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GENERATION OF NEURAL STEM CELLS (NSCs) FROM HUMAN FIBROBLASTS USING QQ-MODIFIED SOX2 AND NEUROD1 PROTIENS

by

ABDULLAH IBRAHIM ABDULLAH ALHOMOUDI

THESIS

Submitted to the Graduate School

of Wayne State University,

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MASTER OF SCIENCE

2020

MAJOR: BIOCHEMISTRY, MOLECULAR BIOLOGY, AND IMMUNOLOGY

Approved By:

_____

Advisor Date

DEDICATION

I dedicate this Thesis to my Father and Mother,

Dr. Ibrahim AlHomoudi and Huyam AlKashi,

Who have always demonstrated, through dedication, how to achieve my dreams;

My wonderful Wife,

Sajedah AlHomoudi,

Who has been my constant support;

&

My daughter,

Shaden AlHomoudi,

Who motivates me to be the best I can be.

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I thank my mentor, Dr. Jianjun Wang, for his supervisions in my research. I enjoy Dr. Wang's company for his honest advice in life and this field. I have grown tremendously since starting this thesis in 2018, and Dr. Wangs influences will never be forgotten.

I would also like to thank Dr. Qianqian Li for her expertise in the laboratory. Additionally, I would like to thank Dr. Robert Akins for graciously agreeing to be a member of my master's thesis committee.

Further, I would like to thank all of the members of Dr. Wang's lab, past and present, for their help and support: Dr. Xiao-Qing Zhao, Mr. Xiuyuan Yang and Mr. Guo Liu. Working in the lab would not have been nearly as rewarding without each member and their contributions.

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1.1 Introduction

1.1.1 Stem Cells

Stem cells have amazing potentials to develop into many different cell types during growth, especially early in life. Most importantly, many of these stem cells serve as internal repair systems, dividing without limit to replenish other cells and tissues (1). When stem cells divide, they can become another type of cell with a more specialized function, such as a brain cell or they can remain a stem cell. Stem cells have three notable characteristics: they are unspecialized, can self-renew, and under physiological or experimental conditions, they can be induced to become organ-specific cells (1). Scientists used to only have two kinds of stem cells to work with: embryonic stem cells (ESC) and non-embryonic adult stem cells.

In mammalian development **(Figure 1-1)**, only the zygote and early blastomeres are totipotent and can generate the whole organism (2). ESCs are derived from the inner cell mass (ICM) of mammalian blastocysts which have unlimited ability to self-renew and are pluripotent.

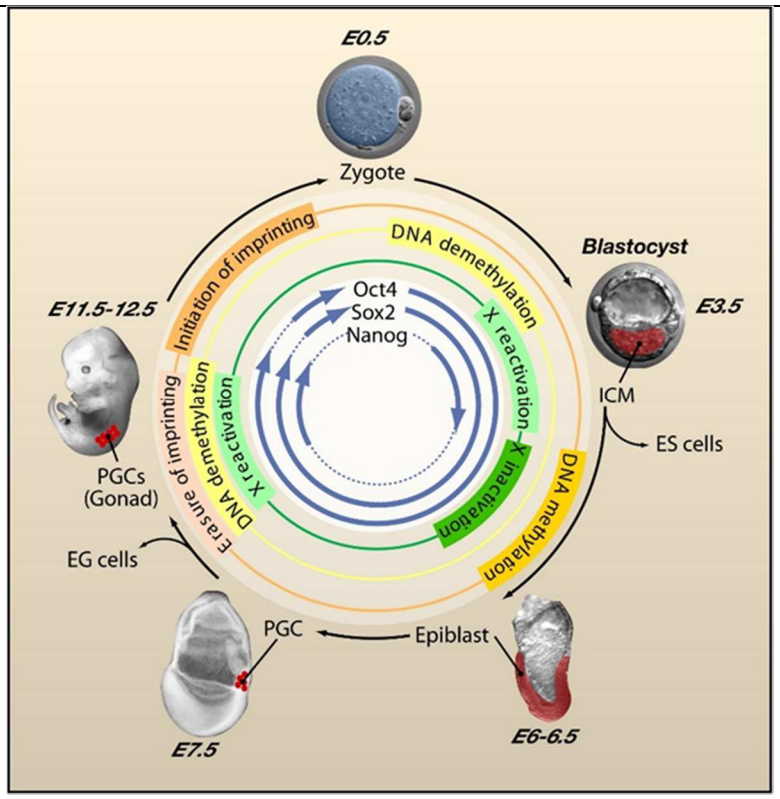

Figure 1-1: Diagram of Genetic and Epigenetic Regulation of Pluripotency during Mouse Development. The totipotent zygote contains maternally inherited epigenetic modifiers and transcription factors, including Oct4, Sox2, and Exh2. These together with the embryonic transcripts regulate development to the blastocyst stage, where the pluripotent cells are established in the inner cell mass (ICM). Deletion of Oct4 and Nanog comprises the development of the ICM (8-10). In the post-implantation embryo, pluripotent epiblast cells are controlled by diverse repressive mechanisms during their differentiation into somatic and germ cell lineages (the latter of which undergo specification following repression of the somatic program). The early germ cells exhibit epigenetic and transcriptional states that are associated with pluripotency, and the ensuing epigenetic reprogramming within this lineage re-generates totipotency. The figure depicts the main epigenetic changes occurring during critical stages of development (2).

The term pluripotent means that cells can differentiate into any cell of three germ layers: ectoderm, endoderm, and mesoderm (2-8). Pluripotent cells may differentiate to become multipotent progenitor cells, such as neural stem cells (NSCs), which then may differentiate into cell types of the neurogenic lineage only (1). Moreover, unipotent cells, such as skin cells, can only differentiate into only one cell type (3). A summary of the different types of potency can be found in **Table 1-1**.

Table 1-1: Definitions of the Different types of Potency (3).

Potency	**Sum of developmental options accessible to cell**
Totipotent	Ability to form all lineages of organism; in mammals only the zygote and the first cleavage blastomeres are totipotent.
Pluripotent	Ability to form all lineages of body. Example: embryonic stem cells
Multipotent	Ability of adult stem cells to form multiple cell types of one lineage. Example: hematopoietic stem cells
Unipotent	Cells form one cell type. Example: spermatogonia stem cells (can only generate sperm)
Reprogramming	Increase in potency, dedifferentiation. Can be induced by nuclear transfer, cell fusion, genetic manipulation
Transdifferentiation, plasticity	Notion that somatic stem cells have broadened potency and can generate cells of other lineages, a concept that is controversial in mammals.

In 1981, scientists discovered a way to derive ESCs from early mouse embryos, which led to the 1998 discovery of a technique to derive stem cells from human embryos to grow in the lab and are referred to as human embryonic stem cells (hESCs) **(Figure 1-2)** (9, 10). The first cultured hESC line was established and revealed the promise for stem cells in future clinical therapies.

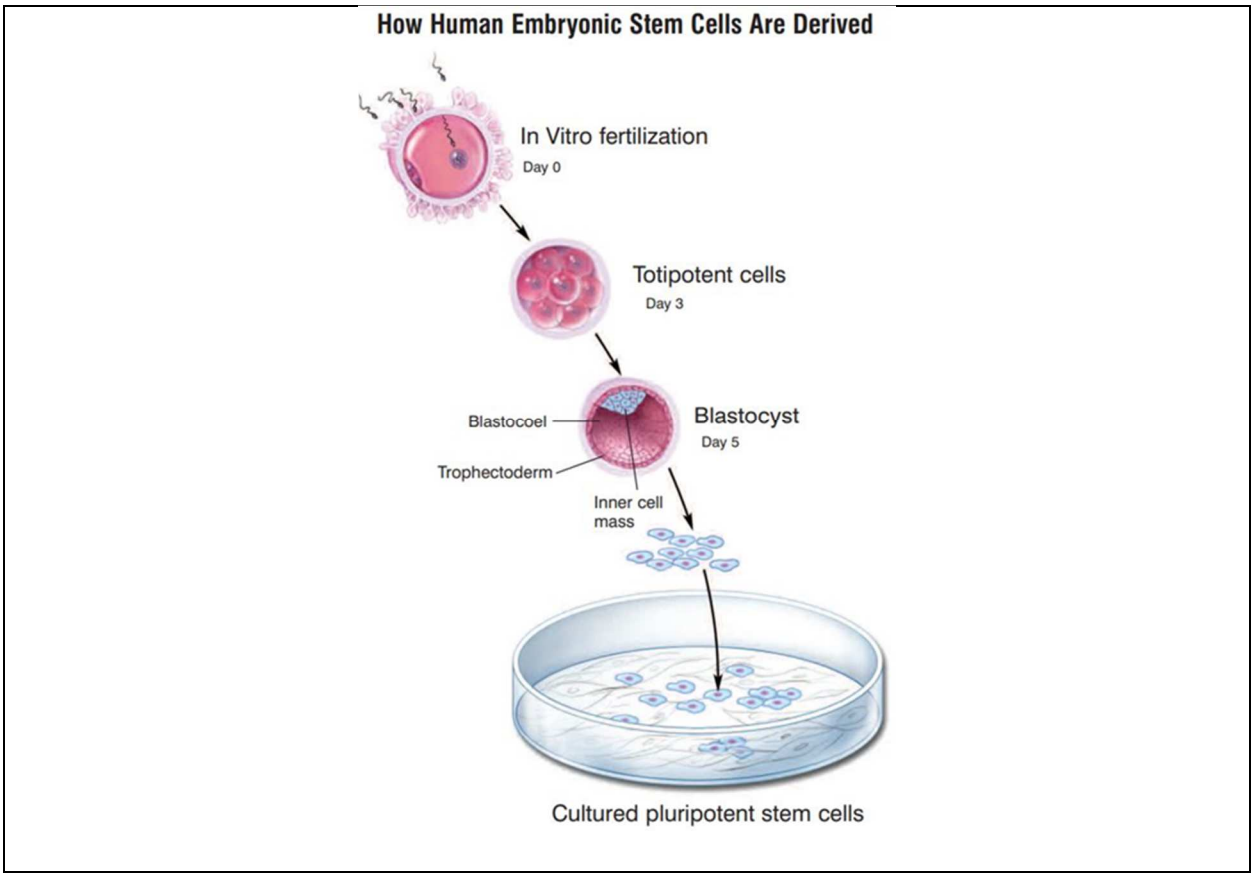

Figure 1-2: How Embryonic Stem Cells are Derived. Adopted from the National Institute of Health (NIH) Web site www.stemcells.nih.gov (7).

1.1.2 Neurodegenerative Diseases and Stem Cell Potential

ESCs have been used as tools for studying developmental biology (11). More recently, ESCs have become of great interest in generating transgenic animals, drug screening, and potential cell therapies for regenerative medicine to address degenerative diseases (11). It has been realized that pluripotent cells possess the distinct potential to be utilized in the treatment of neurodegenerative diseases through the differentiation to neurogenic cells for transplantation (**Figure 1-3**).

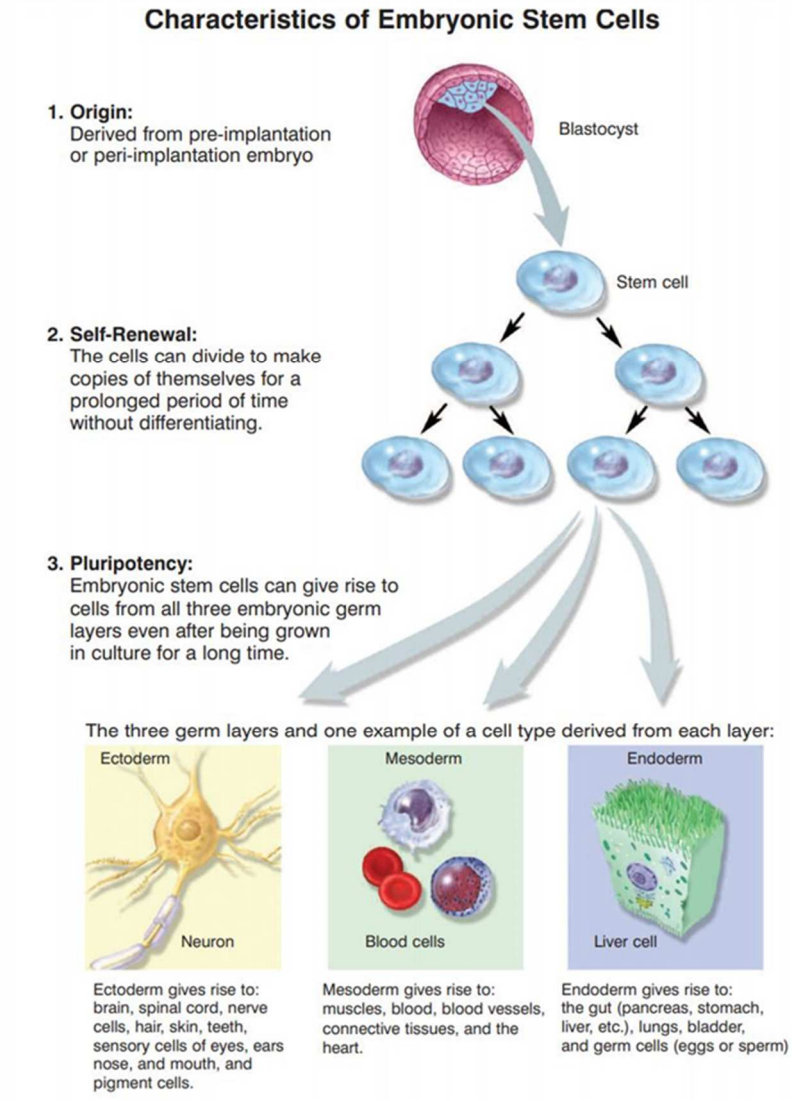

Figure 1-3: Characteristics of Embryonic Stem Cells. Adopted from the National Institute of Health (NIH) Web site www.stemcells.nih.gov (7).

Neurodegeneration is a complex process that causes neuronal death in the central nervous system (CNS) and the peripheral nervous system (PNS), resulting in damage or dysfunction (12). "This damage further causes oxidative stress, axonal transport deficits, protein oligomerization, aggregation, calcium deregulation, mitochondrial dysfunction, abnormal neuron–glial interactions, neuroinflammation, DNA damage, and aberrant RNA processing" (12). There are hundreds of different neurodegenerative diseases, however, the most attention is paid to

Parkinson's disease, Huntington's disease, schizophrenia, amyotrophic lateral sclerosis, Alzheimer's disease, and spinal muscular atrophy (5, 13). Many of the less broadcasted neurodegenerative disorders, though not less devastating, have been technically ignored (13).

Fascinatingly, increasing age is the most consistent risk factor for developing a neurodegenerative disorder (11). Today, the growth rate of the population aged 65 and over has very much surpassed than that of the whole population combined (14, 15). Thus, many anticipate that, over the next generations, the proportion of elderly citizens will double. As expected, the proportion of persons suffering from neurodegenerative disorders will also be doubled, triggering an increased emotional, physical, and especially financial burden on society (15).

The ability to isolate human embryonic stem cells (hESCs) is key for research and clinical applications. However, there are ethical concerns that come behind the use of human embryos for studies and cell therapies (5). One must also consider the complications of potential immune responses that some patients undergo during allogenic therapies (5). For these reasons, the ability to generate patient-specific somatic cell-derived induced pluripotent stem cells is of great interest to medical and scientific communities (5). In 2006, researchers made a discovery by classifying conditions that would allow some specialized adult cells to be genetically reprogrammed to assume an embryonic stem cell-like state called induced pluripotent stem cells (iPSCs) (5). From then on, scientists have developed techniques towards discovering more effective methods to reprogram somatic cells to other highly valuable stem cells.

1.1.3 Network and Molecular Mechanisms of Pluripotency

Mechanisms of pluripotency are still not entirely understood. However, scientists have clarified the master regulators of the pluripotency network; Sox2, Oct4, and Nanog (SON) proteins (16). Oct4 (octamer-binding protein) is a transcription factor and a key regulator of stem cell

pluripotency (5 & 17). It has also been discovered that the expression of Oct4 is limited to the inner cell mass (ICM) in a developing embryo and is maintained in adult germ cells but downregulated in differentiated somatic cells (16 & 17). When scientists knockout Oct4 in embryos, they die after the blastocyst stage and fail to develop an ICM indicating its essential role in normal mammalian embryonic development (18). Moreover, reduced expression levels of Oct4 in embryos leads to the spontaneous differentiation into the trophoblast lineages (18). This indicates the identity of Oct4 as part of a pluripotent maintenance mechanism (18). Furthermore, an increase in the expression levels of Oct4 in embryos, induces differentiation into primitive endoderm and mesoderm stages (19-21). Therefore, the Oct4 gene expression level precisely regulates the pluripotent state of ESCs.

Sox2, (SRY-related HMG-box) is also a transcription factor that is highly expressed in the pluripotent lineage of the early embryos (22). Like Oct4, Sox2 plays an important role in maintaining pluripotency and its expression is confined to the ICM, epiblast of early embryos, and germ cells (22). In contrast to Oct4, however, Sox2 is also expressed in the extraembryonic ectoderm and in precursor cells in the early and adult nervous system (22 & 23). Moreover, Sox2 knockout embryos fail to develop epiblast and die at the implantation stage (24). Additionally, a reduced level of Sox2 in ESCs is associated with a loss of pluripotency and a tendency to differentiate (25 & 26). Furthermore, it's been reported that Sox2 and Oct4 work synergistically to control the expression of specific ESC genes by forming protein-heterodimers (26-28) and regulate expression of Oct4 itself (25). Moreover, Sox2 is a key component of the transcriptional regulatory networks, which maintains the totipotency of the cells during embryonic preimplantation period, the pluripotency of embryonic stem cells, and the multipotency of neural stem cells (NSCs) (30).

Nanog (homeobox protein) is also a transcription factor that was discovered based on its ability to maintain ESC self-renewal in the absence of leukemia inhibitor factor (LIF). LIF is used to maintain cultured mouse ESCs in an undifferentiated state (12 & 31). For instance, Nanog-null mouse embryos fail to maintain a pluripotent epiblast, while disruption of Nanog *in vitro* results in an increased tendency of ESCs to spontaneously differentiate (31 & 32). Nanog has been shown to act as a strong activator of Oct4 (33). "When the ESCs experience a drop in Nanog levels, they cause upregulation of transcription factors that are involved with trophectoderm differentiation, indicating that Nanog helps sustain pluripotency by repressing these differentiation factors" (32). Other reports suggest that Nanog is upregulated in embryonic based tumors (10). However, Nanog is not strongly correlated with cancers in human adults (20, 34). Sox2, Oct4, and Nanog, serving as master regulators, make up the pluripotency autoregulatory circuit, which maintains the pluripotency of ESCs (30) (**Figure 1-4**).

The roles that SON transcription factors play during early development suggest that these master regulators are critical to a transcriptional regulatory mechanism that controls ESC identity (6). To find genes that are occupied by SON, scientists have utilized genome-wide location analysis and chromatin immunoprecipitation (ChIP) in hESCs (6). They found significant insights on the ability of these transcription factors to regulate the pluripotency of cells (10, 36). Firstly, SON proteins bind to their own promoters and to each other's promoters to form an autoregulatory circuit (**Figure 1-4**) (6). Secondly, as regulators, SON mostly occupy their target genes to maintain pluripotency and repress differentiation. The autoregulatory circuit of the SON regulator system ensures that gene expressions are maintained in ESCs so that pluripotency can be maintained.

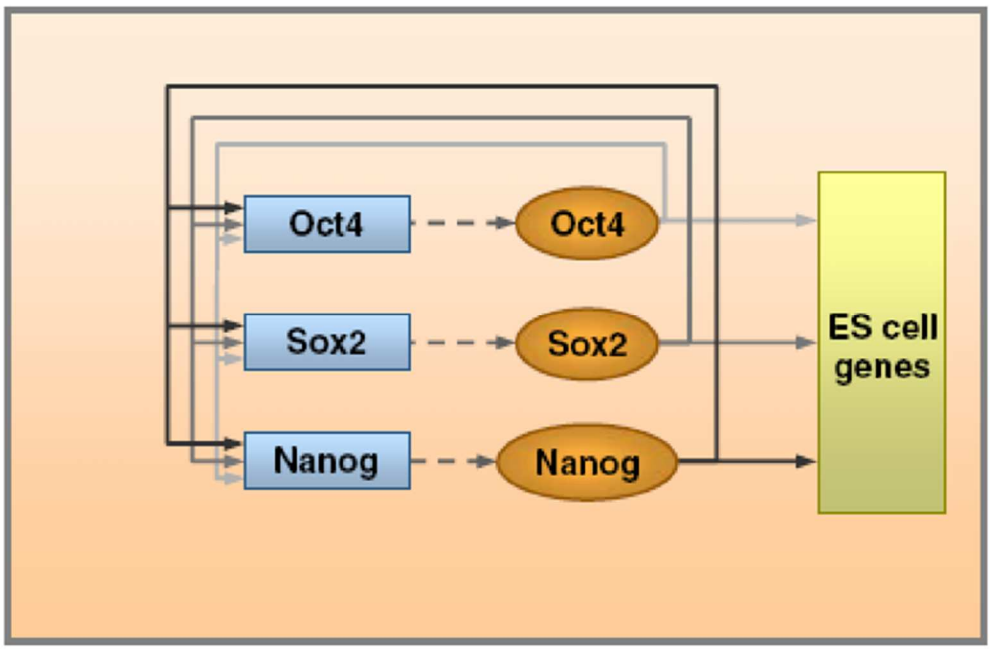

Figure 1-4: Schematic Diagram of Autoregulatory Circuit. SON form an interconnected autoregulatory circuit, by targeting both their own promoters and those of each other. In this way, the three key transcription factors can maintain their own expression, thereby contributing to the maintenance of pluripotency. Proteins are depicted as circles, gene promoters as rectangles. (6)

SON factors have also been identified to target hundreds of other genes in ESCs (6) (**Figure 1-5**), mainly including two types of ESC genes. The first type of gene family contains pluripotency genes, where the SON factors bind to the promoter regions of these genes to activate their gene expression. The second sets of genes include differentiation genes that remain silent until cell differentiation (10). During the embryonic state, the SON factors bind to the repressors of these second set of genes set to silence their gene expression. The activation of gene expression of this second set of genes causes differentiation and the SON factors bind to the promoter regions of these 'silent genes' to activate their gene expression for induction of differentiation. Therefore, the SON master regulator factors regulate a cell's fate through the interactions with the promoters and repressors of specific genes (10). It has also been discovered that the SON master regulator

autoregulatory circuit responses to external stimuli, which implies that pluripotency could be maintained through developmental stimuli (10).

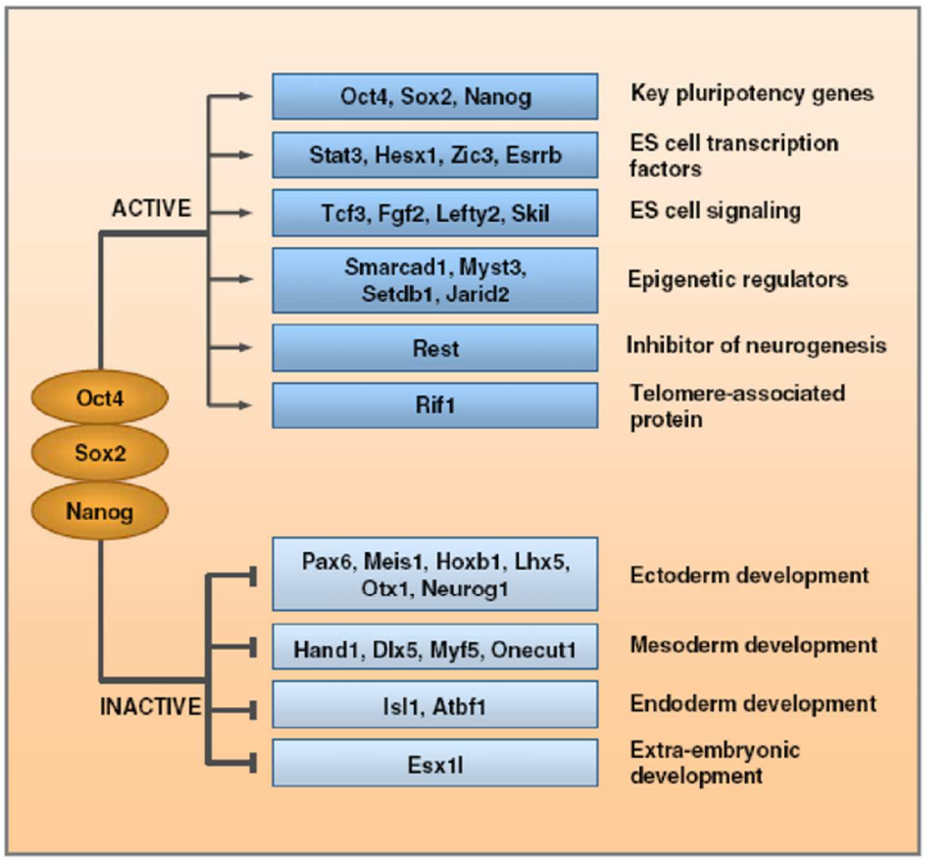

Figure 1-5: Diagram of SON Transcriptional Network. The SON trio contribute to ESC pluripotency by repressing genes linked to lineage commitment and activating genes involved in pluripotency. Active genes include, besides Oct4, Sox2, and Nanog themselves, ESC-specific transcription factors, components of ESC signaling cascades, and epigenetic enzymes. In contrast, repressed genes comprise regulators of differentiation into the three germ layers and extraembryonic development. Proteins are depicted as circles and gene promoters as rectangles (6).

SON co-occupy different genes, meaning that they work together to maintain pluripotency (3-6). STAT3, SKIL, ZIC3, and REST are a few of the transcription factors that are transcribed for pluripotency. Chromatin-modifying enzymes such as SET or signal transduction components such as DKK of the Wnt pathway or Lefty2 of the TGF-β pathway are other genes for pluripotency (10, 36). SON are involved in epigenetic complexes that further represses the transcription of

'silent' differentiation genes that translate to proteins of the Polycomb group, chromatin remodeling complexes, and histone deacetylase complex (37). Moreover, expressed factors are all known to maintain the pluripotency of ESCs and their self-renewal, while the silent genes, such as Pax6, are involved with differentiation of the cells to specific lineages (10, 36). The expression of silent genes that translate to developmental transcription factors like Pax6, are associated with the differentiation to neurogenic lineage commitment. These factors have established the formation of models to uncover the downstream of cellular differentiation.

1.1.4 Most Effective Stem Cells for Neurodegenerative Disorders

Over the past 14 years, cell reprogramming techniques have been widely applied to generate induced pluripotent stem cells (iPSCs) (5, 38, 39). Many patient-specific iPSC-lines have been established for specific diseases such as Parkinson's disease (40, 41, 46), Huntington's disease (40) schizophrenia (42), amyotrophic lateral sclerosis (43), Alzheimer's disease (44), and spinal muscular atrophy (45). However, for therapeutic application, iPSCs need to be efficiently differentiated into the desired specific lineage cell type (**Figure 1-6**).

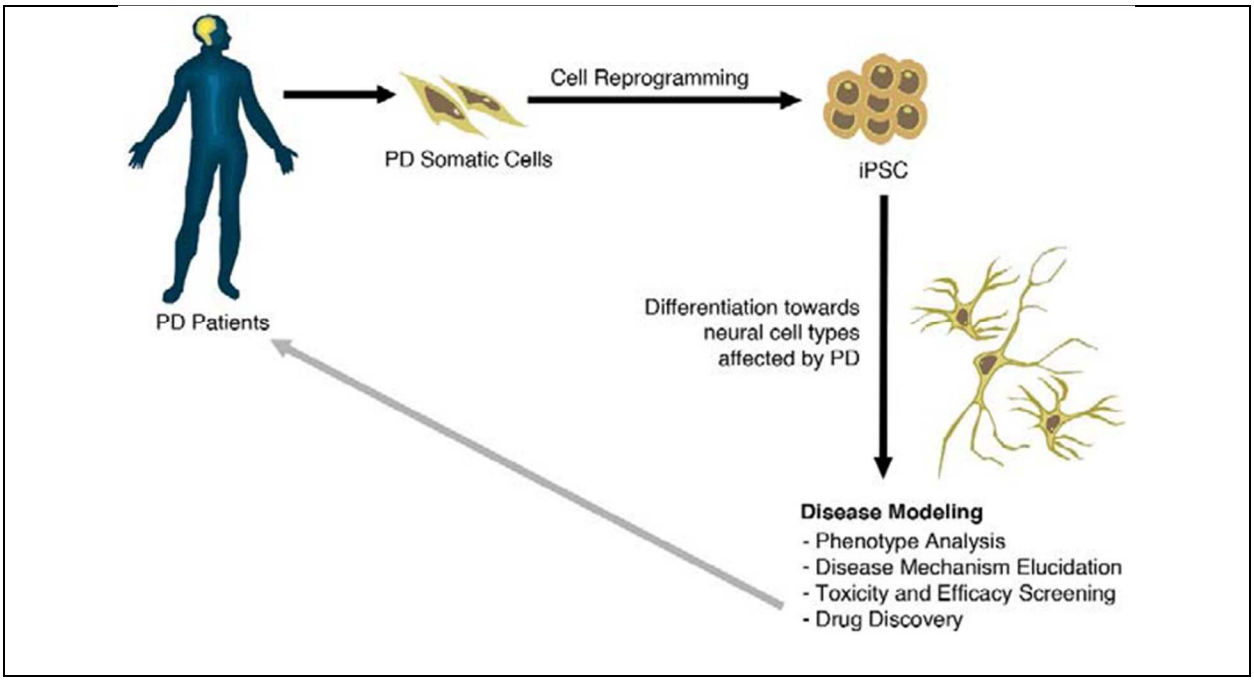

Figure 1-6: Generation and use of iPSC Modelling in Parkinson's Disease (PD). Somatic cells from a diseased patient are isolated and then reprogrammed to a pluripotent state (iPSCs). iPSCs can be maintained in culture or induced to differentiate along tissue- and cell-type specific pathways. Differentiated cells can be used to elucidate disease mechanism pathways, as well as for the development of novel therapies (46).

