Therapeutic Future of iPS

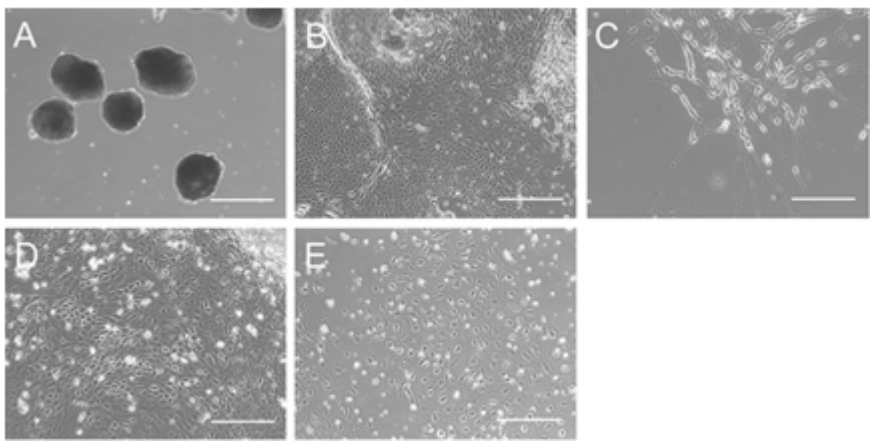
Arsani William
BIO 118Q
Professor Douglas Brutlag
15 March 2009

It was in 2006 when Shinya Yamanaka and Kazutoshi Takahashi uncovered the four key pluripotency genes that would revolutionize the field of stem cell research as we know it. In his laboratory at Kyoto University, retroviruses were used to transfect mouse fibroblast cells and transform them into functional pluripotent stem cells. These induced pluripotent stem cells (iPS) were both morphologically and biochemically identical to the embryonic stem cells, which have been the target of increasing controversy in recent years. Yamanaka's success in the pluripotent reprogramming of somatic skin cells was a victory for researchers endlessly seeking equivalent alternatives; due to previous Executive constraints on embryonic stem cell (ES) research. Surprisingly, induced pluripotent stem cells were quickly accepted by the mass public and researchers alike. Pro-life opponents of ES research praised the ethicality of iPS; researchers acknowledged their practical advantages in advancing therapeutic options. The revolutionary facets of iPS involve their ability to bypass the limitations of immune rejection in existing stem cell therapy. Nevertheless, the question remains: *do these cells hold a future in the therapeutic treatment of neurodegenerative disease?* Moreover, *what needs to be done to ensure a viable therapeutic future for iPS?* There are several key areas that must be analyzed to provide an ample and accurate discussion of the future of iPS. First, we will examine the original and follow-up studies on iPS to note the revolutionary advances in the field. We will then look into the present and future limitations of iPS in its contributions to regenerative medicine, before

ultimately putting these cells to the test and examining their potential therapeutic uses in neurodegenerative diseases like Parkinson's.

