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iPS CELLS AS A POTENTIAL REPLACEMENT FOR EMBRYONIC STEM CELLS

An Interactive Qualifying Project Report

Submitted to the Faculty of

WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements for the

Degree of Bachelor of Science

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ABSTRACT

The purpose of this project was to investigate whether induced pluripotent stem cells (iPSCs) can potentially replace the more ethically controversial embryonic stem cells (ESCs) in therapies. An extensive literature review was performed followed by personal interviews with key stakeholders in the stem cell field. Our findings indicate that several potential problems exist with iPSCs before they can be used in human therapies, including their slow expansion if c-Myc is not used in the reprogramming mix, the potential for cancer at the injection site if c-Myc or integrating viruses are used for the reprogramming, and the potential for immune-rejection of the injected cells. We identified several best-practice procedures for overcoming each of these problems, but conclude that more studies should be performed directly comparing the effectiveness of iPSCs versus ESCs for specific therapies.

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We would also like to thank Dr. David Adams for advising our project. His uncompromising dedication and interest in this project was not only helpful but commendable. He managed, organized, and carefully advised each member of our team on both the topic and the project protocols. Professor Adams was not content in merely informing us, but also educated us on the key issues and alternatives. His persistence and untiring spirit taught us how to work efficiently and effectively together to get the work done on time. His hands-on assistance with this project not only helped our research, but also taught us strong work ethic and gave us a newfound appreciation for the sciences. Overall, Professor Adams made the IQP experience educational and unforgettable. We look forward to working with Professor Adams again in the near future.

EXECUTIVE SUMMARY

Stem cells can self-renew and can differentiate into other more specialized cells, so have been researched for a variety of applications in regenerative medicine. A large variety of stem cells exist, each with different potencies that specify how many different types of tissues they can become. The higher the potency, the more tissues they can become.

There are two main categories of stem cells: embryonic stem cells (ESCs) and adult stem cells (ASCs). ESCs are isolated from the inner cell mass of a 5-day old blastocyst embryo prepared by *in vitro* fertilization (IVF) at a fertility clinic. Isolation of the inner cell mass destroys the embryo. Because an IVF embryo represents potential life (if implanted into a uterus), individuals who believe that life begins at conception, or who believe in minimizing harm to any life, are against the use of ESCs. Thus, ESCs are ethically controversial. Because of these ethical concerns, the federal funding for embryo (and ESC) research has varied considerably over the past three U.S. presidential administrations. Under the Bush administration, federal funding was banned for any experiments proposing to derive new ESC-lines, leaving a relatively small number of ESC-lines available for research purposes. During this era, scientists complained that the lack of cell lines for research severely hindered research progress. Obama's administration is more favorable to embryo research, but with the stipulations that the embryos must be prepared at IVF clinics for reproductive purposes, must represent surplus embryos no longer needed by that family, and must be provided for research purposes with donor consent. Due to the ethical concerns and inconsistent funding of ESCs, many scientists sought alternative sources of stem cells for their therapies.

ASCs are isolated from adult tissues or from umbilical cord blood, not from embryos, so have been the subject of much research. ASCs include hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), neuronal stem cells (NSCs), epithelial stem cells, etc. ASCs have different problems associated with their use: they are harder to grow than ESCs, they are rare in the body (so are harder to identify and isolate), and they are typically less potent. However, one type of ASC, the induced pluripotent stem cell (iPSC) has received considerable attention in the past few years; these cells appear to be pluripotent (like ESCs) but do not destroy an embryo to obtain them. For these reasons, our team chose to investigate the topic of iPSCs as potential replacements for ESCs in therapies.

The discovery of iPSCs by Yamanaka's group in 2006 (Takahashi and Yamanaka, 2006) introduced to the world the idea that there might be more pluripotent alternatives to cell therapeutic stem cell practices than extracting ESCs from embryos. Based on the pioneering work of John Gurdon in the 1950's, who showed that skin fibroblast nuclei can be reprogrammed by microinjecting them into oocytes, Yamanaka's experiments showed that skin fibroblasts can be reprogrammed to a pluripotent state by transfecting genes encoding key transcription factors inside the cells. The cells were capable of growth, and could differentiate into all three germ layers, so were thought to be pluripotent. If such cells could be prepared from a patient's skin cells, his/her iPSCs could be used to generate new tissue to treat the disease. Our research identified several potential problems associated with iPSCs that must be solved prior to their use in human therapies, including: 1) iPSCs may not be as *potent* as ESCs, 2) iPSCs may not necessarily be *immunologically compatible* with the person providing the skin cells for reprogramming, 3) iPSCs may not grow well if the c-Myc is omitted from the reprogramming

mix, and 4) iPSCs may cause *cancer* at the injection site. For each of these potential problems, our research identified best-practice procedures for minimizing the problem.

Potency is measured by the ability of the stem cell to create other types of cells. The original four reprogramming genes used in Yamanaka's experiments were deemed pluripotent because they formed teratomas at the injection site comprised of the three main germ layers (endoderm, mesoderm, and ectoderm) which together can form all tissues in the body. Other scientists investigated their ability to differentiate into a large number of cell types following injection into an implanted blastocyst; for example, Boland et al. (2009) showed that iPSCs injected into a blastocyst can form an entire adult mouse, indicating they were truly pluripotent. But the tendency to form teratomas prevents the use of the cells in therapies, so scientists developed newer reprogramming methods to eliminate the use of the c-Myc component (which is an oncogene). However, eliminating c-Myc slowed the growth of the iPSCs. Our research identified a best-practice method for resolving this problem (Hanna et al., 2007); if c-Myc must be included to provide expansion of the cells for therapy, the c-Myc gene can subsequently be removed using Cre-recombinase delivered by an adenovirus treatment. And in all cases, any cells to be used for therapy should be thoroughly screened to ensure the c-Myc has been silenced and there is no abnormal growth. Our findings indicate that cell potency is not homogenous and may actually vary between cells in a given batch due to "intrinsic variability", differences that exist between reprogrammed cells perhaps due to random DNA damage during the reprogramming (discussed below). Our findings also show that cell potency can be varied by altering the *timing* of addition and the *type* of reprogramming factors added, so this research should be expanded in the future.

With respect to *immunogenicity*, one of the main assumptions of iPSC research is that the cells reprogrammed from the skin cell will be immunologically compatible with the patient who donated the skin cell. This *autologous* therapy would be comparable to a patient providing his own bone marrow cells for therapy. However, our research found that some scientists, for example Zhou et al. (2011), observe rapid immune-rejection of the injected iPSCs from the mouse, while other labs, such as Araki et al. (2013) showed no differences in transplantation success (no differences in immune rejection) when iPSCs were directly compared to ESCs. The latter lab also directly assayed for immune responses, and observed no unusual T-cell infiltration. Our research indicates the area of immunogenicity is under-investigated, and recommends that more studies be performed directly assaying T-cell infiltration near the iPSC injection site.

Cancer is one of the most serious potential problems associated with iPSCs (or ESCs). The c-Myc oncogene problem was discussed above, but two other events are also important to cancer: the use of integrating viruses to deliver the reprogramming genes (the random integration can cause cancer), or the DNA damage caused by the reprogramming. The early experiments used retroviruses to deliver the reprogramming genes because of their high transduction efficiency, but due to cancer formation subsequent experiments focused on delivering the genes using non-integrating viruses or plasmids, delivering transcription factor proteins instead of genes, or using small molecule drugs to mimic the transcription factor proteins. These advances should be pursued further and hopefully will decrease the percent of cancer incidence while still allowing the cells to expand and remain fully potent. In addition to cancer formation caused by c-Myc or the use of integrating viruses, cancer can also be caused by DNA damage. The DNA damage includes point mutations (Gore et al., 2011), copy number variations (Hussein et al., 2011), or alterations in epigenetic modifications (such as DNA

methylation) (Lister et al., 2011). The DNA mutations often occurred in growth regulating genes (likely selected for in the cell expansion process) and in tumor suppressor genes (such as p53). Nonetheless, our interviews consistently pointed to a best-practice approach for minimizing the effects of any DNA damage by rigorously screening the cells using karyotyping (to look for gross chromosomal alterations such as translocations), and performing growth curves (to avoid any cell batches with uncontrolled growth patterns). Unfortunately, the cell assays can also be time consuming, but we conclude they must be performed prior to infusing the cells into any patient.

Overall, echoed continuously throughout all the iPSC and ESC research articles and interviews is that more research must be done prior to reliably using either cell type in patients. Both types of stem cells have the potential for form tumors or become immune-rejected. Our group is strongly in favor of continuing research on iPSCs, as are many other researchers, but we also believe that it is imperative to also continue research with ESCs, in spite of their inconsistent funding and ethical issues. We conclude that few, if any, studies have been done directly comparing the effectiveness of iPSC versus ESC therapies, and we agree that such studies must be done prior to deciding whether to replace one cell type with another. If both stem cell types are found to work equally well for a specific disease, we give the nod to iPSCs due to their fewer ethical issues.

AUTHORSHIP

Author	Areas Covered
Alex Freilich	Stem cell definition iPSC ethics and funding
Andrew Galanis	ESC funding issues iPSC history iPSC immune rejection
Theodore Kostopoulos	Example stem cell uses ESC funding issues iPSC potencies
Michelle Mulkern	Stem cell definition, types, and potencies Current state of iPSC reprogramming
Luisanna Paulino	ESC ethical issues iPSCs and tumors DNA damage

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PROJECT GOALS

Due to ethical issues and inconsistent federal research funding for embryonic stem cells (ESCs), researchers need a replacement for these cells in therapies. The overall goal of this project is to identify potential problems associated with using an alternative type of pluripotent stem cell, induced pluripotent stem (iPS) cells, and evaluate whether the problems are serious enough to prevent their use as a substitute for ES cell therapy.

Specific Objectives:

1. **Document** and evaluate the ethical and funding concerns associated with the use of ES cells and how research scientists may need replacements for these cells in therapy.
2. **Develop** a comprehensive assessment of the origins, inducing technology, and current uses of iPS cells.
3. **Characterize** what key stakeholders believe are potential problems associated with using iPS cells for therapy and their suggestions for potential solutions.
4. **Evaluate** the problems and risks associated with the use of iPS cells.
5. **Recommend** potential solutions to remaining problems associated with iPS cell usage.