Moreover, the cells derived from iPSC and undifferentiated iPSCs exhibit potential tumorigenic risks (29, 47, 48, 49) and limit their direct use in cell transplantation applications. This makes PSC differentiation mechanisms highly attractive. With that said, Pluripotent stem cells such as ESCs and iPSCs can form teratomas *in* vivo, whereas multipotent, lineage-restricted stem cells such as induced neural stem cells (iNSCs) and hematopoietic stem cells do not (29, 47, 48, 49). "Reprogramming somatic cells into less potent, lineage-restricted cells will sidestep the difficulty of differentiating iPSCs, thus lowering the risk of teratoma formation, while complementing iPSC technology" (29).

Transcription factors have been previously used to induce a change in cell fate from one type of cell to another (5, 29, 38, 39, 50, 51, 54, 55). Induced multipotent adult stem cells such as iNSCs, induced cardiac progenitor cells, induced functional neuronal cells, and cardiomyocytes

have been generated from somatic cells. In particular, the generation of induced neuronal cells (iNCs) from somatic cells provided a new avenue for research in transplantation therapies for neurodegenerative disorders. Although iNCs have typical neuronal cell properties such as exhibiting proper electrical function in culture and can be generated with high efficiency (5%-20%), clinical applications must consider the inability of iNCs to self-renew in culture because they are terminally differentiated (29). Terminally differentiated means that there is a restriction in cell fate to only a few neuronal sub-types. Moreover, current reprogramming methods generate a mixture of neuronal cells and other unknown types of cells, limiting the use of iNCs for transplantation therapy (**Figure 1-7**). In figure 1-7 iNCs are referred to as neural progenitors.

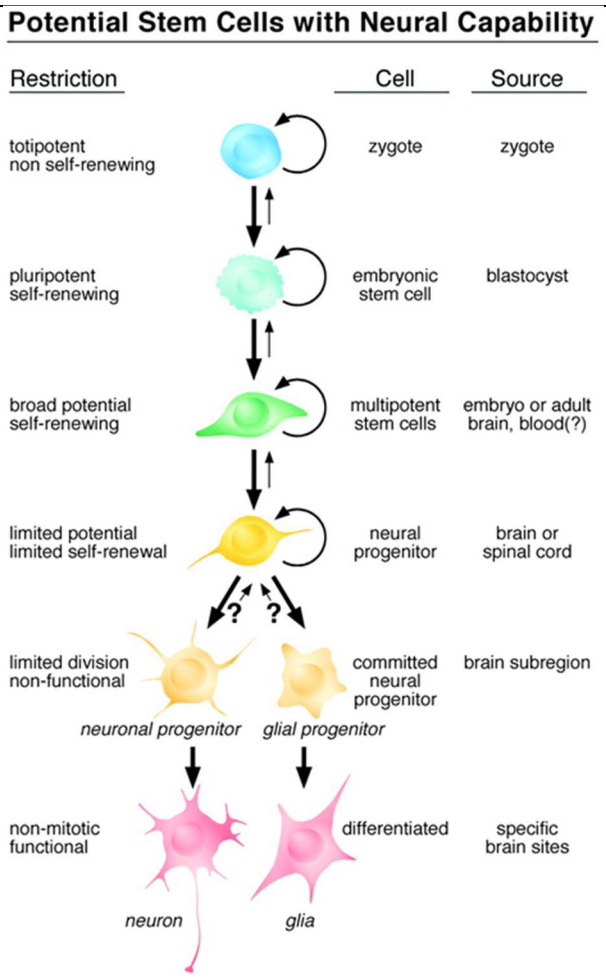

Figure 1-7: Potential Stem Cells with Neural Capability. An illustration proposing the classes of mammalian stem cells that can give rise to neurons presented as a hierarchy beginning with the most primitive and multipotent stem cell and progressing to the most restricted. The restrictions of fate at each step and examples of sites in the body where they can be obtained are also presented. As our understanding of the true potential and nature of stem cells is still unfolding, modifications will be added. For example, the small arrows pointing up suggest the potential, although not well documented, dedifferentiation of the more restricted cell below (52).

Although many studies have shown the generation of iNCs from mouse and human somatic fibroblast cells (53, 54, 55, 56, 57), these cells are terminally differentiated which results in a restriction in cell fate to only a few neuronal subtypes. In all, the generation of patient-derived multipotent iNSCs is more advantageous than the generation unipotent (iNCs) because multipotent cells express greater self-renewing capacity and passaging ability, along with limited potential (**Figure 1-7**).

1.1.5 Neural Stem Cells and Neurogenesis

NSCs were first identified within the CNS in the year 1992 (58). Overall, the ability to efficiently reprogram somatic cells into self-renewable, multipotent, nontumorigenic, and neural-linage restricted iNSCs, may have major implications in regenerative medicine for neurodegenerative diseases. “The most common brain cells are neurons and glial cells” (59). Glial cells are the astrocytes and oligodendrocytes of the CNS. NSCs are very powerful model systems for neurodegenerative disease transplantation therapies because they are only able to make three types of cells; astrocytes, oligodendrocytes, and neurons (**Figure 1-8**). “Furthermore, NSCs secrete therapeutic molecules like soluble factors, including neurotrophic factors, growth factors, and cytokines, that protect existing neural cells against damage *in situ*” (60). Neurotrophic factors regularly released from NSCs improve a lesion’s microenvironment, therefore generating suitable conditions for damaged neural tissue repair (60, 61, 62, 63).

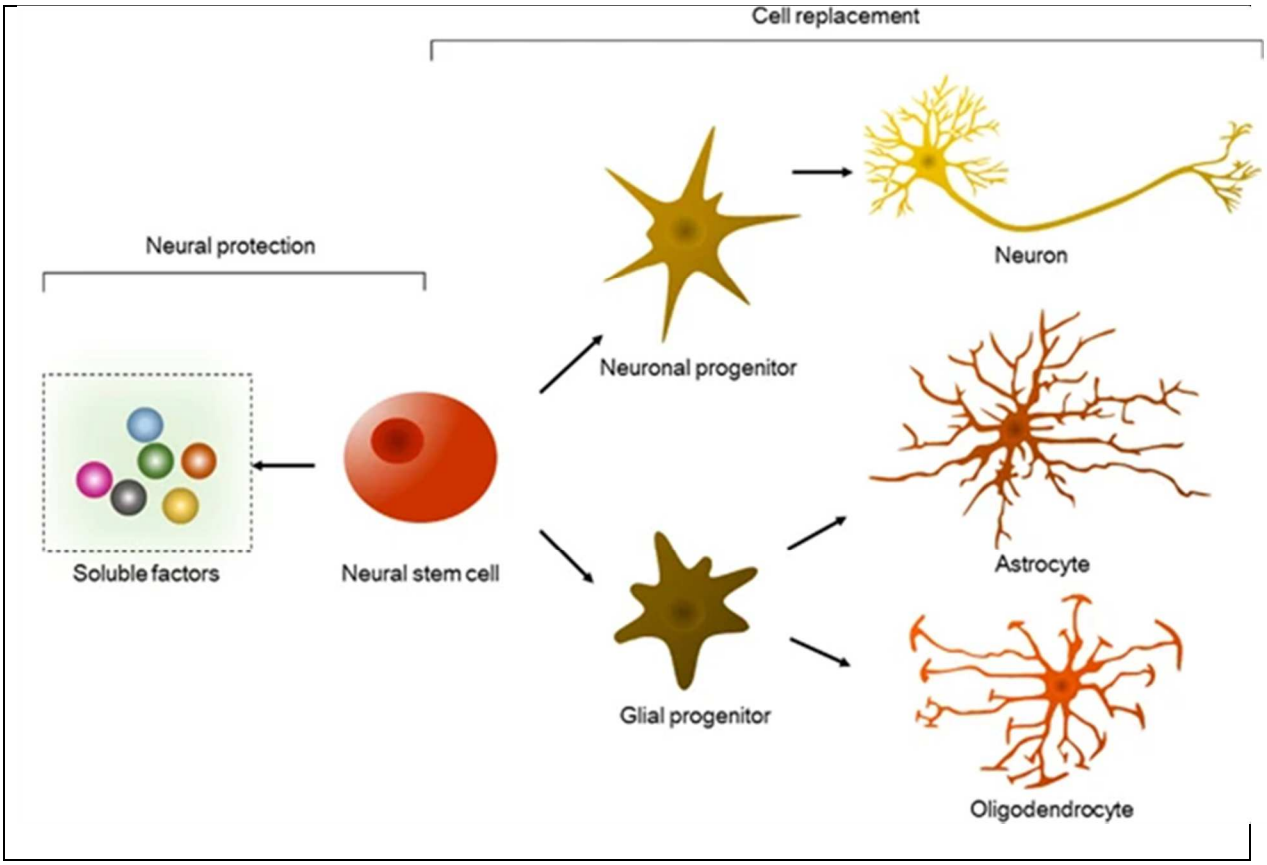

Figure 1-8: NSC Properties for Therapeutics. NSCs secrete soluble factors, including neurotrophic factors, growth factors, and cytokines, thus protecting existing neural cells against damage *in situ*. Furthermore, they differentiate into neurons, astrocytes, and oligodendrocytes via committed progenitor stages to replace lost neural cells. Either neural protection or cell replacement may aid in neurological functional recovery after acute or chronic injury via neural regeneration (60).

Multipotent adult stem cells, which are adult stem cells such as hematopoietic stem cells, mesenchymal stem cells, and NSCs, exist throughout the adult human body and play important roles in tissue maintenance and repair. Until now, the reason for the age-associated decline in self-renewing capability and differentiation potential of multipotent stem cells in tissues is unclear. Like pluripotent stem cells, multipotent stem cells can self-renew and proliferate. However, multipotent stem cells can only develop into multiple specialized cell types for specific tissue or organ versus pluripotent stem cells that can generate any cell in all three germ layers. The discovery of stem cells that can directly generate neural tissue has raised new possibilities for

repairing the CNS. NSCs, with self-renewal ability that are multipotent and able to generate multiple neural lineages, including glia, and neurons, are derived from regions throughout the adult CNS (64).

The major function of neurons is the transmit information mostly through biochemical mechanisms. The typical neuron consists of a cell body, dendrites specialized for receiving information from other neurons, and an axon specialized to conduct impulses away from the cell body. Astrocytes function as physical, and metabolic supporters for neurons, through detoxification, guidance during migration and regulation of energy metabolism. Moreover, astrocytes also serve as electrical insulation for unmyelinated axons. Astrocytes are also thought to play a vital role in regulating NSC proliferation and differentiation. Oligodendrocytes mainly function to provide support and insulation for axons in the CNS by creating the myelin sheath.

Neurogenesis is the continuous natural growth and development of nervous tissue. Adult neurogenesis is an active process, where adult neural progenitors differentiate and mature into neuronal cells that integrate into the existing neural circuitry. In mammals, CNS neurogenesis occurs in two discrete locations called neurogenic regions: the subgranular zone (SGZ) of the dentate gyrus in the hippocampus for dentate granule cells and the subventricular zone (SVZ) of the lateral ventricles in the forebrain for interneurons in the olfactory bulb (64) (**Figure 1-9**).

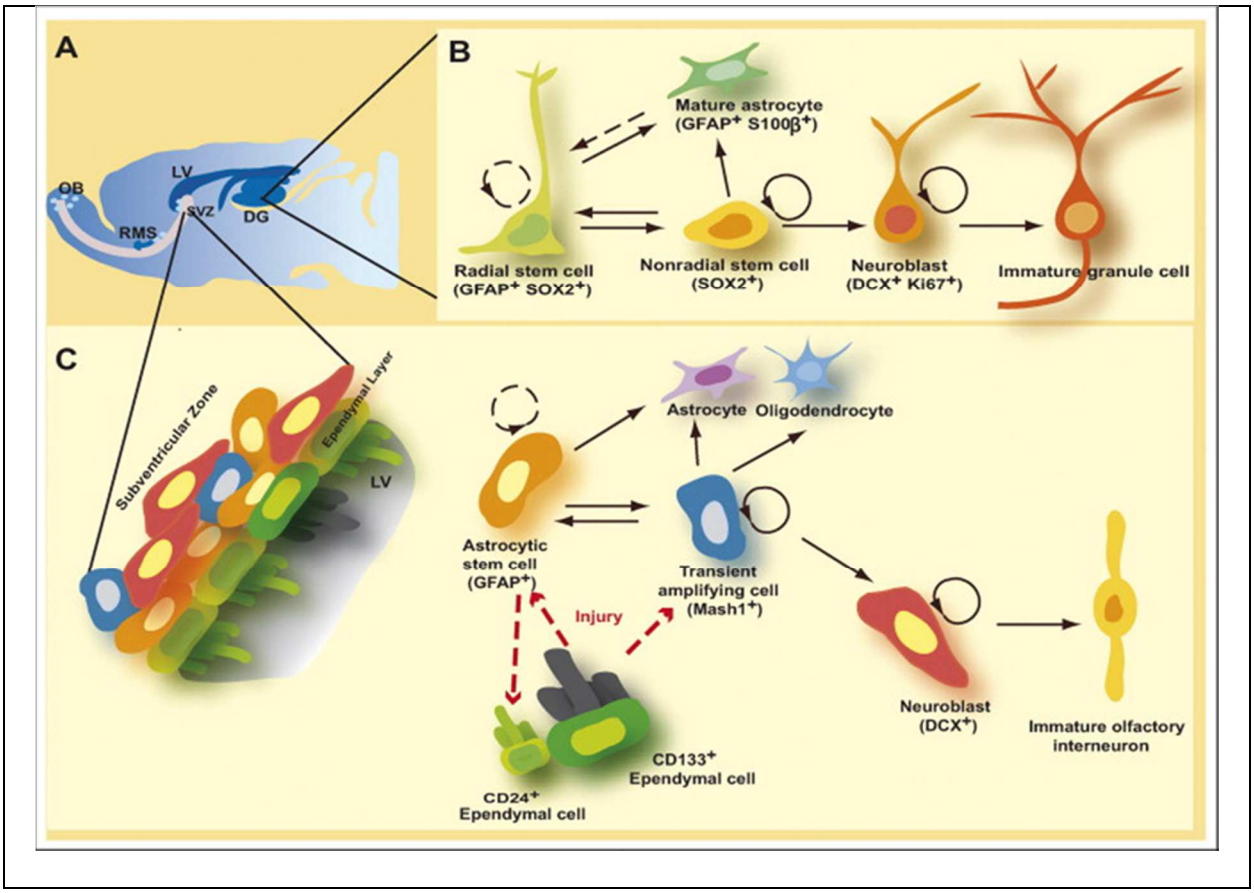

Figure 1-9: Models on the Identities of Potential Quiescent NSCs in the Adult Brain. (A) Two neurogenic regions in the adult brain: the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (LV). (B) Potential lineage relationships in the adult SVZ. (C) Potential lineage relationships in the adult SGZ (64).

The development of the mammalian cerebral cortex follows a stepwise production of neurons, then glial cells, including astrocytes and oligodendrocytes from NSCs (65). During embryonic development, cells of the central nervous system are derived from the neuroectoderm, which is arranged as a neural tube which later invaginates to form structures including the prosencephalon. Then the telencephalon is diencephalon emerge from the prosencephalon. The cerebral cortex arises from the dorsal telencephalon and the ventral telencephalon gives rise to the basal ganglia (65). NSCs from the dorsal and ventral telencephalon are critical to the generation of neurons.

Injuries, such as stroke, activate the neurogenesis mechanism outside of the described neurogenic regions. However, whether and the extent of the active neurogenesis occurs outside the two neurogenic regions in the mammalian CNS *in vivo* remain debatable. Neurogenesis is reduced in the progression of aging, which is thought to contribute to age-related cognitive issues (66). The mechanism of age-dependent decline in neurogenesis remains largely obscure.

1.2 Induced Neural Stem Cells (iNSCs)

The generation of neurons or glial cells to replace damaged tissue is a key step in stem cell-based therapies designed to treat neurodegenerative disease and neurological disorders (29). Although the mechanisms that establish and maintain the multipotency of neural stem cells are unclear, the generation of expandable NSCs is a promising therapeutic approach for treating neurodegenerative diseases or injuries. One approach is to obtain NSCs by differentiation of pluripotent embryonic stem cells (ESCs) as indicated on the **right side of Figure 1-10**. The second approach is to reprogram the skin fibroblast cells from the patients into expandable induced NSCs (iNSCs) as indicated in the left of Figure 1-10. The obtained expandable NSCs serve as the source of neural cells for stem cell-based therapies designed to treat neurological disorders (**Figure 1-10**).

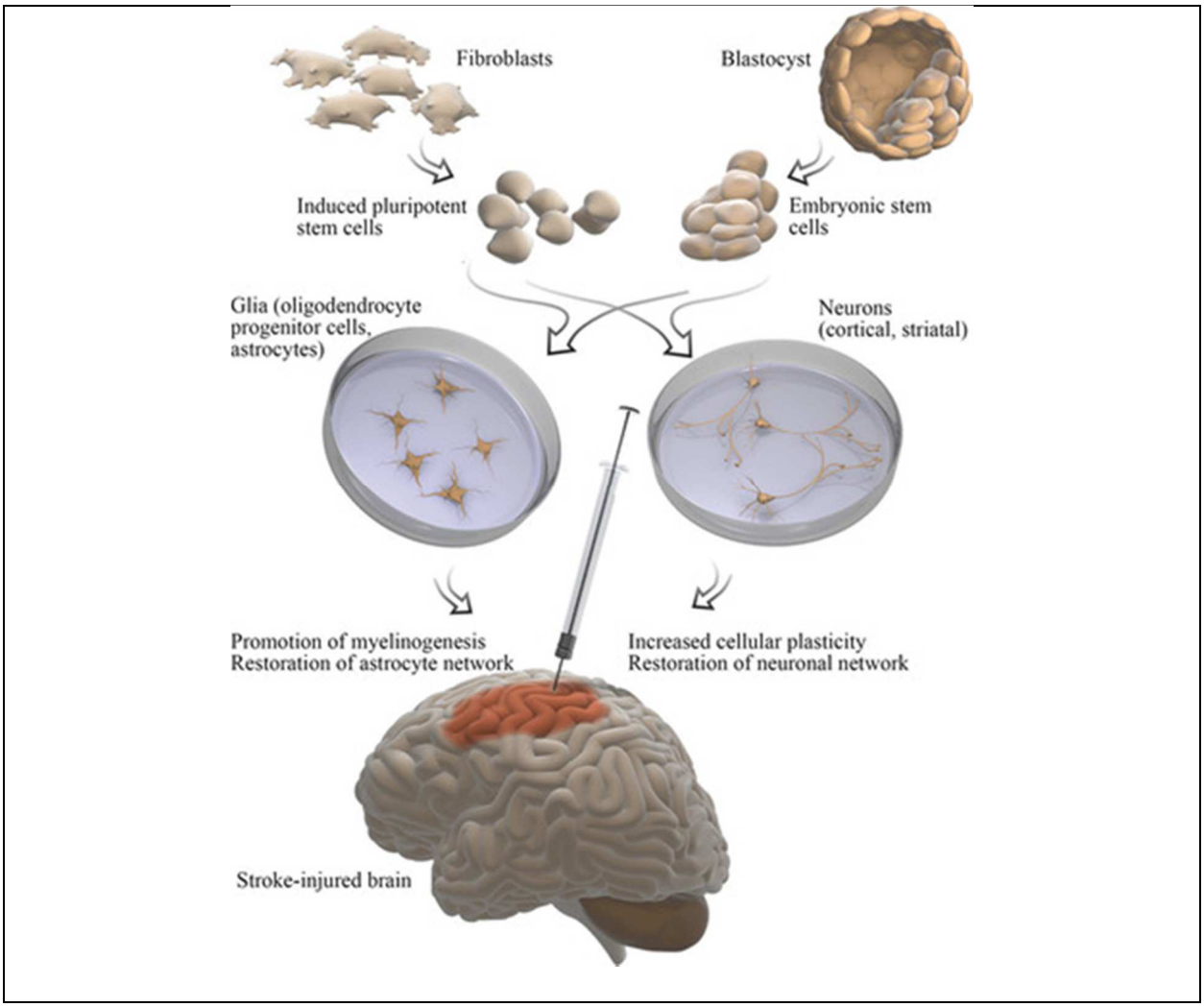

Figure 1-10: Application of Pluripotent Stem Cells in Cell Therapy for Stroke-injured Brain. (1) Pluripotent stem cells can be derived from blastocyst (embryonic stem cells [ESCs]) or through reprogramming of postmitotic somatic cells, most commonly fibroblasts, generating induced pluripotent stem cells (iPSCs). (2) Both ESCs and iPSCs can be treated in vitro to generate glia and neurons. (3) Transplantation of glial and neuronal cells at the early stages of their development into stroke-injured brain can lead to functional recovery through the promotion of myelinogenesis and restoration of astrocyte network (glia) or by increasing cellular plasticity and restoring neuronal network (neurons). OPC an indicates oligodendrocyte progenitor cell (67).

1.2.1 Generation of iNSCs using Defined Transcription Factors

Certain Transcription factors (TFs) are lineage-determining factors, which activate necessary genes that determine a cell's fate such as Sox2, Oct4, Klf4, and c-Myc factors. Yamanaka et. al., reprogrammed somatic fibroblast cells into ESC-like pluripotent stem cells, iPSCs. Induction of differentiation of the generated iPSCs to NSCs could be accomplished by

overexpressing certain neural-lineage TFs. Despite the differentiation capabilities of pluripotent stem cells, they were found to have ethical and practical issues (49). Specifically, the origin of ESCs and the ability of iPSC derived cells to form tetratomic cells, respectively (49). From this point on, many researchers have turned their focus towards reprogramming somatic cells to adult stem cells such as NSCs, which do not form tetratomic cells.

Their et al. used the same reprogramming cocktail as Yamanaka (Oct4, Sox2, Klf4, and c-Myc), but with the initial absence of Oct4 when reprogramming fibroblasts (68). In this case, cells are thought to abandon the initial pluripotent fate and enter the NSC multipotent fate by being overwhelmed by the overexpression of transcription factors (69). This result indicated that the TF-driving cell reprogramming technique could be used to generate adult stem cell fates such as NSCs from somatic cells without going through the pluripotent state. Since then, scientists have spent major effort in searching different TFs to ensure a gain of insight on the minimal combinations of lineage-factors required to convert fibroblasts in iNSCs.

NSCs show characteristic properties that differentiate them from other types of adult stem cells. For example, NSCs form self-renewable neuro-spheres, which can be serially passaged and assayed using different techniques such as immunostain for assaying NSC marker proteins and qRT-PCR for assaying NSC marker mRNAs. More importantly, NSC's have the capability of differentiation into tri-neural lineage cells, including neurons, astrocytes, and oligodendrocytes, which is a key feature that can be used to characterize NSC's.

Recently, two studies have shown that the combination of Sox2, Klf4, and c-Myc or Brn4, Sox2, Klf4, c-Myc, and E47/Tcf3 can reprogram fibroblasts into iNSCs (70, 71). It is crucial here to omit Oct4 form the combination because the aim is to only induce NSC's without pluripotency. By screening 11 lineage-determining factors, Lujan et al., and Han et al. pinpointed three to four-

factors that induce the NSC fate. The results from both reports showed impractical long conversion time of around 3 weeks, and very low conversion efficiencies of induced NSC's from fibroblast cells (as low as 0.1%) (70, 71).

Despite the low conversion efficiency, they demonstrated that when the potent c-Myc oncogene is overexpressed, the iNSCs can self-renew and generate functional neurons *in vitro* and integrate *in vivo* (70). In some cases, iNSCs established high self-renewal capacity with up to 130 passages in culture (70). It seemed that c-Myc overexpression is the key factor for iNSC conversion. However, as seen in transplant studies, the overexpression of the c-Myc oncogene is correlated with increased brain tumorigenesis (72). Moreover, Lujan et al. were also able to generate iNSCs with the use of FoxG1 and Brn2 only (71). Further studies suggest that the successful induction of NSCs is due to the activation of the Sox2 gene expressions by FoxG1 (70, 72).

1.2.2 Sox2 and NeuroD1 are Two Key Factors for iNSC Fate Determination

Up to now, it is still unclear which linage-determining factors are needed to efficiently reprogram fibroblasts into high-quality iNSCs. Regardless of what approach it takes to generate iNSCs from fibroblast cells, Sox2 seems to be a key factor. Sox2 is a master regulator factor that is highly used for NSC identity and maintenance (73). Sox2 expression can reprogram fibroblasts to multipotent NSCs (73).

One report used only one transcription factor (Sox2) to generate iNSCs from human and mouse fibroblasts (29). In this study, iNSCs were shown to generate functional neurons *in vitro* and can survive *in vivo* without the formation of tumors or tetratomic cells (29). Results of this study also shows mouse-iNSCs (miNSCs) were passaged over 40 times, and around 20 passages for human-iNSCs (hiNSCs) (29). This experiment also flaunted the formation of homogenous tri-

potent populations, instead of a heterogeneous population containing different neural progenitor cells (29).

In a cell, contrary to c-Myc overexpression, Sox2 overexpression was observed to become silent over time. This implies that iNSCs can switch, on or off, the endogenous expression of NSC fate-determining genes to maintain cell fate (29). Furthermore, Ring et al., also confirmed the advantages of NSCs over PSCs by transplanting mouse brain cells induced by Sox2 and observing the lack of tumor formation. (29).

As discussed above, many cell reprogramming techniques and/or combinations can be performed to induce NSCs from fibroblasts, however, they all suffer from several of the following major challenges. First, they show very low reprogramming efficiency, probably partly due to the combination of TFs and partly due to the current gene-delivery based reprogramming technology. Second, all studies above used invasive methods such as a virus to force the gene expression of lineage- determining factors. “The use of a virus may have a major safety risk and possibly cause undesired tumor formation, genotoxicity, and mutagenesis” (74). Third, the current existing cell reprogramming procedures are less efficient, complicated, and expensive for practical use.

Neuronal Differentiation Factor 1 (NeuroD1), is a basic helix-loop-helix lineage-determining transcriptional factor (75). NeuroD1 is required for regulating the transcription of genes that cause neurogenesis and early differentiation of adult-born neurons (75). The combination of TFs along with NeuroD1 can be used to reprogram mouse fibroblasts into iNSCs with self-renewing ability (55, 68, 70, 131, 76). Moreover, one factor (Sox2) is enough to generate self-renewable, multipotent, nontumorigenic, and lineage-restricted iNSCs (29). However, these groups have demonstrated practical issues and very low conversion efficiencies, implying that the

addition of neuronal fate-specifying factors to the reprogramming cocktail can influence the ability to generate specific neuronal subtypes with much higher conversion efficiency (54, 77).

In my thesis project, I applied a patented reprogramming technology called QQ-protein-induced pluripotent stem cell (QQ-piPSC) technology, developed in Dr. Jianjun Wang's laboratory. This piPSC technology allows for protein-induced cell reprogramming to generate piPSCs from somatic cells with over 90% conversion efficiency (137). I used this breakthrough technology to reprogram fibroblast cells into protein-induced NSCs (pi-NSCs) and eliminated the need for any invasive and unsafe methods in the traditional gene-based cell reprogramming techniques. Second, we believed that the addition of the NeuroD1 factor along with the Sox2 factor would lead to significantly enhanced efficiency of reprogramming fibroblasts into piNSCs with high quality and less safety risk. Third, we demonstrated high differentiation potential of the generated pi-NSCs into high quality and safe specialized neurons, astrocytes, and oligodendrocytes *in vitro.*

1.2.3 NSC Differentiation

Here, we focus on the early steps of ESC neurogenesis, in which multiple protocols have been established that produce pluripotent cell□derived iNSCs. Recent studies suggest that ESC□ derived NSCs can have the properties of embryonic radial glia, that can proliferate and generate all types of neural derivatives, neurons, astrocytes, and oligodendrocytes (78). During early development, the radial glia cells are the primary neural stem cells (NSCs) that generate all the neurons as well as the glial cells and ependymal cells that persist in the CNS (79). In the embryo, neurogenic radial glial cells are present throughout the developing CNS (79).