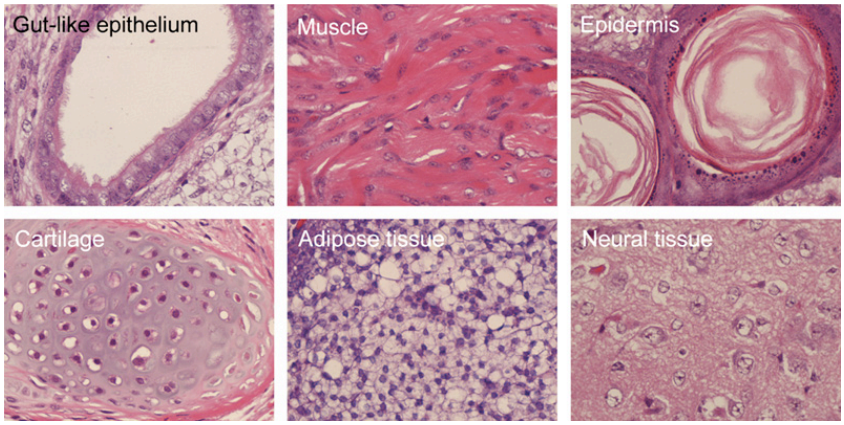
THE YAMANAKA STUDY

Yamanaka and his colleagues were able to successfully reprogram mouse fibroblasts into pluripotent cells that displayed exceptional similarity to human embryonic stem cells. Yamanaka concluded that his induced pluripotent cells (iPS) were similar in "morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity" (Takahashi and Yamanaka 10). Yamanaka identified a complement of four genes essential for inducing the pluripotency state: Oct3/4, Sox2, Klf4, and c-Myc. He then introduced these genes via retroviral infection, and used a selectable marker in the Fbx15 locus to select for colonies. In 25 days, colonies of iPS cells emerged that appeared identical to human embryonic stem cells—both morphologically and biochemically (Takahashi and Yamanaka 9). Western blotting and chromatin immunoprecipitation were conducted (among other studies) to confirm that the iPS cells expressed numerous undifferentiated stem cell marker genes, including telomerase reverse transcriptase at similar levels to human ES. It was also found that iPS histone modifications were *comparable* to human ES in their expression. While Yamanaka's cells displayed only partially-demethylated promoter regions in Oct3/4, Sox2, and Nanog, they were still found to proliferate *in vivo* (Takahashi and Yamanaka 10).



To assess for differentiation, the iPS cells were cultivated in suspension (Figure 1A).

Cells found to attach showed various "morphologies, such as those resembling neuronal cells (Figure 1C), cobblestone-like cells (Figure 1D), and epithelial cells (Figure 1E)."



In testing for pluripotency *in vivo,* injection of iPS cells into immunodeficient mice showed teratoma formation containing various tissues. Histological examination of tumors induced by iPS included fully differentiated cells of gut-like epithelial tissues, striated muscle, cartilage, neural tissues, and epidermal tissues (see Fig. 2). Further studies examined for differentiation specificity. iPS cells were cultured with activin A and bone morphogenetic protein (BMP) 4 to determine whether differentiation could be directed toward cardiac cells. RT-PCR confirmed that the cells expressed markers for cardiomyocytes, and closer observations revealed contractile abilities! Further RT-PCR analysis on tissue extracted from iPS induced tumors in mice revealed various differentiation markers for all three dermal layers (Fig. 3). (Yamanaka 9)

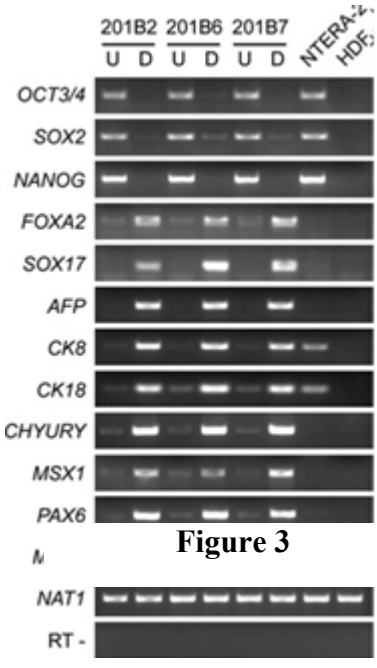


Figure 3

Interestingly, Yamanaka observed that the four factors he initially introduced into the cultured fibroblasts—were ironically silenced in human embryonic stem cells. This observation held two major implications: 1) The reprogramming of iPS cells could allow for the expression of self renewal transgenes, in the absence of the expressed *pluripotency* genes. 2) The continued expression

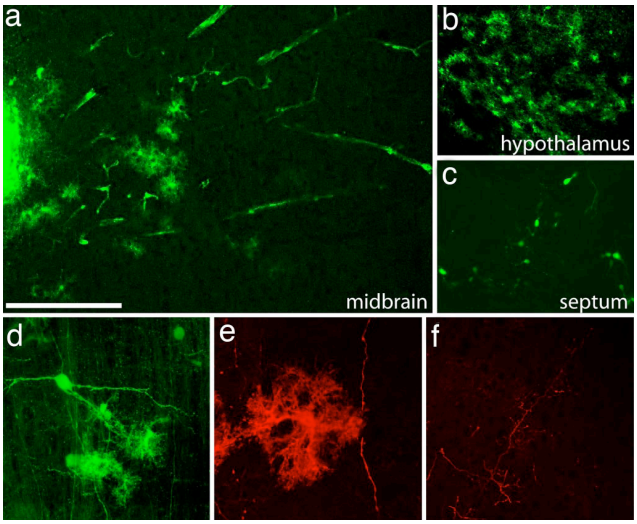
of the four *pluripotency* factors could possibly explain the differences observed between iPS and ES, especially among the methylated promoter regions.