LITERATURE REVIEW

Stem Cell Introduction

Stem Cell Definition

Although there are several different subcategories under the broad umbrella of stem cells, at the fundamental level all stem cells are long-lived unspecialized cells in the body that do not have any tissue-specific functions (McKay, 2000; Kirschstein and Skirboll, 2001). These cells are continuously dividing and renewing into new stem cells. Since they are not programmed to perform a specific task in a particular type of tissue, some of these unspecialized cells target tissues to replace damaged tissues other areas of the body, becoming specialized through several differentiation stages. During normal development, stem cells divide, renew, and develop into entire functioning organisms. Thus, stem cells are different than most other cells in the body, and are relatively unspecialized. Due to their ability to self-renew and to create new tissues, they are the focus of the entire field of regenerative medicine.

Stem Cell Types and Potencies

Stem cells are divided into two distinct groups, known as embryonic stem cells (ESCs) and adult stem cells (ASC), and are further classified into their levels of potency along the path of differentiation. Stem cells can be totipotent, pluripotent, multipotent, or unipotent. As previously mentioned, stem cells become specialized into different types of specific tissue cells during cell differentiation. Their ability to form new types of cells specifies their potency, the higher the potency, the greater the number of cell types they can form.

Newly fertilized eggs through about the 8-cell stage are considered to be *totipotent*. When the sperm and egg unite, the zygote that is formed has the ability to form all the tissues of the future adult organism and its extra-embryonic tissues like the placenta. Totipotent cells are the easiest type of stem cell to isolate, as scientists simply take them from 1-8 cell embryos prepared by in vitro fertilization (IVF) (Murnaghan, 2010). In the case of humans, a totipotent cell can create the placental tissue and the complete anatomical tissue of the human. Another example of a totipotent cell found in nature is a fungal spore which can create the entire organism.

The first few cells that divide out of the zygote are said to be totipotent, but after a few days of development, some of the cells begin to specialize into a blastocyst containing roughly 100-150 cells with two layers of cells. The outer cell layer is called the trophoblast, which eventually forms into the placenta. Inside the trophoblast is a mass of cells called the inner cell mass, which generates the fetus and contains embryonic stem cells (ESCs) (From Fertilization to Blastocyst, 2009). ESCs are said to be pluripotent because they are able to differentiate into all types of cells in the body; from bone to smooth muscle to nerves to skin, it all begins with a pluripotent blastocyst. But they are not totipotent, and cannot generate placenta.

As the embryo continues to grow, a network of more highly specialized cells, called *multipotent* cells are formed. These cells have a specific predisposition, allowing them to form groups of related cells, but usually not cells outside the group. For example, a hematopoietic stem cell (HSC) can form into a variety of red and white blood cells and platelets, but does not usually, for example form a nerve cell or cardiac cell. This level of potency is also similar to a mesenchymal stromal cell, which is found in the bone marrow, and which can create bone, cartilage, and marrow fat cells, but is usually limited only to these possibilities. Exploration of

the different types of multipotent stem cells found in the body has recently been expanded and debated by the scientific community as potential replacements for ESCs, as they retain medically relevant levels of potency without the problems associated with ESCs (discussed below).

Unipotent stem cells have the lowest level of potency. They are able to self-renew but only into the same type of cell. Cells in this category are sometimes later proven to be multipotent, but a common example is intestinal stem cells (Kirschstein and Skirboll, 2001).

Adult Stem Cells

Adult stem cells (ASCs) are any type of stem cell that is not an ESC. This is a very broad category of stem cells, also called somatic stem cells, and includes multipotent stem cells in the body that are used to replace damaged tissues. In addition to the aforementioned types of multipotent stem cells (hematopoietic and mesenchymal stromal cells), there have been numerous recent discoveries of many other types of adult stem cells, including neural stem cells, epithelial stem cells, cardiac stem cells, intestinal stem cells, lung stem cells, and kidney stem cells.

As an example of a discovery of a new type of adult stem cell, Temple (1989) was the first to isolate adult neural stem cells from rat forebrain. The cells were grown *in vitro*, and were observed to form new neurons and glial cells. Temple theorized that the cells forming new neural tissues were adult brain stem cells. In 1993, Morshead et al. validated the earlier 1989 Temple experiments, and isolated neural stem cells from mouse forebrains. The neural stem cells were located in an area of the brain thought to participate in cell renewal, the subependymal cells. Six years later, in 1999, Johnansson et al. in Stockholm found definitive proof that ependymal cell can generate neurons that migrate to the olfactory bulb of the brain in

response to traumatic injury (Johnansson et al., 1999). Other numerous studies subsequently found adult rat and mouse brains to contain several areas with small populations of neural stem cells, including the ependymal areas, forebrain, and hippocampus (Palmer, 1997).

With respect to mesenchymal stem cells (MSCs), those cells are usually isolated from bone marrow, but there has been some discrepancy among professionals at defining which marrow cells are MSCs. These cells are multipotent, and can differentiate into different types of bone and cartilage tissues, but not usually anything else. In their 2006 paper, Dominici et al. set clear boundaries for what qualified as a MSC, including the requirement for the stem cell to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* (Dominici et al., 2006).

Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) are derived from adult stem cells, and are the main subject of this project and will be discussed in more detail later. Briefly, iPSCs are adult differentiated cells (usually skin fibroblast cells due to their ease of isolation and ease of transfection) induced to de-differentiate back into pluripotent cells by transfecting into them the genes encoding specific reprogramming transcription factors. This technique was first done successfully in mice (Takahashi and Yamanaka, 2006) and a year later with human skin (Takahashi et al., 2007). The transcription factors chosen by Yamanaka's group themselves help maintain pluripotency in ESCs. In the initial 2006 study, four transcription factors were used (c-Myc, Klf4, Oct4, and Sox2), chosen because they were known to switch on genes that maintain the defining properties of embryonic stem cells (Takahashi and Yamanaka, 2006). However, the reprogramming techniques are constantly being improved, using fewer factors and not using DNA but the factors themselves to avoid potential chromosome problems for therapies in

humans. Because some research groups claim iPSCs are as potent as ESCs, they do not destroy an embryo to obtain them, and they can be prepared to be immunologically similar to the patient donating the skin cell, iPSCs are the subject of much current research. In 2012, John Gurdon and Shinya Yamanaka were awarded the Nobel Prize in Medicine or Physiology for their work on cell reprogramming (NobelPrize.org, 2012).

Example Stem Cell Medical Uses

Due to their ability to self-renew and form new more specialized tissues, stem cells are currently intensely researched for potential uses in treating several diseases. Stem cells have been used to treat a variety of animal disease models, including leukemia, diabetes, Parkinson's disease, stroke, myocardial infarction, spinal cord injuries, lung cancer, scleroderma, macular degeneration, etc. The animal models have been tested with both ESCs and ASCs. To date, human patients have been treated with two types of adult stem cells: HSCs for treating leukemia and other blood disorders, and HSCs or MSCs for treating patients with myocardial infarction and stroke (discussed below).

HSCs have been used since 1957 to treat human leukemia patients in bone marrow transplants (Thomas et al., 1957), so HSCs are the most characterized of all stem cell types. Adult stem cells (HSCs and MSCs) have also been used to treat stroke and myocardial infarction patients. A heart attack occurs when coronary arteries become restricted due to an accumulation of fat, cholesterol, and inflammatory substances lining the artery walls, restricting blood flow (American Heart Association, 2013). Bone marrow stem cells have been used since 2002 to treat heart attack patients (Strauer et al., 2002). Mononuclear bone marrow cells (BMCs) were perfused into the heart of 10 patients by intracoronary injection. Their results at 3 months post-

therapy showed that the dead cardiomyocyte cells were able to regenerate, and the infarct size was significantly reduced, while the treatment appeared to be safe (Strauer et al., 2002). Other studies treating human heart attack patients with bone marrow cells include Britten et al. (2003), Lunde et al. (2006), Schächinger et al. (2006), Assmus et al. (2010), and Traverse et al. (2012).

MSCs have also been used to treat heart attack patients (Chen et al., 2004; Hare et al., 2012). In addition to treating heart attack patients with adult stem cells, recent treatments are also being attempted by reprogramming heart fibroblast cells *in vivo* with cardiac transcription factors (Song et al., 2012).

Recently, MSCs have found potential applications for treating musculoskeletal injuries, one of the most common types of injuries in sports or heavy lifting jobs. Drs. Tortland and Kozar, two doctors affiliated with Valley Sports Physicians & Orthopedic Medicine, Inc. (Avon, CT) perform a unique procedure that treats individuals suffering from these injuries with their own MSCs (Kozar and Tortland, 2013). The procedure itself is fairly simple, Novocain is administered at the spot to numb the injection, and then the injection of MSCs is guided by ultrasound to make sure it is administered to the proper location. The operation has a 60-85% success rate, depending on the extent of the injury (Kozar and Tortland, 2013).

Regarding treating patients with ESCs, Geron Pharmaceuticals was leading the way by gaining approval to treat human spinal cord patients with ESCs, but halted their clinical trial in 2011 due to insufficient funds (Baker, 2011). In 2012, the FDA approved former Worcester (MA) biotech company Advanced Cell Biology, Inc. to investigate macular degeneration in patients using ESCs (Lok, 2012). A biotech company in California is near to testing ESCs for treating diabetic patients (Dance, 2012). Type-I diabetes is a disease where the human body's pancreas is unable to produce insulin, the hormone that regulates blood glucose levels (American

Diabetes Association, 2012). ViaCyte Inc. (San Diego, CA) has developed a method for differentiating ESCs into pancreatic cells *in vitro*, then encapsulating them and implanting them under the skin. According to Eugene Bradon, a director for ViaCyte Inc. “We’re essentially creating a replacement pancreas” (Dance, 2012). The method has worked successfully with hundreds of mice, and they are aiming to begin clinical trials in 2013. The company is aided by a \$26 million grant from the California Institute of Regenerative Medicine (CIRM).

ESCs Versus Adult Stem Cells

ESC Ethical Issues

Few topics in biology have resulted in more ethical debates than human embryo research and embryonic stem cells (ESCs). In 1978, the birth of Louise Joy Brown in Great Britain blurred the lines between life and technology because she was the world’s first “test-tube” baby (BBC News, 1978). Louise had been fertilized *in vitro* (IVF) and developed for two and a half days *in vitro*, then was implanted within her mother’s uterus to develop normally. IVF procedures lead the way for many women who were previously unable to conceive to give birth to healthy children. However, the procedure is not perfect, so extra embryos are fertilized for each couple in case additional implants need to be performed. Once the couple has enough children, the debate focuses on what to do with the surplus embryos: implant them into other recipients, destroy them, or use them for research (including deriving new ESC lines). In 2007, it was estimated that more than 50% of IVF clinics discard the embryos (Robinson, 2007). At its infancy, human ESCs were used in research to study human embryonic development, but today they are most commonly used to derive new ESC lines for treating diseases. But the question remains, in cultivating these ESCs is science saving more lives than it is preventing from being

born? Thus, the ESC debate focuses on the status of a 5-day old blastocyst embryo, and whether it should be created in the first place, used for reproduction only, or also used for research.