Two approaches have been identified to make NSCs from ESCs. One fascinating method is to imitate the environment that produces neurectoderm in the embryo by providing appropriate

cell-cell interactions and signals through EB formation (80). It was found that when mouse ESCs were removed from a feeder layer and suspended without LIF growth factor that was a maintenance factor for pluripotency, they aggregate to form an outer layer of hypoblast-like cells (extraembryonic visceral endoderm) surrounding an epiblast-like core collectively referred to as an EB (primitive ectoderm) (81 & 82). The epiblast-like core in EBs can generate cell derivatives of all three primary germ layers (endoderm, mesoderm, and ectoderm) (83). At this point, the EB core continues to express the ESC marker Oct4 and starts to express the primitive ectoderm marker FGF5 (81, 82, 84). The ectoderm is known to develop into the nervous system, among other types of tissues such as hair, nails, eyes, and ears. Furthermore, the EB core may also be committed to the ectoderm which is characterized by the expression of Sox2 (81 & 82). At this point, a morphological transformation occurs to the columnar epithelium and it starts to look like a neural tube, which is then followed by the expression of neurectoderm-specific markers such as Sox1 (81 & 82). Moreover, conditions that direct the differentiation of the EB core towards neuroectoderm will further increase the pool of cells that can differentiate into neurons and glial cells (81 & 82).

It has been identified that signals released from the embryos anterior extraembryonic visceral endoderm (AVE) promote the differentiation of the anterior neuroectoderm and the visceral endoderm, EB derived ESCs behave similarly (85). However, the notochord signals known for structuring the neural tube, are not present in the EB derived from ESCs (86). For this reason, EBs derived from ESCs are able to generate primitive ectoderm, but not neuroectoderm or neurons (81). This is because the signaling molecule Indian Hedgehog, which is required for the formation of the notochord and is secreted by the visceral endoderm, promotes neurectoderm

differentiation (80). Several other studies have shown an additional role of the hedgehog signaling cascade in NSC proliferation in the embryo and adult mouse brain (87, 88, 89, 90).

Other studies show that the addition of retinoic acid (RA) can significantly increase the generation of neural lineage cells. RA has a well-established dorsoventral patterning role during development in CNS tissue (91 & 92), and ESC derivatives (93). Cells treated with RA, yield of up to 40% neuron□like cells, versus just a few percent observed in the cultures without RA (80). Similar methods that utilize RA treatment, increased the yield of neural derivatives, including glutamatergic neurons and motor neurons (92, 94, 95). However, when neural progenitors generated by RA induction showed a limited capacity to differentiate when transplanted into the neural tube of chick embryos, compared with ESC□derived neural progenitors generated in the absence of RA (96). Further drawback includes the presence of a heterogeneous mixture of cells as the final product.

Others have also tried to include other factors, such as the use of MEDII medium which appears to have signals that are capable of inducing neuroectoderm differentiation (80). These NSCs do not appear to have a restricted regional identity and, when subjected to different signaling factors, can be further induced to generate neurons or glia, as well as neural crest cells (82). However, the molecules in the medium responsible for this activity, Hep□G2–conditioned medium, have not been classified and this method has not yet been applied to hESCs.

The EB selection in defined media mimics the embryo environment and this method combines the use of an EB intermediate, in which cells of all three primary germ layers arise, followed by a neural lineage-specific selection step (97-99). During the first several days of culture non□neuro-ectoderm cells, including undifferentiated ESCs, extraembryonic endoderm and mesoderm derivatives, die, leaving a neuro-ectoderm□derived neural progenitor population, based

on the expression of Nestin, Sox1, Sox2, and other NSC markers (97). NSCs generated using this approach have been used to give rise to a variety of specific neural and glial derivatives, including oligodendrocytes (100), dopaminergic neurons, and glutamatergic neurons (101, 95). The strength of this method is the relative purity of the final product and the ability to generate large numbers of NSCs upon FGF2 addition (80). The weaknesses of mimicking the embryo environment in defined media include the extended time it takes to generate NSCs (10-14 days) and, the constant variability in the final quality of the product. The final products are usually contaminated with mesodermal derivatives, likely due to the multiple steps (80) yeilding heterogeneous populations.

Neurogenesis of ESCs provides an *in vitro* culture system that can be used to characterize the progenitor cell types involved in the differentiation, identify the active growth factors, and signaling molecules of Neurogenesis (78). Studies show that Wnts and BMPs retrain ESC neural differentiation, specifically by inhibiting the differentiation of primitive ectoderm and primitive NSCs (78). Leukemia inhibiting factor (LIF) supports this inhibition, probably by supporting the maintenance of ESC and primitive ectoderm survival (78). In contrast, Notch and FGF4 promote the commitment of cells to the neural lineage and FGF promotes the proliferation of committed cells like primitive NSCs (78). RA seems to have a role in ESC neural induction, however, findings that identify its mechanism are not clear (80). Other data shows that VEGFA promotes the survival of mature NSCs and inhibits the survival of the primitive NSCs (78). Which implies that these growth factors and signals function in all stages of development to regulate differentiation, proliferation, and survival of NSCs, neurons, or glial cells (78) (**Figure 1-11**).

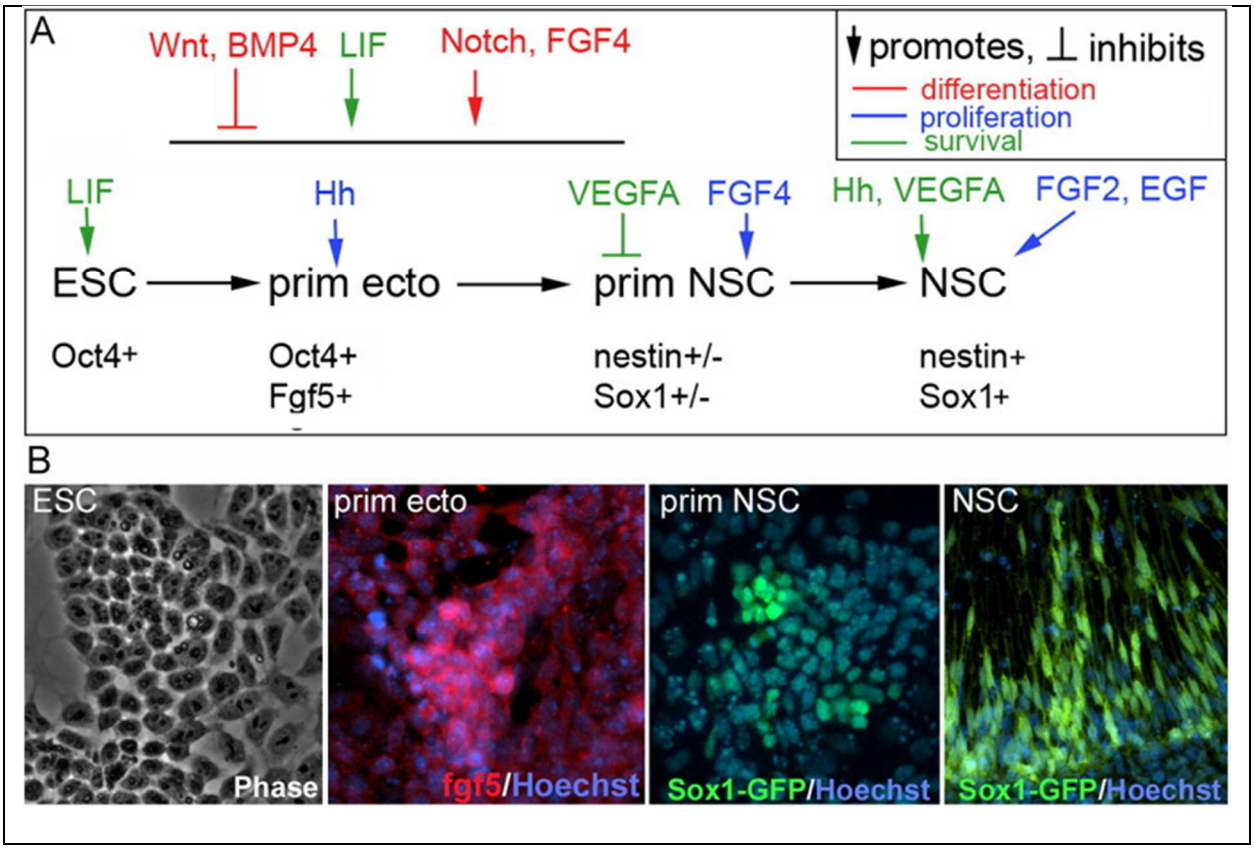

Figure 1-11: Growth Factors and Signaling Molecules Regulate the Multistep Transition from Embryonic Stem Cell (ESC) to Neural Stem Cell (NSC). **(A)** Oct4□positive ESCs first differentiate into primitive ectoderm□like cells (prim ecto), which express fibroblast growth factor□5 (FGF5) as well as Oct4. These cells then differentiate into primitive NSCs (prim NSC), characterized by the expression of moderate levels of nestin and Sox1, and a rounded morphology. These cells differentiate into NSCs with an elongated□radial glia□like morphology that express high levels of nestin and Sox1. LIF, Wnt, bone morphogenetic protein (BMP), Notch, FGFs, Hedgehog (Hh), and vascular endothelial growth factor□A (VEGFA) influence this process as shown. **(B)** Images of ESC, prim ecto, prim NSC, and NSCs generated using the direct defined medium monolayer culture approach (protocol 6) showing expression of FGF5 in prim ecto, Sox1□GFP (green fluorescent protein) in prim NSCs and NSCs. Note the elongated morphology of NSCs. Another method to generate NSCs from ESCs is to the deprive ESCs of both cell–cell interactions and signals by low density culture in serum□free medium, triggering a mechanism for NSC differentiation (78).

It is clear that we have just begun to understand the complex cell inducing interactions that regulate ESC neurogenesis. The addition of specific factors, at specific stages, will allow scientists to optimize the production of NSCs, neurons and, glial cells for transplantation therapies designed to treat nervous system injuries of neurodegenerative diseases.

1.3 Cell Reprogramming Methods

Many studies have been performed to improve the reprogramming methods and to apply technology to understanding potential treatments. As mentioned before, iNSCs complement iPSC technology which includes the molecular techniques used to reprogram to the two types of stem cells. Many have established methods to induce the conversion of fully differentiated cells into iPSCs or iNSCs (**Figure 1-12**) (3).

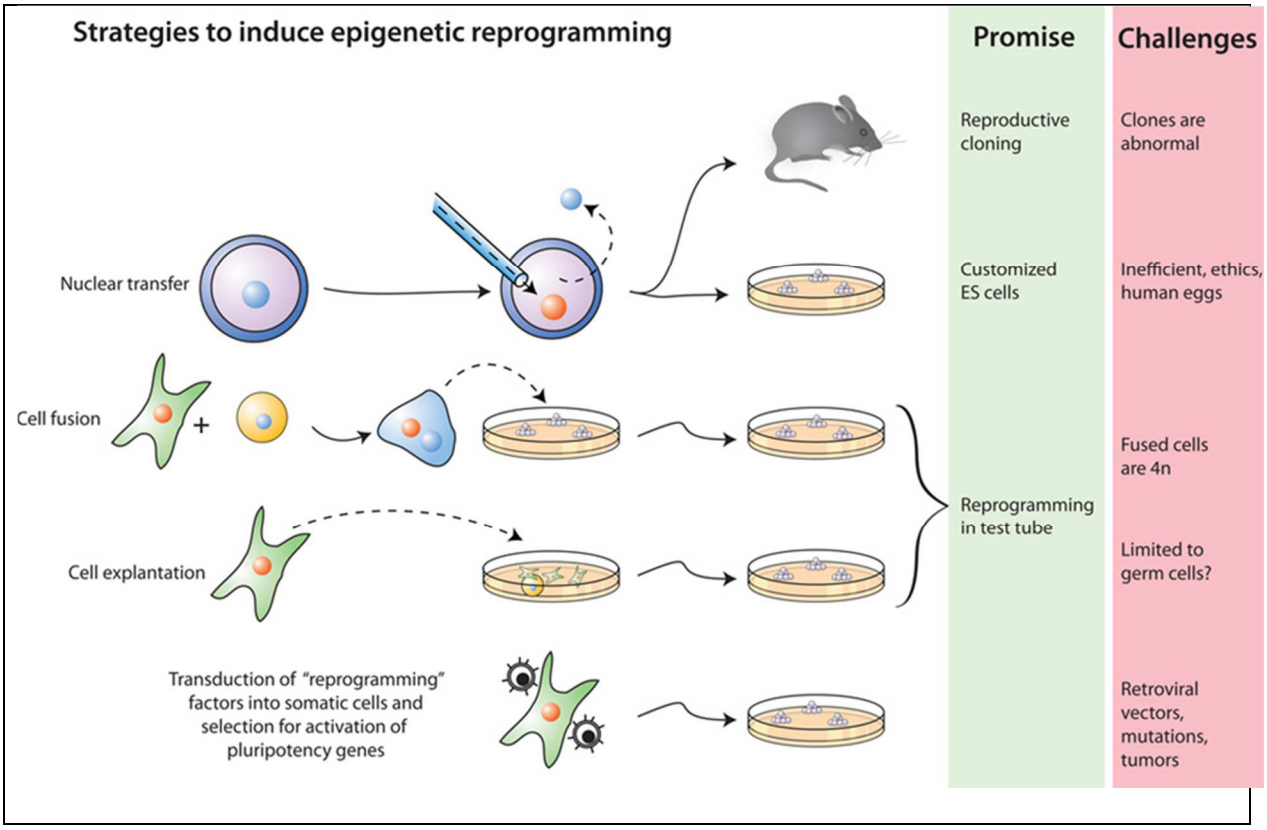

Figure 1-12: Four Strategies to Induce Reprogramming of Somatic Cells. **(i)** Nuclear transfer involves the injection of a somatic nucleus into an enucleated oocyte which, upon transfer into a surrogate mother, can give rise to a clone ("reproductive cloning"), or, upon explantation in culture, can give rise to genetically matched embryonic stem (ES) cells ("somatic cell nuclear transfer", SCNT). **(ii)** Cell fusion of somatic cells with ES cells results in the generation of hybrids that show all features of pluripotent ES cells. **(iii)** Explantation of somatic cells in culture selects for immortal cell lines that may be pluripotent or multipotent. At present, spermatogonial stem cells are the only source of pluripotent cells that can be derived from postnatal animals. **(iv)** Transduction of somatic cells with defined factors can initiate reprogramming to a pluripotent state (3).

Up to now, four methods have been developed to reprogram somatic cells. Nuclear transplantation (NT), the fusion of somatic cells and ESCs, culture-induced reprogramming, and viral transduction. NT is the introduction of a nucleus from a donor somatic cell into an enucleated oocyte to generate a cloned animal (102). The ability to generate cloned animals implicated that the epigenetic status of differentiated cells is not permanent, and cells can be reprogrammed to an embryonic cell that can develop into an organism (3). However, this process is inefficient (3). It was also found that less differentiated cells are more capable of epigenetic reprogramming, greatly reducing the efficiency when reprograming abundant somatic cells such as fibroblasts (3). Further, the cloning of stem cells was found to be much more efficient than differentiated cells (103, 104, 105). Although NT-induced cells show no molecular or biological differences with ESCs fertilized from fertilized embryos (105 & 106). These data implicate that NT ESCs are very useful, but the NT method can only be used with rare unfertilized human oocytes which provides a need for more practical methods when considering ethics (107).

The second method is the fusion of ESCs with somatic cells. This method has been shown to reprogram somatic murine nuclei to an undifferentiated state (107-109). The resulting cells contain many parental embryonic features, such as the activation of Oct4 markers or activation of the somatic X chromosome, suggesting that the pluripotent phenotype is dominant (3). However, the inefficiency of these fussed ESCs with somatic cells has shown to be inefficient and the resulting cell is a tetraploid that limits their potential in clinical applications because of the risk of generating large-scale genetic instability (3).

A third method to reprogram cells is called the culture-inducing reprogramming, which is cell explantation in culture. This involves the removal of living tissue, placed in a medium for expansion in culture which selects for immortal cell lines that may be pluripotent or multipotent

(3). Presently, spermatogonia stem cells are the only source of pluripotency from postnatal animals (3). Other embryonically sourced ESCs from blastomeres or the ECM and embryonic germ cells (EGCs) can be expanded in culture and be functional in germlines (3). However, we are interested in reprogramming somatic cells to generate adult stem cells that are present in postnatal animals. Moreover, it is unknown whether somatic stem cell-derived cells, like those from spermatogonia stem cells, are truly pluripotent since scientists have failed to show significant findings (3).

The fourth cell reprogramming method, viral infection of reprogramming TFs, so-called Yamanaka iPSC technique, which suffers from several major challenges: 1) Very low reprogramming efficiency (< 0.1%); 2) Using integrating virus to deliver pinpointed transcription factor (TF) genes, causing major safety concerns; 3) Very complicated procedure which is expensive for future human clinical applications (134). Moreover, viral vectors may be randomly integrated into the host genome which increases the risk for undesired genotoxicity, mutagenesis, and tumor formation.

1.3.1 Reprogramming by Defined Transcription Factors-Gene Delivery

In 2006, Takahashi and Yamanaka generated mouse ESC-like cells from mouse somatic cells through viral-mediated transduction of the transcription factors Oct4, Sox2, Klf4, and cMyc with selection for the activation of the Oct4 target gene Fbx15. These generated cells were termed as induced pluripotent stem cells (iPSCs). They displayed morphology like that of ESCs (**Figure 1-13A**). Reverse transcription PCR (RT-PCR) analysis of iPSCs revealed the expression of ESC markers Oct3/4, Nanog, Dax1, Zfp296, E-Ras, Cripto, and Fgf4 (**Figure 1-13B**). iPSCs were also confirmed to be pluripotent by their ability to form teratomas (**Figure 1-13D**). Although unable to generate live chimeric mice, iPSC clones were injected into blastocysts and were found to contribute to the growth of the embryo (**Figure 1-13C**).

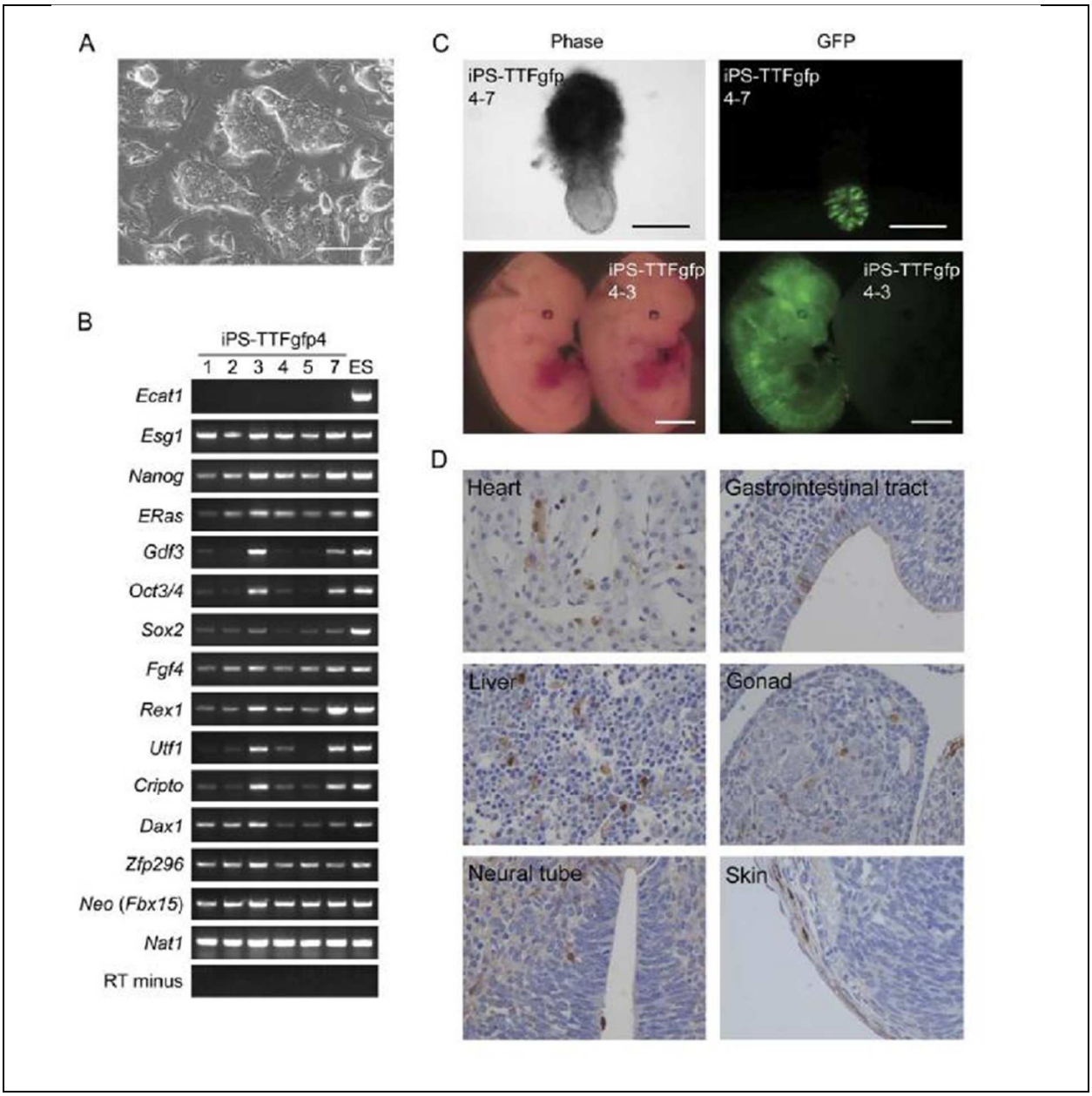

Figure 1-13: Characterization of iPSCs Derived from Adult Mouse Tail Tip Fibroblasts. **(A)** Morphology of iPS-TTFgfp4-3 on STO feeder cells. **(B)** RT-PCR analysis of ES marker gene expression in iPS-TTFgfp4 cells (clones 1-5 and 7). They used primer sets that amplified endogenous but not transgenic transcripts. **(C)** Contribution of iPS-TTFgfp4-7 and iPS-TTFgfp4-3 cells to mouse embryonic development. iPS cells were microinjected into C57/Bl6 blastocysts. Embryos were analyzed with a fluorescence microscope at E7.5 (upper panels, iPS-TTFgfp4-7P) or E13.5 (lower panels, iPS-TTFgfp4-3). Scale bars = 200 μm (upper panels) and 2 mm (lower panels). **(D)** The E13.5 chimeric embryo was sectioned and stained with anti-GFP antibody (brown). Cells were counterstained with eosin (blue) (5).

On the other hand, to maintain the pluripotent state of iPSCs, they require the continuous viral expression of SON genes. Furthermore, it was found that the endogenous Oct4 and Nanog genes were highly methylated and expressed at lower levels or not at all when compared to ESCs. This implicated that these iPSCs and ESCs are similar, but not identical.

The cell reprogramming method used suffers from several major challenges. Firstly, they have found very low reprogramming efficiency (< 0.1%). Secondly, viral transduction procedures are complicated, expensive, and have a major safety concern for future human clinical applications. Pluripotency depends on tightly regulated levels of pluripotency genes and it has been shown that a minimal increase or decrease of Oct4 levels causes differentiation (20). More studies have shown that iPSCs could be successfully generated without viral integration into specific sites (110), such as plasmid transfection (72) or non-integrating adenovirus (111), nevertheless, these methods all suffer very low reprogramming efficiency. Thus, retroviral integration methods are not likely to be the reason for the inefficiency.

In 2007, researchers demonstrated that selection for expression of the endogenous Nanog and Oct4 genes, instead of the Fbx15, resulted in full reprogrammed pluripotent cells unlike Yamanaka's Fbx15-iPSCs just a year before (112 & 113). This implicates that even a slight increase or decrease of Oct4 could change the cell fate. In this case, like ESCs, iPSC expression of the endogenous Nanog and Oct4 genes were found to have hypomethylated Nanog and Oct4 promoters (112 - 114).

Human iPSCs were generated by retroviral introduction, using different sets of transcription factors into fibroblasts (50-51). Takahashi et al. and Park et al. both showed iPSC being generated by using Yamanaka's reprogramming cocktail (Oct4, Sox2, Klf4, and cMyc). Also, in 2007, Yu et al. group utilized a different cocktail (Oct4, Sox2, Nanog, and Lin28) which

generated human iPSCs that were able to differentiate into cell types of all three germ layers in the dish and formed mature teratomas upon injection into mice (39). The iPSC from Yu et al. more closely resemble ESCs through morphology, patterns of DNA methylation and histone modifications, gene expression profiles, and expression of telomerase. Other groups have also reported the successful generation of human iPSCs from somatic cells, offering a great tool for regenerative medicine (40, 43, 115-119).

1.3.2 Reprogramming by Defined Transcription Factors-Protein Delivery

Yamanaka's and related methods cause many concerns. One major concern is the safety of these iPSCs when they are used for human clinical applications. Many modified genetic methods have been developed and have generated iPSCs with less risk (120). However, they still utilized genetic materials and always risked unexpected genetic modifications by the exogenous sequences in target cells (120). A few years later, in 2009, it was found that directly delivering the four recombinant reprogramming proteins (Oct4, Sox2, Klf4, and cMyc) to reprogram somatic cells into ESC-like iPSCs. The resulting pluripotent cells are referred to as protein-induced pluripotent stem cells (piPSCs) (120, 121). Instead of the previous method that depends on the transcription of delivered genes, this method avoids introducing exogenous genetic modifications to the target cells by delivering the transcription factors themselves into the target cells.

The difficulty of accomplishing this direct delivery of proteins is passing the plasma membrane into the cell. This challenge was solved using cell-penetrating peptide (CPP), a short peptide sequence that contains a high amount of basic amino acids (e.g. arginine or lysine) which are attached to the c-terminus of the reprogramming proteins (121). Researchers employed poly-arginine CPPs and determined that the CPP-tagged recombinant proteins readily entered the cell and translocated to the nucleus. They were successful in generating piPSCs.

One group initiated their protein delivery method by first establishing stable HEK293 cell lines that expressed each Oct4, Sox2, Klf4, and cMyc fused with a nine arginine (R) CPP and a Myc tag to surpass the plasma membrane (122). The HEK293 extracts were then incubated with human dermal neonatal fibroblasts (HDFn) and the intracellular translocation of proteins was observed by immunocytochemistry by using antibodies **(Figure 1-14A)** (121). They found that the efficient intracellular translocation of each reprogramming protein and translocated to the nucleus. By testing HEK293 cell extracts they found that repeated protein treatment cycles (16 hours protein treatment followed by 6-day incubation in ES media) yielded human p-iPSCs **(Figure 1-14B)** especially after three to four cycles yielded to iPSC-like morphology **(Figure 1-14C, top panel)**. However, induced cells failed to show alkaline phosphatase (AP) activity, suggesting that the reprogramming was incomplete. However, and approximately half of these colonies were found to be AP positive starting from the sixth cycle **(Figure 1-14D)**.

AP-positive colonies of iPSCs were characterized as similar to hESCs and maintained for more than 35 passages and shared common morphological characteristics and expressed markers such as Oct4 and Nanog similar to hESCs **(Figure 1-14C)**. Furthermore, the qRT-PCR analysis also found similar characteristics such as the high expressions of endogenous mRNAs of ESC markers such as Sox2, Oct4, and Nanog similar ESCs (**Figure 1-15A**). The protein human-induced p-hiPSCs also formed EBs which were able to differentiate into cells of all three germ layers (**Figure 1-15D**). Further testing found global gene-expression analysis to be similar to hESCs and different from HDFn (**Figure 1-15B**). Moreover, after transplantation of p-hiPSCs inside the kidney capsule of mice formed teratomas within 6-8 weeks (**Figure 1-15E**). p-hiPSC lines also showed similar Bisulfite sequencing analyses to hESCs. For instance, p-hiPSCs and hESCs showed demethylated promoter regions of Oct4 and Nanog but methylated Oct4 and Nanog in

HDFn (**Figure 1-15C**). These data confirmed that p-hiPSC are pluripotent both *in vitro* and *in vivo*.