FOLLOW-UP STUDIES

Among researchers at Harvard, MIT, and UCLA, Professor Marius Wernig from Stanford University expanded upon the epigenetic reprogramming of Yamanaka's cells, by modifying the original protocol. Yamanaka's success was slighted by the abnormal differences that persisted between his iPS and ES. Implantation into chimera blastocysts yielded abnormal malignancies and embryonic lethality. Also, *in vitro*, there remained an incomplete reactivation of many pluripotency genes; the Oct4 and Nanog promoters remained methylated. To select for iPS cells displaying full pluripotency expression, Wernig targeted the neomycin gene to the endogenous Oct4 and Nanog loci. Hence, he was able to view the expression of Oct4 and Nanog, whenever neomycin was expressed. A conditional virus expressing siRNA was used to down-regulate the DNMT1 mRNA to demethylate the promoter regions of Oct3/4 and Nanog—resulting in iPS cells that were epigenetically indistinguishable from ES cells (Wernig 12). Wernig uncovered that selection based upon Fbx15 markers isolated only "partially reprogrammed" iPS cells, while Nanog and Oct 3 selection was able to identify "full reprogrammed" colonies.

Wernig also made a key discovery in noting that iPS differentiated neuronal precursors can migrate and differentiate into neurons and glia after transplantation into mouse developing brains. Wernig used a GFP-expressing lentivirus to reprogram iPS cells that were later differentiated into neuronal precursor cells. Transplantation *in utero* into lateral brain ventricles was performed and mice embryos were analyzed for the GFP-expressing brain cells. Strikingly, high densities of cells were found in "septum, striatum, hypothalamus, and midbrain" tissues

Figure 4



(Fig. 4). Moreover, "incorporated cells displayed various complex neuronal and glial morphologies, expressing the neuronal marker proteins NeuN and B-III-tubulin" (Wernig 12). Wernig then induced Parkinson's disease-like symptoms in rats by administering 6-hydroxy dopamine to kill dopamine neurons. iPS cells were then differentiated into neuronal precursors and dopamine neurons and injected in the dorsal striatum of mice. Shockingly, 8 of the 9 mice tested showed stable recovery from the Parkinsonian-like symptoms only four weeks after transplantation. Upon more close morphological observations, Wernig noted that many of the injected cells continued to proliferate, restored the loss of function of endogenous dopaminergic neurons and were morphologically identical to the endogenous neurons in mice brains (Wernig 12).

CURRENT AND FUTURE LIMITATIONS:

Despite the early overwhelming promises that iPS cells have evoked, they nevertheless face major hurdles on their way towards therapeutic application. Among such challenges is the integration of the retroviruses used to introduce the pluripotency genes in the genome. Such viral integration could and have induced the expression of oncogenes that invoke malignancy and disrupt endogenous gene expression (Soldner). Researchers have been searching for alternative means of introducing the *Yamanaka factors* into somatic cells to overcome the problem of eventual tumor growth. Retroviral vectors are feared in therapeutic treatment, because of their