Representatives of all major religions have commented on embryo and hESC research, and have individually made conclusions that follow their specific religious doctrines. Moshe Tendler, an Orthodox Judaism scholar proclaims the biblical positions of Judaism, which states that “the biblical tradition does not grant moral status to an embryo before 40 days of gestation,” (Tendler, 2000). Thus, Judaism places *lower* emphasis on a 5-day old embryo than one at 40-days. Laurie Zoloth in her address to the National Bioethics Advisory Commission (NBAC) further explains that in Judaism, “the task of healing is not only permitted, it is mandated” (Zoloth, 2000) which adds weight to the debate if the embryos are used in healing and is done in the best interests of the patients; in Judaism performing healing is considered a moral obligation.

Islamic leaders also favor the use of hESCs for research, as long as “it is not misused for the purpose of human reproductive cloning, which would result in contamination of progeny and the loss of human dignity” (Singapore Bioethics Advisory Committee, 2002). The Qur’an states that there are guidelines to power. Mufti Muhammad, a public speaker and author of many Islamic books, clarifies the Islamist position on life by stating “according to the jurist (fuqaha), the soul (ruh) enters the fetus at around 120 days (4 months) from conception” (Muhammad, 2011). Thus, Islamic leaders support the use of embryos in research, especially if they are not used for reproductive cloning.

Buddhism provides a much less focused forum for ethical issues such as HESC research because there is no singular spokesperson for that religion. However, it is clear that Buddhism prohibits harm to any sentient beings (Sullivan, 2004). But Buddhists also suggests that destroying an embryo for possible healing does not pose a large ethics issue (Hung, 2006). The

embryo in this case of an IVF embryo not implanted into a uterus is said to be “molecular human parts” such that they “are hardly seen as human beings, thus their destruction in the process of research is not likely to be seen as morally wrong” (Changthavorn, 2003; Hung, 2006). Thus, Buddhist opinions vary depending on how harm is viewed with respect to the embryo, and most Buddhists are in favor of embryo research.

In Hinduism, the “killing a fetus is a sinful act”, however, whether the 14 days old fetus is endowed with all qualities of life is not well regarded” (Singapore, 2002). Thus, the Hindu stance focuses on the fact that a blastocyst lacks most of the features of a newborn baby, but is still against its destruction. This uncertainty of life’s development lead to Hinduism’s “no non-acceptance” approach to hESC research in general, but with the potential exception that focuses on the advancement of life by curing diseases through hESC research (Singapore, 2002).

Taoism, although not a largely populated religion, is adamant on the respect and conservation of life. Ancient documents such as the Laojun Jiejing state “All living creatures that breathe, including those that fly, crawl, should not be killed. Even wriggling creatures also treasure living, even mosquitos and other insects understand the avoidance of death” (Singapore, 2002). In a letter addressed to the Bioethics Commission of Singapore Taoism, advocates stated that the religious group is not supportive of research that goes against nature and that involves killing of another life (Singapore, 2002).

The general opinions of most Christian denominations are opposed to hESC research. The reasons are clearest for the Catholic Church which believes that life begins at conception, and that no embryo should be destroyed. The Pontifical Academy of Life states that “on the basis of biological analysis, the living human embryo is from the moment of the union of gametes- a human subject with a well-defined identity, which from the point of conception

begins its own coordinated, continuous and gradual development” (Pontifical Academy for Life, 2000). Such beliefs are derived from biblical passages and other religious documents, such as the Encyclical’s and The Catechism, which coincide with church teachings. The destruction of an embryo is considered to be destruction of human life (Secretariat of Pro-Life Activities, 2009). American bioethicist Donal P. O’Mathuna, argues that “scripture does not state that some humans are persons and others are not”, which counters the belief that embryos must reach “some stage of biological development” in order to be considered living (O’Mathuna, 1999).

Overall, each religion has its own reasons for supporting or denying hESC research, and this is one of the main reasons scientists are constantly seeking replacements for these controversial cells. The other reason for replacing hESCs in therapy is their inconsistent funding, discussed in the next section.

ESC Funding Issues

Due to the ethical issues associated with ESCs discussed above, politicians have enacted policies that reflect their own moral beliefs. These policies affect the federal funding of embryo research, and in some cases prohibit it, which strongly affects researchers in the ESC field. This inconsistent funding, is another main reason scientists are constantly seeking alternatives to ESC research.

Whether to fund ESC research is not an easy decision for any politician to make, especially within heterogeneous societies containing broad views for and against the research. In the U.S. the executive and congressional leaders of our country have the final say whether we fund this type of research. During the time period following the first use of human IVF reproductive procedures in the mid-1970’s, and the resulting dilemma of what to do with the

created surplus embryos, no laws allowed federal money to be spent on embryo research. So, during this period, most IVF research was funded by private sources (Wertz, 2002).

The Clinton administration (1993-2001) was generally in favor of embryo research, and acted on NIH Guidelines from bioethical committees for funding such research (NIH Revitalization Act, 1993). But before Clinton could act on the NIH Guidelines and issue an executive order, congress enacted the Dickey-Wicker amendment prohibiting federal dollars to be spent on embryo research (Kiessling, 2010).

In 2001, the newly elected Bush administration decided against embryo research and in August 2001 enacted a policy prohibiting the derivation of any new ESC lines after that date (Bush, 2001). He did, however, give 250 million dollars to laboratories that research the placental stem cells, umbilical cord stem cells, and adult stem cells (Bush, 2006). Due to the lack of federal funding for embryo research, by 2004 the United States started to fall behind in ESC science, and scientists began to pressure the government (Cook, 2004). In 2005, congress made an attempt to override Bush's veto on stem cell research (HR 810, 109th), but it was not successful (Stem Cell Research Enhancement Act, 2005). Throughout his presidency, George Bush stuck to his original 2001 view on stem cell research (Fletcher, 2007).

During the presidential race of 2008, all the major candidates regardless of their political party supported embryo research (Stolberg, 2007). When Barak Obama was inaugurated in January of 2009, one of the first issues he addressed was embryo research. He asked the NIH to create new guidelines for ESC research (Removing Barriers...2009). These guidelines made embryo research less complex, allowing them to be taken from surplus reproductive IVF embryos with parental consent. According to Raynard Kington, the NIH's acting director, these guidelines are a "significant new step and will lead, in a relatively short period of time, to a

greatly increased number of ESC lines available for federal funding (Wadman, 2009)." Based on the NIH guidelines, Obama in 2009 enacted a law allowing some types of embryo research (Associated Press, 2009). Although most researchers thought Obama's policy was a victory and a vast improvement over Bush's former policy, other scientists wanted Obama to allow embryos to be created solely for research purposes (Wadman, 2009).

Several groups have attempted to reverse Obama's 2009 legislation. U.S. District Judge Royce C. Lamberth ruled in 2010 that Obama's policy was unconstitutional, initially bringing the funding for ESC research facilities to a screeching halt, holding up millions of dollars for this research until it was appealed (Government Will Appeal... 2010). Supporting the 2010 Lamberth ruling, Alliance Defending Freedom Senior Counsel, Steven H. Aden, said "Americans should not be forced to pay for experiments that destroy human life, have produced no real-world treatments, and violate federal law—especially in economic times like these" (Ertelt, 2009). However, in April of 2011, Lambert's ruling was overturned in the Washington D.C. Court of Appeals by a vote of two to one, restoring hope for federal ESC funding (Appeals Court... 2011). In his second term as president, Obama has held strong to his stance on ESC funding to help continue the advancement of this field (Kaiser, 2011).

These examples demonstrate the uncertainty of funding for ESC research. The history of the funding is full of uncertainty based on various administration beliefs. Jack Fowler, Editor of The National Review said it best as "This is one of the topics where science and politics meet to dictate the future for this research" (Fowler, personal communication, 2013). Based on these inconsistent funding issues and the ethical issues discussed previously, scientists are constantly seeking alternative sources of stem cells to replace ESCs.

iPSCs as Potential Replacements for ESCs

iPSC History

To assess the possibility of replacing embryonic stem cells (ESCs) with induced pluripotent cells (iPSCs), one must understand the history of how iPSCs were first developed. Although the practice of doing cell reprogramming has been around for decades with the pioneering work of John Gurdon and others, the discovery of iPSCs in 2006 was a relatively recent application of the cell reprogramming technology.

In 2006, Dr. Shinya Yamanaka and his team of scientists first discovered iPSCs in mice (Takahashi and Yamanaka, 2006). They transfected the genes encoding 4 key transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) into fully differentiated mouse skin fibroblast cells. The four transcription factors were carefully chosen based on their earlier proven roles to help maintain the stemness of ESCs and stimulate cell division. They bind DNA upstream of specific genes to stimulate the expression of genes that maintain pluripotency within the ESCs. According to Yamanaka, they chose this combination of four genes to “induce pluripotency in somatic cells” and that “by combining four selected factors, we were able to generate pluripotent cells, which we call induced pluripotent stem (iPS) cells, directly from mouse embryonic or adult fibroblast cultures” (Takahashi and Yamanaka, 2006).

Yamanaka’s group experimented with about twenty four different genes to determine the ideal combination for the reprogramming. They determined which genes show increased expression in ESCs and would share the same attributes as those that, “play pivotal roles in the maintenance of ES cell identity” (Takahashi and Yamanaka, 2006). Each of the twenty four genes was used with skin/tail grafts of the mice to develop colonies of cells for subsequent experiments. With so many factors used in the experimentation, the list of factors had to be

narrowed down to a smaller combination. To do this, Yamanaka and his team used a virus to deliver various combinations of the genes encoding several factors into the skin and tail cells of mice, which in theory would change the skin/tail cells to an ESC-like state (Watson, 2004). Once they settled on their combination of Oct4, Sox2, Klf4, and c-Myc, the iPSCs were grown and then injected into IVF blastocysts and implanted into the uterus of other mice to produce ‘chimaeric’ mice (Yee, 2010). When the iPSC-derived mice (tagged with a genetic marker) were mated with normal WT mice, they produced normal mice and iPSC-tagged mice, thus proving that the iPSCs developed normally from the original blastocyst (as if they were pluripotent ESCs), and can be passed down genetically (Yee, 2010).

Yamanaka’s team one year later in 2007 repeated a similar reprogramming experiment with *human* skin to produce iPSCs (Takahashi et al., 2007). They reprogrammed fibroblasts from the facial skin of a 36 year old woman and a 69 year old man using human versions of the same 4 transcription factors used in the earlier mouse experiments.