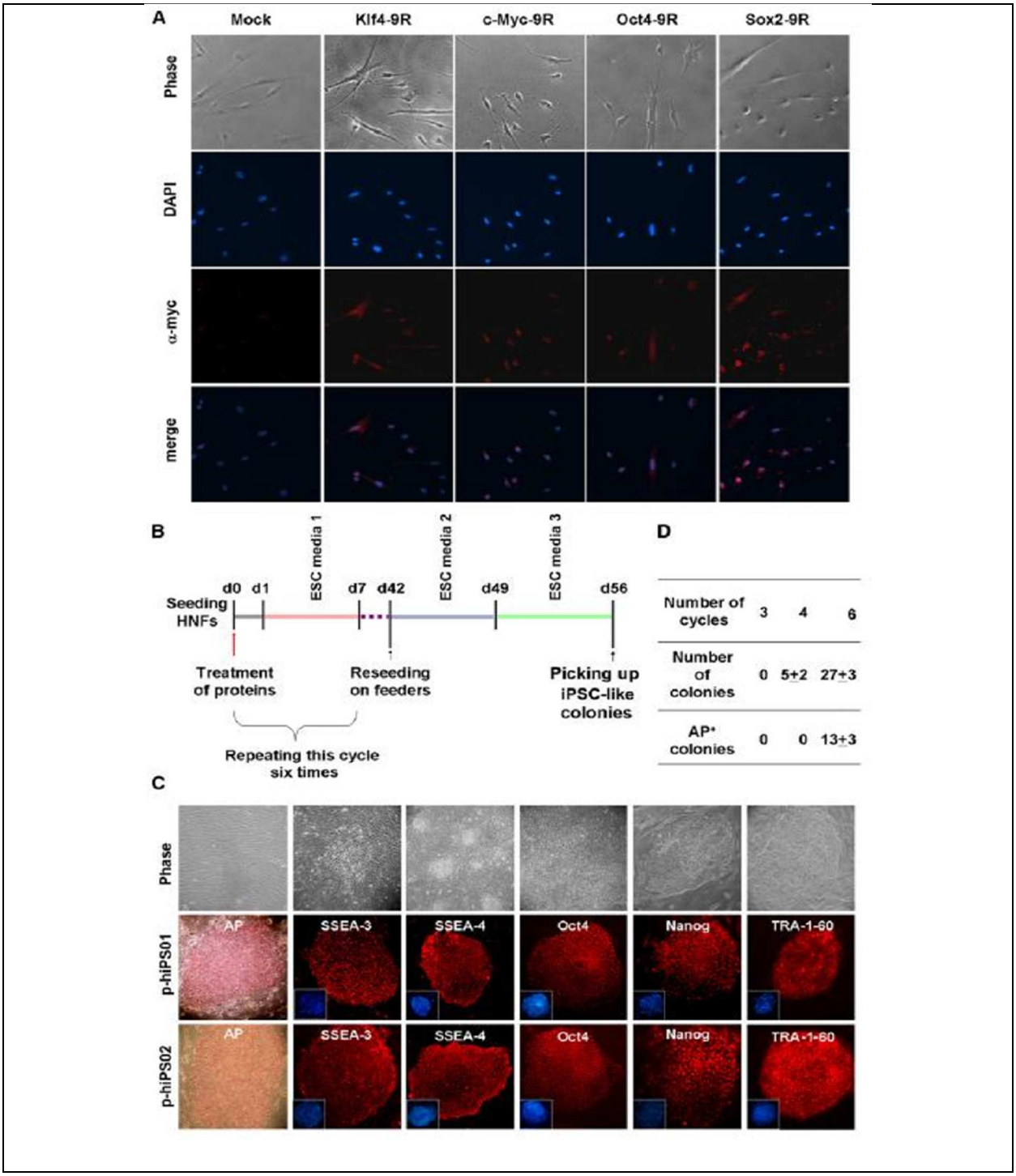

Figure 1-14: Generation of Protein-Induced hiPSC Lines by Direct Delivery of Reprogramming Proteins Fused with 9R as a CPP. **(A)** HNFs were incubated with HEK293 extracts expressing each reprogramming protein and subjected to immunocytochemistry using myc antibodies. Nuclei

were counterstained with DAPI. **(B)** The schematic protocol depicts a repeated process and the timeline for generating phiPSCs from HNFs. **(C)** (Top panel) shown are starting HNFs (first image), morphology after three-cycle protein treatments (second image), and increased colony number after six cycles (third image). Approximately half of these iPS-like colonies stained positive for AP; early morphology after p-hiPSC colonies were transferred to MEF is shown (fourth image), and morphology of established p-hiPSC line is shown at passage number 10 (p-hiPS01 [fifth image] and p-hiPS02 [sixth image]). Immunostaining of p-hiPS01 (middle panel) and p-hiPS02 (bottom panel) clones show expression of hESC markers, including AP, SSEA-2, SSEA-4, Oct-4, Nanog, and Tra1-60. Nuclei were stained with DAPI (blue in the second and third row of the panel). **(D)** Shown is the efficiency of reprogrammed colony formation with iPS-like morphology and AP-positive staining after different numbers of the protein treatment cycle. This is the summary of three independent experiments with the standard error (121).

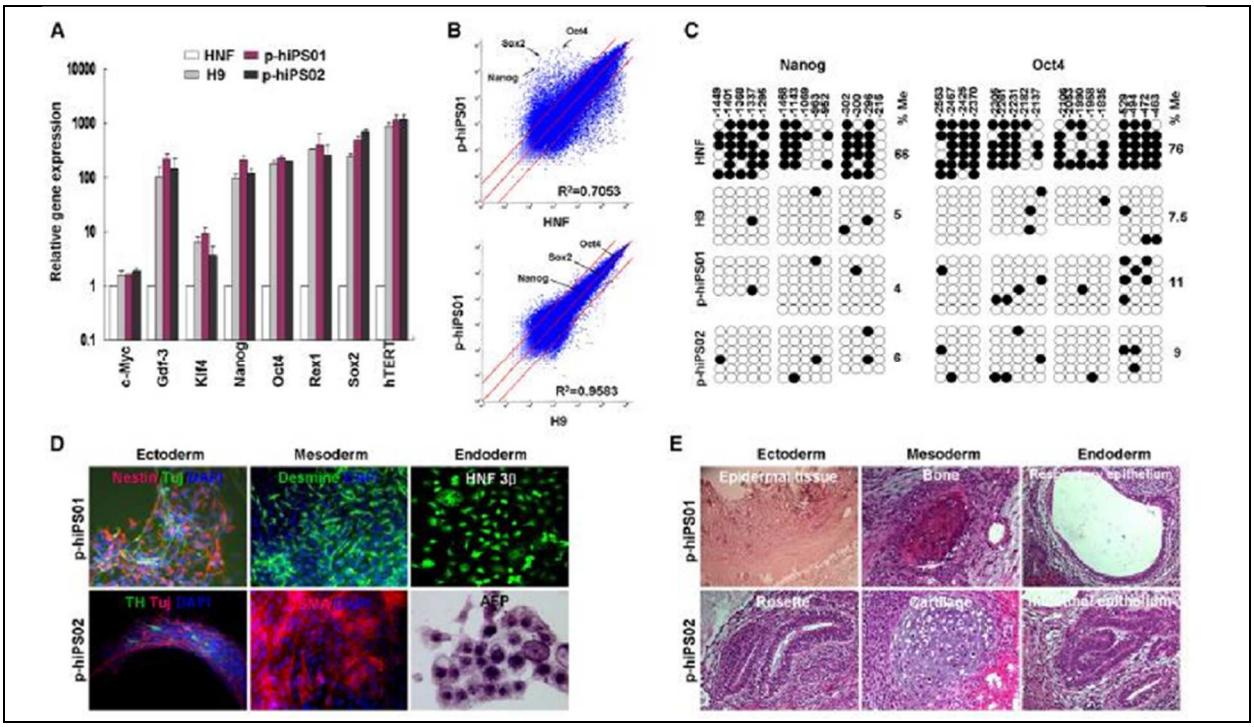

Figure 1-15: Characterization of p-hiPSC Lines. **(A)** Quantitative RT-PCR was performed to assess the expression of c-Myc, Gdf-3, Klf4, Nanog Oct4, Rex1, Sox2, and hTERT in p-hiPS01 and p-hiPS02, hES (H9), and HNF cells. Relative gene expression represents fold changes relative to that of HNF cells normalized to β-actin expression. This experiment (repeated twice in triplicate using independently prepared cDNAs) resulted in almost identical patterns. **(B)** The global gene-expression patterns were compared between p-hiPS01 and HNF, and between p-hiPS01and H9 with Affymetrix microarrays. The red lines indicate the diagonal and 5-fold changes between the paired samples. **(C)** Bisulfite sequencing analysis of the Nanog and Oct4 promoters reveals almost complete epigenetic reprogramming. Open and closed circles indicate unmethylated and methylated CpG, respectively. Numbers on the top show each CpG location. Percentages of CpG methylation (%Me) are shown. **(D)** In vitro differentiation of p-hiPSCs. Immunostaining images (first and second-row panels) show all three germ layer cells at day 24, including neural (ectodermal), muscle and endothelial-like (mesodermal), and endoderm-like cells (endoderm). **(E)** Teratoma formation in immunodeficiency mice by p-hiPSCs. H&E staining was performed for

teratomas. The resulting teratomas contained tissues representing all three germ layers (p-hiPS01, first row; and p-hiPS02, second row): ectoderm, epidermal and neural tissue (rosette); mesoderm, bone and cartilage; and endoderm, respiratory epithelium and intestinal-like epithelium (121).

P-hiPSC technology offers a new and potentially safe method for generating patient-specific stem cells (121). By eliminating the manipulation of the genome and DNA transfection, p-hiPSCs are appropriate for potential clinical applications and research (121). However, the p-hiPSC colonies took double the amount of time it takes with viral transduction, about 8 weeks, and much lower efficiency (~0.001%) when compared to virus-based methods (~0.01%) suggesting that the published piPSC method are not efficient and require the implementation of new methods (38, 39, 123, 134).

1.3.3 Reprogramming by Defined Transcription Factors-RNA Method

In 2010, specific synthetic modified messenger RNA (mRNA) that code for reprogramming factors is delivered directly to cells (124). Through *in vitro* transcription (IVT), mRNA was made by PCR amplicons along with the removal of 5‟ triphosphates to activate PKR to repress protein translation, the addition of guanine cap for successful translation and increase the half-life, and modified ribonucleotides to further the minimization of innate immune responses to transfected RNA. They found that modified RNAs in addition to interferon inhibitor B18R brought upon high protein expression levels and high cell viability (124). By using a cationic vehicle to facilitate endocytosis of modified mRNAs that code for the necessary reprogramming factors, they were able to generate RNA-iPSCs (RiPSCs) from human fibroblasts (124). However, to sustain high levels of protein expression, the transfection of modified RNAs had to be done daily. RiPSC colonies are then mechanically picked and expanded in ESC permissive conditions to establish iPSC lines for characterization. Bisulfite sequencing showed demethylation patterns like ESCs and distinct from fibroblasts. By using qRT-PCR and global gene expression profiles,

RiPSCs displayed the expression of pluripotency genes such as SON and revealed RiPSCs having a molecular signature like ESCs and distinct from fibroblasts.

The pluripotency-associated transcript genes including Sox2, Oct4, Nanog, Rex1, Lin28, and DNMT3B of RiPSCs were upregulated in the RiPSCs compared to fibroblast lines. The embryonic bodies generated for RiPSCs were able to differentiate into cell types of all three germ layers. Lastly, the pluripotency of these cells was confirmed by their ability to form teratomas when injected into the kidney capsules of mice. This method of inducing PSCs yielded within 17 days and higher reprogramming efficiency (~2%), versus the viral methods which usually take 4 weeks for colonies to emerge. In all, this method also eliminates the risk of genomic integration and insertional mutagenesis. However, this method is very complex and is very difficult to be used for human clinical applications.

1.4 Methods for Characterization of iNSCs

To prove that iNSCs have been generated, it is necessary to fully characterize them and show the similarities to naturally occurring NSCs. It is also necessary to show their distinct differences from the cell type they originated from. Many methods can be used to determine whether iNSCs are multipotent, with some of these methods have been mentioned previously in this chapter; here is a summary of the common methods.

1.4.1 Colony Formation of iNSCs

The observance of ESC-like morphology is commonly used as the initial form of characterization for iPSCs. ESCs display round shaped colonies, large nucleoli, and scant cytoplasm (4) and display the ability to form closely packed colonies with a clear edge, distinct from the surrounding cells. Accordingly, NSCs form neurospheres, which are similar to ESC colonies. The morphology of these neurospheres is distinct from the spindle-shaped fibroblast cells

that are commonly used as the parental cells as a somatic cell source. During cell reprogramming, the observation of typical iNSC colony morphology, the efficiency of colony formation, and increased doubling time provide good preliminary indicators if the reprogramming protocol is working and if there is any iNSC formation. However, the cells must meet more stringent criteria to be considered multipotent iNSCs.

1.4.2 Immunostaining

Immunocytochemistry of the immunostaining of NSC markers is another technique that is frequently used to characterize iNSCs. This is accomplished by using antibodies to detect the presence of specific NSC marker proteins. The detection of common NSC marker proteins during cell reprogramming of the starting somatic cells into iNSCs may indicate a switch or transition in the transcriptional gene network of the somatic cells to the one that resembles NSCs. Immunostaining of the cells is commonly performed in comparison with both the starting fibroblast cells, as a negative control, and the reprogrammed cells during or after cell reprogramming as an indication of the formation of iNSCs.

1.4.3 Western Blot

Western Blot is also used to detect the presence of NSC marker proteins in the newly generated iNSCs. In a western blot, cell extracts are prepared, and the proteins contained within are typically separated by size using SDS-PAGE gel electrophoresis before being transferred to a membrane. The membrane is then incubated with an antibody against a specific protein for a certain amount of time, and, washed with PBS to remove unbound antibody, then incubated with a secondary antibody, which will bind to the primary antibody. This secondary antibody is linked to a reporter enzyme that, when incubated with substrate, will indicate the presence of the primary antibody and therefore, the presence of the targeted protein.

1.4.4 qRT-PCR

Reverse transcription-polymerase chain reaction (qRT-PCR), is the most well-known technique for absolute and relative quantification of mRNA transcription level (125). The expression of target genes is often standardized to reference genes to eliminate sample-to-sample partialities. Reference genes are genes that are expressed constantly in all cells to maintain essential cellular functions (126). In this study, it is imperative to identify the appropriate reference genes before qRT-PCR, since the accuracy of qRT-PCR is highly reliant on reference genes.

Furthermore, qRT-PCR is another technique used to detect the mRNA levels of up-regulations of the common NSC marker gene expressions. In RT-PCR, mRNA extracted from cell samples is reverse transcribed to cDNA, which is then amplified through the use of primers and a DNA polymerase enzyme, as in traditional PCR. While immunostaining and western blotting detect the presence of specific proteins, this method detects the presence of mRNAs the code for specific proteins. While the qRT-PCR technique serves as an indication for the amount of transcription of a particular gene, it does not guarantee that these transcripts will be properly translated into a fully functional protein. Only a combination of the positive results in immunostaining/western blot and qRT-PCR of a particular NSC marker of the reprogrammed cells indicates the up-regulation of both gene transcription and protein translation of this NSC marker, thus reliably indicating the success of the cell reprogramming of fibroblast cells into iNSCs.

1.4.5 DNA Demethylation Analysis

DNA demethylation analysis is the process of removing a methyl group from the nucleotides of DNA. After an injury to nerves in mammals, DNA demethylation plays an important role in neuro-regeneration (160). DNA methylation occurs on cytosine at CpG sites on a gene promoter that leads to the silencing of gene expression while DNA demethylation of a gene

promoter is associated with transcriptional activation and gene expression (156). In all, these are called epigenetic changes.

DNA demethylation analysis of promoters of the NSC marker genes of the reprogrammed cells is also commonly carried out via bisulfite genomic sequencing analysis to determine the epigenetic state of these cells. In this process, bisulfite is used to determine the methylation pattern of the DNA. Once the fibroblast cells are reprogrammed into iNSCs, the demethylation level of the NSC marker gene is significantly decreased as compared with that of the starting cells, indicating transcriptional activation and gene expression of this NSC marker gene.

1.4.6 In Vitro Differentiation

In vitro differentiation is utilized as a method for determining the developmental potential of the reprogrammed iNSCs. NSCs possess the potential to differentiate into neurons and glial cells, including astrocytes and oligodendrocytes. Therefore, the differentiation of the reprogrammed iNSCs into astrocytes, oligodendrocytes, and neurons indicates as to whether or not they are multipotent. In this process, the reprogrammed NSCs are cultured in the medium that induces neural differentiation.

1.4.7 In Vivo Differentiation

When the GFP-labeled mouse iNSC (miNSC) neurospheres were microinjected into the cortex of pups. Immunostaining revealed that miNSCs survived and differentiated into NeuN-positive neurons with mature-looking dendritic spines, Oligigo2-positive oligodendrocytes, and GFAP-positive astrocytes 5 days after transplantation (**Figure 1-16**). So, miNSCs were shown to be able to differentiate into neurons, astrocytes, and oligodendrocytes *in vivo* (29).

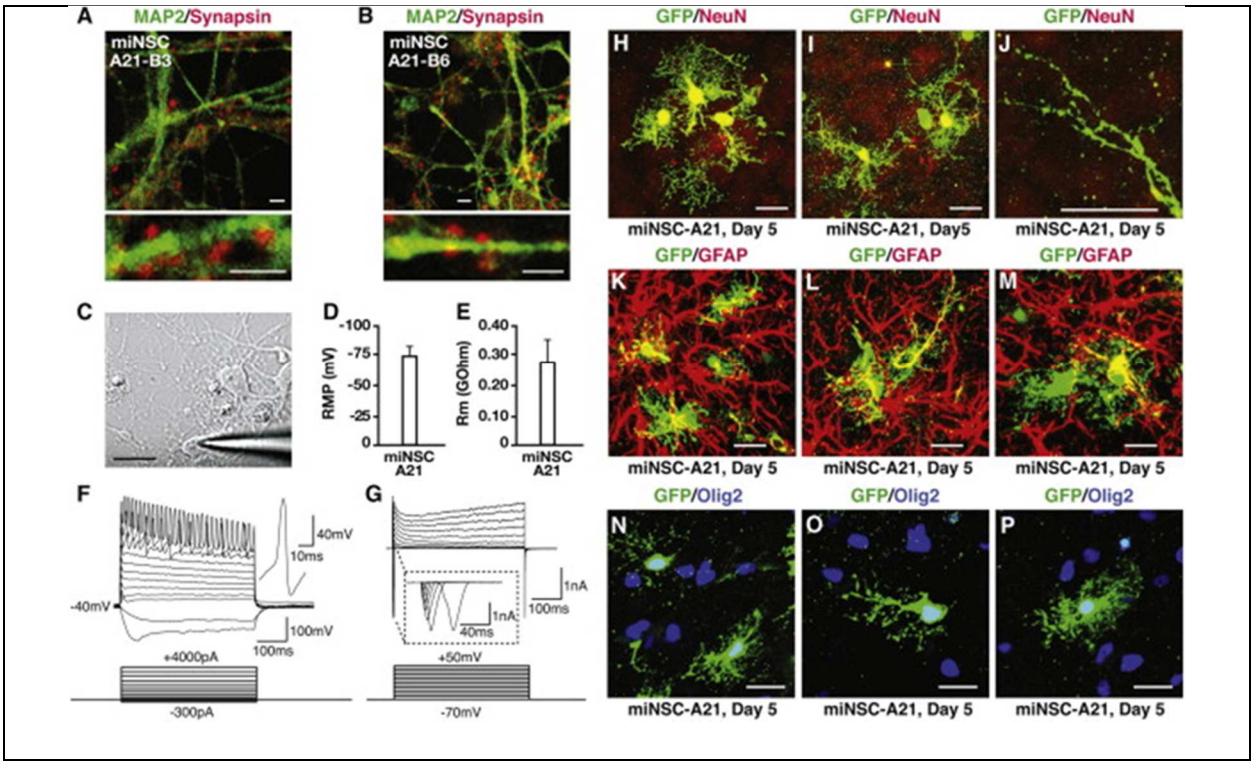

Figure 1-16: miNSC-Derived Functional Neurons In Vitro and Multipotency of miNSCs In Vivo. **(A and B)** Neurons derived from subclones miNSC-A21-B3 or miNSC-A21-B6 at 14 days in culture express MAP2 (green) and Synapsin (red), a presynaptic marker of mature neurons. **(C)** A patched neuron derived from miNSC-A21-B3 at 17 days in culture. **(D and E)** Whole-cell capacitance and membrane resistance of neurons derived from miNSC-A21 were determined from a transient 5 mV hyperpolarizing step from a holding potential of –70 mV. Values are mean ± SEM. **(F)** Current-clamp recordings of neurons derived from miNSC-A21 at –40 mV reveal action potentials with stepwise current injection. **(G)** Voltage-clamp recordings of neurons derived from miNSC-A21 reveal both fast inactivating inward and outward currents indicating functional voltage-dependent Na+ and K+ channels. **(H–P)** GFP-labeled miNSC-A21 were grown in suspension cultures for 1 day to generate small neurospheres and then microinjected into the cortex of P2–P3 wild-type pups. Five days after transplantation, mouse brains were collected, fixed, sectioned, and immunostained. **(H–J)** Immunostainings reveal that miNSC-A21 can differentiate into NeuN+ neurons **(H and I)** with mature-looking dendritic spines **(J)** in vivo. **(K–M)** miNSC-A21 can also differentiate into GFAP+ astrocytes in vivo. **(N–P)** miNSC-A21 can also differentiate into Olig2+ oligodendrocytes *in vivo*. Scale bars represent 2 μm in **(A)** and **(B)** and 10 μm in **(C)** and **(H)–(P)** and include genetic analyses, epidemiology, statistics and studies in animal models. iPSC technology expands the opportunities for research. Patient-derived iPSCs can be used to examine the disease process at a cellular level. iPS cells can be used to test response to possible drugs and might be used to develop patient-specific therapy (29).

Since the generation of iNSCs has been achieved by many investigators (70, 57, 29, 127), a study of *in vivo* long-term survival rates, multilineage differentiation, and the functional integration of iNSCs have been reported (128). A total of 2.25×10^5 iNSCs were transplanted into

the cortex of adult female immunodeficient mice (8 weeks; ~25 g; n = 9; **Figure 1-17**). iNSCs were labeled using retroviral transduction along with a GFP-coding vector to distinguish transplanted cells from the surrounding tissue. Cells were passaged at least two times and three washing steps to make sure no remaining virus particles are present in the iNSCs, which may induce endogenous cells after transplantation.

Six months after transplantation, an overview analysis revealed a significant long-term survival rate in the cortex and hilus and, a densely packed graft core was detected at the injection site and a less densely organized fraction of migrating cells that integrated within the existing network. However, immunostain analysis has revealed that transplanted cells do not express the NSC marker, Nestin, the cell-cycle marker, KI67, or the neural progenitor marker, DCX (**Figure 1-17**) (128). This implies that iNSCs have lost their stem cell identity after grafting and therefore can be used for safe cell transplantation (128).

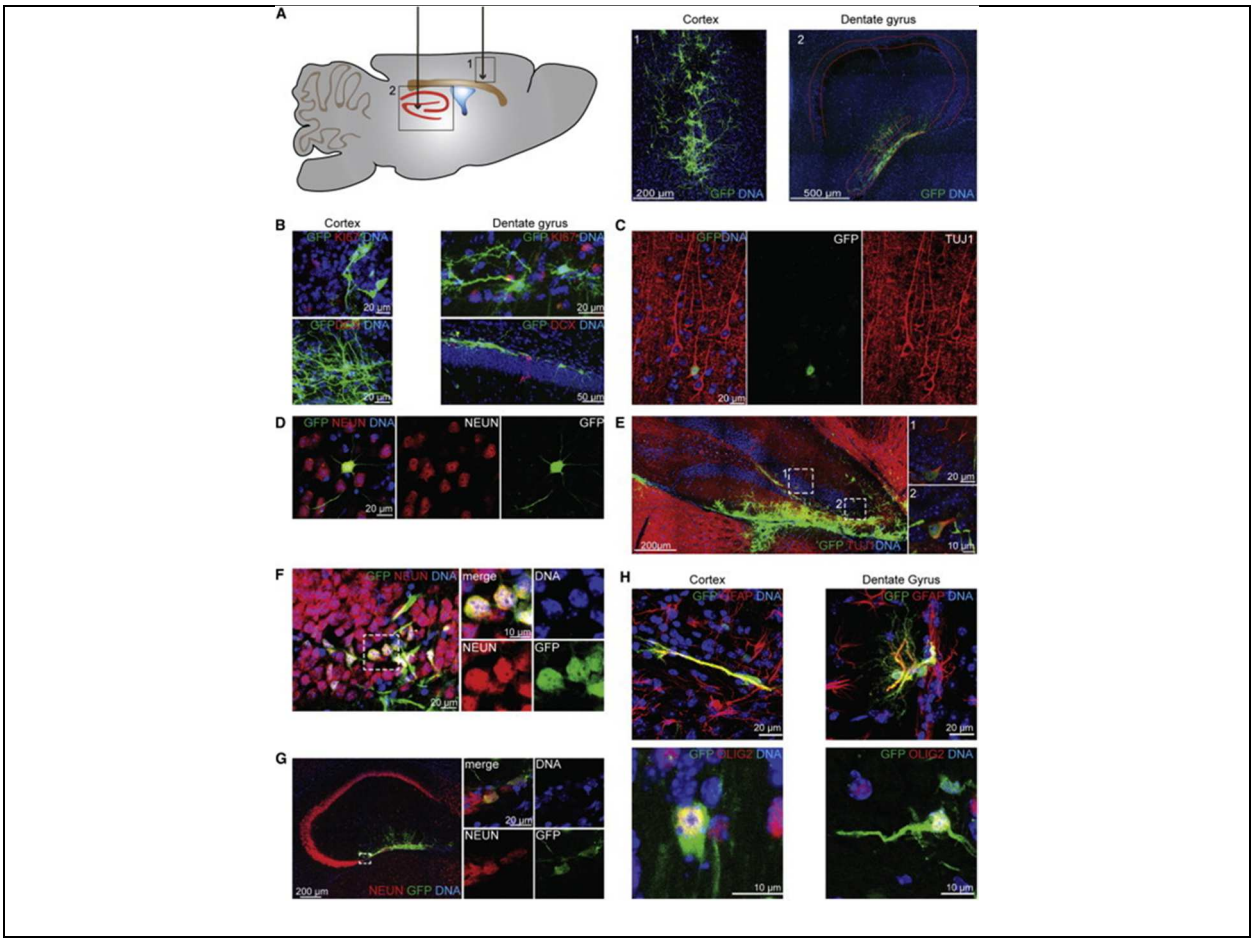

Figure 1-17: iNSC-Derived Cells Show In Vivo Long-Term Survival Rates and a Multilineage Differentiation Potential. **(A)** Schematic overview (left) of the two transplantation target sites in the adult mouse brain: (1) the cortex and (2) the hilus of the dentate gyrus. Six months after transplantation, an immunohistological analysis (right) revealed a sound survival rate of the GFP-labeled iNSCs in both regions. Image 1 displays a maximum intensity projection (MIP). Red dashed lines indicate the hippocampus. **(B)** iNSC-derived cells do not express the cell-cycle marker KI67 (upper panel) or the neural progenitor marker DCX (lower panel). The images represent the MIPs of a confocal z stack. **(C and D)** iNSCs at graft edges or those that had substantially migrated outside of the graft differentiate into TUJ1- **(C)** and NEUN-positive **(D)** neurons and showed an orientation and shape comparable with the neighboring endogenous neurons when transplanted into the cortex. **(E and F)** Transplanted iNSCs of the hilus migrate and integrate into the granule layer of the dentate gyrus and express the neuronal marker TUJ1 **(E)** and NEUN **(F)**. Dashed lines indicate the regions of magnification. **(G)** iNSC-derived NEUN-positive cells transplanted into the hilus integrated into the existing network and extended the CA3 region of the hippocampus. Dashed lines indicate the region of magnification. **(H)** In both regions, iNSCs differentiated into the glial lineage indicated via the astrocyte marker GFAP (upper panel; MIP of a confocal z stack) and the oligodendrocyte marker OLIG2 (lower panel; the left image represents a MIP of a confocal z stack). Nuclei were counterstained with Hoechst (128).

The authors also analyzed the differentiation potential of iNSCs 6 months after transplantation by immunofluorescence staining using neuron-specific class III-beta-tubulin (Tuj1), for immature neurons, and NeuN, which is only expressed in mature neurons (**Figures 1-17**) (128). Moreover, these induced cells integrated into the neuronal network and astonishingly displayed identical orientations and shapes to the surrounding endogenous neurons (128). iNSCs that experienced neuronal differentiation migrated and integrated into the granule cell layer of the dentate gyrus (**Figure 1-17**). This process has been found to be like differentiation of the endogenous neural stem cells of the SGZ (subgranular zone) (129).

1.5 Future Clinical Applications of iNSCs

The ability to reprogram somatic cells into self-renewable iNSCs has major implications for future clinical application to treat neurodegenerative diseases. iNSCs can also serve as a model system for revealing disease pathogenesis, drug screening, toxicity tests, cell transplantation therapies (**Figure 1-18**).