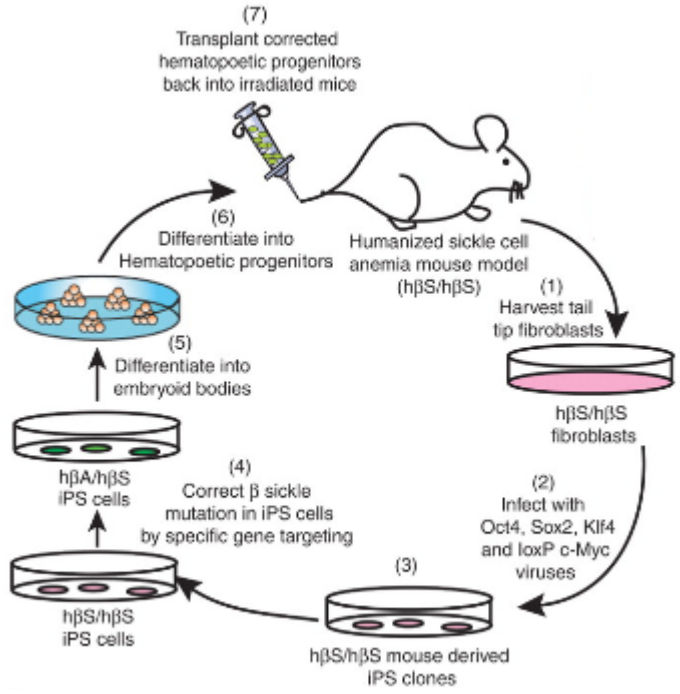
unpredicted abilities to integrate within the genome at many sites where oncogenes may reside. Hence, transplantation could lead to cancer formation. In Wernig's study, 16 of the 36 generated iPS cell chimeras died early because of tumor development. Yamanaka's studies also noted tumor formation via the reactivation of c-Myc transgenes (Wernig 12). In Yamanaka's study, the c-Myc proto-oncogene, which was proven necessary for *efficiently* inducing the pluripotency state, generated tumors in 20% of implanted chimera blastocysts. As a result, various combinations of pluripotent factors have been tested for reprogramming since the original Yamanaka study. Repeated iPS mouse models experiments have consistently shown that reactivation of the c-Myc oncogene induces tumor development. While reprogramming with alternative combinations of factors that exclude c-Myc have been done, reduced efficiency and longer latency are observed. Nanog and Lin28 have been shown to complement the Oct4 and Sox2 activators, without evoking an oncogenic response (Nakagawa). Nonetheless, the majority of complements assayed have failed to produce sufficient numbers of reprogrammed colonies.

There has been an increasing research focus on the means of inducing the silencing of the retroviral inserted genes. After full reprogramming, the expression or activation of the pluripotency genes may lead to tumor growth if the cells are implanted. Also, continuous expression of these factors is noted to interfere with the pluripotency state. For instance, the two-fold expression of Oct-4 causes cell differentiation into the endoderm and mesoderm layers. Down-regulation of Oct-4 results into trophectoderm differentiation (Wernig 11). Hence, the continued expression of the 4 factors could highly interfere with the signaling pathways involved in normal pluripotent stability. Future therapeutic techniques would require the localized iPS cell transfer into targeted regions of human brains. While Wernig's iPS transplants in mice brains succeeded, there remains the possibility that the very factors inducing the neurodegenerative

disorder in the human brain will lead to iPS apoptosis. Further research on the refining of gene complements needed to maintain the pluripotency state with efficiency, needs to be done. To accommodate such, researchers must actively find mechanisms of transplantation that are fully uninhibited by the possibility of tumor formation.

Among conservative and anti-ES research advocates, the initial publications regarding iPS have been hailed as the ultimate solution to the ethical challenges facing ES research. Nonetheless, while the controversy surrounding stem cell research may have waned since the inception of iPS in the scientific community, there remain vast and unresolved differences present between the potential for iPS and ES cells in therapeutic applications. DNA microarray data has identified 1,267 genes that are expressed in vastly differing levels in iPS cells than in ES cells (Gottweis). While the cascade mechanisms and functions of these genes remain unknown, the premature implantation of iPS cells into humans could lead to unexpected, harmful results. Furthermore, iPS reprogramming involves retroviral insertion in over 20 sites—a feat that has largely coincided with tumor mutagenesis (Abeliovich). Further proper research must be done to refine techniques that will make iPS transplantation a safe procedure in humans.

Figure 5

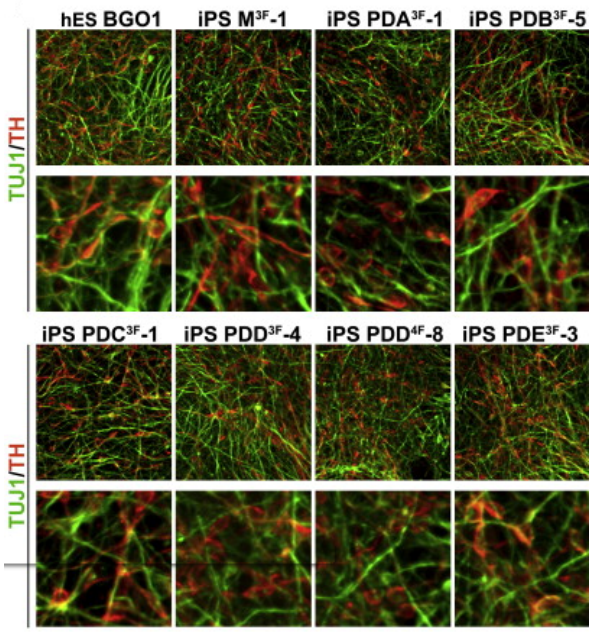


THERAPEUTIC ADVANCES:

Yet, IPS cells have already proven themselves viable as future sources of therapy in regenerative medicine. iPS cells from anemic mice expressing sickled blood cells have been differentiated into normal

hematopoietic cells that in turn cured mice from the disease (Fig. 5). (Hanna) Accordingly, iPS stands as an outstanding candidate for transplant therapy, bypassing the current setbacks of immune rejection. By extracting an individual's skin cells displaying 'self antigens,' researchers can in the future reprogram these cells, and reinject them into the same individual. Immune rejection has for long been a proven setback for the field of stem cells. Transplanted embryonic stem cells are recognized as *foreign* by the body's immunity, and attacked. Because induced pluripotent cells can be effortlessly extracted from each individual patient, stem cells transplants need not be accompanied by immune-suppressant drugs in the future (Ebert).

FIGURE 6: Human iPS differentiated dopaminergic Neurons from PD Patients. Immunofluorescence staining of neuronal cultures for neuron-specific class III β-tubulin (green) and dopaminergic neuron-specific marker tyrosine hydroxylase.



However, before such therapeutic novelties can be developed, researchers must seek alternative means of introducing iPS into human tissue to avoid the formation of melanomas. On March 6, 2009, researchers at the Whitehead Institute in Cambridge, MA published their findings of how to avoid vector-induced melanomas in iPS transplants. In the Whitehead Institute study, fibroblasts from patients with sporadic Parkinson's disease (PD) were collected and reprogrammed using a DOX-inducible *lentiviral* vector to transmit the pluripotency factors (Soldner & Jaenisch). Specifically, the factor gene sequences were placed within lox-P states that could be excised via cre-recombinase. After excision, it was observed that the factor-free iPS cells were more characteristically identical to ES than were the iPS cells which still retained vector expression. The study showed that after somatic cells were induced in a state of pluripotency, they could remain in such a state in the "complete absence of the exogenous reprogramming factors." This not only affirmed Yamanaka's previous presumptions, but expanded the realms in which these iPS cells could be used. When the iPS cells were injected into mice, they formed teratomas of all embryonic germ layers; some of the cell lines formed were dopaminergic neurons. Researchers stained the dopaminergic neurons with classIII β-tubulin and tyrosine hydroxylase (Fig. 6) to confirm that the iPS cells had indeed differentiated into fully functional neuronal precursors and dopamine producing neurons.

There are two major implications to the study. The first revolves around the therapeutic potential for the generation of dopaminergic neurons that could be introduced into PD patients. The second revolves around using these reprogrammed cells to establish an effective in vitro model by which researchers can understand the pathophysiology of neurodegenerative disease. Neurons cultured in vivo through iPS protocols could be exploited for their generic capacity to test drugs, environmental stresses, and genetic therapy—in turn broadening our understanding of disease, its causes, and treatment (Wernig 13). The Whitehead study identified that regardless of age, fibroblast tissue derived from human PD patients could be reprogrammed into the pluripotency state—bypassing the current setbacks of immune rejection to embryonic stem cell therapy.

Current treatment for Parkinson's involves the transplantation of fetal dopaminergic neurons into PD patients. Dopaminergic neurons obtained from human fetuses of 8-9 weeks have been shown to produce sustained synthesis and storage of dopamine in the grafted areas. Reduction in patient rigidity and bradykinesia were also noted (Shah). Nevertheless, at large, the estimated survival rate for these transplanted cells is only 18 months, and research revolving around the clinical improvement of more lasting solutions has slowly advanced. Indeed, the transplanted dopaminergic fetal cells succumbed to degeneration and resulted in a loss of graft function. Autopsies conducted on patients who had received fetal midbrain cells showed alpha-synuclein inclusions normally associated with Parkinson's pathology (Shulz). This has raised concerns about whether the transplantation of fetal cells can be a standardized or sustainable solution. Required immune-suppression and surgical implantation are among the negative attributes of the surgical intervention. Indeed, mesencephalic dopamine neurons from fetuses did

restore dopamine synthesis and significantly reduced PD symptoms in *some* individuals (Shah). However, the therapy has not been proven successful across many trials. While the transplanting of healthy iPS cells to replace neural damage is indeed the target aim for future therapy, the more immediate uses of iPS revolve around its ability to test the effects of new drugs in lab dishes. Indeed, more time is needed for researchers to accurately assess the various reprogramming factors involved in inducing full and identical ES pluripotency before transplanting these cells in human tissue.