Yamanaka’s team reprogramming efforts appear to produce pluripotent cells without destroying an embryo, so scientists are excited these cells might be used for therapies instead of the more controversial ESCs. Their goal was to develop a new series of cells that would share the same qualities as ESCs without the boundaries set by the use of human embryos. However, as will be discussed in detail in subsequent report sections, like all medical applications, precautions must be taken before these new iPSCs can safely be used in humans. Very recently, a Japanese team received permission to treat age-related macular degeneration (AMD) with iPSCs (Grant, 2013). “Scientists at the Riken Center for Developmental Biology and the Institute of Biomedical Research and Innovation Hospital will take adult skin cells from six AMD patients and reprogram them to into a stem-like state, before injecting them back into the subjects’ retinas

to treat the disorder” (Grant, 2013). Experiments like these will allow scientists to continue to investigate the potential of iPS cells.

Current State of iPSC Programming

The strategies for reprogramming iPSCs continue to evolve as scientists seek to minimize cancer caused by the original c-myc component, and seek to eliminate the use of viruses to deliver the genes in anticipation of the use of the cells in human therapies.

Various delivery systems have been used to insert the reprogramming genes, including lentiviruses (HIV retroviruses), adenoviruses, and plasmid transfection. However, not all of these methods are efficient, and those that insert the genes into the host chromosomes have major concerns. One of the first serious concerns noted when iPSCs were first injected into mice was they sometimes cause cancer. This was thought to result from the inclusion of the c-myc oncogene component which was used to facilitate cell division, or from the use of the retrovirus used to insert the genes. Retroviruses are RNA viruses that use reverse transcriptase to copy their RNA genome and convert it to DNA to insert into the host cell’s DNA. The insertion point with retroviruses is random, so can sometimes insert next to a growth gene and cause tumors. Because of this, new methods for reprogramming cells are constantly being explored. In 2009, a research team developed a method of reprogramming cells without using a viral vector in hopes of eliminating genetic dysfunction (Kaji et al., 2009). This team showed that using a non-viral single plasmid that expresses proteins c-Myc, Klf4, Oct4 and Sox2 can reprogram mouse and human fibroblasts. Even more importantly, the plasmid could be removed after the reprogramming, to remove the added DNA decreasing its chances of later integration. As specified by the standards set by the Mesenchymal and Tissue Stem Cell Committee of the

International Society for Cellular Therapy, the cell lines expanded successfully. Thus, this system successfully eliminated uncontrolled gene expression and efficiently reprogrammed adult somatic cells back to their pluripotent state (Kaji et al., 2009).

A similar approach was used in 2008 by Matthias Stadtfeld and his team when they used adenoviruses to deliver the reprogramming genes. They generated mouse iPSCs through both skin fibroblast cells and liver cells. Using an adenoviral vector, iPS cells were created with consistent, efficient expression and without DNA integration into the host genome using the four factors Oct4, Sox2, c-Myc and Klf4 (Stadtfeld et al., 2008).

Other scientists have developed reprogramming methods that use fewer than the original 4 transcription factors. In 2008, a Japanese team of researchers were able to use genes encoding only two transcription factors, Sox2 and c-Myc, supplemented with exogenously added Oct4 and c-Myc proteins to generate iPSCs from neural stem cells (Kim et al., 2008). They claim that this two-factor approach is compositionally similar to ESCs at the molecular level, but hasten the clinical application of iPS cells more than a four-factor or viral approach. They confirmed the pluripotency of their cells by testing their ability to form embryoid bodies; the bodies contained all three germ layers, and expressed markers appropriate for each layer (Kim et al., 2008).

One popular approach uses a programming method with 3 factors encoded by a retrovirus. Numerous studies, including one completed by Thomas Vierbuchen and his research group, claim that genes *Ascl1*, *Brn2*, and *Myt1l* can induce the generation of mouse neural stem cells expressing multiple neuron-specific proteins, generate action potentials, and even form functional synapses (Vierbuchen et al., 2010). Masato Nakagawa and his research team in Japan completed a similar feat using adult dermal fibroblasts, but omitted the c-Myc gene. They explained that eliminating the use of the c-Myc transcription factor decreased the possibility of

tumor generation, and their results showed that c-Myc-negative iPSCs did not develop tumors during the study period (Nakagawa et al., 2008).

Hou et al. (2013) recently devised a new method for cell reprogramming using small molecule compounds. The scientists screened a library of over 10,000 small molecules (smaller than transcription factor proteins) to identify a mixture capable of reprogramming mouse fibroblast cells into pluripotent cells at a frequency of 0.2%. The small molecules they identified to do the reprogramming included forskolin (FSK), 2-methyl-5-hydroxytryptamine (2-Me-5HT), D4476, VC6T, Prostaglandin E2, and Rolipram. When delivered sequentially, the small molecules induced cell transcriptional profiles equal to those treated with the usual reprogramming transcription factors. The authors called their cells chemically induced pluripotent stem cells (CiPSCs), and argue that because they do not use viruses or genes to do the reprogramming, the cells will be more suitable for human therapies. The CiPSCs also had transcriptional profiles that equaled ESCs.

In general, as Pera explains in his paper titled Low Risk Programming, as iPSCs move into human clinical trials, the best methods of reprogramming adult somatic cells will have the best control of cell division (no cancer induced by an added component), high programming efficiency (to produce the high number of cells required for human therapies), and produce the lowest risk of host oncogene activation (by retroviral insertion) (Pera, 2009). Different stakeholders in the field have varying opinions on the best method to achieve this, and some of these opinions will be sorted out in the project interviews.

Potential iPSC Advantages: Ethics

As discussed above, ESCs have strong ethical disadvantages associated with their use, especially for individuals arguing that life begins at conception and the destruction of a 5-day old IVF blastocyst is murder. Although not everyone holds these views, enough individuals have issues with ESCs to withhold federal funding from their research in the U.S. depending on the political administration, so ethics strongly affects the debate about whether iPSCs hold an advantage over ESCs. The hope of iPSC research is to provide pluripotent cells without destroying an embryo.

Because iPSCs are directly reprogrammed from skin fibroblasts, the problems associated with egg donation can be avoided. This process is currently performed at IVF reproductive clinics as mandated by Obama's 2009 policy in agreement with NIH Guidelines, and cannot be performed solely for research purposes. Reproductive IVF procedures are performed to isolate several eggs, fertilize them, grow them to the blastocyst stage, and then either implant them into the donor or store them for later use. Once the couple has enough children, the surplus embryos are either destroyed, or with the couple's consent can be used for research purposes, including deriving ESC lines. With the use of iPSCs, the surplus IVF embryos would not be needed for deriving new ESC lines, and egg donation would be minimized, a process that is a type of invasive surgery considered painful with considerable health risks.

Due to the ease and inexpensive methods involving iPSC reprogramming, new ethical issues have come to light. The potency of iPS cells is currently being debated (discussed in detail in a subsequent section), but some evidence shows that mouse iPSCs are strongly potent and can form entire mice (Boland et al., 2009). If this can be shown with human iPSCs, a human could be cloned from their own skin cells. Entire countries have already created policies to effectively

stop human cloning (Cohen and Brandhorst, 2008). This particular ethical issue would create far worse complications for the iPS research community than ESCs, because the latter cannot create individuals. In addition, if iPSCs are found to be totipotent and could create a baby upon implantation into the uterus, individuals could have babies far longer than pre-menopause, and DNA damage to the skin cell might create problems for the baby. The far off, but real, possibility to become a parent at any age, using tissue from any age person living or dead creates new problems (Lehrman, 2010). However, most scientists believe that iPSCs are not totipotent, but are pluripotent (discussed below), so more research should be done to clarify this point.

If skin donors are used other than the original patient for providing iPSCs to treat a patient's disease, new ethical issues appear regarding the skin donor. The donor's entire medical history (genetically present in the skin nucleus) would be constantly linked with the patient, so the patient would need to know the entire medical history of the donor and such records might be difficult to keep private (Lehrman, 2010).

Due to the method of reprogramming the skin cell (over-expressing stem-maintaining genes), some scientists argue the ESC-like genes are actually expressed stronger in iPSCs than in ESCs themselves (Yu et al., 2007; GEN, 2011). "Embryonic stem cells all do more or less the same tricks, but some iPS cells express just a few markers of pluripotency, and some express all," so the "resulting differentiated cell types will presumably differ as well" (Cyranoski, 2008).

In addition, some scientists have shown that iPSCs can contain chromosomal damage (Apostolou and Hochedlinger, 2011; Gore et al., 2011; Hayden, 2011; Pera, 2011) and may not be immunologically identical to the skin cell donor (Zhao et al., 2011), so these important issues are discussed below in more detail.

But overall, most scientists are in favor of using iPSCs for therapies. In an interview with Dr. Baylis of the Hastings Center for Bioethics, she stated that “the main obstacles in using iPSC cells as replacement for ES cells are scientific, not ethical (and thus may disappear as the relevant science improves) (Baylis, 2008). Right now it is scientists who are arguing that it is important to proceed with both iPSCs and ESCs for therapies until they know more about similarities and differences” (Baylis, 2008).

Potential iPSC Advantages: Research Funding

Inconsistent federal funding for ESC research is a potential negative for using these cells in research. In the United States, the funding of embryo research in general, and ESC research specifically, has varied considerably over the terms of three presidents, Bill Clinton, George W. Bush, and Barak Obama. Bills promoting embryo research under one President, have been vetoed and removed by his successor, and cause frustrations among ESC researchers, increasing the drive to find replacements for ESCs.

President Clinton created the National Institutes of Health Revitalization Act of 1993 (NIH Revitalization Act...1993), which gave him and Congress the authority to direct federal funding to human embryo research. Many scientists argued this would significantly improve their ESC research. After the declaration of the NIH Revitalization Act, a panel of experts in ethics, science, and public relations was formed to oversee embryo research. Their main jobs were to determine where the federal funds would go, while maintaining proper moral and ethical conduct. The panel of experts deemed that any embryos used for research must use excess embryos prepared for reproductive in vitro fertilization (IVF). With the embryo source identified, and with the promise of heavy federal funding and the assembled panel, the future

possibilities for embryo research seemed endless. However, these aspirations were short lived when the Republican-controlled Congress led by Republican Representatives Jay Dickey and Roger Wicker in 1996 passed the Dickey-Wicker Amendment (Genetics and Public Policy Center, 2011) whose SEC 509 states that:

- (a) None of the funds made available in this Act may be used for--
 - (1) the creation of a human embryo or embryos for research purposes; or
 - (2) research in which a human embryo or embryos are destroyed, discarded, or knowingly subjected to risk of injury or death greater than that allowed for research on fetuses in utero under 45 CFR 46.204(b) and section 498(b) of the Public Health Service Act (42 U.S.C. 289g(b)).