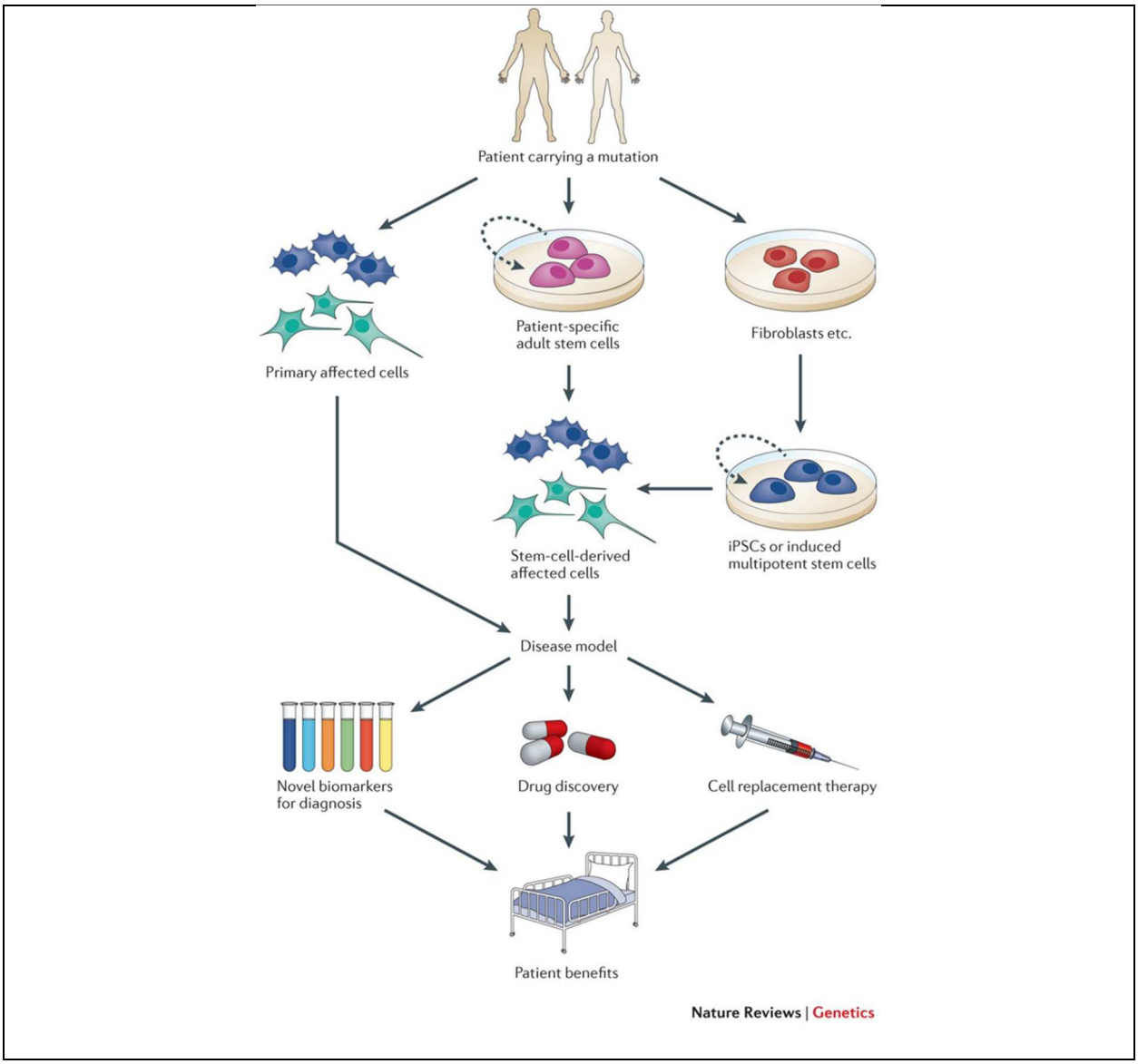

Figure 1-18: Overview of Different Approaches to Disease Modeling. Generation and Applications of patient-specific disease models. Primary cells of patients can be used for disease modeling, but they are not easily available and cannot be expanded in culture. Adult stem cells or induced pluripotent stem cells (iPSCs) can be expanded in culture and differentiated into the disease-affected cells that can be used to recapitulate disease pathogenesis in vitro. Patient-specific disease models can be used to identify new biomarkers for improved diagnostic procedures, such as earlier detection of disease onset. For example, hyperphosphorylated mitogen-activated protein kinases (MAPKs) and microtubule-associated protein tau (MAPT) have recently been associated with early susceptibility to neurodegeneration using patient-specific models of Parkinson's disease with mutant leucine-rich repeat kinase 2 (LRRK2)29. These disease models can also be used to identify compounds that alleviate disease pathology in vitro, which can be further developed into novel drugs. Such compounds can be identified by targeting a novel pathway that is dysregulated in the disease model or, alternatively, by carrying out a phenotypic screen using the disease model to identify compounds that may act through novel mechanisms. Stem-cell-derived cells can also form the basis for cell replacement therapies (130).

1.5.1 Cell Replacement for Regenerative Medicine

NSCs, which exist in various regions of the CNS throughout the mammalian lifespan, can be expanded and induced to differentiate into neurons and glia in vitro and in vivo. Because of these characteristics, there have been increasing interests in the identification and characterization of NSCs and neural progenitor cells both for basic developmental biology studies and for therapeutic applications to the nervous systems. Transplantation of NSCs or their derivatives into a host brain and the proliferation and differentiation of endogenous stem cells by pharmacological manipulations are potential treatments for many neurodegenerative diseases and brain injuries, such as Parkinson's disease, brain ischemia, and spinal cord injury. Continued progress in NSC research is providing a new future for brain repair.

1.5.2 NSC Drug Discovery and Toxicology Research

Neurological diseases such as Alzheimer’s disease and Parkinson’s disease are emerging major problems for human health, as average life expectancy is increasing globally. Drug discovery for neurological disease remains a major challenge. Poor understanding of disease pathophysiology and incomplete representation of human disease in animal models hinder therapeutic drug development. Advances with iNSCs can enable modeling of human diseases on a personal basis with the patient derived NSCs and neural cells. Utilizing iNSC-derived neurons also advances drug compound screening and evaluation of drug efficacy. These cells have the genetic backgrounds of patients that more precisely model disease-specific pathophysiology and phenotypes. Therefore, applications of human iNSCs or iNSC-derived neurons are a new direction for drug discovery in neurodegenerative diseases.

Drug testing identifies potential therapeutic compounds and increases understanding of drug absorption, distribution, metabolism, and excretion (ADME) properties. The development of

ADME NSC models is thought, throughout the medical community, to have a strong impact on drug discovery and development. Human NSCs could be used for testing different drugs and toxic environmental agents in developmental neurotoxicity studies (131). Mature neurons provide useful drug safety screens for neurotoxicity and synaptic dysfunction (131). In the presence of reliably expandable and physiologically relevant models, drug discovery processes become less costly. Benefits also include more relevant research for the clinic.

Patient-specific neural cells could change the world of drug development for neurodegenerative disease treatment. An estimated 40% of drug candidates are abandoned throughout the development pipeline due to unforeseen adverse reactions in humans (132) and adverse drug reactions are still the fourth leading cause of death in the United States, indicating that current testing practices do not sufficiently predict clinical toxicity (131). Since the human nervous system displays unique complexity and patterns of gene expression, the potential use of these patient-derived neural cells could increase the predictability of clinical trials from preclinical studies because current animal models used for toxicity are not always predictive of human response. Also, these patient-derived NSCs and neurons precisely record the patients' genetic background which is critical for drug developments in neurodegenerative disease treatment since patients' genetic background may show the pathology of the patients at the molecular level. In 2002, the research community found that human neurons react differently to toxin, as compared with mouse brains in a vaccine clinical trial for Alzheimer's disease plaques. The vaccine is AN-1792, showed severe cases of meningoencephalitis in humans that were not shown to occur in rodent safety studies (133).

In all, in vitro toxicology assays from human iNSCs and their derivatives can offer significant advantages over current animal models. The *in vitro* assays based on rodent and/or

human transformed cell lines, largely due to improved relevance and greater versatility. More relevant human NSC-based assays should translate into safer drugs, lower attrition rates, and a better understanding of disease and toxicity mechanisms.

CHAPTER 2: MOLECULAR CLONING AND RECOMBINANT PROTIEN PRODUCTION

2.1 Goal

The application of **i**nduced **N**eural **S**tem **C**ells (iNSCs) in science and medicine, in place of iPSCs or ESCs, not only eliminates the controversy of utilizing human embryos to derive stem cells (4) but also the potential to increase the efficiency of obtaining the desired cell. In contrast with allogenic approaches that use a single source of cells to treat many patients, patient-specific somatic cell-derived iNSCs avoids the risk of immunologic rejection in regenerative medicine studies (39). Protein-based iPSC (piPSC) technology has provided safer opportunities for biomedical research and clinical application (134). Complimenting piPSC technology with even safer piNSCs with fewer differentiation options further increases the magnetism of this technology. Furthermore, our protein-induced reprogramming technique eliminates DNA transfection and genome manipulation, which results in human piNSCs that are appropriate for drug discovery, disease modeling, and potential human clinical applications for neurodegeneration (121).

The viral method of reprogramming described in the previous chapter has been used to reprogram somatic cells into iNSCs but faces many challenges. Firstly, this method is time-consuming, taking 35 to 65 days to achieve cell reprogramming of human somatic cells into iNSCs (120, 121). The Extended timeline renders the current iNSC technology useless for the treatment of the disorders that demand rapid treatment. Secondly, the increased culture time makes the current iNSC technology expensive for patients (120, 121). The current induced reprogramming technology also faces major challenges, such as a very low conversion efficiency of 0.01% of somatic cells into human piPSCs (120, 121) or hpiNSCs. Low conversion efficiencies are primarily caused by the CPP-fusion protein delivery method employed. The CPP protein delivery method

suffers several setbacks (135, 136). The CPP fusion method has low protein delivery efficiency because the delivered proteins are sensitive to intracellular proteases, causing degradation before the delivered proteins reach their target intracellular compartment to carry out their functions (135). CPP fusion method also lacks the capability to deliver the proteins into the nuclei for cell reprogramming (135). The resulting low efficiency of protein-induced reprogramming impedes the study of the mechanism of reprogramming and prevents its application in the clinical setting.

The goal of this study was to develop a simple, fast, and efficient piNSC technology using four reprogramming proteins. We reduce current reprogramming protocols to one simple incubation step of somatic fibroblast cells directly with our four bacterially expressed transcription factors to achieve the generation of piNSC within one week. We also sought to achieve nearly ~90% cell conversion efficiency by utilizing our QQ-protein delivery technology, a high yield bacterial expression protocol, and an in vivo protein refolding system.

The QQ-protein delivery technology is developed in our lab that solves the problems associated with the CPP-protein delivery method (137, 138). This technology involves an incubation step of our proteins with the QQ-reagent which is polyethyleneimine (PEI) based (137). The QQ-reagent noncovalently associates to the protein surface to maintain the proteins original configuration. The QQ-reagent modified protein has targeting capability to deliver the protein to the specific intracellular compartments based on the localization signal sequences carried by the delivered proteins. QQ-modification of the protein also protects the delivered proteins form degradation by intracellular proteases (137).

Our lab has a very high-efficiency bacterial expression method, which allows us to prepare high yields of pure proteins (139). Our method uses a tightly controlled induction by IPTG and utilizes both rich and minimal media to achieve a very high cell density for the production of

recombinant proteins. This method obtained a much higher yield of recombinant proteins than the bacterial expression system or the human cell expression system employed by the other groups (120, 121). Our high-efficiency bacterial expression method can reduce the cost of reprogramming.

The bacterially expressed protein may not be folded properly. The *in vitro* protein refolding protocols have been developed by many groups to properly refold the functional proteins (120). However, these *in vitro* protein refolding techniques require an additional purification step that is inefficient, making the reprogramming proteins expensive. Our QQ-protein delivery technology gives us the capability of in-cell protein refolding, which is a technique that enables the direct delivery of bacterially expressed proteins to the correct intracellular compartment of mammalian cells for intracellular folding machinery to efficiently refold the proteins (134, 137 & 138). In the case of cell reprogramming, we can directly deliver the QQ-modified recombinant reprogramming proteins into the human dermal neonatal fibroblast (HDFn) cells, refold the proteins, and then transport them to the cell nuclei to initiate cell reprogramming to generate piNSCs (134, 137 & 138).

As described above, Sox2 was found to reprogram fibroblasts to iNSCs (29). However, further neural linage differentiation factors such as NeuroD1 are necessary to effectively differentiate iNSCs to astrocytes, oligodendrocytes, and neurons. In our reprogramming protocol, QQ-modified SON pluripotent reprogramming factors are used as a tool to quickly drive the human somatic fibroblasts to a transient pluripotent state, followed by Sox2 to generate iNSCs. The generated iNSCs shall rely on endogenous gene expression of the NSC factors to express NSC markers and resemble wild-type NSCs in their morphology, self-renewal, and gene expression profiles. Next, NeuroD1 is used to guide neural-lineage specific differentiation and the generated piNSCs indicate multipotency with the capability of differentiating into neurons, astrocytes, and

oligodendrocytes, as confirmed by the cell morphology and their specific biomarker protein expressions.

In all, we apply our cell reprogramming technology to generate protein-induced neural stem cells (pi-NSCs) from fibroblasts using defined TFs. Using Sox2, we will generate pi-NSCs from human fibroblasts that are self-renewable, multipotent, nontumorigenic, and lineage-restricted cells. Secondly, we will optimize our cell-reprogramming procedure specific for NSC generation to achieve greater than 90% conversion efficiency. Lastly, we will use NeuroD1 to differentiate the generated pi-NSCs into neurons, astrocytes, and oligodendrocytes *in vitro* and *in vivo*.

2.2 Molecular Cloning - Materials and Methods

The molecular cloning of Sox2, Oct4, Nanog, and NeuroD1 genes into an in-house high-level bacterial expression vector of pET30a-sHT was previously done in Dr. Jianjun Wang's lab by a previous graduate student in a paper to induce iPSCs. I was not involved in this molecular cloning work. Here I just go through the process of molecular cloning of all four proteins. The selected reprogramming genes were amplified by PCR and subcloned into a high-level protein expression vector pET30a-sHT. The correct sequence vectors were then transformed into bacterial cells for bacterial expression. The expression of the bacterial proteins was carried out following the traditional IPTG induction method. This is then followed by a purification process that uses a His-tag column followed by QQ-modification.

2.2.1 Genes for Subclone, and Bacterial Expression Vector

Four genes were chosen for subclone-Oct4, Sox2, Nanog, and NeuroD. Each of the four genes (Oct4, Sox2, Nanog, and NeuroD) were obtained from Addgene. Oct4, Sox2, Nanog were chosen because they are the three proteins utilized by Dr. Wangs group to generate iPSCs.

NeruroD1 was chosen because it's a neural-linage differentiation factor with an ability to sufficiently induce neuronal differentiation of neural progenitors (140).

Oct4 (**NM_002701**), is a member of the octamer-binding (Oct) family of transcription factors. Oct4, as described in the previous chapter is a key regulator of stem cell pluripotency (17). Although Oct4 is also a known oncogene and its abnormal expression is implicated in dysplastic growth and the formation of several types of cancer (141), it is essential for mammalian development (17).

Sox2 (**NM_003106**), is an SRY-related HMG-box (Sox) transcription factor. Sox2 is highly expressed in the pluripotent cells of the early embryo and plays an important role in maintaining potency of stem cells (11, 142). Sox2 is also a master regulator gene for NSC identity and maintenance, as shown by the fact that knocking down Sox2 expression leads to immediate cell cycle exit and terminal differentiation of NSCs (143, 73). In ESCs, Sox2 has been found to form heterodimers with Oct4 to synergistically control the expression of ESC-specific genes (27, 28, 134). Multipotent cell lineages in early mouse development also depend on Sox2 function (142). Mutations of Sox2 genes are involved with cancers and many other pathological human disorders (134, 144-146).

Nanog (**NM_024865**) is a homeobox transcription factor and is a key master regulator of pluripotency. In ESCs, low levels of Nanog cause the upregulation of transcription factors associated with differentiation, implicating that Nanog may help sustain the pluripotent state by repressing these factors (33 & 147). Sox2, Oct4, and Nanog (SON) bind to their own promoters and to each other's as an autoregulatory circuit (6).

NeuroD1 (**NM_028456**), is a basic helix loop helix transcription factor expressed in the brain. This protein was chosen for its involvement in the differentiation of nervous system cells. it

heterodimerizes with the products of the E2A gene and controls the transcription of a variety of genes by identifying and binding E boxes in their promoter region. NeuroD1 could convert Xenopus ectoderm into fully differentiated neurons and that it could prematurely differentiate neural precursor cells in the nervous system (148). NeuroD1 is sufficient to induce neuronal differentiation of neural progenitors in regions that normally do not show the addition of new neurons (140).

For bacterial expression of the reprogramming genes, our lab used an engineered pET30a expression vector by Novagen **(Figure 2-1)**. The pET30a was mutated via PCR using specifically designed PCR primers; the long His-tag was replaced by a short His-tag (six histidine residues) and a Factor Xa cleavage site could be inserted, adding the capability of removal of short his-tag from the reprogramming proteins (134, 149). Replacement of the long his-tag with a short his-tag reduces the risk of the tag interfering with the proper folding of proteins (134, 149). When interference in proper folding and function of the protein occurs, we turn to our option to remove the His-tag by utilizing factor Xa cleavage sites and this vector was named pET30a_sHT **(Figure 2-2)** (134).

Along with a short His-Tag coding sequence, pET30a_sHT vector contains, a T7 *lac* promoter, and for gene insertion, a multiple cloning site (MCS) that contains cleavage sites for different and unique restriction enzymes (NcoI, EcoRV, BamHI, EcoRI, SacI, SalI, HindIII, NotI, and XhoI) (134). Downstream of the T7 promoter, there is a *lac* operator, which allows us to induce the expression of proteins using Isopropyl β-D-1-thiogalactopyranoside (IPTG) reagent (134). The function of IPTG is to molecularly mimic allolactose. In our case, a lactose metabolite triggers the transcription of the *lac* operon to induce protein expression.

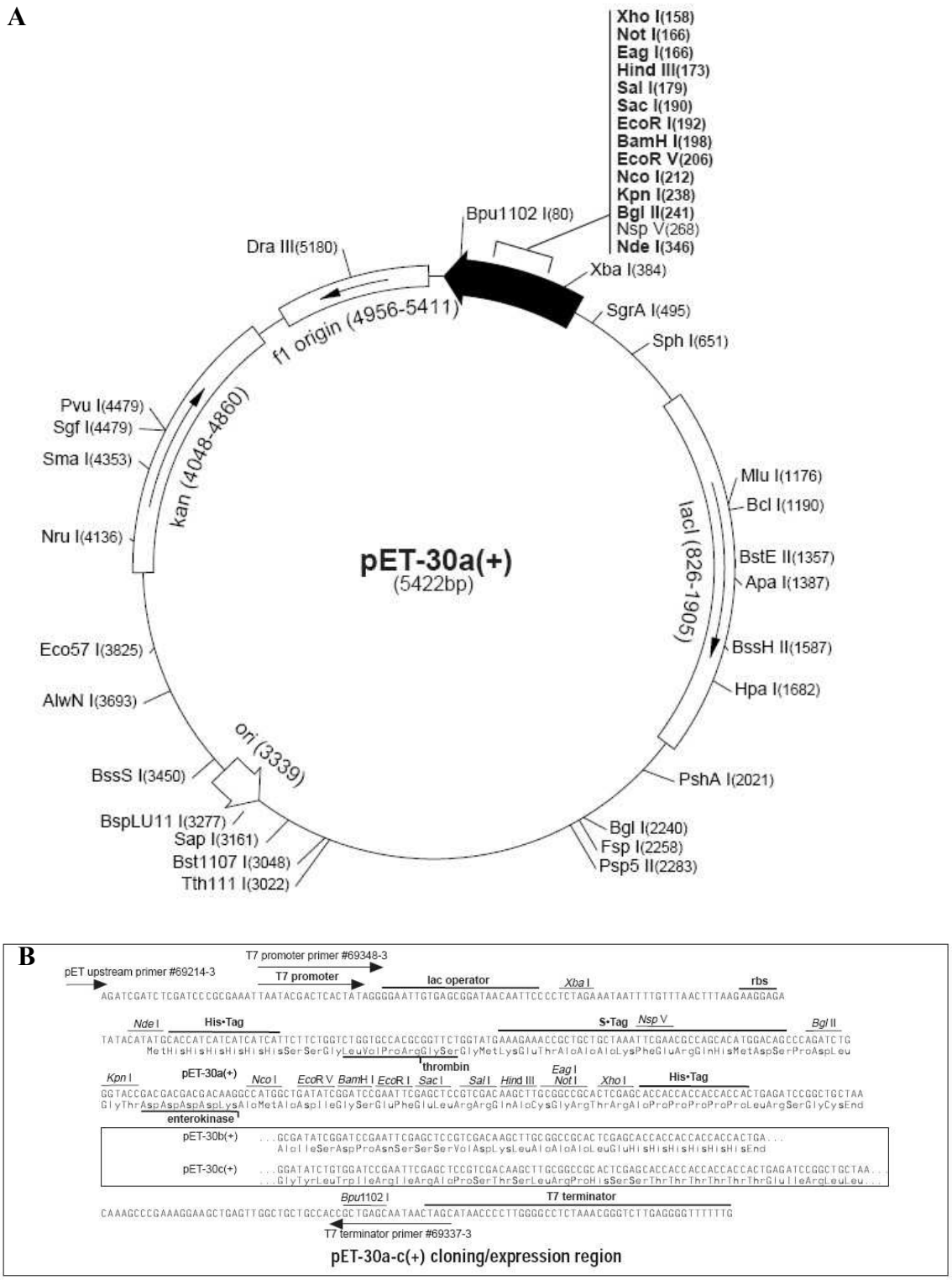

Figure 2-1: pET30a Expression Vector (Novagen). **(A)** Diagram of pET30a expression vector. **(B)** Sequence map of the MCS of the pET30a expression vector (150).

pET30a_sHT

Nco I EcoRV bamHI EcoRI

*atgcaccatcatcatcattcttct*AAGGCCATGGCTGATATCGGATCCGAATTC

Sac I Sal I HindIII Not I Xho I

GAGCTCCGTCGACAAGCTTGCGGCCGCACTCGACACCACCACCACCACCACTGA

Figure 2-2: Sequence map of engineered pET30a_sHT (134).

2.2.2 Primer Design and PCR

For primer design, they began with two single-stranded RNA primers, a forward 5‟ primer, and a reverse 3‟ primer. For amplification, the reverse 3‟ primer contained a sequence of bases that are the reverse complement of the 3‟ end of the DNA coding sequence and the forward 5‟ primer was designed to contain a base sequence identical to the 5‟ end of the DNA coding sequence. When conducting PCR on genes for subcloning, restriction enzyme (RE) cleavage sites that are present in the vector, must be coded into the forward and reverse primers for digestion later in the process to achieve the ligation reaction (134).

Forward and reverse PCR primers are designed for each of the four genes. The forward primers contain bases that are identical to the 5‟ end of the coding DNA strand, and the reverse primers were designed to contain a sequence of bases with reverse complementarity to the 3‟ end of the coding DNA sequence, and are encoded with restriction enzyme cleavage sites that will be used for digestion by the specific restriction enzyme later in the process.. They were designed in this fashion to safeguard the proper annealing to the 3‟ end of the non-coding DNA, and coding strand respectively. To safeguard proper binding of restriction enzyme, six extra bases ware encoded onto the 5” or 3” end of each primer. PCR is then conducted for each gene in an Eppendorf

Mastercycler Personal PCR Machine using the following the PCR recipe (**Table 2-1**) and PCR process (**Figure 2-3**).

Table 2-1: The PCR Recipe for PCR Reaction.

PCR grade water (39 µl)
reverse primer (1.5 µl)
forward primer (1.5 µl)
template DNA (1.0 µl)
dNTP (1.0 µl)
DNA polymerase enzyme (1.0 µl)
10 x Buffer (5.0 µl)

PCR Program	
Initialization step	96°C for 3 min
Cycle	
Denaturation	94°C for 1.5 min
Annealing	60°C for 1.0 min
Elongation	68°C for 2.0 min

Figure 2-3: PCR Program. This program was followed for PCR amplification of each of the four genes-Oct4, Sox2, Nanog, and NeuroD1. The Eppendorf Mastercycler Personal PCR Machine was programmed to these specifications and the cycle was repeated 30 times for each amplification reaction (134).

2.2.3 Restriction Enzyme Digestion and Purification of PCR Products

To remove any DNA fragments from the PCR products, they are purified through a Clonetechchroma spin +TE-400 column. At this point, the products are digested by the specific restriction enzymes encoded in their primers, and samples of pET30a_sHt vector were also digested with an identical restriction enzyme for ligation (134, 150). The DNA was then incubated for approximately 3 hours in a 37ºC water bath with restriction enzyme, a corresponding buffer, BSA, and water.

After digestion, each of the digestion products was run on an agarose ethidium bromide gel by electrophoresis to separate the digested DNA from other DNA fragments and purified using

a Qiagen-QIAEXII Gel Extraction Kit and run on a 10% agarose ethidium bromide gel by electrophoresis for confirmation.

2.2.4 Ligation of Genes into Expression Vector and Transformation of the Ligated DNA into Competent Bacterial Cells

In a ligation reaction, an enzyme referred to as DNA ligase links two sticky ends of each gene covalently (150). The ligation reactions occur overnight in an incubator at 4ºC of the purified digested PCR gene product, the purified digested vector, 10 x ligase buffer, PCR grade water, and DNA ligase enzyme. Electrophoresis is then run to confirm the presence of ligation products.

The ligated DNA is then transformed into E. coli cells. Our lab used the heath shock method for transformation (150). Here, the ligated DNA is transferred ER2566 E. coli competent cells through temperature changes for DNA to undergo transformation into E. coli cells. The cells are incubated (37°C) in plates rich LB medium with kanamycin so that only those bacterial cells that successfully taken up our vector with the resistance gene can survive. The next day, colonies are selected and placed in rich LB medium with kanamycin until the optical density 600nm (OD_{600}) is ~1. The culture is then combined with $1/4^{th}$ the amount of glycerol to make glycerol stocks and placed in -80°C for protein expression later in our study.

2.2.5 Screening for Positive Colonies and DNA Sequencing

Here members of my lab utilized the QIAprep Spin Miniprep Kit for the isolation of plasmids from bacteria cells to determine if plasmid DNA contains each of the genes of interest. The silica-gel-membrane technology can bind DNA, from which the DNA can be eluted with. The resulting DNA solutions are then run on a 1% agarose ethidium bromide gel by electrophoresis and subjected to digestion by the corresponding restriction enzymes used for ligation to confirm

gene size. The digestions follow the same digestions listed for PCR. The plasmids are then sent away for DNA sequencing for DNA sequence conformation.

2.3 Molecular Cloning – Results (This was previously done by Wang's lab)

2.3.1 Primer Sequences and PCR Products

The designed forward and reverse PCR primers for each reprogramming gene based on the gene sequences **(Figure 2-5)**. All forward and reverse primers contain six initial 5‟ bases to serve as spacer nucleotides to allow for binding of the restriction enzyme (150). Following the six spacer nucleotides, a restriction enzyme cleavage site was encoded for each. The rest of the 24 bases of the forward primer are designed to be identical to the first 24 bases of the gene sequence. The reverse primer followed a similar design but used the reverse complement of the 3‟ end of the coding gene sequence, for proper annealing to the coding DNA strand.

The restriction enzyme maps for the Oct4 gene indicates that there were no cleavage sites for NcoI, BamHI, EcoRI, SacI, or SalI present in the gene sequence. We then selected the highly efficient NcoI for the forward primer and EcoRI for the reverse primer (**Table 2-2**). The restriction enzyme map of the Sox2 gene indicated that there were no cleavage sites within the gene sequence for BamHI, EcoRI, SacI, and SalI, HindIII, NotI, and XhoI. For the forward primer, we selected was the BamHI cleavage site and HindIII for the reverse primer **(Table 2-2)**. The restriction enzyme map of Nanog indicates that there were no cleavage sites within the gene sequence for EcoRI, SacI, SalI, NotI, and XhoI. For the forward primer, we selected EcoRI, and XhoI for the reverse primer (**Table 2-2**). The restriction enzyme map of the NeuroD1 gene indicated that there were no cleavage sites within the gene sequence for EcoRI, SacI, SalI, NotI, and XhoI. For the forward primer, we selected EcoRI, and XhoI for the reverse primer (**Table 2-2**).

Table 2-2: Forward and Reverse PCR Primers for each of the Four Reprogramming Proteins.

Gene	**PCR Primer (Forward/Reverse)**	**Forward PCR Primer Sequence**	**Reverse PCR Primer Sequence**
Sox2	BamHI/HindIII	5' cca att gga tcc atc gaa ggt cgt atg tac aac atg atg gag acg gag 3'	5' cgg ttt aag ctt tca cat gtg tga gag ggg cag tgt 3'
Oct4	NcoI/EcoRI	5' cca att gcc atg gct atc gaa ggt cgt atg gcg gga cac ctg gct tcg gat 3'	5' cgg ttt gaa ttc tca gtt tga atg cat ggg aga gcc 3'
Nanog	NcoI/XhoI	5' cca att gaa ttc atc gaa ggt cgt atg atg gtg gat cca gct tgt ccc 3'	5' cgg ttt ctc gag tca cac gtc ttc agg ttg cat gtt cat gga 3'
NeuroD1	NcoI/XhoI	5'cca att gaa ttc atc gaa ggt cgt atg atg gtg gat 3'	5' cgg ttt ctc gag tca cac gtc ttc agg ttg cat 3'

2.3.2 Digestion of PCR Products and Ligation

In order to successfully ligate the reprogramming genes into the pET30a_sHT expression vector, each gene and a sample of the vector had to be digested with restriction enzymes (150). Proper restriction enzyme digest results in the formation of sticky ends on each gene and vector, giving the ability for sequences to line up for proper ligation. Each gene, and a sample of pET30a_sHT, are digested with the corresponding restriction enzymes that were coded for in the PCR primers.

We then use agarose gel electrophoresis of the digested product for purification of these digested products including both digested PCR product and digested vector. After purification, the purified DNA will be used to run an agarose gel to visualize the purified DNA fragments for the PCR product and vector to decide the amount of each DNA fragment used in the ligation reaction.

2.3.3 Plasmid Prep of Transformed Ligation Products and Positive Colony Screening

After identifying the ligation products on an agarose gel, they are transformed into BL21[DE3] competent E. coli cells. The transformed cells were grown up on LB plates containing kanamycin to select for E. coli colonies that have taken up the pET30a_sHT expression vector with the kanamycin resistance gene. Plasmid prep was then performed on colonies that showed growth to verify the uptake of the expression vector. This was done for each of the four reprogramming genes, Sox2, Oct4, Nanog, NeuroD1.

2.3.4 DNA Sequencing

For each of the four genes, two of the plasmids that contained a gene of appropriate size based on the gel electrophoresis results were sent for DNA sequencing to confirm the inserted gene identity. Positive plasmids were identified for each of the four genes, and the bacteria containing these positive plasmids were then used for protein expression. Glycerol stocks of each of the positive bacterial cultures were properly labeled and stored in -80°C for use in protein expression.

2.3.5 Conclusions-Summary of Results

Forward and reverse PCR primers were designed for each of the four reprogramming genes-Sox2, Oct4, Nanog, and NeuroD, based on the gene sequences, the efficiency of available restriction enzymes on either of the four genes or the multiple clone sites of the pET30a-sHT. PCR was then carried out for amplification of each gene sequence. The PCR products are then digested

by restriction enzymes encoded in their forward and reverse primers of the PCR products and with the vectors of pET30a_sHT.

The Sox2 PCR product was digested with BamHI and HindIII, resulting in the removal of 18 base pairs. Oct4 PCR product was digested with NcoI and EcoRI, resulting in the removal of 19 base pairs. Nanog PCR product was digested with EcoRI and XhoI, resulting in the removal of 18 base pairs. The NeuroD1 PCR product was digested with NcoI and HindIII, resulting in the removal of 19 base pairs. Once the had pure digested PCR product samples of Sox2, Oct4, Nanog, NeuroD1, and samples of pET30a_sHT vector which were then exposed to ligase and ligated with their specific digested pET30a_sHT vectors with kanamycin resistance gene. When the ligation products are obtained, they are transformed into BL21[DE3] E. coli cells and cultured in LB plates containing kanamycin.

Cell colonies that are positive for the uptake of pET30a_sHT vectors containing the four genes and kanamycin resistance are subject to plasmid preps. The resulting plasmids are then digested with their specific restriction enzymes for verification of the target gene. Correctly sized plasmids are then subjected to DNA sequencing to confirm the identity of genes, and no mutations had occurred for protein expression and purification.

2.4 High Cell-Density Bacterial Expression - Materials and Methods

2.4.1 IPTG Induction Method

For bacterial expression, I used the high cell-density IPTG-induction **(Figure 2-3)** method developed in Wang's lab (139) to obtain the quantities needed for the reprogramming experiments.

Figure 2-3: A Schematic Diagram of the High Cell-Density IPTG-Induction Method of Protein Expression. Cell Culture temperature and time after IPTG-induction are obtained by optimization of the protocol (139).

This expression method uses a rich medium to achieve high cell density. We started bacterial expression using a rich LB medium, at 37°C. Once the cell density reaches a cell density that is in the middle of the growing phase before saturation, we switched the cell culture by gently spinning down cells and resuspending the cell pellet into the same volume of minimal M9 minimal medium. We found that bacterial cells reach saturation at cell densities of OD_{600} of ~8-10 in LB

and OD_{600} of ~10–15 in 2xYT by using a WPA Biowave© cell density meter. Thus, bacterial cells are in the middle of the growing phase with a cell density of OD_{600} of 3–5 in LB. After switching the medium, we cultured bacterial cells for another 1.0–1.5 h without adding IPTG to allow the bacterial cells to get used to M9 minimal medium and to ensure that bacterial cells go back to the growth phase, at the optimized temperature that is used for the cell culture after IPTG induction. After this 1.0-1.5 h incubation without IPTG, the OD_{600} usually increases by 0.6-1.0 OD_{600} units. IPTG (1mM) was then added to induce protein production. The cell culture is incubated at the same optimized temperature for a period that is optimized for different proteins before cell harvest. We found that the OD_{600} value at the end of cell culture increased by 2.0- to 2.5-fold compared with the OD_{600} value at IPTG-induction. Therefore, before cell harvest, the bacterial cells can reach to OD_{600} of 6-9 with a starting medium using LB and OD_{600} of 8-10 using 2× YT. The cells are then harvested, and the protein purified (100). A schematic diagram of this protocol can be seen in **Figure 2-3**.

2.4.2 Preparation of Starting Culture

Starting cultures for protein expression were prepared by adding 1 µl of glycerol stock for each gene into LB media (**Table 2-3**). These cultures were then incubated in an incubator shaker for ~5 hours at 37°C and 160-180 rpm.

LB rich medium was prepared using the following recipe:

Table 2-3: Recipe for 1-Liter Rich LB Medium used for the preparation of starting culture.

Sterile Deionized H_2O (dH_2O)	**10x LB Stock (7.8 pH)**	**Vitamins**	**Kanamycin**	**Glycerol Stock**
900 mL	100 mL	1 mL	1 mL	~25 µL

10 x LB medium stock was made using 1000 g LB powders (**Invitrogen**) followed by sterile deionized distilled water (dH_2O) until 4 liters total, with an adjusted pH of 7.8. Once the ingredients are added, the solution is heated and stirred until the components are completely dissolved. The solution was poured into smaller bottles with loosened caps and autoclaved at 15 lb/in^2 for 15 min. After the bottles cool to below 40°C, the caps are tightened, and the concentrated LB rich medium is stored at room temperature.

2.4.3 Protein Induction

Once the cell density reaches a cell density that represents the middle of the growing phase (OD_{600} 3-5 for LB and 5-7 for 2xYT), we switched the cell culture by gently spinning down cells and resuspending the pellet into the same volume of minimal M9 medium. M9 minimal medium was made using stock 5 x M9 salts (**Table 2-5**) to make the final M9 medium used in the protein expression and purification process (**Table 2-4**). After switching the medium from rich LB medium to minimal M9 media, we cultured bacterial cells for another 1.0–1.5 h without adding IPTG, at the optimized temperature to allow for the discharge of unlabeled metabolites. During this period, the OD_{600} of the cell culture should increase by 0.5-1 OD_{600} units. IPTG (1mM) was then added to induce protein production. Various IPTG concentrations and temperatures were tested to find the optimal values for each. The induced cultures were then incubated in the 20ºC shaker for ~16 hours to achieve maximum protein expression. We found that the OD_{600} value at the end of cell culture increased by 2.0- to 2.5-fold compared with the OD_{600} value during IPTG-induction.

Table 2-4: Recipe for 1-Liter M9 Minimal Medium used for Bacterial Culture Protein Expression.

Sterile Deionized H_2O (dH_2O)	**5 x M9 Salts (7.8pH)**	**Vitamins**	**Kanamycin**	**20% Glucose**	**1M $MgSO_2$**	**1M $CaCl_2$**
800 mL	200 mL	750 µL	1 mL	20 mL	2 mL	100 µL

M9 is a minimal, low osmolarity media for *E. Coli*, resulting in a slower growth rate of these cells. 5 x M9 salts were prepared by adding the following to a 2-liter flask (**Figure 2-9**).

Table 2-5: Recipe for 1-Liter 5 x M9 Stock Solution used to make M9 Minimal Medium.

1M Na_2HPO_4	**1M KH_2PO_4**	**1M NH_4Cl**	**1M NaCl**	**1M $CaCl_2$**	**dH_2O**
34.0 g	15.0 g	5.0 g	2.5 g	15.0 mg	Top To 1.0 L

Once the ingredients are added, the solution is heated and stirred until the components are completely dissolved. The solution was poured into smaller bottles with loosened caps and the autoclave at 15 lb/in2 for 15 min. After the bottles cool to below 40°C, the caps are tightened, and the M9 minimal medium is stored at room temperature.

2.4.4 Protein Purification Procedure

Upon completion of protein expression described above, the resulting cultures were then spun down by centrifugation at 8,000 rpm for 10 minutes (7,438 G) and the supernatant was discarded. The cell pellets were then resuspended in 1X His Binding Buffer with 6M urea (**Figure 2-9**) and subjected to multiple rounds of sonication to disrupt the cell membranes while releasing the expressed protein into the supernatant. The sonication solutions were then centrifuged and the supernatant, containing the expressed protein, was applied to a column for purification. After purification, the four proteins of interest are eluted from the column and subjected to dialysis to remove any residual salts from the purification process. The resulting protein solution was then freeze-dried by lyophilization to obtain pure protein powder.

2.4.4.1 Preparation of Buffers

Stock Buffers for purification were prepared by the following recipes:

Table 2-6: Recipes for Column Protein Purification Buffers.

1 x His-Binding Buffer (4 L)	8 x Charge Buffer (500 mL)	Elution Buffer (2 L)	Triton-100 Wash Buffer (1 L)	Wash Buffer (1 L)
1440 g Urea 35.06 g 1M NaCl 400 mL 10X PBS Top dH2O to 4L final volume Adjust pH to 7.4-7.6 Pass through filter paper	68.08 g Imidazole 1 x Binding buffer to 500 mL final volume	68.08 g Imidazole Binding buffer to 2 L final volume.	900 mL Binding Buffer 25 mM Imidazole 150 mM NaCl 100 mL 0.2% Triton	1 L Binding Buffer 35 mM Imidazole 150 mM NaCl

2.4.4.2 Preparation of His-tag Column

The His-tag purification columns were prepared by the addition of ~10 ml of clean Ni-NTA resin slurry (**ThermoFisher Scientific R90110**) to a clean 30 ml column. Once silica his-binding beads settle and form a lightly packed base, they were charged using the following our step-by-step column recharge protocol described in **Table 2-7** for used Ni-NTA column.

Table 2-7: Steps to Recharge Column for Protein Purification.

Step	Solution
1	200 mL 0.1 M EDTA
2	400 mL dH2O
3	200 mL 2% SDS
4	200 mL dH2O
5	100 mL 1M NaOH
6	1 L dH2O
7	1 L dH2O
8	1 L 1X Charge Buffer
9	1 L 1X Charge Buffer
10	100 mL 6 M Urea

2.4.4.3 Sonication of Bacterial Cells

To release the expressed protein from bacterial cells, the IPTG-induced bacterial cell cultures are spun down at 8,000 rpm for 10 minutes at the end of protein expression. The supernatant was discarded while the cell pellets were kept. For 1-liter of bacterial expression, the resulting cell pellets were then resuspended in ~30 ml 1 X His Binding Buffer/6M urea and subjected to three rounds of sonication to disrupt the cell membranes and release the expressed proteins. Each round of sonication consisted of three exposures to high power sound waves directed through an ultrasonic probe (**ThermoFisher Scientific Sonic Dismembrator Model 500**) for 30 seconds at a power level of 21. The supernatant was always placed back on ice for 1 minute between each sonication to prevent overheating that may degrade the protein. The cell pellet was then resuspended in 10 ml 1X His Binding Buffer 6M urea and subjected to a subsequent round of

sonication. This was repeated three times. After three sonication cycles, the cell pellets were spun down for 10 minutes 12,000 rpm (17,211g) and the supernatant lysate was applied to the purification column. A total of ~50 ml of lysate was added to each purification column. The solutions were applied to flow through the column at a slow pace (~1 drop per second) to allow the target protein to bind to the charged Ni-NTA beads. Before loading, we collected a sample of the lysate (100ml, **Lysate**), which was properly labeled. After loading the lysates on to the column, we also collected a flow-through sample (100ml, **Flow-through**), which was properly labeled. Both samples were placed in a -20°C freezer for SDS-PAGE.

2.4.4.4 Column Wash

The column was then washed with 1L 0.2% Triton-100 to remove bacterial endotoxin. A triton wash sample was collected and properly labeled (100 µL, **Triton**) for SDS-PAGE. The column was then washed with 1L 1X His Binding Buffer with 6M urea and 25 mM 1M imidazole to remove any bacterial junk proteins. These conditions were followed for each of the four reprogramming factors except for NeuroD1. NeuroD1 required the imidazole concentration in the wash to be lowered to 15 mM because the target protein would begin to elute at 20 mM. A sample was collected in each of the washing steps for SDS-PAGE (100 µL, **Wash**). All of the samples collected for SDS-PAGE were properly labeled and placed in a -20°C freezer for SDS-PAGE.

2.4.4.5 Elution of Purified Proteins

After washing the purification column and removing the impurities, the target protein was eluted from the beads with 1 x His Binding Buffer/6M urea and 1M imidazole. The multiple elution samples were collected at different elution volume and were properly labeled (50 µl, **Elution X**) and placed in a -20°C freezer for SDS-PAGE. This elution was subjected to dialysis to remove any residual salts from the purification process with at least 8 changes of dialysis water. After dialysis,

the elution was placed on a lyophilizer to obtain the pure protein powders. The protein powders obtained for each reprogramming gene were run on 10% SDS polyacrylamide gels to check their purity. A Western Blot was then performed on each protein powder using antibodies specific to each protein to confirm their identity.

2.4.5 Western Blot

Western blot using specific antibodies was performed on each of the protein powders obtained to verify the identity of each protein. I followed the western blot nitrocellulose membranes protocol for provided by **Bio-Rad**.

The first step in the Western blotting procedure is to separate the macromolecules using gel electrophoresis (151). Samples of each protein powder were dissolved in 50 µl 1X SDS loading buffer and placed on a 90ºC heat block for 9 minutes. The samples were then removed from the heat block, allowed to cool to room temperature, and each sample was incubated with 3 µl of dithiothreitol (DTT) for 1 hour at room temperature to reduce any disulfide bonds. The samples were then used to run 10% SDS-PAGE by gel electrophoresis.

Following separation by gel electrophoresis, the SDS-PAGE gel was transferred or blotted onto a 0.2 µm PVDF nitrocellulose membrane (**Bio-Rad 1620097**). To do this, the Nitrocellulose membrane is soaked in methanol because extremely hydrophobic which may hinder the movement of aqueous buffer and protein binding in the membrane during membrane transfer. So, PVDF membrane is hydrated with 100% methanol to facilitate effective transfer. Then we combine the SDS-PAGE gel, two pieces of filter paper, and the nitrocellulose membrane between two sponge pads and incubate in transfer buffer. The SDS-PAGE gel and membrane were stacked together between the two pieces of filter paper (**Mini Trans-Blot Filter Papers**) and placed on a transfer

block (referred to as a transfer sandwich). The transfer chamber was set to a maximum voltage of 150. Transfer took approximately 2.5 hours to be completed in a cold room.

Next, the membrane was incubated in 5% milk blotting blocker for 15 minutes to prevent nonspecific binding of antibodies to the surface of the membrane, followed by overnight incubation with the primary antibody at 4ºC in a cold room. The primary antibodies, an antibody specifically against the target protein, was diluted 1:1000 in 5% milk diluted in 1 x TBST as a blotting blocker. The next morning, the primary antibody was removed, and the membrane was subjected to four (5 minutes) washes with PBS, followed by 2-hour incubation with the secondary antibody at room temperature. The secondary antibodies were diluted 1:1000 in 5% milk diluted in 1 x TBST. The membrane was once again subjected to four (5 minutes) washes with PBS to remove any excess antibody.

Finally, the membrane was soaked with chemiluminescent substrate and enhancer in a dark room and the light produced by the reaction between the substrate and the enzyme conjugated to the secondary antibody was detected on film. Indications of light transferred to the film verified the presence of the specific proteins.

2.5 High Cell-Density Bacterial Expression – Results

2.5.1 SDS Gel – Purification Samples

The samples collected during protein purification of Sox2 and NeruoD1 (Pre-IPTG, Post-IPTG, Lysate (sonication solution), Flow-through, Triton-100, Wash, and Elution X) are run on SDS-PAGE gel electrophoresis. Seven Samples of each of the two proteins are subject to centrifugation and the supernatant is discarded and, sample cell pallets are then incubated in 100 µL 6M urea and stored in -20°C immediately after sample collections. After all, samples are collected, they are quickly sonicated (2-5 seconds, power level 21) and combined 50 µl 1 x SDS

loading buffer and placed on a 90ºC heat block for 10 minutes. The samples were then removed from the heat block, allowed to cool to room temperature, and each sample was incubated with 3 µl of dithiothreitol (DTT) for 1 hour at room temperature to reduce any oligomers. The samples were then run on 10% SDS gels by gel electrophoresis.

Figure 2-5 displays the results of the SDS-PAGE for each sample of the purified bacterially expressed reprogramming proteins. For each gel, a molecular weight marker was run to indicate size. In **Panel A**, Sox2 (34.3 kDa) is shown in the elution sample. While the predicted molecular weight of the Sox2 protein is 34.3 kDa and ran to a point on the gel just above halfway between the 34 kDa and 43 kDa indicator bands, consistent with this size. **Panel B** displays the SDS-PAGE results for the purified bacterially expressed NeuroD1 purification samples. The predicted molecular weight of the NeuroD1 protein is 39.9 kDa and on the gel, its elution sample band is displayed just above the 34 kDa indicator band, consistent with this size.

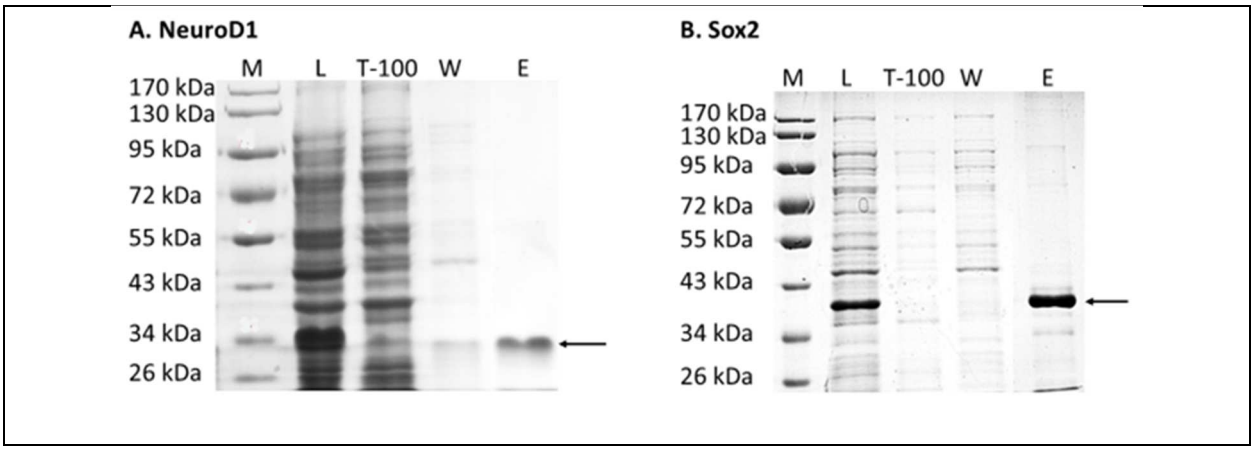

Figure 2-5: SDS-PAGE of Reprogramming Protein Purification Samples. 10% SDS gel of purification samples for Sox2 (34.3 kDa) and, purification samples for NeuroD1 (39.9 kDa). For each gel, a molecular weight marker was run to indicate size; arrows indicate the position of each protein band; (M) is molecular weight marker, (L) is lysate or sonication solution, (T-100) is the triton-100 buffer, (W) is the wash buffer and (E) is a sample of the elution.

2.5.2 SDS Gel – Protein Powder

Samples of each of the two protein powders were dissolved in 50 µl 1X SDS loading buffer and placed on a 90ºC heat block for 10 minutes. The samples were then removed from the heat block, allowed to cool to room temperature, and each sample was incubated with 3 µl of dithiothreitol (DTT) for 1 hour at room temperature to reduce any oligomers. The samples were then run on 10% SDS gels by gel electrophoresis.

Figure 2-6 displays the results of the SDS-PAGE for each of the purified bacterially expressed reprogramming proteins. For each gel, a molecular weight marker was run to indicate size. In the **right two lanes**, Sox2 (34.3 kDa) is shown. While the predicted molecular weight of the Sox2 protein is 34.3 kDa and ran to a point on the gel just above 34kDa between the 34 kDa and 43kDa indicator bands, consistent with this size. **Left two lanes** to display the SDS-PAGE results for the purified bacterially expressed NeuroD1 (39.9 kDa) protein powder. The predicted molecular weight of the NeuroD1 protein is 39.9 kDa and on the gel, its band is displayed just above the 34 kDa indicator band, consistent with this size.

To confirm the identity of each protein powder, Western blot analysis was next carried out on samples of each protein powder.

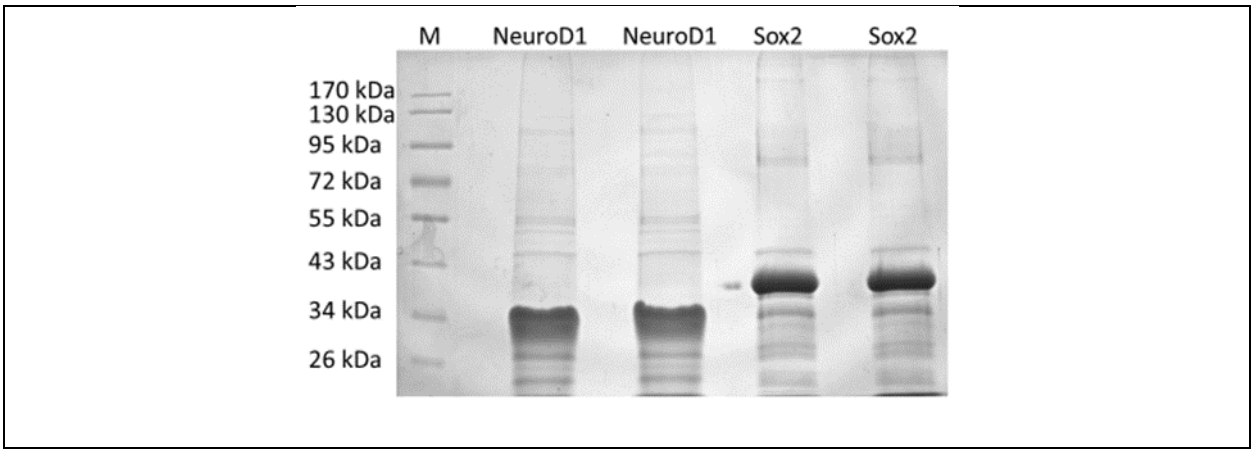

Figure 2-6: SDS-PAGE of Reprogramming Protein Powders. For each gel, a molecular weight marker was run to indicate size; arrows indicate the position of each protein band. **(A) left** 10% SDS gel of purified bacterially expressed Sox2 (34.3 kDa), **(B) right** 10% SDS gel of purified bacterially expressed NeuroD (39.9 kDa).

2.5.3 Western Blot

Western blot analysis was carried out on samples of each of the purified bacterially expressed reprogramming proteins to confirm their identities. **Figure 2-7** shows the western blot results for each of the two proteins I purified, Sox2, and NeuroD1. **Figure 2-8** shows the western blot results of SON factors. Primary antibodies specific to each protein were used to confirm the identity of each protein powder.

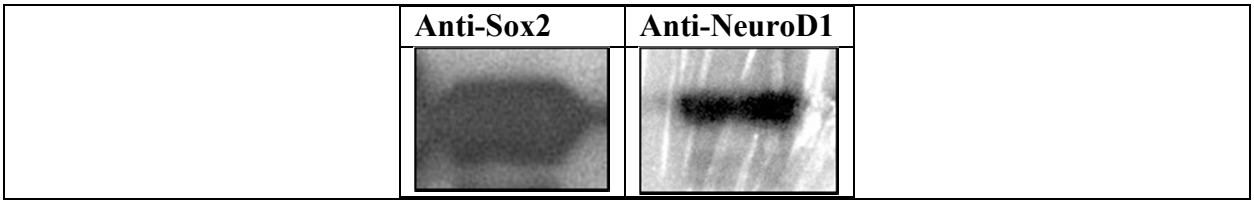

Figure 2-7: Western Blot Analysis Results for Sox2 and NeuroD1 Purified Recombinant Proteins using Anti-Sox2 and Anti-NeuroD.

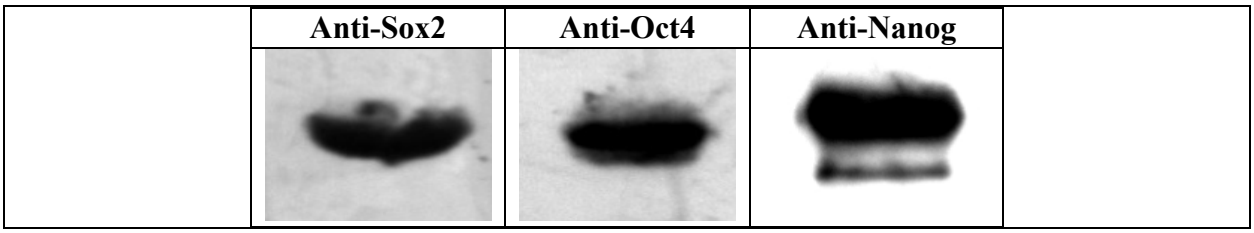

Figure 2-8: Western Blot Analysis Results for the Three iPSC Reprogramming Proteins. Western blot results for Sox2, Oct4, and NeuroD purified recombinant protein using anti-Sox2, anti-Oct4, and anti-NeuroD antibodies, respectively (134).

2.5.4 Protein Quantification and QQ-Modification

After the confirmation of the identities and purity of Sox2 and NeuroD1 proteins using SDS-PAGE and Western Blotting, we had to finally perform QQ-reagent modification to the proteins to generate QQ-Sox2 and QQ-NeuroD1, so that we could apply our patented QQ-protein delivery technology. This was achieved by incubation of the protein: Sox2 and NeuroD1 with pre-made QQ-reagent respectively for overnight at room temperature with slow gently shaking to mix well of protein and QQ-reagents. The QQ-reagent is a mixture of polyethyleneimine (PEI) and lipid emulsion. The final concentration of QQ-reagent was 0.2mg/ml based on PEI concentration.

A 10% SDS-PAGE gel of QQ-Sox2 and QQ-NeuroD1 shows distinct Sox2 and NeuroD1 bands with even more distinct QQ-reagent bands (**Figure 2-9A**). With this combination, the proteins are now referred to as QQ-Sox2 and QQ-NeuroD1.

In order to optimize our reprogramming protocol, the QQ-modified reprogramming proteins were quantified. Before quantification, reprogramming proteins were filtered using a 0.2 μm filter for sterilization. Bovine serum albumin (BSA), a protein from bovine, or cows, was used as a biological protein concentration standard. BSA was purchased in crystalline. The stock BSA solution was diluted to span the 0.1-1 mg/mL range as shown in **Table 2-8**.

Table 2-8: Standard BSA Solution Preparation.

[BSA] μg/mL	**Volume (μL) of 10 mg/mL BSA Stock**	**Volume (μL) of MilliQ Water**
100	5	495
200	10	490
300	15	485
400	20	480
500	25	475
600	30	470
700	35	465
800	40	460
900	45	455
1000	50	445

Both Sox2 and NeuroD proteins and the BSA solutions were used to run a 10% SDS-PAGE. By comparing band intensities of the target protein to the BSA, it revealed an estimated concentration of 0.4 mg/mL for QQ-NeuroD and 0.2 mg/mL of QQ-Sox2 (**Figure 2-9 B**).

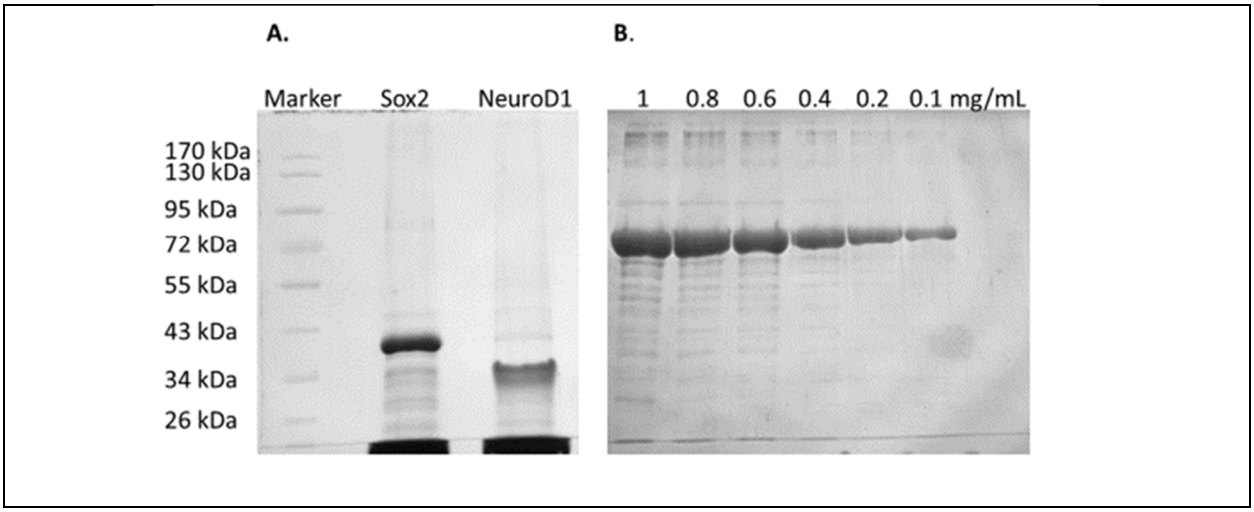

Figure 2-9: SDS-PAGE of QQ-modified Reprogramming Proteins, QQ-Sox2, and QQ-NeuroD1, with BSA Concentration Standard.

A Nanodrop spectrophotometer was used to confirm our BSA protein concentration results of QQ-Sox2 and QQ-NeuroD1. Using Beers Law Spectrophotometry results revealed and confirmed a protein concentration of 0.2 mg/mL of QQ-Sox2 and 0.45 mg/mL of QQ-NeuroD. Equations for calculated concentrations are shown below tables.

$$QQ.Sox2\ Calculated\ Concentration = \frac{Average\ Absorbance}{Sox2\ Extinction\ Coefficent}$$

$$= \frac{0.215}{1.02\ \left(\frac{mg}{mL}\right)^{-1} cm^{-1}} = 0.21\ mg/mL$$

$$QQ.NeuroD1\ Calculated\ Concentration = \frac{Average\ Absorbance}{NeuroD1\ Extinction\ Coefficent}$$

$$= \frac{0.283}{0.63\ \left(\frac{mg}{mL}\right)^{-1} cm^{-1}} = 0.45\ mg/mL$$

2.5.5 Conclusions-Summary of Results

Both reprogramming proteins Sox2 and NeuroD1 were bacterially expressed in E. coli using the high cell density IPTG-induction method. The bacterial cultures were then sonicated, and the expressed protein was subsequently purified using a His-tag column. Washing the column allowed us to remove most of the impurities to obtain purified proteins of interest. The proteins were then eluted from the column and subjected to dialysis to remove the salts that resulted from the purification process. The eluted protein solutions after dialysis were then freeze-dried on a lyophilized to obtain pure protein powders. The protein powders were then run on 10% SDS-PAGE and the size and purity of each protein were confirmed. The identity of each of the protein powders confirmed using Western blot analysis. The proteins were then QQ-reagent modified to generate QQ-Sox2 and QQ-NeuroD1 and quantified using BSA as a concentration standard and Nanodrop spectrophotometry. With the above procedure, we have successfully produced QQ-Sox2 and QQ-NeuroD1 proteins, ready for our cell reprogramming experiment of human fibroblast into iNSCs.

2.6 Discussion

Our laboratory previously has successfully sub-cloned the genes of my two reprogramming proteins: Sox2, and NeuroD1 into the pET30a_sHT expression vector, allowing me to perform bacterial expression of the proteins using BL-21[DE3] E. coli cells. Through rounds of successful protein bacterial expression and column purification, over 60 mg of pure protein powder was obtained for both proteins whose identities were confirmed by Western blot analysis. After numerous rounds of protein purification, I was able to isolate high-quality Sox2 and NeuroD1 protein powder to carry out the rest of the research.

Throughout the duration of these experiments, I was able to acquire effective experimental skills. I have learned how to perform protein expression and purification, SDS-PAGE electrophoresis, and Western Blotting at an advanced level. These molecular biology skills will certainly allow me to repeat these protocols in future experiments and, perform troubleshooting, and optimize conditions when problems arise. Now, I'm also able to review literature at a more skilled level than when I began in 2018. I have also learned how to effectively communicate, collaborate, and report findings much more clearly and applicably. Most importantly, I have intensified my skill of working independently in the lab through the organization, multitasking, problem solving, and budgeting time. All these experiences have given me a strong self-disciplined background to accompany my passion for this field.

CHAPTER 3: GENERATION AND CHARACTERIZATION OF PROTIEN INDUCAED NEURAL STEM CELLS (pi-NSCs)

3.1 Goal

The goal of this chapter was to apply a protein-induced pluripotency stem cell (piPSC) technology developed in Dr. Wang's laboratory (Patents) to produce safe, high quality, and homogeneous protein-induced neural stem cells (piNSCs) from neonatal human dermal fibroblast cells (HDFn). The current reprogramming technology uses viral-gene delivery techniques that suffer from imperative challenges such as low efficiency, time-consuming, complicated, expensive, and major safety concerns. These reprogramming methods often show a conversion efficiency of 0.01% (5). The reprogramming procedure developed in Dr. Wang's laboratory includes a simple incubation step of somatic fibroblast cells with the bacterially expressed transcription factors in media to achieve piPSC generation within 10-days with near 90% conversion efficiency (134). We applied this reprogramming procedure to generate piNSCs within 1-2 weeks, followed by differentiation of the generated piNSCs into astrocytes, oligodendrocytes, and neurons within two weeks.

Our reprogramming procedure utilizes a QQ-protein delivery technology, a high yield bacterial expression protocol, and an in-cell protein refolding system. The QQ-protein delivery technology delivers reprogramming proteins to cross the cell membrane and enter into the nuclei of somatic fibroblast cells (137). Based on localization signal sequences carried by the reprogramming proteins, this technology also allows us to deliver these proteins to the nucleus so that these transcription factors can interact with the promoter and repressors of different genes on the chromosome to regulate cell fate changes. Lastly, the QQ-reagent also protects the reprogramming proteins from degradation by proteases (134).

Furthermore, a low cost and high yield bacterial expression protocol, allowed us to produce over 60 mg of recombinant Sox2 and NeuroD1 proteins. To ensure that our reprogramming proteins were properly folded, we utilized an in-cell protein refolding technique developed in our laboratory (152), allowing the intracellular protein folding machinery to efficiently refold the proteins that are directly delivered into the correct intracellular compartment of the HDFn cells (137). The folded proteins are then targeted to the cell nuclei to initiate cell reprogramming of HDFn.

Chapter 2 outlines the subcloning and protein expression and purification of two reprogramming proteins Sox2 and NeuroD1; resulting in the production of at least 60 mg pure protein for each reprogramming proteins. This chapter will discuss the QQ-modification and delivery of these proteins into HDFn, protein-induced cell reprogramming to generate piNSCs, characterization of the generated, as well as differentiation of the piNSCs into neural tri-lineage cells including astrocytes, oligodendrocytes, and neurons.

Previous studies have used various reprogram cocktails, with different combinations of the transcription factors, in the reprogramming experiments to generate iPSCs. The cocktails included the combinations of the traditional Yamanaka factors (Oct4, Sox2, Klf4, and cMyc), as well as other combinations with new factors (Oct4, Sox2, Nanog and Lin28; Oct4, Sox2, and Nanog; Oct4 and Nanog; Nanog only) to induce iPSCs. A study has also found that only a single factor is enough to reprogram HDFn into the neural linage (29). One of our goals is to examine new combinations of transcription factors that allows us to eliminate oncoproteins and to provide safer piNSCs for regenerative medicine.

3.2 Characterization of piNSCs

In order to confirm that reprogrammed cells are multipotent, the generated piNSCs need to be fully characterized. To achieve this goal, we used the visualization of morphology and colony formation under the microscope and immunostaining techniques.

Neural stem cells give rise to all three neural lineages, have a capacity to self-renew (153). *In vitro* differentiation is the induced differentiation of piNSCs in the neural differentiation medium. Typically, this process involves the formation of neurons, and glia. The successful differentiation of these cell types indicates that the cells have the developmental potential of multipotent NSCs.

The visualization of morphology and colony formation under the microscope is commonly used as the initial form of characterization during cell reprogramming of HDFn into piNSCs. NSCs proliferate in neurosphere-like structures which can differentiate into complex astrocytes, oligodendrocytes, and neurons (68). Astrocytes display heterogeneous fibrous morphologies and are the most abundant glial cells in the central nervous system (CNS) and participate in synaptic, circuit, and behavioral functions (154). Oligodendrocytes display highly complex structures with extraordinary large myelin sheath extensions out of the cell membrane (155). Neurons display a con-like cell body called the soma, a long thin axon, and numbers and dimensions of axonal and dendritic branches (160). These cells, especially piNSCs, are distinct from the spindle-shaped fibroblast cells. The observation of typical NSC, neuron, and glia cell morphology, the ability of piNSCs cells to form colonies, and quick reprogramming time serve as good indicators that the reprogramming protocol has been successful. However, these cells must face more rigorous characterizations to be considered identical to wildtype CNS cells.

Immunostaining for common NSC, neuron, and glia markers provides further confirmation of successfully induced multipotency of piNSCs and their differentiation. Immunostaining includes the use of fluorescently labeled antibodies to detect the presence of particular proteins inside cells. The detection of common proteins in these desired cells indicates a switch from a somatic transcriptional network to that of a multipotent NSC.

Quantitative reverse-transcription PCR (qRT-PCR) is a technique that is used to measure the level of expression of a target gene (mRNA) (126). This is done by combining the reverse transcription of mRNA with PCR amplification (126). PCR is technically another method to find neural progenitor markers, serving as another indication that the cells have changed from somatic cell gene expression patterns to NSC, neuron, and glial gene expression patterns. This technique also serves as a method to compare gene expression patterns in different cell types, including induced and wildtype. We originally plan to perform qRT-PCR to our piNSCs samples as well as the samples of the induced differentiation of piNSCs into neurons, astrocytes, and oligodendrocytes, however, due to the Covid-19 pandemic, we could not complete these experiments.

Electrophysiology is to study the electrical properties of biological cells and tissues. Neurons exhibit electrophysiological properties. Functional neurons derived from piNSCs, under the conditions of primary neuron culture, express synapsin, suggesting synaptic formation *in vitro* (29). A patch-clamp technique is a technique in electrophysiology to show electric currents of an individual cell, tissues, or different areas of a cell's membrane. Testing different areas of one cell membrane is referred to as, whole-cell patch-clamp with voltage-clamps and current-clamps. Voltage clamp recordings of mouse iNCS-derived neurons revealed hyperpolarized resting membrane potential (~40 to -80 mV) with membrane resistance properties (29). Current clamp

mode can elicit action potentials by depolarizing the membrane (29). Therefore, neurons derived from piNSCs shall exhibit the functional membrane properties of wildtype neurons. Due to the lack of technical expertise and equipment, we did not plan to perform patch-clamp experiment.

The formation of complex organs depends on precise spatial and temporal controls of gene expression (156). Therefore, epigenetic mechanisms have been frequently attributed to playing a central role in controlling cell fate determination (156). DNA demethylation analysis is an epigenetic marker to confirm if this gene is activated for expression. DNA methylation immunoprecipitation followed by microarray analysis of piNSCs can visually show the differences between all types of cells used in this experiment. We did not plan to perform DNA demethylation analysis of our piNSCs.

Reynolds and Weiss demonstrated the presence of NSCs in mouse brain by employing a novel serum-free culture system termed the neurosphere assay (58). Neurosphere assay is a culture system with floating clusters of NSCs and provides a method to investigate neural precursor cells *in vitro*. This is achieved by suspending NSCs in medium with growth factors that lacks adherent substrates. This method allows NSCs to form into clusters that contain a small percentage of NSCs (158). Neurosphere assay focuses on three imperative characteristics of neural stem cells: self-renewal, proliferation, and multipotency (158). This method is beneficial because it shows the ability of iNSCs to integrate themselves into host CNS without disrupting normal function.

In vivo differentiation is a more stringent method for determining the developmental potential of iNSC. To examine the *in vivo* developmental potential of iNSCs, researchers transplanted iNSCs into the brains of neonatal myelin-deficient (md) rats (68). Two weeks after transplantation, they could detect murine neural marker M2 and oligodendroglia proteolipid protein (PLP) by immunostaining, which is deficient in the md rat brain. This *in vivo*

study revealed that the implanted iNSCs might be differentiated into M2-positive cells with astrocyte morphology in a variety of host brain regions including cortex and striatum. Many of the M2-positive profiles could be double immunostained with an antibody against GFAP. While these data need to be complemented by other glial markers and an assessment of *in vivo* neuronal differentiation, they clearly demonstrated that iNSCs survive and give rise to differentiated neural cells *in vivo*, which makes this method highly beneficial (68).

For this thesis, we examined the morphology of the generated piNSCs as well as the differentiated neurons, astrocytes, and oligodendrocytes under a light microscope and performed immunostaining to detect various markers of piNSCs and their differentiated neurons, astrocytes, and oligodendrocytes. We also cultured piNSCs for an extended period (2-3 months) to show their ability of long-term self-renewal and proliferation. Furthermore, we performed *in vitro* differentiation assays and staining to show the development of potential neurons and glial cells.

3.3 Generation and Characterization of piNSCs - Materials and Methods

3.3.1 Preparation of Starting Cells

Human neonatal dermal fibroblasts (HDFn) were purchased from ThermoFisher. These cells were plated onto gelatin-coated tissue culture plates in DMEM medium with 10% FBS containing antibiotics to reduce the risk of infection. HDFn were incubated in a 37ºC (5% CO_2) incubator and the medium was changed as needed until their growth reached approximately 50-70% confluence.

3.3.2 QQ-Protein Delivery Method

To deliver the reprogramming proteins into the cells, we used QQ-protein delivery technology developed in our lab (137, 138, 152). The QQ-reagent efficiently delivers the protein into the cell, protects the protein from degradation by intracellular proteases, and allows the protein

to be targeted to the specific intracellular compartment based on the localization signal sequence carried by the specific protein (137, 138, 152).

The process of QQ-modification of the proteins consisted of a simple overnight incubation of the reprogramming proteins with the QQ-reagent, a polyethyleneimine (PEI), and lipid emulsion. A stock solution of the QQ-reagent (molecular weight 1,300) was prepared in 50 mM NaPi buffer at 25 mg/ml at pH 7.4. Approximately 1 mg of each of the reprogramming proteins was dissolved in the same concentration of NaPi, resulting in protein concentrations between 0.5 mg/ml and 1 mg/ml. The exact protein concentration for each of the reprogramming proteins was determined by electrophoresis with BSA as a concentration standard and confirmed by nanodrop spectrophotometry following QQ modification. The protein was mixed with QQ-reagent, drop by drop, and incubated in at 4 ºC overnight. The final concentration of QQ-reagent in the QQ-protein was 0.1mg/ml. The following day, the QQ-modified protein solutions were centrifuged at high speed to remove any precipitate that resulted from the modification process. The supernatant was removed from the precipitate if there was any and directly used for cell reprogramming; no further purification was needed. The QQ-modified reprogramming proteins were then mixed in DMEM medium at specific concentrations to make reprogramming medium.

For protein-induced cell reprogramming, the delivered reprogramming proteins need to enter the nuclei of cells to bind to different DNA promoters and repressors. The reprogramming proteins bind to the repressors of somatic differentiation genes to silent their expression, as well as bind to the promoter's neural multipotent genes to activate their gene expression to initiate cell reprogramming (10). Using a fluorescence microscope, we showed that QQ-modified reprogramming protein, Sox2, reached to the nucleus of the majority cells within a few hours of protein delivery **(Figure 3-1)**. Reprogramming proteins were also observed in the cytosol of the

cells since imaging was performed during the protein delivery cycle and the proteins were in the dynamic process of reaching the nuclei via the cytosol after being delivered across the plasma membrane.

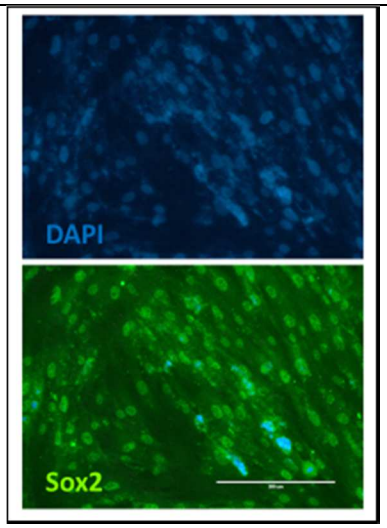

Figure 3-1: Fluorescence Images of the Nuclear Targeting of the QQ-delivered Reprogramming Protein Sox2. Immunostaining results for Sox2 3-5 hours after protein delivery through incubation with HDFn. (Top) DAPI nuclear stain. (Bottom) Sox2 shown in Green.

3.3.3 piNSC Reprogramming Protocol

Our reprogramming protocol is a straightforward protocol that includes the preparation of bacterially expressed proteins, quantification, QQ-modification, and 8-14 cycles of cell reprogramming (**Figure 3-2**). Each cycle is applied in the growth medium or 5-16 hours per cycle. The optimized protocol for HDFn cells begins with tree cycles of incubation with QQ-SON factors. Each cycle is followed by incubation with regular medium for 24 hours with no QQ-modified proteins within freshly replaced growth medium. The three QQ-SON cycles allow the fibroblasts to be driven into a transient pluripotent state (4 Days for HDFn to iPSC-like state). This is then followed neuronal differentiation cycles of QQ-Sox2 and QQ-NeuroD1. QQ-Sox2 (3 cycles, 4-8 days) is used to induce NSCs and, QQ-NeuroD1 (2-6 cycles, 3-20 days) is used to induce terminal

neuronal differentiation of our iNSCs into astrocytes, oligodendrocytes, neurons. An optimized protein concentration of 0.1 µg/ml was used for all proteins.

The cells are incubated in regular cell culture medium (10% FBS) so that they could grow and become confluent in the dish throughout the QQ-SON induction. Once the cells reached 80-90% confluence and have developed into a transient pluripotent state cell, the entire dish was passaged, 50% to a new dish, and the remaining 50% was kept in the original dish. With the application of QQ-Sox2, the cells are incubated in NSC medium, and with the application of QQ-NeuroD1 the cells are incubated in NSC differentiation medium. In all, Throughout the reprogramming process, media is switched between cycles, beginning with regular media, then NSC medium, and finally differentiation media. Continuous passaging was performed along with the medium alterations yields differentiated astrocytes, oligodendrocytes, and neurons within 19-22 days after QQ-SON induction of HDFn (**Figure 3-2**).

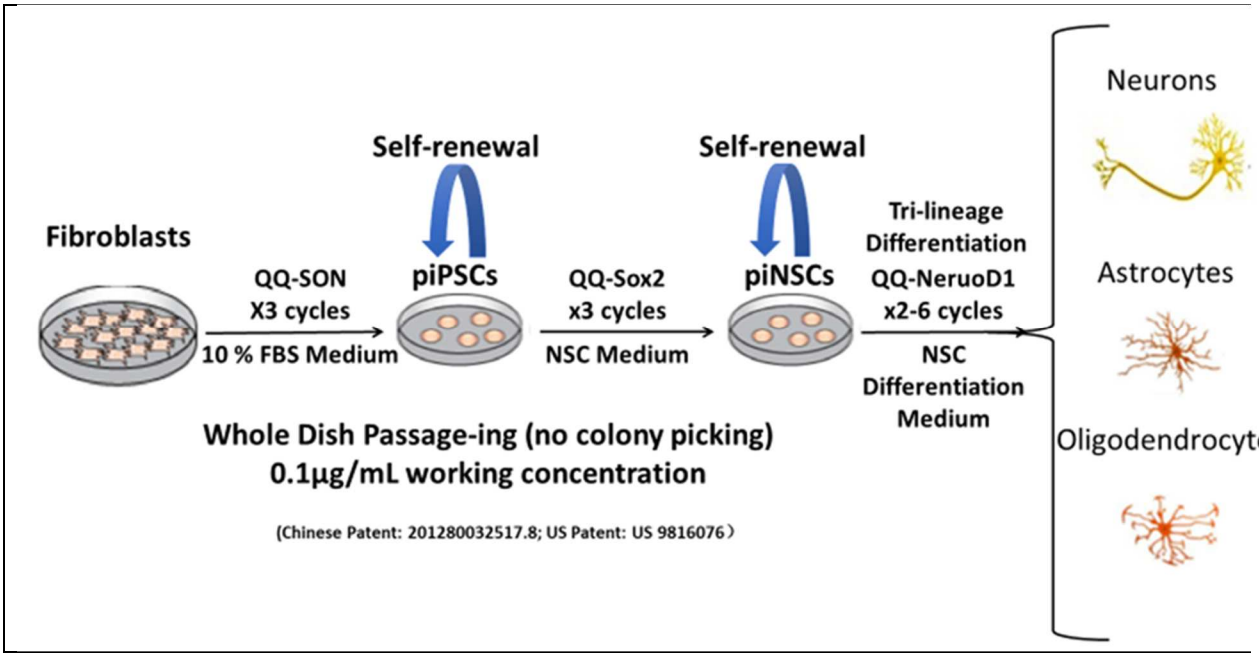

Figure 3-2: Schematic Diagram of the Reprogramming Protocol; including Three Different Reprogramming Cycles of QQ-SON, QQ-Sox2, and QQ-NeuroD. Involving a few rounds of 5-16 hours incubation. Starting HDFn cells.

3.3.4 Characterization of piNSCs, Neurons and Glial Cells

Numerous methods are available today to characterize piNSCs and determine if they are fully reprogrammed into a multipotent state. These characterization techniques assess the similarities between the generated piNSCs and naturally occurring NSCs. The following characterization techniques were utilized in these reprogramming experiments to determine the success of each: visualization of morphology under the microscope, immunostain for multipotency markers, and immunostain for *in vitro* differentiation.

3.3.4.1 Visualization of Morphology

The morphology of each of the reprogramming experiments was visualized by examination under a light microscope as the initial measure for the success of the reprogram. NSCs display proliferates in neurosphere-like structures which can differentiate into complex astrocytes, oligodendrocytes, and neurons (153, 142). Astrocytes display heterogeneous fibrous morphologies and are the most abundant glial cells in the central nervous system (CNS) and participate in synaptic, circuit, and behavioral functions (154). Oligodendrocytes display highly complex structures with extraordinary large myelin sheath extensions out of the cell membrane (155). Neurons display a cell body called the soma, a long thin axon, and numbers and dimensions of axonal and dendritic branches (159). These cells are distinct from the spindle-shaped fibroblast cells that are commonly used as the parental cell in reprogramming experiments.

3.3.4.2 Immunostaining for NSC Markers

Immunostain using specific antibodies was carried out on the final piNSCs of each reprogramming to detect the common NSC markers for an indication of successful reprogramming. The iNSCs were examined for the nuclear markers, Sox2, Pax6 as well as the intermediate filament, Nestin. For immunostain, the cells were passaged to 12-well culture wells

attached to and incubated in the 37ºC CO_2 incubator. The cell culture medium was then removed, and the cells were washed twice with PBS for 5 minutes each time. For fixation, the cells were treated with 10% formalin for ~10 minutes at room temperature. The fixed cells were then washed 3 times with 1 x PBS for 5 minutes each time. Next, the cells were treated with a penetration buffer, consisting of 0.2% Triton-100, and 0.2% BSA for 2 hours at room temperature to allow the entrance of the specific antibodies into the cells. The cells were then incubated overnight (~16 hours) at 4ºC with the primary antibody. Anti-Sox2 (1:300**, Invitrogen**), anti-Pax6 **(**1:300, **Invitrogen**), and anti-Nestin (1:300, **Invitrogen**) were used. The cells were then washed 3 times with the wash buffer and then incubated with the secondary antibody, which is fluorescently labeled for 2 hours at room temperature. Alexa Fluro mouse anti-rabbit IgG (1:2000, **Invitrogen**), Alexa Fluro goat anti-mouse IgG (1:2000, **Invitrogen**), and Alexa Fluro rabbit anti-mouse IgM (1:2000, **Invitrogen**) were used. Following a final round of 3 washes with the wash buffer, the cells were mounted with medium containing DAPI (1:5000, **Invitrogen**) to stain for the nucleus, the slides were taken to the microscope imaging system for viewing of florescence. As negative controls, the starting HDFn cells were also performed using the same immunostain procedure.

3.3.4.3 In Vitro Differentiation

In order to examine the developmental potential of piNSCs, the *in vitro* differentiation was performed. The generated piNSCs were passaged to 12-well culture plates and incubated in NSC medium (DMEM contain: 10% FBS, bFGF 20ng/ml, 0.1 mM non-essential amino acid, 2 mM L-glutamine) for 3 weeks in the 37ºC CO_2 incubator. The Medium was changed as needed. The generated piNSCs were also cultured in a special NSC differentiation medium (DMEM contain: 2-5% FBS, BFGF 10ng/ml, EFGF 10ng/ml, 0.1 mM non-essential amino acid, 2 mM L-glutamine) that promotes differentiation into neurons and glial cells. Differentiation of the piNSCs into

various cell types was observed under the microscope through changes in cell morphology and was confirmed by immunostaining for specific cell markers: Tuj1 and ChAT for neurons, GFAP for astrocytes and O4 for oligodendrocytes.

3.3.4.4 Culture of piNSCs - Long-Term Self-Renewal

The reprogrammed cells were dissociated as a single cell suspension using trypsin (0.05%) and transferred onto new wells with NSC medium containing 1X DMEM (**ThermoScientific, 11885084**) with 0.1 mM Nonessential amino acid **(ThermoScientific Gibco, 11140050)**, 2 mM L-glutamine **(ThermoScientific Gibco, 25030081)**, 2% FBS (**ThermoScientific Gibco, 16000044**), 20 ng/mL bFGF (**ThermoScientific Gibco, PHG0360**). Many neurospheres were observed during self-renewal. The cells were passaged every 5 – 8 days when the dish was 80-90% confluent and were cultured for over 10 passages for 2 months. The medium was prepared fresh medium every other week and kept this medium in a cold room (4°C).

3.4 Generation and Characterization of piNSCs - Results

3.4.1 Morphology Change During Reprogramming

NSCs display proliferation in neurosphere-like structures that can differentiate into complex astrocytes, oligodendrocytes, and neurons. The piNSCs generated by the use of our reprogramming cocktail displayed NSC-like morphology **(Figure 3-3)**. The reprogramming cocktail includes QQ-SON, and QQ-NeuroD1, and QQ-Sox2. The neurospheres pictured in **Figure 3-3** resulted within the first week after whole dish passaging. For each reprogramming cocktail, a large number of NSC-like cells with neurosphere morphology was observed at about the same timeline.

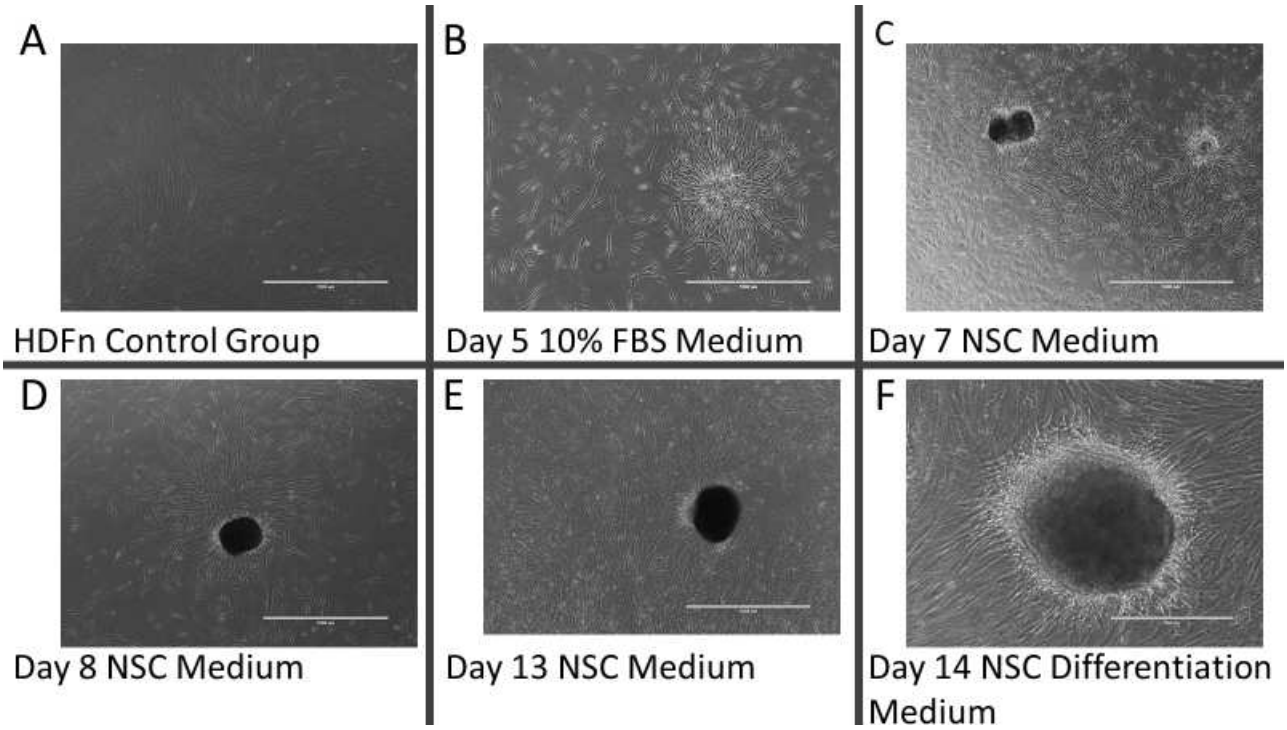

Figure 3-3: Pictures of piNSC Neurospheres Resulting from QQ-SON, and QQ-Sox2 Reprogramming Cocktail. (A) Untreated human dermal neonatal fibroblasts (HDFn) are shown as a negative control. (B-F) Neurospheres. (B) Day 3. (C) Day 5. (D) Day 8. (E) Day 10. (F) Day 13.

3.4.2 Immunostaining for the Reprogrammed Cells

Immunostains were carried to detect the NSC markers: Sox2, Pax6, and Nestin. Identification of these markers inside piNSCs provides an indication of the reprogramming of unipotent fibroblast cells to multipotent piNSCs. To estimate conversion efficiency, we intentionally prepared monolayer cells for these immunostains. As a negative control, the same immunostains were performed using HDFn with the same multipotency markers and these starting HDFn showed negative stains.

3.4.2.1 Immunostaining for Neural Stem Cells

Immunostaining for the nuclear multipotency markers Sox2, Pax6, and Nestin were carried out on the piNSCs generated using QQ-SON and QQ-Sox2 only **(Figure 3-4)**. Staining was performed following the sixth passage after reprogramming. In **Figure 3-4**, blue DAPI stain was used to label the nuclei of cells. The presence of Sox2 is indicated by green fluorescence. while

the presence of Pax6 is indicated by red fluorescence. Sox2 and Pax6 must be visible inside of the nucleus of the cell because they are transcription factors with the blue DAPI stain for the nuclei. Co-staining indicated the cell was positive. Nestin is an intermediate filament involved in the radial growth of axons, and is its stain shows through the cell and not limited to areas like Sox2 and Pax6 which are localized to the nucleus. A large percentage of positive cells were identified.

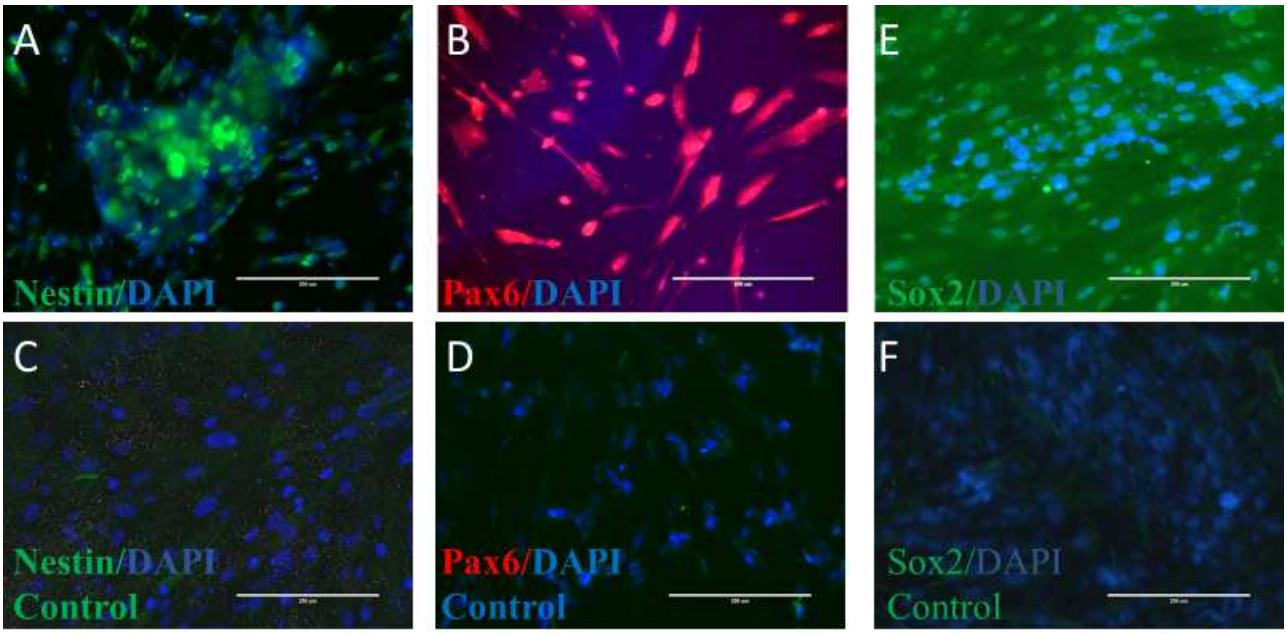

Figure 3-4: Fluorescence Images of Single Immunostains for NSC Markers Sox2, Nestin, and Pax6. All cells were in the 6th passage after reprogramming cycles. **(A)** Nestin is indicated by green. **(B)** Negative control of Nestin. **(C)** Pax6 indicated in red. **(D)** Negative control of Pax6. **(E)** Sox2 is indicated in green. **(F)** Negative control of Sox2. Blue DAPI stain was used to indicate the nuclei of cells. Negative controls are starting HDFn cells.

3.4.2.2 Cell Conversion Efficiency of iNSCs

The conversion efficiency to iNSCs from HDFn using a reprogramming protocol, including cell reprogramming using QQ-Sox2, QQ-Oct4, and QQ-Nanog (QQ-SON) for three cycles, followed by the guided neural differentiation using QQ-Sox2 alone, has been determined based on immunostain data **(Table 3-1)**. The immunostain data of markers Sox2, Pax6, Nestin, and Tuj I, were analyzed. The numbers of positively stained cells *versus* the total number of cells

were counted to determine the percentage of iNSCs. The percentages for each of the markers were then averaged to determine a percentage for the overall efficiency of reprogramming.

Table 3-1 displays the results of the conversion efficiency analysis. As indicated in the table, each of the four NSC markers indicated high cell conversion efficiencies about 80% using the reprogramming protocol described above. While each of the NSC markers shows very similar efficiencies, **the average conversion efficiency is 78.8±4.** While the NSC conversion efficiency of the currently published literature is only ranged 0.1-0.5%, our conversion efficiency of the piNSCs using this described reprogramming protocol above is extremely high.

Table 3-1: Efficiency of Reprogramming. For the generation of NSCs of the reprogramming cocktail of QQ-SON followed by QQ-Sox2 alone. Average reprogramming efficiency determined based on the percentage of cells that stained positive for the multipotency markers Tuj1, Sox2, Pax6, and Nestin.

Markers	QQ-SON & QQ-SOX2 to obtain NSCs
Tuj1	81±1
Sox2	78±5
Pax6	76±2
Nestin	80.3±1
Average	78.8±2.34

3.4.3 In Vitro Differentiation of NSCs

To examine the developmental potential of piNSCs, *in vitro* differentiation was performed. When piNSCs were subjected to a neural differentiation medium that contained QQ-NeuroD, they readily differentiated into various neural-linage restricted subtypes. **Figure 3-5A** shows the pictures that indicate the morphology of undifferentiated piNSCs that have been generated using a reprogramming protocol of the two separate cycle sets of QQ-SON, and QQ-Sox2 alone as described above in Figure 3.2. Differentiation was carried out by incubation with the differentiation medium for 14 days using the fifth passage of piNSCs. Pictures of control HNFs and piNSCs are also included in the figure for comparison of morphology. The HDFn, as well as

non-differentiated piNSCs, showed very different morphologies as compared to the differentiated neural-lineage cells, which show morphologies of neural-like outgrowths.

Differentiation of piNSCs was further confirmed by visualization of morphology. When piNSCs were incubated with special NSC differentiation medium they readily differentiated into astrocytes, oligodendrocytes, and neurons **(Figure 3-5)**. These results indicated that the piNSCs generated have the developmental potential to develop into various cell types, aided in the visual confirmation of their multipotency.

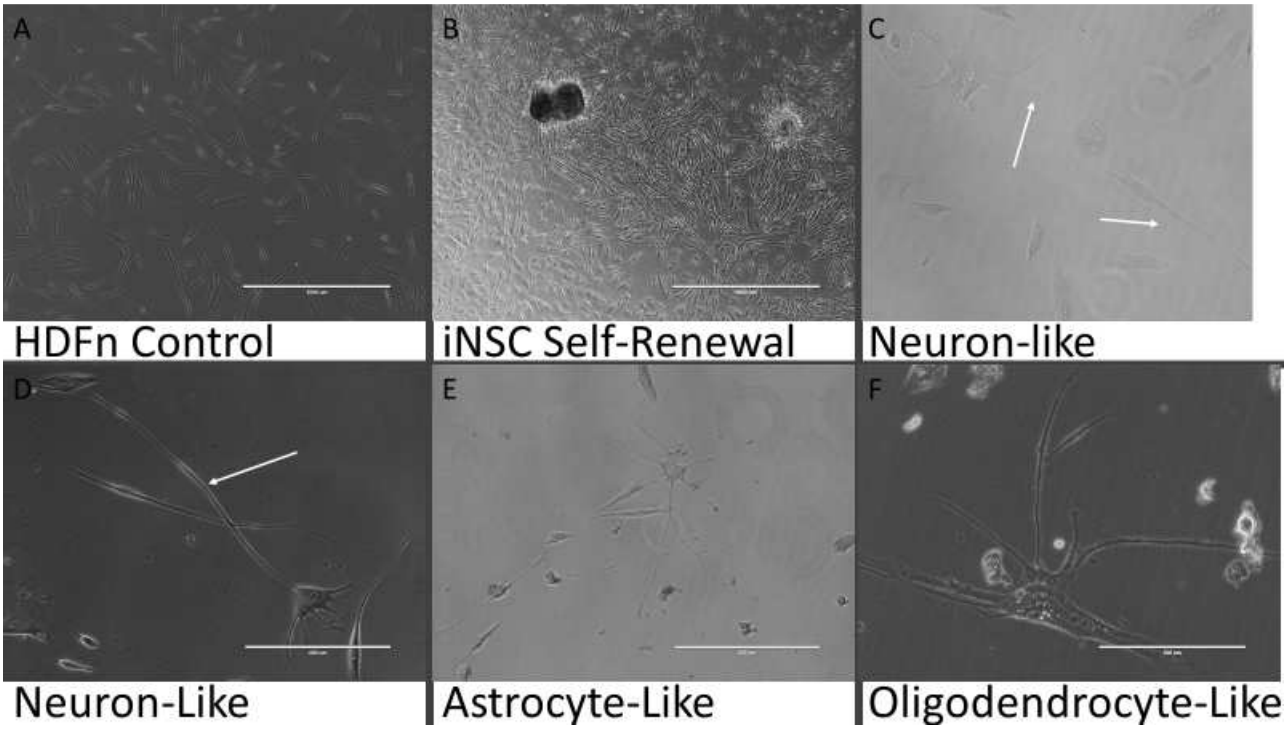

Figure 3-5: Morphology of HDFn, piNSCs and the Differentiated piNSCs. **(A)** Morphology of the starting HDFn. **(B)** Morphology of the generated piNSCs. **(C-F)** Morphologies of the differentiated neural-lineage restricted cells. **(C, D)** Neuronal-like morphology. **(E)** Astrocyte-like morphology. **(F)** Oligodendrocyte-like morphology. Long neuronlike extensions are indicated by arrows.

3.4.3.1 Immunostaining for Neurons

Immunostain for immature and mature neurons was carried out on cells that have been induced differentiated into neural tri-lineage cells in a medium that contained QQ-NeuroD1 **(Figure 3-6)**. Staining was performed for the cells generated following three passages after the last

QQ-NeuroD1-induced differentiation cycle. In **Figure 3-6**, blue DAPI stain was used to label the nuclei of cells. For TUJ 1 (**Figure 3-6 A-D**), NSE (**Figure 3-6 E-F**), and ChAT (**Figure 3-6 I-L**) stains are distributed throughout the cells. A large percentage of positive cells were identified after our reprogramming protocol versus HDFn controls with significant visual differences. The expression of these immature (Tuj1) and mature neuron markers provides strong indication of successful reprogramming.

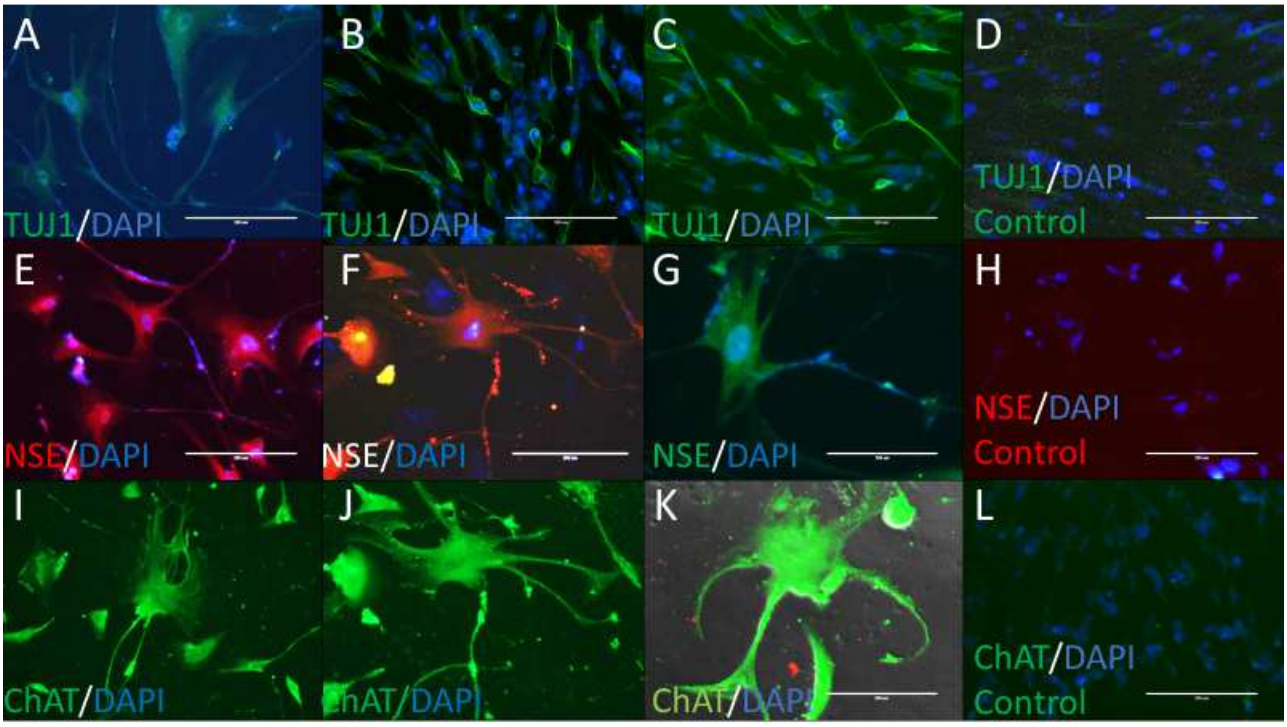

Figure 3-6: Fluorescence Images of Single and Double Immunostain for Neuronal Markers TUJ1, NSE, and ChAT. All cells were in the third passage after QQ-SON, QQ-Sox2, and QQ-NeuroD reprogramming cycles. **(A, B, C)** Tuj1 shown in green. **(D)** Negative control for Tuj1 immunostain. **(E, F)** NSE shown in red. **(G)** NSE shown in green. **(H)** Negative control for NSE immunostain. **(I, J, K)** ChAT shown in green. **(L)** Negative control for ChAT. **(F, J)** Double immunostain of NSE in red and ChAT in green. Negative controls of immunostains using starting HDFn. Blue DAPI stain was used to indicate the nuclei of cells.

3.4.3.2 Immunostaining for Astrocytes and Oligodendrocytes

Immunostaining for astrocytes and oligodendrocytes was carried out on cells that have been induced differentiated into neural tri-lineage cells in a medium that contained QQ-NeuroD1 **(Figure 3-7)**. Like neuron immunostaining, this staining was performed following three passages

after the last QQ-NeuroD1-induced differentiation cycle. Immunostain for the astrocyte marker GFAP, and the oligodendrocyte marker O4 were carried out on the differentiated cells and control cells **(Figure 3-7)**. Blue DAPI stain was used to label the nuclei of cells. The presence of O4 is indicated by red fluorescence (**Figure 3-7 F-H**), while the presence of GFAP is indicated by green fluorescence (**Figure 3-7 I-J**). O4 and GFAP are visible throughout the cells in order to be considered positive. A large percentage of positive cells were identified, which provides a strong indication of successful differentiation of the piNSCs into astrocytes and oligodendrocytes. Phase-contrast images are displayed directly about immunostains (**Figure 3-7 A-E**). Negative controls are also stained with O4 and GFAP markers to show unexpressed biomarkers in HDFn (**Figure 3-7 K-L**).

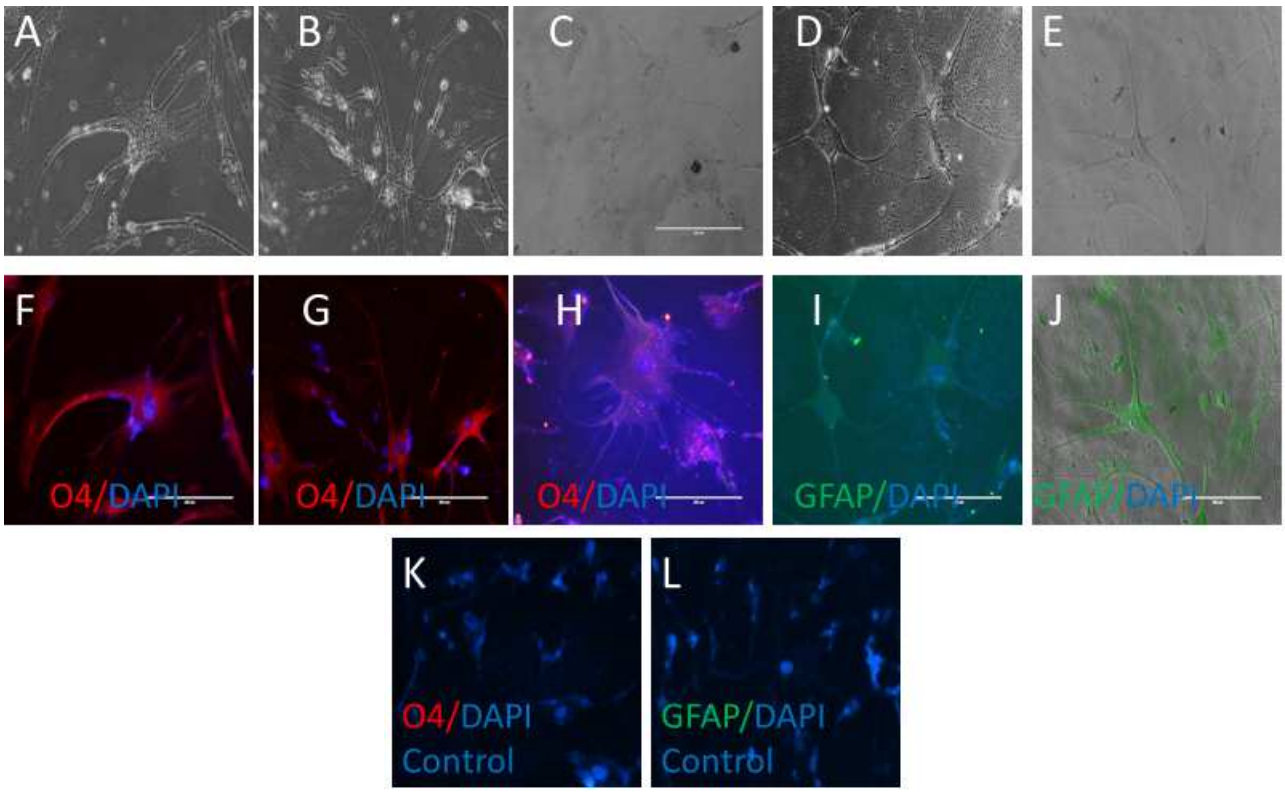

Figure 3-7: Fluorescence Images of Single Immunostain for Astrocytes (GFAP) and Oligodendrocytes (O4). All cells were in the third passage after the QQ-NeuroD1-induced differentiation cycles. (**A-E)** Phase-contrast image of the stains directly beneath. **(F-H)** O4 shown

in red. **(I-J)** GFAP shown in green. **(K-L)** Negative controls of immunostains using starting HDFn. Blue DAPI stain was used to indicate the nuclei of cells.

3.4.4 Conclusions-Summary of Results

In this chapter, we investigated the generation and characterization of multipotent NSCs with NSC properties from human fibroblasts using three reprogramming factors (QQ-Sox2, QQ-Oct4, QQ-Nanog). The conversion efficiency of piNSC generation from fibroblasts in this experiment was 78% as determined from positive immunostaining results for NSC biomarkers (Nestin, Pax6, and Sox2). Continuous whole-dish passaging of human reprogrammed cells showed self-renewal capacity in the presence of growth factors, reprogramming factors, and suitable medium. piNSCs were also stably expanded for over 10 generations in a feeder-free culture system for 2 months. These piNSCs formed NSC-like neurospheres.

piNSCs Displayed tri-lineage developmental potential through *in vitro* differentiation Immunostaining experiments using QQ-NeuroD1 alone. The expanded piNSCs readily differentiated into various neural-linage restricted cell types such as neurons, astrocytes, and oligodendrocytes. Characterization of piNSC tri-lineage differentiation capacity was investigated using visual analysis of morphology and immunostaining for specific lineage biomarkers (Tuj1, ChAT, NSE, O4, and GFAP). ChAT and NSE stain for neurons, GFAP for astrocytes and, O4 for oligodendrocytes.

3.5 Discussion

The transplantation of neurogenic precursors into the CNS for protection and neurodegeneration in humans with injury or disease has not achieved widespread testing and implementation. NSCs serve as a tool for studying neural development and neurological disorders (57). The ability to isolate NSCs is very difficult, and currently, no methods have been well-established to reproducibly, efficiently, and safely isolate large numbers of NSCs. Moreover, the

ability to induce NSCs from abundant somatic cells eliminates the controversy of using human embryos to derive stem cells.

Like NSCs, human iNSCs can undergo unlimited self-renewal, proliferation, and are multipotent cells that are capable to differentiate into neurons and glia. However, unlike NSCs, iNSCs can be derived from a patient's own somatic cells, evading the risk of immunologic rejection for in patient-specific therapies (39). iNSCs, therefore, display the potential to be utilized in a broad range of applications such as cell replacement therapy for regenerative medicine, pathology of neurodegenerative disease in a dish, and research of toxicity and drug discovery for neurodegenerative disease, without the controversy and difficult isolation involved with using human NSCs (29).

While work has been accomplished in developing iNSCs, the current techniques cause cell reprogramming to be a random process, obstructing the study of reprogramming and the applications for iNSCs. Gene delivery methods for iNSC generation are time-consuming, low efficiency, and unsafe for clinical applications due to the high risk of genetic mutations. The current protein-delivery techniques use the CPP-fused reprogramming proteins, which are not metabolically stable and do not possess nuclear targeting capability (120, 121). In addition, these reprogramming protocols require the delivery of high concentrations of protein furthering the expenses required for reprogramming cells.

The reprogramming highlighted in this thesis reprograms somatic cells through the direct delivery of reprogramming proteins, which evades the safety risks associated with the gene-delivery methods. Our lab has developed an efficient QQ-protein delivery technology, to solve the technological challenges faced by the current protein delivery technology. Our lab has also developed a high-level bacterial expression and purification system of the reprogramming

proteins. This was followed by an *in vivo* refolding protocol which, after QQ-delivery of the proteins, employed mammalian cell machinery to properly refold the proteins. The QQ-protein delivery technology allowed us to efficiently deliver reprogramming proteins across the cell membrane and into the cytoplasm (137, 138, 152). The QQ reagent non-covalently binds to the delivered proteins and protects them from degradation by intracellular proteases. This ensures that the delivered proteins refold into their native form and possess the metabolic stability to carry out reprogramming (137). QQ-delivered proteins can specifically localize in the targeted intracellular compartments based on their sequence localization signals (137). Once the delivered reprogramming proteins were properly refolded, they were targeted to the nucleus where they carried out their functions as transcription factors.

Using this protocol, we have successfully generated piNSCs through incubation of HDFn with QQ-modified SON (3 cycles, 5 hours each cycle) in 10% FBS medium and QQ-modified Sox2 alone (4 cycles, 16-24 hours each cycle) in NSC medium. We further differentiated the generated piNSCs to neurons, astrocytes, and oligodendrocytes under the differentiation medium that contained QQ-NeuroD1 protein (4 cycles, 16-24 hours each cycle).

Following a whole-dish passaging method, the piNSCs formed neurospheres. These piNSCs were stably and homogenously expanded for 2 months. Characterization of piNSCs by immunostaining to confirm the expression of NSC makers. An In-depth analysis of immunostaining data verified high cell conversion efficiencies about 80%.

Further characterization of piNSCs by *in vitro* differentiation confirmed the developmental potential and multipotency of these piNSCs. Characterization of the multipotency of piNSCs was done using immunostaining for neurons, astrocytes, and oligodendrocytes.

Our method uses a tightly controlled induction by IPTG and utilizes both rich and minimal media to achieve a very high cell density for the production of recombinant proteins. This method obtained a much higher yield of recombinant proteins than the bacterial expression system or the human cell expression system employed by the other groups (120, 121). Our high-efficiency bacterial expression method can reduce the cost of reprogramming

Our reprogramming technology offers an efficient and fast method to generate human piNSCs that are safe for potential human clinical applications. This technology directly delivers bacterially expressed proteins for cell reprogramming, making this method simple and inexpensive (134). The speed at which piNSCs are achieved with this protocol will allow for the affordable generation of patient-specific piNSCs for transplantation. Previously used methods show very low iNSC conversion efficiencies (0.001-0.01%) versus our QQ-protein delivery technology which displayed high piNSC conversion efficiency (78%).

Furthermore, our reprogramming technology features an *in vivo* protein refolding mechanism that enables direct delivery of bacterially expressed protein to the correct mammalian intracellular compartment for intracellular refolding machinery to efficiently refold the proteins. This *in vivo* refolding technology reduces cost when compared to previously used *in vitro* methods because *in vitro* protein refolding protocols require an additional purification step that is inefficient, making the reprogramming proteins expensive (120). QQ-modification of the protein also protects the delivered proteins form degradation by intracellular proteases, which reduces the frequency and quantity needed to reprogram fibroblasts to piNSCs.

In all, piNSCs from human dermal fibroblasts using QQ-SON pluripotent reprogramming as a tool to quickly reset the time clock of the human somatic fibroblasts to a transient pluripotent state, followed by QQ-Sox2 and QQ-NeuroD1-guided neural-lineage specific differentiation using

our patented QQ-protein delivery technology. The generated piNSCs rely on endogenous gene expression of the NSC factors to express NSC markers and resemble wild-type NSCs in their morphology, self-renewal, and gene expression profiles. Furthermore, the generated piNSCs indicate multipotency with the capability of differentiating into neurons, astrocytes, and oligodendrocytes, as confirmed by the cell morphology and their specific biomarker protein expressions. Thus, self-renewable and multipotent piNSCs without tumorigenic potential can be generated from fibroblasts by our method.

3.6 Future Directions

The generation of clinically safe piNSCs presents a great opportunity to the scientific and medical communities. Further investigation may be done to elucidate the mechanism of reprogramming as well as the potential applications of these cells. Specifically, I would like to apply transcriptomic technologies to study the transcriptome of the induced cells, the sum of all the RNA transcripts, and compare to naturally occurring neurogenic cells. Other characterization experiments such as DNA demethylation analysis, karyotyping, and qRT-PCR could further show the presence of neurogenic markers. Furthermore, studies should be employed to examine various reprogramming cocktails and reprogramming technologies to determine which achieve the highest quality neurogenic cells. These experiments will clarify the molecular mechanisms of reprogramming for a bigger picture of what is truly occurring during reprogramming.

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ABSTRACT

GENERATION OF NEURAL STEM CELLS (NSCs) FROM HUMAN FIBROBLASTS USING QQ-NEUROD1 AND QQ-SOX2 PROTIENS

by

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The generation of induced neural stem cells (iNSCs) and induced neuronal cells (iNCs) from somatic cells provides new avenues for basic research and potential transplantation therapies for neurological diseases. However, clinical applications must consider the tumor formation capabilities of the implanted cells, the inability of iNCs to self-renew in culture, and reprogramming methods that use retroviral transduction which permanently alter genetic network of the cells. Here we report the generation of protein-induced neural stem cells (piNSCs) from human dermal fibroblasts using QQ-SON pluripotent reprogramming as a tool to quickly reset the time clock of the human somatic fibroblasts to a transient pluripotent state, followed by QQ-Sox2 and QQ-NeuroD1-guided neural-lineage specific differentiation using our patented QQ-protein delivery technology. The generated piNSCs rely on endogenous gene expression of the NSC factors to express NSC markers, and resemble wild-type NSCs in their morphology, self-renewal, and gene expression profiles. Furthermore, the generated piNSCs indicate multipotency with the capability of differentiating into neurons, astrocytes, and oligodendrocytes, as confirmed by the cell morphology and their specific biomarker protein expressions. Thus, self-renewable and

multipotent piNSCs without tumorigenic potential can be generated from fibroblasts by our method.

AUTOBIOGRAPHICAL STATEMENT

When I began studying in America, only one thing was clear; I have an opportunity that many from my hometown don't. In fact, the most educated person in my village was once my father, whom also graduated and worked as a professor at Wayne State University (WSU). Others from our village deal with limited access to facilities and educational opportunities. Most families, like mine, sell their farming produce near the entrance of my city. Customers are those that travel from all over the Arabian Gulf to buy unique fruits and vegetables from the world's largest oasis, AlAhsa Saudi Arabia. Today much more is clear, I know that I deserve this opportunity to showcase my hard work to WSU.

In the late 1980s, my father received a full scholarship by Saudi Arabian Cultural Mission (SACM) to attend university in America. He quickly established his interests, and within a few years, I was able to attend the Crestwood School District, volunteer at the Young Muslim Association, and become an Eagle Scout with the Boy Scouts of America right here in Michigan. This was followed by SACM funding me to attend Eastern Michigan University (EMU), and Wayne State University (WSU). Throughout my life, I have faced many unexpected and challenging experiences, all of which have shaped me to become the man I 'am today.

My research experiences led me to discover that I truly enjoy theory work. Graduate research taught me about writing a thesis and dealing with the hardships that follow. It also gave me the chance to become more acquainted with the essentials. Furthermore, when I began my master's degree, I was specifically targeting a challenging research project, one that could allow me to flaunt my knowledge and determination. I was seeking an advisor that was agile and resourceful yet aggressive in their teaching style, like my father. I knew if I could satisfy Dr. Wang, others wouldn't question my hard-working ethic. Moreover, after some time working in the Wang lab, I realized that although my interest in science began ten years ago, my obsession initiated during this graduate research.