Nevertheless, advances in iPS research are occurring rapidly, even as the potential for standardized therapeutic iPS treatment is still in the earliest of stages. Research on the various types of molecules and potential carrier proteins that could replace viral introduction has become a rising priority. While the Whitehead study confirmed that excising the vector genes minimizes oncogenic activation, successful excision has nevertheless failed in many trials. Yet, even with pending limitations, the iPS breakthrough has dramatically pacified the flaming controversy surrounding stem cell research. Anti-ES research advocates are overwhelmingly in support of iPS research and funding. Even influential religious denominations, as the Catholic Church, are praising the successful alternative to embryonic stem cells. The National Catholic Bioethics Center has called induced pluripotent stem cells "ethically pristine" (Vinnedge). Yet, the left-wing push for legalizing and reversing previous executive constraints on ES research remains. While pro-life opponents of ESC research have hailed iPS as an ethical standard for research, many proponents of ESC research argue that ES cells still remain as the "gold-standard," by which research is most likely to produce therapeutic results. Pursuing multiple lines of work could provide insight into the value each cell type holds in the field of regenerative medicine.

Yet, this is a novel break between the left and the right that will only resolve itself with time as the iPS cells approach the standards required for therapeutic transplantation.

DISCUSSION & SUMMARY:

Up until now, the iPS breakthrough has been at the forefront in addressing both the ethical and practical concerns associated with embryonic stem cells. iPS cells hold the advantage of transforming a patient's own somatic cells into stem cells that could be differentiated to any tissue form. Stem cells succumb to immune rejection and have become overtly controversial because of their destruction of human embryos. iPS cells appear at the forefront of advancing regenerative medicine in both its understanding and treatment of disease. While it may be premature to suggest that iPS could replace ES as the ultimate, standardized source for future stem cell research, it appears as if such a suggestion is highly plausible. Generating large colonies of iPS cells could allow researchers to test the molecular effects of drugs or even environmental triggers of disease through the introduction of stressors including chemical neurotoxins in lab dishes. Before these induced pluripotent cells can be utilized for therapeutic assays, researchers must identify what triggers the switching between endogenous and exogenous gene expression during the pluripotency state.

Nonetheless, great strides have been made in the short lived history of iPS. Yamanaka's Fbx15 selections expressed the retroviral transgenes that mechanistically impeded the achievement of the full pluripotent state. Wernig and other researchers found that viral transgenes can interfere with iPS reprogramming by either becoming silenced before reprogramming is complete, or maintaining their expression after the endogenous gene are expressed. Moreover, it was found that in the absence of the oncogenic c-myc factor, iPS cells

colonize at a much lower rate and efficiency. Alternative factors that can induce the same transformation effects should optimize the induction of pluripotency without the associated harm of malignancy. This is an area where further research is needed.

No doubt has iPS addressed the ethical challenges confronting embryonic stem cell research. In the absence of controversy, full-pledged public support and financing could mean advanced therapeutic applications in the near future for iPS. Models for Parkinson's and neurodegenerative disease like muscular dystrophy could soon enable scientists to uncover the pathophysiological mechanisms for the triggering of such diseases. These models could then be studied in petri dishes for therapeutic purposes in advancing appropriate regenerative solutions. Hence, researchers could use iPS to attain colonies of specifically differentiated cells from which they could observe the pathology of triggered diseases; they could then observe responses to various methods of therapy and drugs. This was previously seen in studies where mice induced with human sickle-cell anemia were injected with reprogrammed mouse fibroblasts. After selection, these fibroblasts were differentiated into hematopoetic bone marrow adult stem cells and transplanted. These precursor cells soon differentiated to replace the defective blood carrying the mutant Beta-HB locus and cured the mice from the sickle cell trait (Hanna).

Nevertheless, despite the overwhelming successes of iPS, the field remains extraordinarily young. Yamanaka isolated the four key pluripotency master genes only 3 years ago. Critical research must continue to assess the safety of transplanting iPS into humans and for measuring the future therapeutic potential of these cells. Better delivery systems that avoid the harmful changes retroviruses can have on the genome must be studied and reviewed. Undoubtedly, continued research on the many novel therapeutic applications of iPS will only bring about revolutionary solutions in the field of regenerative medicine. Conclusively, to say

that the future of iPS looks ‘bright’ is an abhorrent understatement. But to say that Induced Pluripotent Stem Cells will bring about revolutionary therapeutic changes in the years to come—is indeed reality.

WORKS CITED

1. Abeliovich, Asa, and Claudia Doege. Reprogramming Therapeutics:iPS Cell Prospects for Neurodegenerative Disease NEURON. DOI 10.1016/j.neuron.2009.01.024

2. Ebert, A.D., Yu, J., Rose, F.F., Jr., Mattis, V.B., Lorson, C.L., Thomson, J.A., and Svendsen, C.N. (2009). Induced pluripotent stem cells from a spinal muscular atrophy patient. Nature 457, 277–280.

3. Gottweis, Herbert and Stephen Minger. iPS cells and the politics of promise. Nature Biotechnology. 26, 271-272 (2008). Doi: 10.1038/nbt0308-271

4. Hanna, J., Wernig, M., Markoulaki, S., Sun, C.W., Meissner, A., Cassady, J.P., Beard, C., Brambrink, T., Wu, L.C., Townes, T.M., et al. (2007). Treatment of Sickle Cell Anemia Mouse Model with iPS Cells Generated from Autologous Skin. Science 318, 1920–1923.

5. Lowry, W.E., Richter, L., Yachechko, R., Pyle, A.D., Tchieu, J., Sridharan, R., Clark, A.T., and Plath, K. (2008). Generation of human induced pluripotent stem cells from dermal fibroblasts. Proc. Natl. Acad. Sci. USA 105, 2883–2888.

6. Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., Okita, K., Mochiduki, Y., Takizawa, N., and Yamanaka, S. (2008). Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat. Biotechnology. 26, 101–106.

7. Schulz, J.B. (2008). Update on the pathogenesis of Parkinson's disease. J. Neurology. 255 (Suppl 5), 3–7.

8. Soldner, Frank, Dirk Hockemeyer, Caroline Beard and Rudolf Jaenisch. Parkinson's Disease Patient-Derived Induced Pluripotent Stem Cells Free of Viral Reprogramming Factors. CELL Volume 136, Issue 5, 6 March 2009, Pages 964-977

9. Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663–676.

10. Takahashi and Yamanaka. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Cell (2007). doi:10.1016/j.cell.2007.11.019

11. Wernig, M., Meissner, A., Cassady, J.P., and Jaenisch, R. (2008). c-Myc is Dispensable for direct reprogramming of mouse fibroblasts. CELL, Stem Cell. 2, 10–12.

12. Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B.E., and Jaenisch, R. (2007). In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 448, 318–324.

13. Wernig, Marius, Jian-Ping Zhao, Jan Pruszak, Eva Hedlund, Dongdong Fu, Frank Soldner, Vania Broccolis, Ole Isacson, and Rudolf Jaenisch. Neurons Derived from Reprogrammed Fibroblasts Functionally Integrate into the Fetal Brain and Improve Symptoms of Rats with Parkinson's Disease Feb. 21, 2008. SCIENCE.

14. Shah, A.K., G.K. Steinberg, Kelly S., and T.M. Bliss. Transplanted Human Fetal Neural Stem Cells Survive, Migrate, and Differentiate in Ischemic Rat Cerebral Cortex. PNAS. July 27, 2004, doi: 10.1073/pnas.0404474101.

15. Soldner, F, Hockemeyer D, and Jaenisch R. Parkinson's Disease Patient-Derived Induced Pluripotent Stem Cells Free of Viral Reprogramming Factors. CELL. (2009) March 6; 136(5):964-77.

16. Vinnedge, Debi. Reprogramming Stem Cells –Or Pro-Lifers' Minds? http://www.cogforlife.org/stemcelltruth.htm. January 2009.