- (b) For purposes of this section, the term `human embryo or embryos' includes any organism, not protected as a human subject under 45 CFR 46 as of the date of the enactment of this Act, that is derived by fertilization, parthenogenesis, cloning, or any other means from one or more human gametes or human diploid cells. (Kearl, 2010)

The Dickey-Wicker amendment effectively stopped all federal funding for ES cell research, leaving private donations as the only way to fund this research.

In 1999, following the first isolation and growth of human ESCs by James Thompson, Harriet Rabb, Thompson's private funder, found a loophole within the Dickey-Wicker Amendment, that ESCs are not embryos as defined by Dickey-Wicker (Rabb, 1999; Dunn 2005). This letter gave the Clinton administration a potential opening for receiving federal funding for ESC research. But before such funding could be initiated, in 2001 George W. Bush was elected into office.

Soon after taking office, Bush ordered a review of Rabb's legal decision. Then, informed the NIH to stop reviewing ESC grant applications. These actions "saddened, angered, and frustrated supporters of human embryonic stem cell research" (Dunn, 2005). On August 9th, 2001, President George W. Bush announced that federal funds may be awarded for research using human embryonic stem cells only if the following three criteria are met:

1) The derivation process (which begins with the destruction of the embryo) was initiated prior to 9:00 P.M. EDT on August 9, 2001. 2) The stem cells must have been derived from an embryo that was created for reproductive purposes only, and was no longer needed. 3) Informed consent must have been obtained for the donation of the embryo, and that donation must not have involved financial inducements.” (NIH.gov, 2001).

Two days after his August 9th executive decision, George W. Bush made a radio address effectively bashing all types of ESC research calling it “further destruction of human embryos that have at least the potential for life” (Bush, 2001). President George W. Bush within eight months of being in office had effectively stopped all federal funding for ES cells and reduced the number of available qualified ES cell lines to those derived prior to 2001. The only testing lines remaining available for research were the sixty lines he had agreed to, however these so called sixty lines were actually far less, as scientists later proved that many were actually duplicates or were defective for research (Holden and Vogel, 2002).

Later attempts to override the Bush 2001 mandate were made by NIH in 2005 and 2007 with their Stem Cell Research Enhancement Acts, but Bush vetoed each of them (Stem Cell Research Enhancement Act, 2005, 2007). President Bush vetoed the bill, stating: “scientific advances now allow researchers to pursue the potentially lifesaving work without destroying human embryos” (Fletcher, 2007). With this veto however he was becoming an enemy to Congress, who then made sure that any further stem cell enhancement bills were given to the next administration.

In 2009, Obama was inaugurated President, and immediately set out to overturn Bush’s policies. He asked the NIH to form new guidelines for embryo research, and then made an executive decision to adopt their guidelines (Hayden, 2009). The new policy allows embryo research using surplus reproductive IVF embryos with donor consent, and open up far more ESC lines than under Bush. But regardless of the new funding allowed by Obama, political tides

change, and this funding may disappear when another republican is elected president. So, this inconsistent federal funding for ESC research drives the need to use other types of cells for research.

Potential iPSC Disadvantages: Potency

If iPSCs are to replace ESCs in therapies, they must be as pluripotent as ESCs, or at least they need to create the necessary tissues to treat a specific disease with the same efficiency as ESCs. But currently, labs do not agree on the potency levels of iPSCs. In fact, the potency may vary depending on the reprogramming methods used by a particular lab.

Embryonic stem cells (ESCs) have a number of advantages for regenerative medicine, including their ease of isolation and strong growth, but perhaps their best advantage is their pluripotency (Amit et al., 2000). As potential replacements for ESCs, induced pluripotent stem cells (iPSCs) must also be potent and be able to produce large numbers of cells for therapies. Currently, this is not the case for most research labs. The number of fibroblast cells reprogrammed in a typical the experiment are a fraction of a one percent, only one out of every ten thousand cells becomes a functioning stem cell (Yamanaka, 2012). The bright side of this reprogramming process is it can be applied to almost any differentiated cell in the body to potentially produce a stem cell (Yamanaka, 2012).

As discussed previously, when he first developed iPSC technology with skin fibroblast cells, Dr. Shinya Yamanaka inspected thirty different potential reprogramming genes, narrowing the number down to a combination of four transcription factors: Oct3/4, Sox2, c-Myc and Klf4 (Induced... 2011). But the viral method of delivering the genes was inefficient, so most of the skin fibroblast cells did not turn into iPS cells (Trounson, 2012). The genetic factor Myc also

caused a tumor to grow in one of the mice (Yee, 2010). Due to the programming inefficiency, cancer hazards, and cost they tried to skip a step omitting one of the genes, Myc, but this led to even fewer somatic cells growing into iPSCs (Hayden, 2011). So, iPSCs programmed with c-Myc are often used for drug screening and validation studies, but must be used with extreme caution in living organisms (Yee, 2010).

As discussed previously, *potency* is the ability of a stem cell to form other cells. The higher the potency, the larger the number of cell types formed. ESCs are considered pluripotent because they can form all the cells of the adult organism, but are not totipotent and cannot form placenta. Cells are considered pluripotent if they are able to form all 3 main germ layers (endoderm, mesoderm, and ectoderm). Several studies have indicated that iPSCs are pluripotent, as the reprogrammed cells were able to form teratomas containing all three germ layers (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Aoi et al., 2008; Kim et al., 2008; Boland et al., 2009). In these studies, evidence for the formation of all three germ layers was obtained by staining for marker proteins specific for each germ layer, or for allowing the cells to differentiate into more specialized tissues (i.e. fat cells, muscle cells, etc.) and then staining those cells for mature markers.

One way scientists have learned to manipulate potency is by changing the time and place of the cell reprogramming. For example, Aoi et al. (2008) created pluripotent cells from mouse liver cells using an albumin promoter to switch on the expression of the reprogramming factors. Intrinsic variability is another factor that affects potency. Intrinsic variability is the random difference in reprogrammed cells compared to those in the natural habitat (Yee, 2010). The teratoma tumors sometimes seen with reprogramming protocols including c-Myc are an example, as some times the tumors formed, and sometimes they did not. During therapy protocols, this

variability must be controlled by assaying each batch for tumor formation prior to infusing into a patient (Yee, 2010).

Also, experiments have been performed to make the iPSC cells more pluripotent. A 2007 study used Nanog instead of Fbx15, and found that Nanog produced a larger percentage of cells with pluripotency, and most the cells integrated into the host without mutated genes (Okita et al., 2007). However, the created iPSCs still did not have the same high integration rate as ESCs (Okita et al., 2007).

Scientists have also examined the relationship between “secondary neurospheres” (SNS) and iPSC potency. A neurosphere is a cluster of free forming neural stem cells derived *in vitro* (Reynolds and Weiss, 2002). The scientists tested how various types of iPSCs turned into neurospheres that could be used to treat neural problems. The results indicated that iPSCs originating from tail-tip fibroblasts had the highest SNS formation success rate, followed in order by mouse embryonic fibroblasts and gastric epithelial cells (Miura et al., 2009).

Scientists continue to investigate the potency of iPSCs, developing procedures for maintaining pluripotency while minimizing the formation of tumors. Cell reprogramming has come a very long ways since the early 1950’s experiments by John Gurdon with frog tadpoles, which showed that skin cell nuclei could be reprogrammed by injection into enucleated eggs, and reprogramming research has a long ways to go. Further research with iPSCs should help to fully unlock the potential of these cells to improve patient’s lives.

Potential iPSC Disadvantages: Cancer

From the very beginning of the iPSC discovery (Takahashi and Yamanaka, 2006) cancer has sometimes been observed at the injection site. The first tumor formations observed were

teratomas which actually serve as a potency marker because teratomas contain a variety of tissues within the three main germ layers (endoderm, ectoderm, mesoderm), which proved that those particular iPSCs could successfully differentiate into all the main tissues so were pluripotent (Takahashi and Yamanaka, 2006). But the findings set major shortcomings for the research in terms of therapies because cancer formation would only further obstruct a patient's health.

One of the main causes of tumor formation is currently believed to be the c-Myc component of the original reprogramming mix of four transcription factors. c-Myc is a well characterized oncogene (Cartwright et al., 2005), and was added to help the reprogrammed skin fibroblast cells proliferate better. The other three factors were added due to their known functions in helping embryonic stem cells (ESCs) maintain their stem-like properties; those factors were (as discussed in the iPSC history section) Oct3/4 (Nichols et al., 1998), Sox2 (Avilion et al., 2003), and Klf4 (Li et al., 2005). However, the progeny developed tumors directly related the expression of the c-Myc oncogene (Miura et al., 2009), so (as discussed in the current iPSC reprogramming section) subsequent protocols were developed to omit the c-Myc component. Kim et al. (2008) showed that pluripotent cells could be created using only Oct-4 and Klf-4, without causing tumor formation in the offspring. In this experiment, the authors generated an 8-cell mouse IVF embryo, implanted it into a host uterus, and followed the tissues formed. The iPS cells reprogrammed with only Oct-4 and Klf-4 were very potent and did not appear to affect the regular development of the mouse embryo. The genetically distinct donor cells created a chimera with the host cells, further indicating their strong normal potency.

Similarly, Nakagawa et al. (2008) showed that using three factors (Oct3/4, Sox2, and Klf4) did not produce tumors in mice, although the proliferation of the cells was reduced. In this

study, tumor formation was ruled out when the generated mouse donor-host chimeras survived 100 days after birth with no development of teratomas. So, these studies show that the incidence of cancer can be reduced by omitting the c-Myc factor in the reprogramming mix.

Studies where iPSCs have been used to treat mouse disease models also serve as an excellent method for monitoring potential tumor formation. Hanna et al. (2007), a key landmark paper in iPS research, treated a mouse model of sickle cell anemia and did not see any tumor formation. What is most remarkable about this study is that all four of the original reprogramming factors were utilized in the study, including c-Myc. Importantly, the authors used a pre-screening process to help ensure that no cells used for therapy were cancerous. The original reprogrammed cells were skin fibroblast cells, and all of the selected reprogrammed cells expressed ESC markers AP, SSEA1, and Nanog (Hanna et al. 2007). The iPSCs used for therapy were karyotyped to eliminate any cells with obvious chromosomal damage, and were infected with an adenovirus encoding the Cre-recombinase gene to remove the floxed c-Myc gene included as part of the original programming. So the c-Myc gene included in the original reprogramming mix to help ensure growth of the reprogrammed cells was removed from the iPSCs prior to therapy. To treat the sickle cell anemia, the screened iPS cells were treated with the normal wild-type globin gene by electroporation (Hanna et al., 2007). Morphological analysis of the treated mice at 12 weeks post-therapy showed few or no sickled cells. These findings serve as a best practice as to how c-Myc can be included to improve iPSC proliferation, without subsequently contributing to cancer formation. However, Hanna et al. (2007) also suggest that further studies must be done to move to clinical trials.

In addition to these findings, other scientists argue that the original use of retroviruses (lentiviruses) to efficiently deliver the reprogramming genes into the skin fibroblast cells

occasionally caused tumor formation by randomly integrating in the host genome near an oncogene site to activate it. As the reprogramming technology evolved, scientists used adenoviruses to deliver the genes or directly delivered the transcription factor proteins themselves instead of using viruses for delivery (Baker, 2007; Check and Baker, 2009; Stadtfeld et al., 2008; Kaji et al., 2009; Yu et al., 2009).

DNA damage has also been linked to tumor formation in iPSCs. In particular, scientists have identified a link between damage to the gene encoding the p53 tumor suppressor and the proliferation of competent reprogrammed iPS cells. DNA damage mediated by a faulty p53 is frequently shown to cause tumors (Marion et al., 2009). The normal function of p53 in the DNA repair pathway is to halt the replication of cells with damaged DNA until the repair pathway proteins fix the damage. Without a functional p53 to help ensure the reprogrammed cells have intact genomes, the DNA damage accumulates until tumors form. Thus, a much greater effort is currently being made to ensure p53 is functional before cells are used for therapies.

Gore et al. (2011) studied DNA damage under various reprogramming techniques and showed that on average each batch contained at least five point mutations located within their sampled regions. About half of those mutations existed in the original fibroblast cell, while the other half was derived from the reprogramming process. Further analysis of the iPSC reprogrammed mutations showed they were especially enriched in cancer causing genes, so these specific mutations could have been amplified in the colony picking and cell growth processes.

A related study was conducted in the same year by Hussein et al. (2011) in which gene copy number variations (CNV) were identified in iPSCs after reprogramming. The CNV's were found to be two-fold higher in iPSCs compared to ESCs or differentiated fibroblast cells.

Other studies have identified changes in DNA epigenetic reprogramming in iPSCs, such as alterations in DNA methylation patterns (Lister et al., 2011). Epigenetic changes are expected in any reprogramming protocol, but they can also lead to cancer if not monitored closely. So, iPSCs should be screened extensively before being used in regenerative research.

The tumor formation problem is not restricted to iPSCs. It is far worse with ESCs that frequently form teratomas at the injection site, so the tumor formation problem is not specific to iPSCs. Tumor-like cells have been observed in ESCs in which the oncogene Ras (ERas) is increased in expression and causes oncogenic transformations (Takahashi et al., 2003).

Altogether, these studies suggest that both iPSCs and ESCs have the potential to create tumors in the patient, so these cells should be assayed for known oncogene expression and p53 anti-tumor activity prior to use in patients.

Potential iPSC Disadvantages: Immune Rejection

In theory, iPSCs should not be rejected by the host if they are transplanted back into the same patient providing the skin cells that were reprogrammed. It was just assumed that autologous cells transplanted back into the same patient would not be rejected, similar to autologous bone marrow stem cell transplants into the same patient. Most scientists argued the DNA in the donated fibroblast cells would encode histocompatibility proteins that would be viewed as self by the patient. If so, this would provide a strong incentive for performing therapies with iPSCs instead of ESCs. Any immunological complications for implanted stem cells would strongly diminish engraftment. This section reviews the studies that analyzed iPSC immune rejection.

In 2011, Dr. Yang Xu and his team explored the possibility of immune rejection of iPSCs in B6 mice. Their findings showed that iPSCs were not as effective as ESCs at surviving and differentiating into new cells iPS cells (Zhao et al., 2011). This was the case whether the cells were reprogrammed by viruses or by plasmids. It is interesting to note that when Dr. Yamanaka and his team performed the initial iPSC experiments in mice in 2006 (Takahashi and Yamanaka, 2006), he used immune-deficient mice so that the chances of immune rejection would be very small (Deng, 2010). Thus, Yamanaka's initial studies did not address the potential immune rejection issue.

However, other researchers argue that the immune rejection of iPS cells is of little concern and has a small chance of happening. Recent experiments in 2013 suggest that iPSCs were not rejected by the host. Araki et al. (2013) showed that with C57 mice there were no differences in the transplant success when comparing reprogrammed skin cells, bone marrow cells, and ESCs. The authors observed limited no immune responses, including no T-cell infiltration, and observed no increase in genes related to immune responses. So, this 2013 study with C57 mice provides evidence that immune responses may be negligible with iPSC therapies (Baker, 2013). In addition, new studies suggest "...that the reprogramming process causes fewer mutations than previously thought" (Baker 2013) which would support the use of iPSCs for therapies. However, Yang Xu still has his concerns, stating that "...the new work does not dispel all concerns about the immune response provoked by iPS cells" because the cells used for therapy were derived by reprogramming cells from chimeric mouse embryos instead of directly culturing the iPSCs (Baker, 2013).

Regardless of how the immunogenicity of iPSCs turns out with future research, one must not forget that ESCs when used in therapies have shown strong signs of immunity (Yee, 2010).

Thus, one must consider the fact that iPSC technology will eventually create cells with less immune problems than ESCs.

METHODS

The specific objectives of this project were to **1) document** and evaluate the ethical and funding concerns associated with the use of ES cells and how research scientists may need replacements for these cells in therapy. **2) Develop** a comprehensive assessment of the origins, inducing technology, and current uses of iPS cells. **3) Characterize** what key stakeholders believe are potential problems associated with using iPS cells for therapy and their suggestions for potential solutions. **4) Evaluate** the problems and risks associated with the use of iPS cells. **5) Recommend** potential solutions to remaining problems associated with iPS cell usage.

To accomplish **objectives 1 and 2**, we performed an extensive review of the current research literature, including reputable academic journal articles, relevant books, scholarly websites, and other pertinent materials. Objective-1 was also supplemented with interview material.

To accomplish **objective-3** (and supplement objective-1), we conducted an extensive set of semi-structured, in-depth interviews with various stakeholders in the iPS and ES fields to determine their range of opinions on problems associated with the use of ES cells, and potential solutions to the use of iPS cells for therapies. The stakeholders included academic experts on iPS cells and ES cells. The interviewees also included academic ethicists to help address issues associated with iPS cells. Some of the stakeholders were initially identified by referral from the project advisor, Dave Adams, but the majority of stakeholders were identified from the academic and related literature, and by referral from the initial interviewees (to develop a referral “snowball”).

Whenever possible, interviews were conducted in person, but the majority of interviews were by email. Interviewees were initially contacted by email and/or phone. In cases receiving no response, we followed up with a second email or phone call one week later. Interview questions were developed based on our background research. A preliminary set of questions is shown in the report Appendix. We tailored the questions asked according to the expertise and initial responses of the interviewee. The questions asked evolved based on what the interviewee said, so the exact questions were adjusted on the spot based on the direction of the emails or conversations.

With respect to the method of the interview, when interviews were in person, whenever possible two team members were involved, so that one member could ask questions while the other member wrote detailed notes, and vice versa. For email, phone, or in-person interviews, we informed the interviewee about the purpose of the project, and asked for permission to quote them (see draft interview preamble in the Appendix). If required to protect proprietary interests, we gave them the right to review any quotations used in the final published report, explaining that the interview is voluntary, and explaining that the interviewee may stop the interview at any time or refuse to answer any question. After the interview, we asked each interviewee for permission to follow-up with them at a later date if needed to fill in any gaps in the information. And, as mentioned above, asked them to recommend other potential stakeholders we might interview, to further increase our number of interviews.

We continued our interviews until we had sufficient information to represent all stakeholder positions, when sufficient information had been obtained to represent all sides of the problem, and when all unclear points had been clarified.

To accomplish **objectives-4 and 5**, the group synthesized all of the information collected in the literature research, interviews, and follow-up interviews to create recommendations and alternative solutions for replacing ES cells with iPS cells for therapies.

RESULTS/FINDINGS

The discovery of induced pluripotent stem cells (iPSCs) by Yamanaka's group in 2006 (Takahashi and Yamanaka, 2006) introduced to the world the idea that there might be more alternatives to cell therapeutic stem cell practices than extracting embryonic stem cells (ESCs) from embryos. Based on the pioneering work of John Gurdon in the 1950's, who showed that skin fibroblast nuclei can be reprogrammed by microinjecting them into oocytes, Yamanaka's experiments showed that skin fibroblasts can be reprogrammed to a pluripotent state by transfecting genes encoding key transcription factors inside the cells. The cells were capable of growth, and could differentiate into all three germ layers, so were thought to be pluripotent. If such cells could be prepared from a patient's skin cells, his/her iPSCs could be used to generate new tissue to treat the disease. And if iPSCs prove to be truly pluripotent, perhaps they could be used instead of ESCs without destroying an embryo.

ESCs are isolated from the inner cell mass of a 5-day old blastocyst embryo prepared by *in vitro* fertilization (IVF) at a fertility clinic. Isolation of the cells destroys the embryo. Because the embryo represents potential life if implanted into a uterus, those individuals who believe that life begins at conception, or who believe in minimizing harm to any life, are against the use of ESCs. Thus, embryonic stem cells have been the subject of much debate, due to the potential development of an embryo into a human fetus. Most scientists argue that iPSCs have few ethical concerns relative to ESCs. For example, in a 2008 interview with Dr. Francois Baylis of the Hastings Center for Bioethics, she stated that "the main obstacles in using iPS cells as replacement for ES cells are *scientific*, not ethical (and thus may disappear as the relevant

science improves) (Baylis, 2008). So, this project focused on some of the main scientific reasons that may need to be solved prior to using iPSCs in human therapies (discussed below).

ESCs also have received highly *inconsistent* support from the federal government over the past 20 years. In the U.S., the amount of federal funding for ESC research has coincided with the change in political parties over the last three presidencies. President Clinton created the National Institutes of Health Revitalization Act of 1993, which gave him and Congress the authority to direct federal funding to human embryo research (NIH Revitalization Act, 1993). However, before President Clinton could implement his executive order, in 1996 Congress enacted the Dickey-Wicker amendment banning federal funding for embryo research (Rabb, 1999; Dunn 2005). In 2001, newly elected President George W. Bush within eight months of being in office modified the federal ban to restrict federal funding to only those ESC lines derived prior to August, 2001. This 2001 act significantly reduced the number of ESC lines available to researchers; the only testing lines remaining available for research were approximately sixty lines agreed upon to meet Bush's restrictions. In reality, the number of ESC lines was far less because scientists later showed that many of these cell lines were actually duplicates or defective for research (Holden and Vogel, 2002). In January of 2009 when Barack Obama was inaugurated into office as president, he immediately set out to overturn Bush's restrictive policies on ESC research, asking the NIH to form new guidelines for embryo research. He then made an executive decision to adopt their guidelines (Hayden, 2009). Obama's new policy allows embryo research using surplus reproductive IVF embryos with donor consent, and open up far more ESC lines than allowed under Bush. The funding that was initially intended by President Clinton was revoked by the 1996 Dickey-Wicker amendment, was restricted to ESC lines derived prior to 2001 by President Bush was reinstated by President Obama. So, although

ESC research currently receives federal funding under President Obama, history has shown that this funding can be revoked in a very short period of time. This leaves many researchers seeking alternative sources of stem cells for their research, including iPSCs.

In an interview with Maria Borowski, former Relations Director at the University of Massachusetts Medical School Stem Cell Bank (Worcester), she stated that the Stem Cell Bank was closed due to a lack of funding (personal communication, 7-30-13). She also believes there are two main reasons stem cell research is hard to obtain funding for: 1) many politicians do not understand that not all stem cells destroy embryos, and 2) stem cell research is relatively new, so the successes are not well documented yet.

Also with respect to funding, in an interview performed with Nancy Kavanaugh of Cell Signaling Technologies, she pointed out that economic concerns nationwide impact the funding that all stem cell research receives (whether for ESCs or iPSCs) (Kavanaugh, personal communication, 7-30-13). Although this global funding statement is true, due to ESC ethical issues their funding situation is still more inconsistent.

With respect to using iPSCs in therapy, unfortunately iPSCs may have several problems that need to be solved before they can be used in patients. Our research of the scientific literature pointed to three main problems that may hinder iPSCs from replacing ESCs: 1) iPSCs may not be as *potent* as ESCs, 2) iPSCs may not necessarily be *immunologically compatible* with the person providing the skin cells for reprogramming, and 3) iPSCs may cause *cancer* at the injection site. Each of these findings is discussed below.

Potential Problems with iPSC Potency

One of the main reasons many scientists work with ESCs is their *pluripotency*. Truly pluripotent cells have the ability to form any tissue in the adult body. So, if scientists are to replace ESCs with iPSCs, the latter perhaps should be pluripotent. Potency is measured by the ability of the stem cell to create other types of cells. The higher the potency, the larger the types of cells formed. The original four reprogramming genes used in Yamanaka's reprogramming experiments were deemed by that group to be pluripotent based on their ability to form teratomas at the injection site. Teratomas are a type of tumor comprised of the three main germ layers (endoderm, mesoderm, ectoderm) which together can form all tissues in the body. So, the presence of teratomas indicated the reprogrammed iPSCs were pluripotent. As discussed below under current reprogramming methods, subsequent experiments showed that the teratomas likely formed due to the presence of an oncogene (c-Myc) present in the original mix of four reprogramming genes, so today most scientists eliminate this factor when the cells are to be injected *in vivo* (even though this slows cell division), and cells reprogrammed with with c-Myc are often used for drug screening or validation studies (Yee, 2010).

Other scientists to measure iPSC potency injected their reprogrammed iPSCs directly into the cavity of a mouse blastocyst IVF embryo, implanted the embryo into a foster mother uterus, and observed the extent of tissue formation as a measure of potency. For example, Boland et al. (2009) showed that the injected cells were able to help form an entire adult mouse, indicating they were pluripotent. No tumors were observed in the mouse derived from the blastocyst, so not all programming methods observe cancer. Future research should focus on why.

Our findings indicate that cell potency is not homogenous and may actually vary between cells in a given batch due to "intrinsic variability", differences that exist between reprogrammed

cells perhaps due to random DNA damage that occurs during the reprogramming (discussed below in the cancer section). Our findings also indicate that cell potency can be varied by altering the *timing* of addition and the *type* of reprogramming factors added (discussed below in the current reprogramming section). The timing and type of factors added also affect cell engraftment success which relates to therapy, which scientists do not fully understand yet and has not been fully utilized in applications. Some scientists have shown that altering the types of reprogramming factors can affect the percent of pluripotent cells in a specific batch of cells. For example, Okita et al. (2007) showed that using Nanog instead of Fbx15 produced a larger percentage of pluripotent cells. So, this type of potency research should be expanded further.

Potential Problems with iPSC Immunogenicity

One of the main assumptions of iPSC research is that the cells reprogrammed from the skin cell will be immunologically compatible with the patient who donated the skin cell. The therapy would be *autologous* and comparable to a patient providing his own bone marrow cells for therapy. If this proves to be true, it would provide a strong incentive for pursuing iPSC therapies instead of ESC therapies; in the latter the embryo donated for research certainly will be immunologically different than the patient.

Yamanaka's original reprogrammed cells were injected into immuno-deficient mice, so those early experiments were not able to address whether the cells were immunologically compatible with the host. However, a subsequent study was done in Yang Xu's lab at the University of California, San Diego (Zhao et al., 2011) who examined iPS cell immunogenicity in C57 mice, and showed the injected cells were rapidly rejected by the host. They hypothesized that the cells were rejected because the C57BL/6 mice (donor and recipient) were not genetically identical. Zhao was quoted as saying "the immunogenicity of therapeutically valuable cells

derived from patient specific iPSCs should be further evaluated before any clinical application of these autologous cells into the patients” (Zhao et al., 2011). He did not advise against using iPSC cells entirely, but believes the risks of using the cells should be minimized to the fullest extent before any clinical use.

The 2011 study was followed by a more thorough study in Masumi Abe’s lab at the National Institute of Radiological Sciences in Japan (Araki et al., 2013) who also focused on immunogenicity in C57BL/6 mice, but went beyond the 2011 study by actually measuring immune responses. Their data showed no differences in transplantation success (no differences in immune rejection) when iPSCs were compared to ESCs, and no unusual immune responses were observed, including no T-cell infiltration. In addition, Hanna et al. (2007) used iPSCs to treat a mouse model for sickle cell anemia. The injected cells established normoglycemia in the model indicating little immune rejection. So, based on the relatively small amount of literature focused on immunogenicity, it appears that some scientists observe a problem while others do not, so more research should be investigated into which systems show the worst immune-rejection and why. One must examine each case separately, making sure to fully assay for T-cell proliferation, the most common mechanism of injected cell death.

Potential Problems with iPSC Cancer

One of the most important problems hindering the use of iPSCs in therapy is their ability to form cancer at the injection site in some experiments. As mentioned above, teratomas were observed in the earliest iPSC experiments, likely due to the presence of the c-Myc gene used in the reprogramming mix. In addition, when the early experiments used retroviruses to deliver the reprogramming genes, cancer was also thought to result from the random integration of the viral

genome into the host genome. So, subsequent iPSC experiments focused on leaving out c-Myc from the mix (although this decreases cell growth), delivering the genes using non-integrating viruses or plasmids, delivering transcription factor proteins instead of genes, or using small molecule drugs to mimic the transcription factor proteins. These advances hopefully will decrease cancer formation in iPSC lines used for therapies.

Like many researchers, Tanja Dominko, associate professor of Biology at Worcester Polytechnic Institute, agrees that iPSC research was a “very important discovery” but the reprogramming process itself makes her nervous. She stated in an interview that iPSCs are “unpredictable, uncontrollable” and therefore “do not have a therapeutic future” (personal communication, 6-25-13). Similar to the current state-of-the-art iPSC reprogramming efforts that avoid the use of reprogramming genes or that use small molecules for the reprogramming (discussed below), Professor Dominko’s research on induced regeneration competent (iRC) cells focuses on a more natural approach than gene delivery to do the programming. In her approach, skin fibroblast cells are cultured through environmental manipulation (hypoxia and FGF-2 addition) which “prevents DNA damage and chromosomal damage” and tumor formation. The iRC cells do not seem to form tumors, a distinct advantage similar to the most recent iPSC experiments using no genes. However, the natural reprogramming process also prevents the iRC cells from achieving true pluripotency. Although the iRC cells do not appear to be pluripotent, they were successful enough to regenerate muscle cells in a mouse model of muscle damage (cite Tanja’s paper with George Pins showing the use of iRC cells in muscle damage models). So, Professor Dominko questions whether “we really need pluripotent cells for a specific regeneration.” Although very compelling research, the apparent limited potential of iRC cells

might limit which types of therapies they can be used for, especially when treating patients needing multiple types of tissues formed.

With respect to iPSCs, some lab groups do not always see cancer even when using c-Myc as part of the reprogramming mix. Dr. Fen-Biao Gao, a principal investigator at the University of Massachusetts Medical School, utilizes iPSCs to study the molecular pathways of Frontotemporal Dementia (FTD) patients. Dr. Gao's lab uses four transcription factors for reprogramming (SOX2, OCT4, c-MYC and KLF4). He sometimes "sees tumors when the iPSCs are injected into immune-compromised mice" (personal communication, 6-27-13). The teratomas show that "the cells are pluripotent". When asked why c-Myc was used knowing that it can cause tumors, Sandra Almeida, a researcher in the Fen-Bio Lab, stated that they can "reprogram cells without c-Myc, but the process is not as efficient, meaning we...get fewer iPSC colonies" (personal communication, 6-27-13). Thus, the c-Myc is initially used in their lab to optimize cell growth. But importantly, this does not mean the cells cannot be used for therapy. After expanding their iPSCs, they routinely assay the karyotype of the cells to make sure the chromosomes look normal. "Chromosomal abnormalities are also more frequent than in other cell types (and similar to ES cells) because these cells are highly metabolically active, so for this reason the iPSCs are frequently karyotyped". In addition, they only use cells for therapy in which all four reprogramming genes have become silenced (including c-Myc). They are concerned however that the c-Myc might become reactivated, so they are now using reprogramming techniques where the 4 factors do not integrate. The selection process can take months, but is worth the time. In regards to iPSCs potentially replacing ESCs, Almeida states that "there is still a ways to go before we can use either of these cells safely in therapies for neurological diseases, but every day we get a little bit closer", and also to us "iPSCs and ESCs

are equally useful and important in research”. However, in therapies, we must also take into consideration the ethical and funding issues of ESCs. If both cell types are equally useful, ultimately iPSC would be more practical considering the funding and ethical uncertainties associated with ESC.

Hanna et al. (2007) also demonstrate how careful regulation and selection of their iPSCs post-programming allows their use in therapy even when using all four original transcription factors. They avoid tumors by karyotyping their cells to avoid cells with clear DNA damage, by eliminating the c-Myc gene using Cre-recombinase (to remove a floxed c-Myc gene), or by avoiding its use altogether. Their iPSCs are pluripotent enough to provide a long term production of normal blood cells months after injection, with no presentation of teratomas. Further research should be done testing the potency of iPSCs programmed with only two or three factors, or using techniques that do not integrate the genes.

Our findings also indicate that iPSC tumors can result from DNA damage or alterations caused by the reprogramming process itself. The DNA damage ranges from point mutations (Gore et al., 2011) to copy number variations (Hussein et al., 2011) to alterations in epigenetic modifications (such as DNA methylation) (Lister et al., 2011). The DNA mutations often occurred in growth regulating genes (likely selected for in the cell expansion process) and in tumor suppressor genes (such as p53). Nonetheless, in best-practice protocols, a rigorous screening of the cells (including karyotyping to look for gross alterations, and growth curves to avoid those with uncontrolled growth patterns) helps to avoid those cells with the potential to form tumors. Unfortunately, the cell assays can be time consuming. Dr. Gao’s lab at the University of Massachusetts Medical School indicated that they routinely karyotype all their iPSC batches to select against those with obvious DNA damage (Gao, personal communication,

6-27-13). Hanna et al. (2007) also used karyotyping to screen their iPSCs prior to use in therapy to treat animal models of sickle cell anemia.

What is echoed continuously throughout all the iPSC and ESC research articles and interviews is that more work still needs to be done prior to reliable clinical use for either type of stem cell. Most current evidence shows that iPSCs not only morphologically look like ESCs but also function similarly. Both iPSC and ESC display tumor formation at the injection site if not properly screened prior to injection. It is interesting to note that although human ESCs were first grown in 1998 (Thompson et al., 1998) no clinical trials have concluded with these cells (although one or two are currently underway), while iPSCs were discovered eight years later in 2006 and have already produced more therapy evidence than ESCs. There is no doubt that scientifically both stem cell types are useful, but when presenting both chromosomal problems and tumor formation, ultimately the most practical and easily obtainable alternative should be used.

Current State of iPSC Reprogramming as Related to iPSC Potential Problems

Our findings indicate that the method used to reprogram iPSCs dictates their subsequent properties, including their potency and their ability to cause cancer. So, the topic of how to improve programming remains a major focal point of iPSC research. Each method has problems and benefits. The main programming challenge is to maintain pluripotency while minimizing cancer. The c-Myc component of the programming mix is added to facilitate growth of the iPSCs, but the presence of this oncogene tends to cause cancer. Cancer is also caused by the random integration of retroviruses sometimes used to reprogram the cells. So, scientists have developed protocols (summarized in the Lit Review) for 1) eliminating c-Myc from the mix, 2) using non-integrating plasmids or adenoviruses to deliver the genes, 3) delivering proteins

instead of genes, or most recently 4) using small molecules to mimic the addition of transcription factor proteins (Hou et al., 2013).

In general, the consensus among leading scientists is that the absence of viruses will allow a more controlled, targeted integration and thus a more stable and predictable reprogramming of cells. But in some cases, viruses remain the most efficient method of gene introduction. In an interview on August 15th, Destin Heilman of WPI stated that he personally uses viral integration for his stem cell lines. He prefers non-lentiviral (non-integrating) vectors because they are commercialized and relatively inexpensive compared to lentiviruses. In addition, the type of research Heilman is conducting does not require stable, focused gene integration; his cell lines are sacrificed in 48 hours, so a completely stable and focused integration does not matter (Heilman, personal communication, 8-15-13). However, he states that he would not recommend using viral vectors for integration; there are other non-viral methods to use when preparing cells for therapy.

Laurie Boyer, an associate professor of Biology at MIT, is also against using viruses to deliver reprogramming genes. In an interview she stated, "In my opinion, the safest methods to reprogram adult cells to iPS cells should NOT involve genetic manipulation (e.g. no viral vectors) of these cells. Current advances using exogenously added small molecules or chemicals to the medium have showed potential for improving the cells for therapeutic applications" (Boyer, personal communication, 8-5-13). Scientists that continue to use integrating viruses or c-Myc as a reprogramming gene need to perform a rigorous screening of the formed cells to eliminate those with obvious chromosomal abnormalities or unusual growth characteristics.

When asked on his overall opinion on the use of iPSCs for therapies, Professor Heilman of WPI believes that with some improvement, they should play a role in regenerative medicine.

He believes that although iPS cells are relatively "new", the research is getting better at developing new techniques for reprogramming, including the direct use of DNA binding transcription factors (Heilman, personal communication, 8-15-13). But in spite of these advances, he currently believes that ESCs remain the best method of treatment. He used a very effective analogy, comparing iPSCs to a chalkboard. A clean chalkboard that has never been used before is a pure, clean slate; it is easy to draw all over and use. However, a dirty chalkboard must be erased to be used (reprogrammed), and when it is drawn over again, the prior chalk dust is still left on the board. Obviously, it is much easier to use a clean board. Heilman believes that the best solution is using the surplus IVF embryos discarded at fertilization clinics. Since they are already being discarded, these embryos could be instead be used to derive new ESC lines for therapy or research. "However, until funding for ESC research expands, the best bet for regenerative medicine is to invest in improving reprogramming methods for iPSCs".

CONCLUSIONS / RECOMMENDATIONS

Based on the findings of our research, we are able to make several conclusions and recommendations. Our investigation of iPSC experiments in animals have identified several problems that must be addressed prior to using iPSCs in humans. The most serious potential problems include: 1) the slow growth of iPSCs if c-Myc is not used, 2) the potential of cancer at the injection site if c-Myc or integrating viruses are used, and 3) the potential for immune-rejection of the injected cells.

With respect to *tumor formation*, the best practice with respect to cell growth appears to include the c-Myc oncogene in the reprogramming mix; but after expanding the cells to obtain a sufficient quantity for therapy, the c-Myc gene should either be excised using a Cre-recombinase gene delivered by an Adenovirus removed a floxed c-Myc gene, or the cells should be screened to ensure the c-Myc has been silenced. Several programming methods use fewer than the original 4 programming factors, but more research should be performed to ascertain whether these cells are as proliferative as those treated with c-Myc, and if so, eliminate the use of c-Myc. To decrease the chance for tumor formation, all cells post-programming should be screened for lack of c-Myc expression, lack of obvious chromosomal abnormalities (by karyotyping), and presence of normal growth characteristics. These tests should be performed prior to infusing the cells into any patient.

With respect to *potency*, we conclude that cell potency is not homogenous within a population of iPSCs and varies due to “intrinsic variability” within the population. This problem should be investigated further to understand how to equalize potency to enhance the efficiency of the therapy. We also conclude that cell potency can be changed by altering the *timing* of

addition and the *type* of reprogramming factors used in the reprogramming added. So these variables should be further investigated.

With respect to *immunogenicity*, our findings indicate that relatively few scientists have focused their research directly on this issue. It appears that some scientists observe a problem of rapid rejection of the implanted cells, while other lab groups do not. So, more research should be performed on which systems show the best or worst immune-rejections, and why. And each donor-host system must be examined separately, making sure to fully assay for T-cell proliferation, the most common mechanism of injected cell death.

While most of the research focuses on improving iPSC programming methods, a key interview with Nancy Kavanaugh of Cell Signaling (personal communication, 7-30-13) reminded us that simpler factors are of equal importance, such as devising standardized procedures for ensuring the iPSCs remain uncontaminated prior to therapy.

Although our group is strongly in favor of continuing research on iPSCs, as are many other researchers, we also believe that it is imperative to continue research with ESCs, in spite of their inconsistent funding and ethical issues. We conclude that few, if any, studies have been done directly comparing the effectiveness of iPSC versus ESC therapies, and also conclude that such studies are critical for determining whether iPSCs can replace ESCs. If both types are found to work equally well for a specific disease, we give the nod to iPSCs due to their fewer ethical issues.

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APPENDIX

LIST OF INTERVIEW QUESTIONS

General Questions for All Stem Cell Researchers:

1. Can you tell me a little more about your current position and how you became interested in working with stem cells?
2. Which type of stem cells do you work with, and on which diseases?
3. Open-ended question: what do you see as some of the major obstacles to using iPS cells for therapy?

The interviewee's response to the opening questions was used to decide which follow-up questions best applied to that particular subject. For example, if the subject brought up the issue of replication problems for iPS cells, we asked their opinion on some of the scientific articles showing similar problems, and asked their opinion of potential solutions.

Questions for iPS Cell Researchers:

1. Which cells do you reprogram, skin fibroblasts?
 - a. Why did you choose those cells, because they are easy to obtain and easy to reprogram?
2. How do you reprogram your cells?
 - a. How many different transcription factor genes do you use to reprogram your cells?
 - b. Do you use cMyc which has been shown by some scientists to cause tumors at the injection site?
 - c. What is the least number of genes shown to be effective at reprogramming in your lab?
 - d. Some scientists claim they can get reprogramming with only 2 genes, are those cells as potent as cells reprogrammed with more genes?
 - e. Do you use viruses to deliver the reprogramming genes? Have you noticed any problems associated with viral delivery, such as integration problems?
3. How potent do you think your iPS cells are?
 - a. Do you think your cells are totipotent, pluripotent, or multipotent?
 - b. Does the potency alter with the number of genes used for transformation?
 - c. What is your opinion of the studies showing that mouse iPS cells can be used to create entire organisms? Does that data argue those cells are totipotent or pluripotent? If such cells are later shown to be totipotent, would they ethically be equivalent to using ES cells?
4. Have you observed any tumors at the injection sites?
 - a. If so, what might cause the tumors?
 - b. What might prevent the tumors?
5. Have you observed any chromosome damage with your iPS cells?
 - a. If so, do you think the chromosome damage would hinder their use in human patients?
 - b. How might the chromosome damage be mitigated?

6. Have you observed any slow growth of your iPS cells?
 - a. If so, would this hinder their use in human patients?
 - b. How could the slow growth be mitigated?

Questions for ES Cell Researchers:

1. Do you think that intermittent federal funding of ES cell research hinders ES cell research and increases the need to derive cell replacements?
 - a. Do you think that U.S. ES cell research lagged during the Bush administration years when federal funding could not be used to derive new ES cell lines?
 - b. Do you think that the U.S. has a sufficient number of ES cell lines for doing thorough research studies?
2. