the expression system of Yamanaka factors can be substituted for mammalian expression system, such as CHO cells to overcome post-translation modification and folding problems.

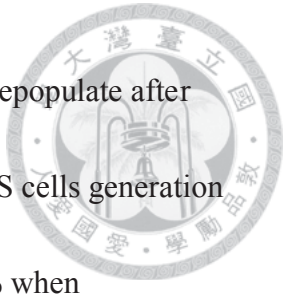
Overall, nanoparticles combine with proteins as delivery vectors may have wide applications besides reprogramming, if the safety and stability of this method can be proved by further experiments, proteins and cationic nanoparticles together may have promising future in medical and reprogramming area.

Chapter five Conclusion

iPS cells are artificial stem cells originated from reprogrammed somatic cells by specific transcription factors, such as Sox2 and Oct4. In order to develop a novel DNA-free method for the generation of iPS cells, proteins were considered to be the proper reprogramming vectors combine with cationic gelatin-PEI nanoparticles. Proteins are able to modulate the gene expression of the host cells, easier to obtain than other DNA-free vectors, and can be simply modified by plasmid construction. Also, proteins are overall negatively charged, so cationic nanoparticles may able to interact with them and carry them into the cells as well as DNA.

In this study, eGFP and Yamanaka factors were fused with 6 x His-tag and successfully overexpressed by *E.coli* overexpression system. 6 x His-tag was added because it provides an affinity tag to purify those target proteins. Also, the proper induction time, which should be between 9 to 12 hours, was found as the result indicated. Although His-Yamanaka factors were not expressed as many as His-eGFP, these factors were still possible to be obtained by *E.coli* overexpression system. Furthermore, His-eGFP and all His-Yamanaka factors were able to be purified by Ni-NTA affinity columns for nanoparticle encapsulation.

On the other hand, cell viability assay showed that the cytotoxicity of gelatin-PEI



nanoparticles was durable with HS68 cell line, and these cells could repopulate after three days application, which indicated that repeated cycles during iPS cells generation are possible. Also, cellular uptake efficiency would reach almost 90% when nanoparticle concentration is 50 μg/ml, and it is enough for further applications.

At the end of the study, the protein delivery result showed that eGFP were able to cross the membrane with the presence of gelatin-PEI nanoparticles. Although it is unclear whether these proteins were in the endosomes or not, the result still indicated that using gelatin-PEI to deliver Yamanaka factors is possible in the future.

In conclusion, gelatin-PEI nanoparticles were able to carry proteins, such as eGFP in this research, into human fibroblast cell line. Combining cationic nanoparticles and proteins is a high potential way to become a stable and integration-free reprogramming method in the future. For further study, repeat applications of Yamanaka factors combine with gelatin-PEI nanoparticles should be performed and the reprogramming efficiency should be measured and compared with other methods.

Name	Sequence (5'- 3')
Adaptor forward	GATCACTCGAGTGCAGCAGCAG
Adaptor reverse	GATCCTGCTGCTGCACTCGAGT
Sox2 forward	GCGAATTCATGTATAACATGATGGAGAC
Sox2 reverse	AAGCGGCCGCTCACATGTGCGACAGGGGC
Klf4 forward	GCGAATTCATGAGGCAGCCACCTGGC
Klf4 reverse	AAGCGGCCGCTTAAAAGTGCCTCTTCAT
Oct4 forward	GCGAATTCATGGCTGGACACCTGGCTTC
Oct4 reverse	AAGCGGCCGCTCAGTTTGAATGCATGGG
c-Myc forward	GCGAATTCATGCCCCTCAACGTGAAC
c-Myc reverse	AAGCGGCCGCTTATGCACCAGAGTTTCG
eGFP forward	TTGCGGCCGCTTATGGTGAGCAAGGGCGA
eGFP reverse	AAGCGGCCGCTTATCTAGATCCGGTGG

Table 1. Primer List of Yamanaka Factors, eGFP and Adaptor for Yamanaka Factors.

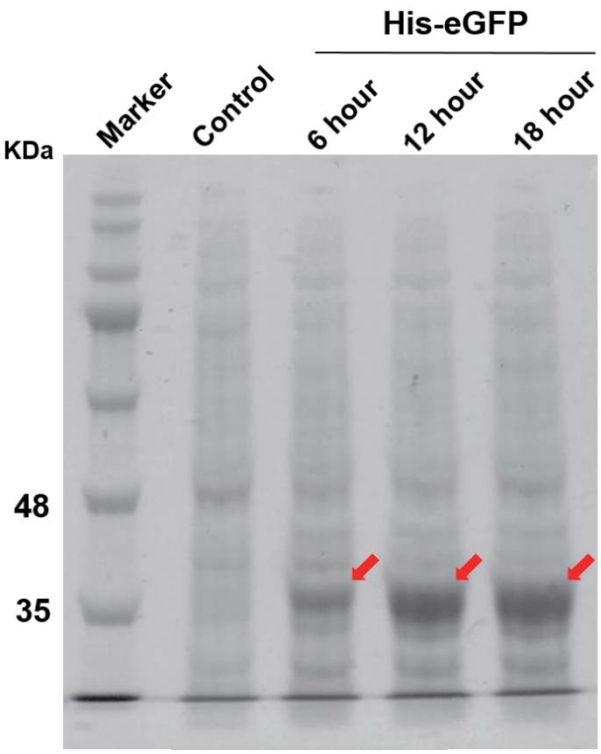


Figure 1-1. eGFP Overexpression Verification by CBR Staining. His-eGFP after 6 hour, 12 hour and 18 hour IPTG induction in BL21 were detected by CBR staining. **Control**: empty pETDUET-1 vector induced with IPTG for 6 hours in BL21.

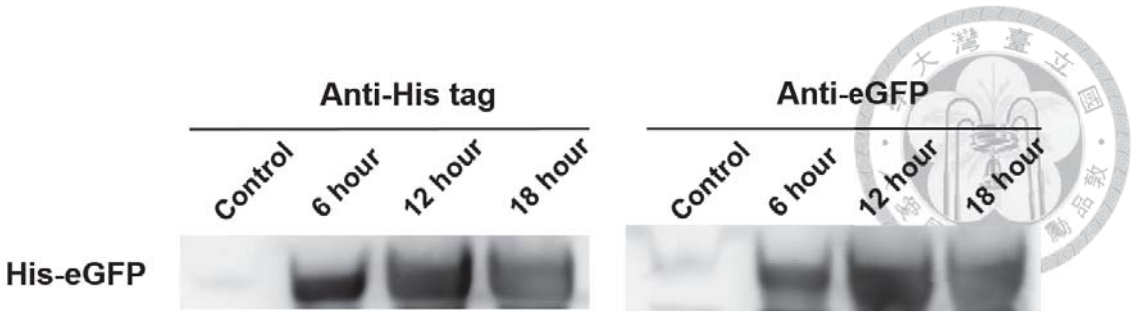


Figure 1-2. eGFP Overexpression Verification by Western Blot Analysis.

His-eGFP fusion proteins were detected with anti-His tag antibody and anti-eGFP antibody after 6 hour, 12 hour and 18 hour IPTG induction in BL21. **Control**: empty pETDUET-1 vector induced with IPTG for 6 hours in BL21.

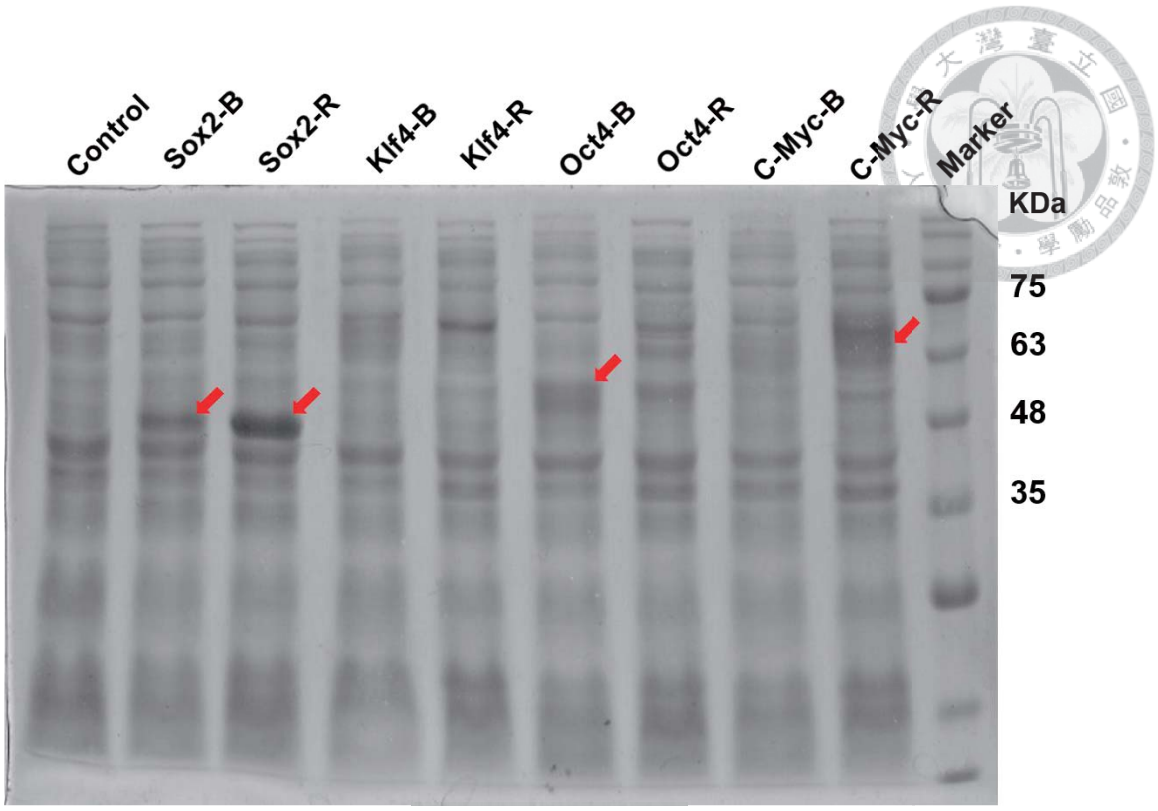


Figure 1-3. Sox2, Klf4, Oct4 and c-Myc Overexpression Verification by CBR Staining. His-Sox2 fusion proteins were detected below 48 kDa, His-Oct4 was detected above 48 kDa and His-Myc was detected at 63 kDa after 6 hour IPTG induction by CBR staining. No Klf4 overexpression pattern could be seen with CBR staining. **Control**: empty pETDUET-1 vector induced with IPTG for 6 hours in BL21. **R**: Rosetta™ competent cells. **B**: BL21 competent cells.

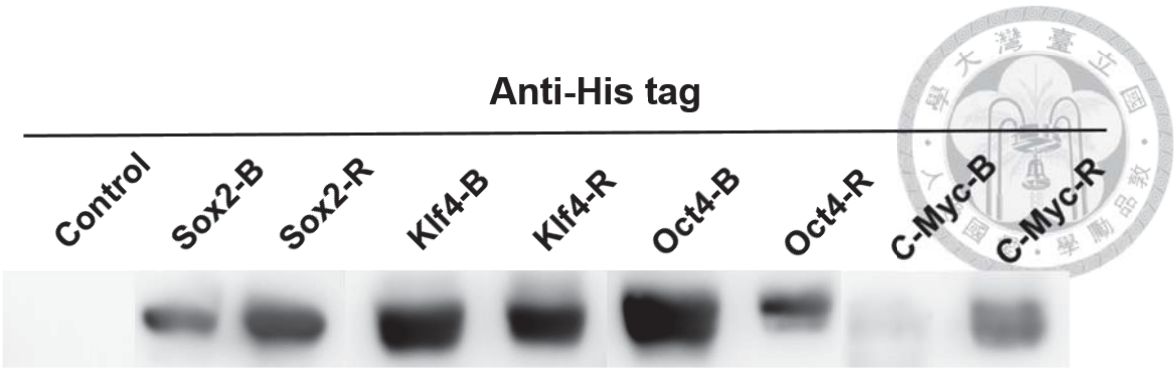


Figure 1-4. Sox2, Klf4, Oct4 and c-Myc Overexpression Verification by Western Blotting Analysis. His-Sox2, His-Oct4, His-Myc and His-Klf4 were detected with anti-His tag antibody after 6 hour IPTG induction in BL21. **Control**: empty pETDUET-1 vector induced with IPTG for 6 hours in BL21. **R**: Rosetta™ competent cells. **B**: BL21 competent cells.

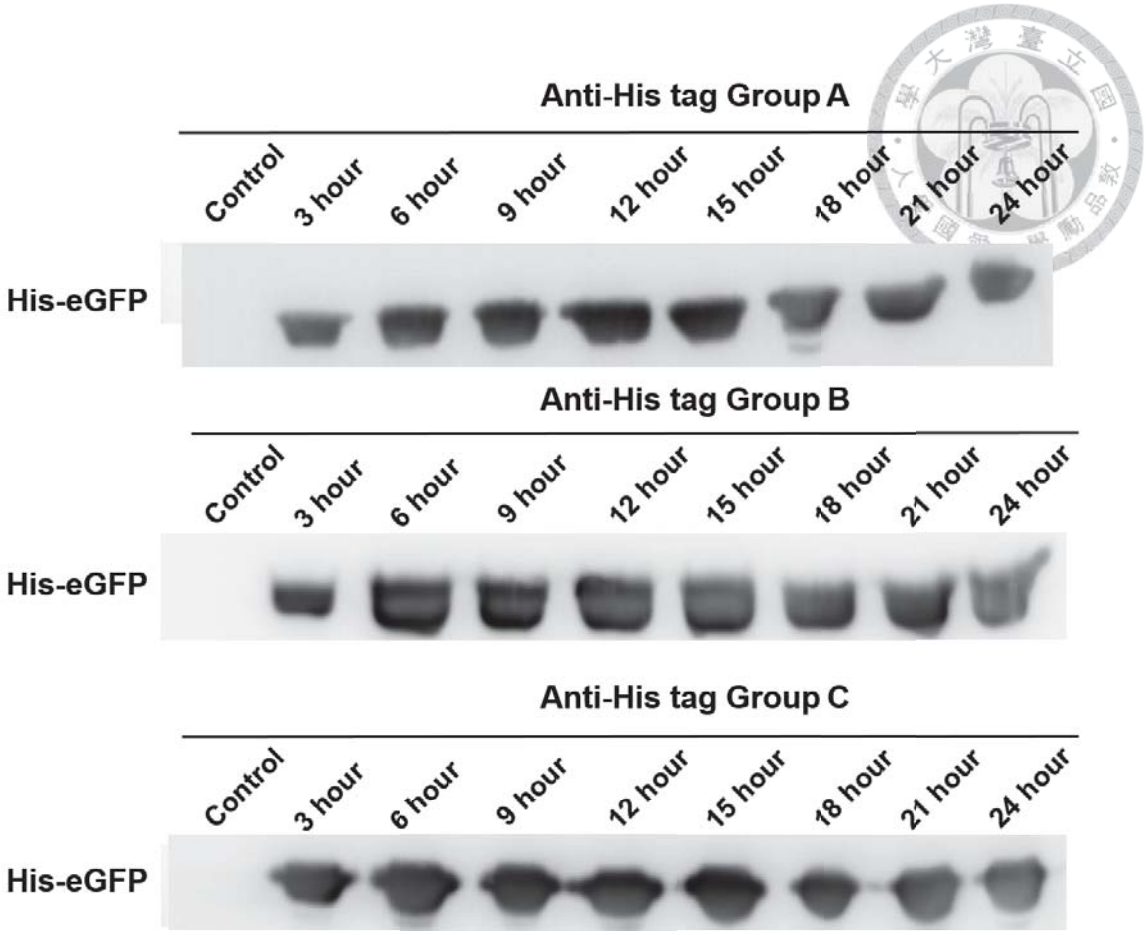


Figure 2-1. Induction Time Optimization of His-eGFP. His-eGFP with different IPTG induction time were able to be detect by anti-His tag antibody, and signal could be seen after only 3 hour IPTG induction. Group A, B and C refer to three different replicates of His-eGFP sample which overexpressed in BL21. **Control**: empty pETDUET-1 vector induced with IPTG for 6 hours in BL21. Different time scale (from 3 hour to 24 hour) indicated different IPTG induction time. Total protein loading amount = 30 μg.

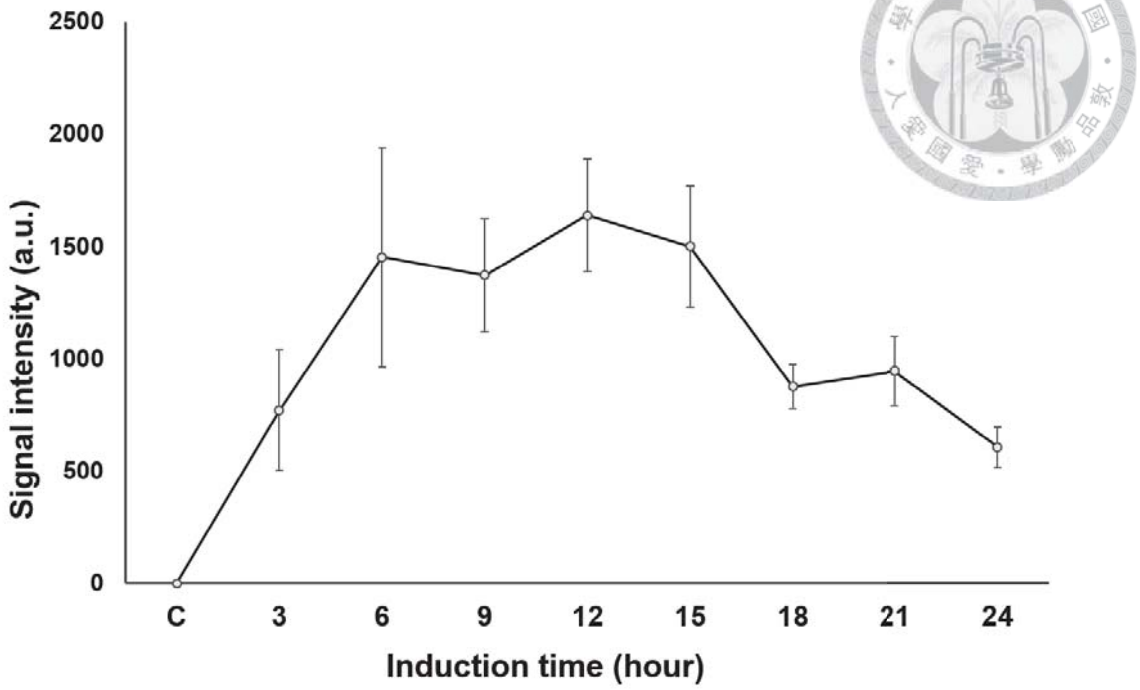
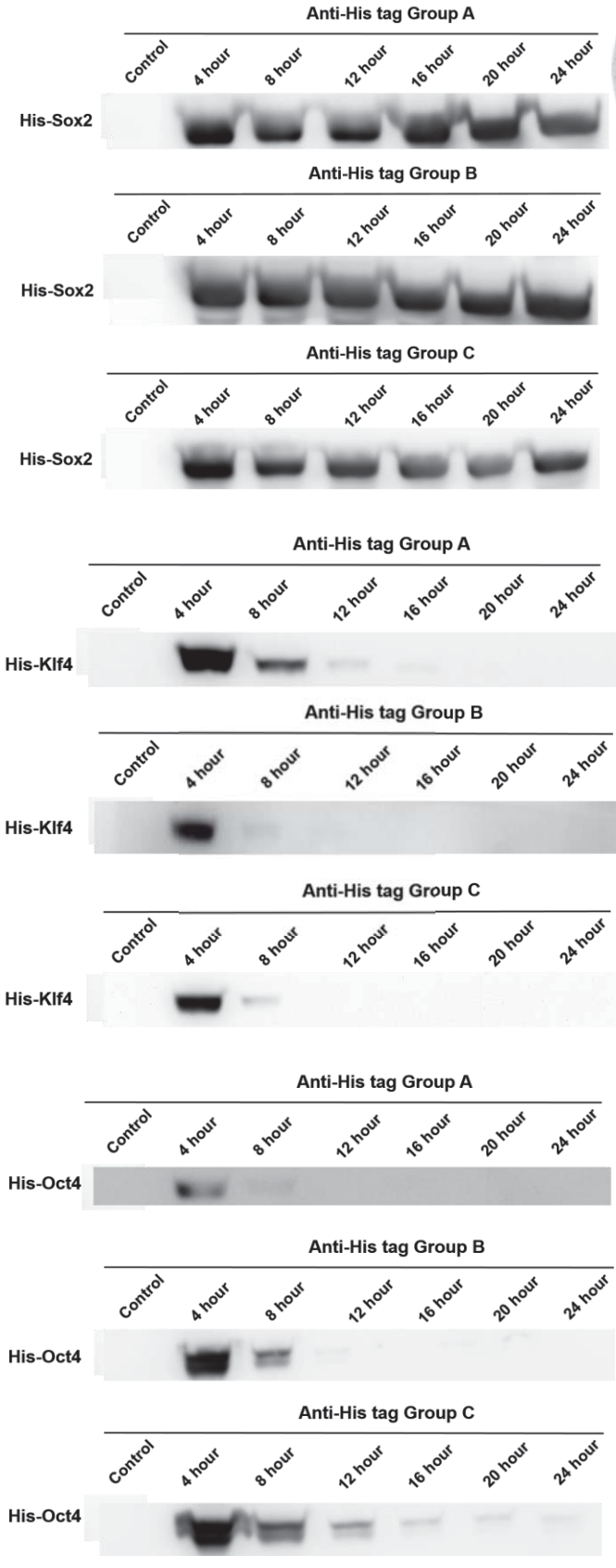


Figure 2-2. Quantification of His-eGFP Overexpression Level with Different IPTG Induction Period. Highest His-eGFP expression level was detected between 9 to 15 hours after IPTG induction in BL21. Western blotting quantification was done by ImageJ. **Control**: empty pETDUET-1 vector induced with IPTG for 6 hours in BL21. Different time scale (from 3 hour to 24 hour) indicated different IPTG induction time. Total protein loading amount = 30 μg.


Anti-His tag Group A
Control
4 hour
8 hour
12 hour
16 hour
20 hour
24 hour
His-Sox2
Anti-His tag Group B
Control
4 hour
8 hour
12 hour
16 hour
20 hour
24 hour
His-Sox2
Anti-His tag Group C
Control
4 hour
8 hour
12 hour
16 hour
20 hour
24 hour
His-Sox2
Anti-His tag Group A
Control
4 hour
8 hour
12 hour
16 hour
20 hour
24 hour
His-Klf4
Anti-His tag Group B
Control
4 hour
8 hour
12 hour
16 hour
20 hour
24 hour
His-Klf4
Anti-His tag Group C
Control
4 hour
8 hour
12 hour
16 hour
20 hour
24 hour
His-Klf4
Anti-His tag Group A
Control
4 hour
8 hour
12 hour
16 hour
20 hour
24 hour
His-Oct4
Anti-His tag Group B
Control
4 hour
8 hour
12 hour
16 hour
20 hour
24 hour
His-Oct4
Anti-His tag Group C
Control
4 hour
8 hour
12 hour
16 hour
20 hour
24 hour
His-Oct4

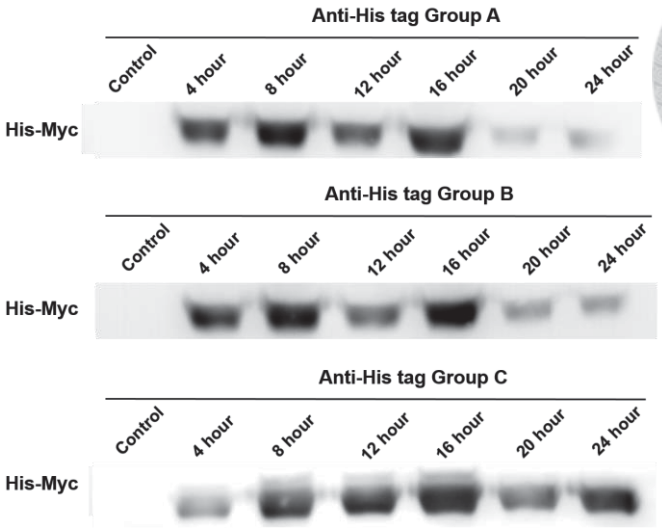
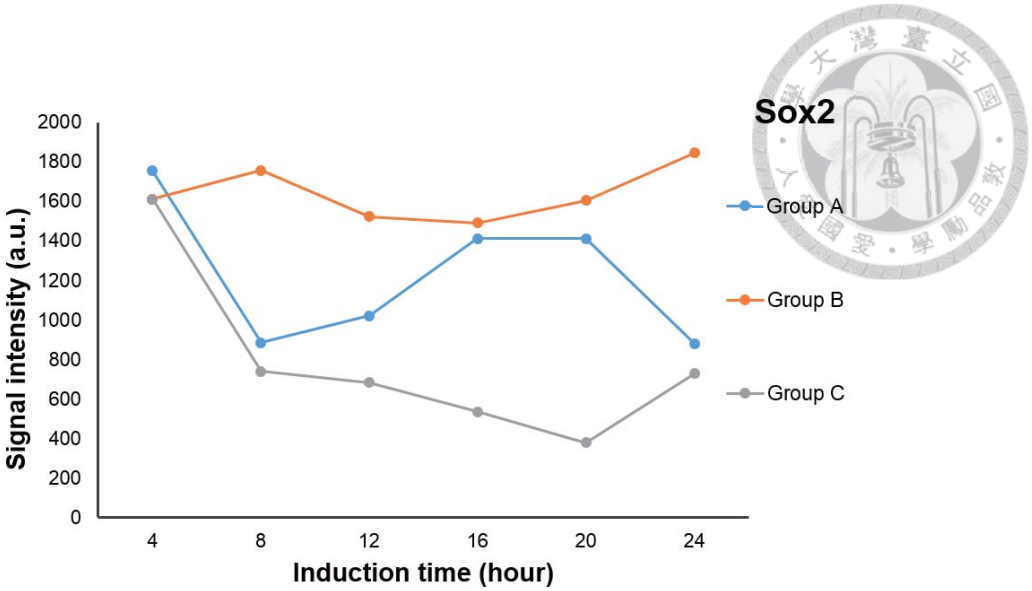
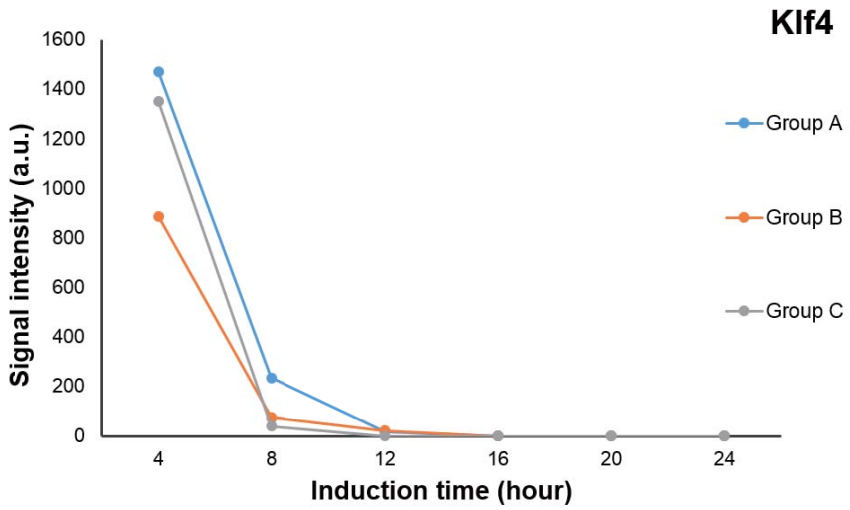
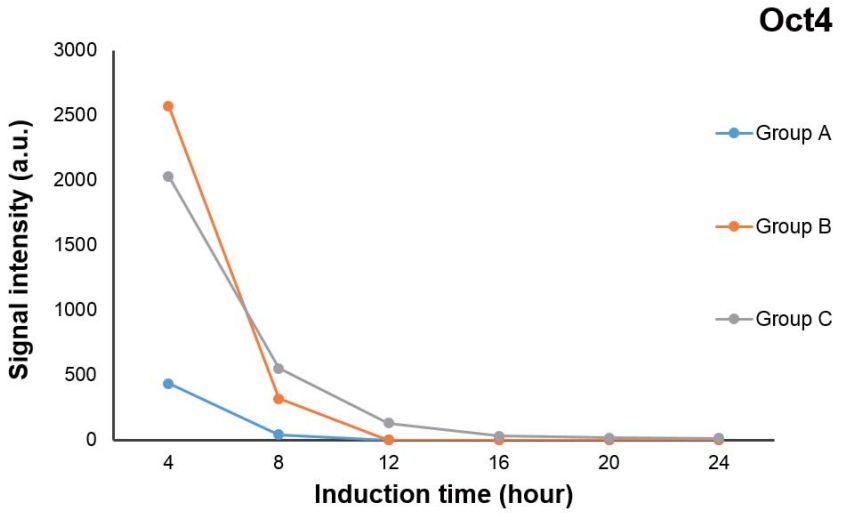


Figure 2-3. Induction Time Optimization of His-Yamanaka Factors. All His-Yamanaka factors were successfully detected by anti-His tag antibody after 4 hour IPTG induction in Rosetta™ competent cells. **Control**: empty pETDUET-1 vector induced with IPTG for 6 hours in BL21. Different time scale (from 4 hour to 24 hour) indicated different IPTG induction time. Total protein loading amount = 30 μg.


Sox2
Signal intensity (a.u.)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
4
8
12
16
20
24
Induction time (hour)
Group A
Group B
Group C


Klf4
Signal intensity (a.u.)
1600
1400
1200
1000
800
600
400
200
0
4
8
12
16
20
24
Induction time (hour)
Group A
Group B
Group C


Oct4
Signal intensity (a.u.)
3000
2500
2000
1500
1000
500
0
4
8
12
16
20
24
Induction time (hour)
Group A
Group B
Group C

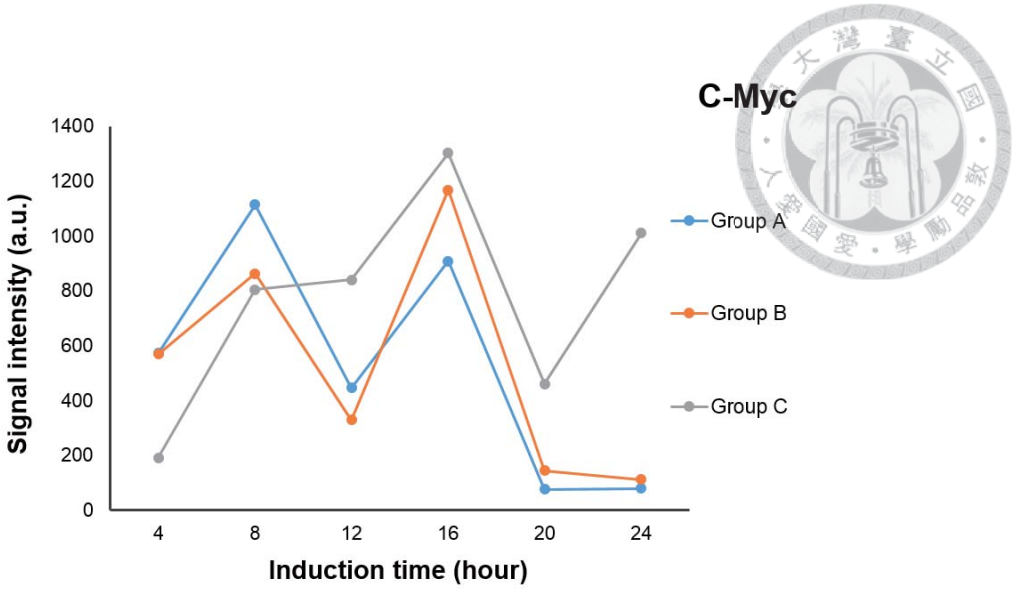


Figure 2-4. Quantification of His-Yamanaka Factors Overexpression Level with Different IPTG Induction Period. 4 hours IPTG induction was sufficient for His-Sox2, His-Klf4 and His-Oct4 but not His-Myc. All His-Yamanaka factors Western blotting result were analyzed by ImageJ. Different time scale (from 4 hour to 24 hour) indicated different IPTG induction time. Total protein loading amount = 30 μg.

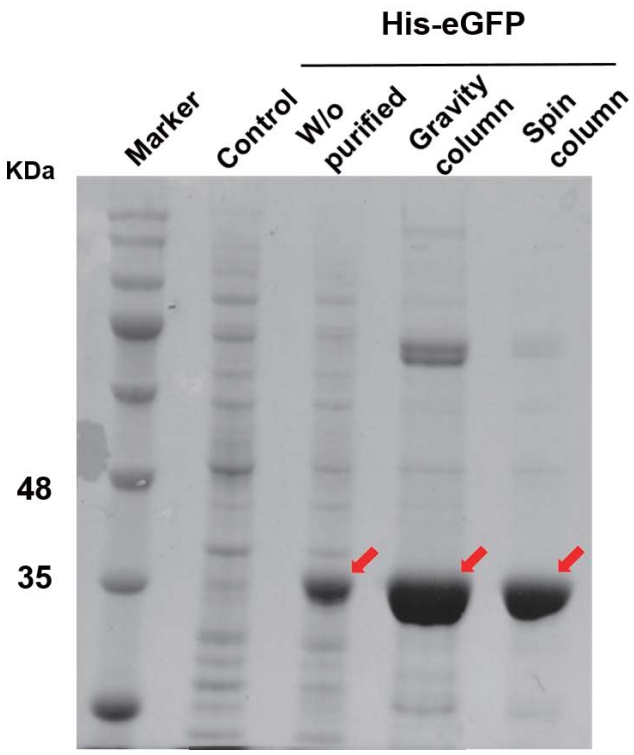


Figure 3-1. His-eGFP Purification was Verified by CBR Staining. His-eGFP could be purified by both Ni-NTA gravity column and Ni-NTA spin column, and detected by CBR staining. **Control**: empty pETDUET-1 vector induced with IPTG for 6 hours in BL21. Total protein loading amount = 30 μg.

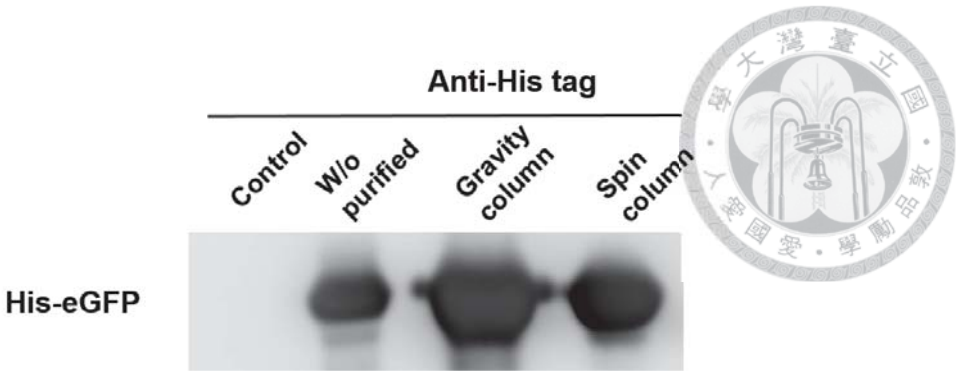


Figure 3-2. His-eGFP Purification was Verified by Western Blotting. His-eGFP could be purified by both Ni-NTA gravity column and Ni-NTA spin column and detected by anti-His tag antibody. **Control**: empty pETDUET-1 vector induced with IPTG for 6 hours in BL21. Total protein loading amount = 30 μg.

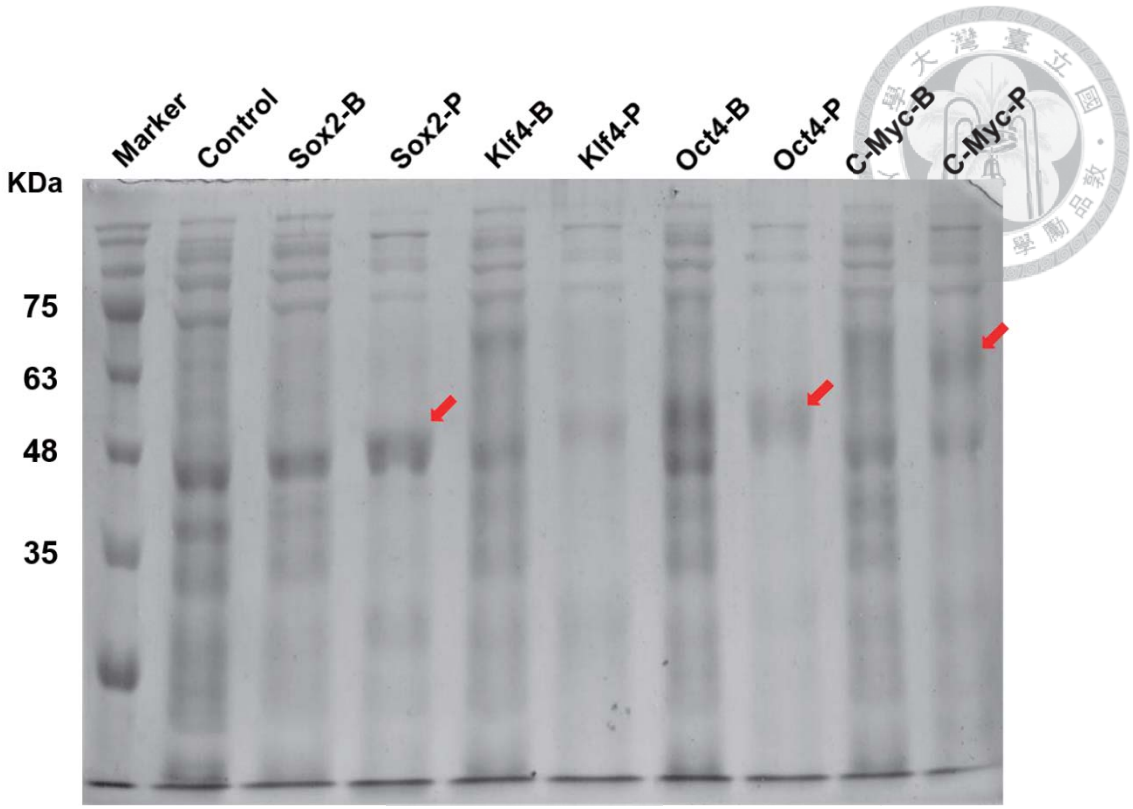


Figure 3-3. His-Yamanaka Factors Purification was Verified by CBR Staining. No specific purified band except His-Sox2 could be observed with CBR staining. **Control**: empty pETDUET-1 vector induced with IPTG for 6 hours in BL21. **B**: Total protein overexpressed in BL21. **P**: Purified total protein overexpressed in BL21. Total protein loading amount = 30 μg.

Figure 3-4. His-Yamanaka Factors Purification was Verified by Western Blotting. Purified and unpurified His-Yamanaka factors were detected by anit-His tag antibody. **Control**: empty pETDUET-1 vector induced with IPTG for 6 hours in BL21. **B**: Total protein overexpressed in BL21. **P**: Purified total protein overexpressed in BL21. Total protein loading amount = 30 μg.

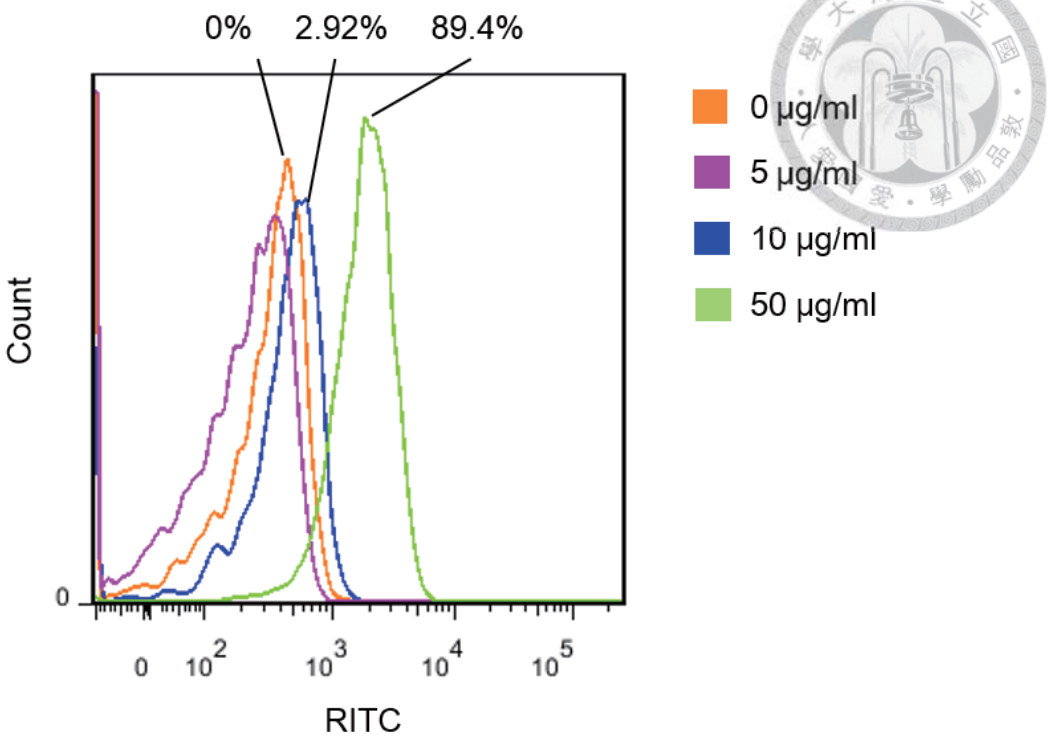


Figure 4. *In vitro* Protein Delivery Efficiency of GR-PEI Nanoparticles in Treating Hs68 Cell Line. Different amount of nanoparticles were applied to HS68 cell line and the protein delivery efficiency was detected by flow cytometry. Data was further analyzed by FlowJo 7.6.1.

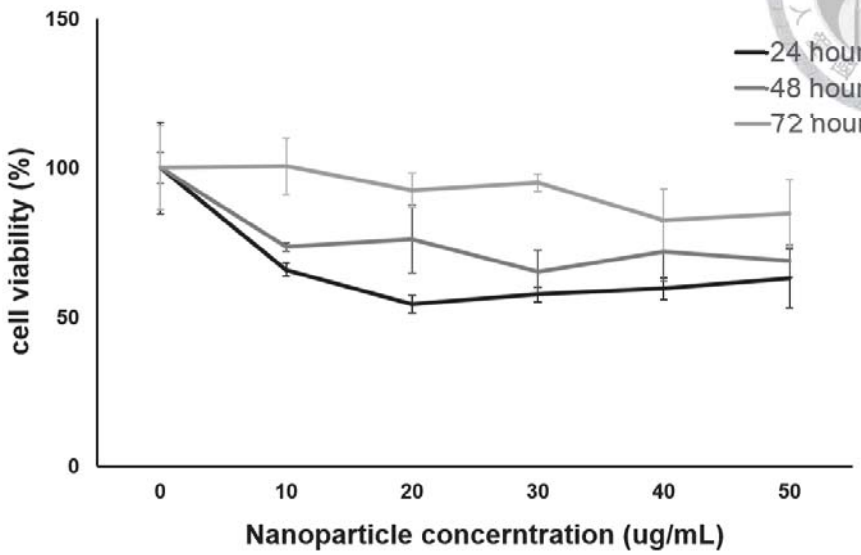


Figure 5. *In vitro* Cytotoxicity Analysis of Gelatin-PEI Nanoparticles in Treating HS68 Cell Line. Different amount of nanoparticles were applied to HS68 cell line with three replicates. Cell viability was measured by MTT assay after different culture period (24 hours, 48 hours and 72 hours), and the absorbance at 570 nm was detected by microplate reader.

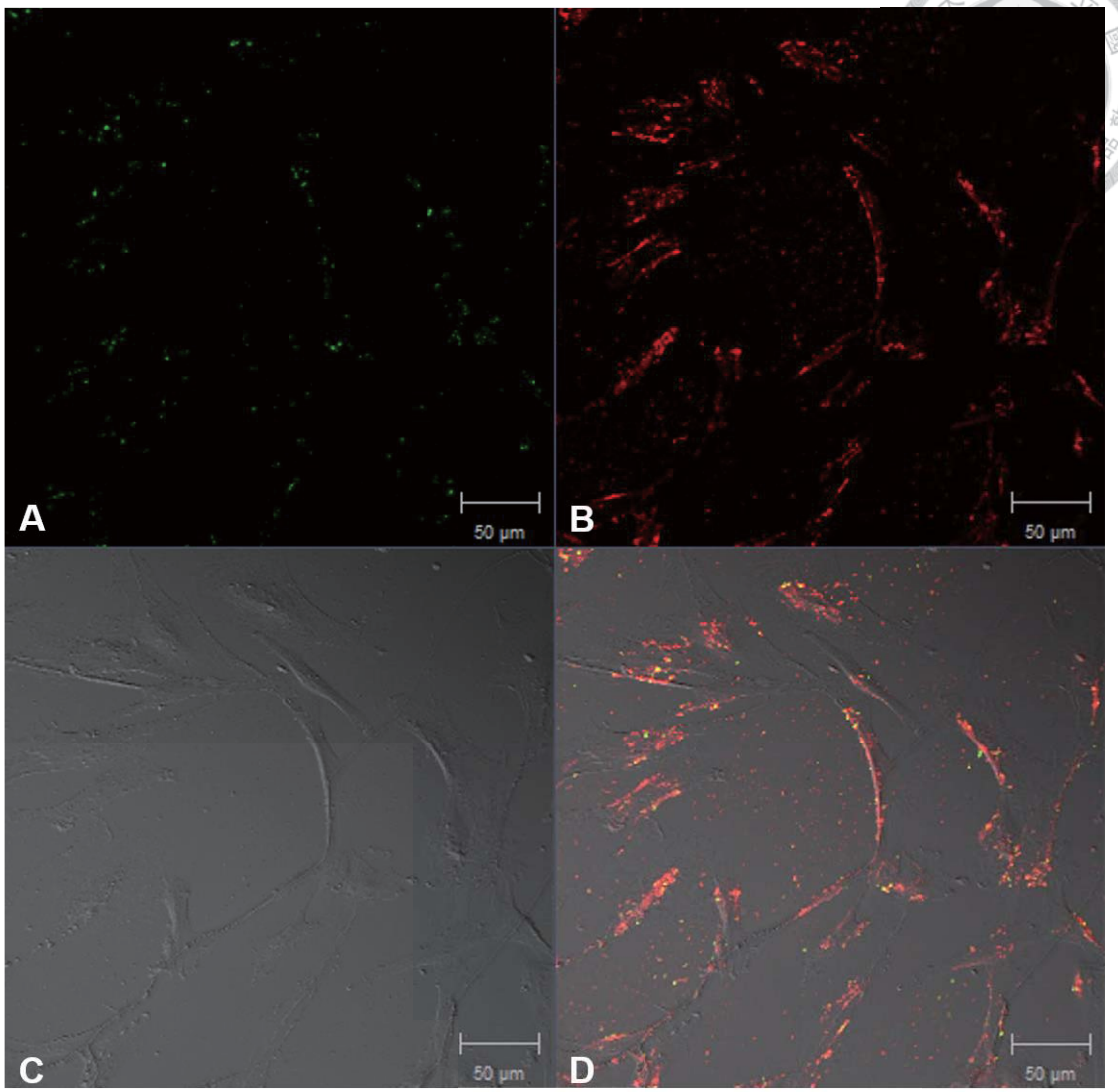


Figure 6. *In vitro* His-eGFP Delivery by Gelatin-PEI Nanoparticles in Treating HS68 cell line. 15 μg/ml His-eGFP was mixed with 30 μg/ml gelatin-PEI nanoparticle and then applied to HS68 cell line. Pictures were taken by confocal microscopy. A: green fluorescence. B: red fluorescence. C: bright field. D: mix. Scale bar = 50 μm.

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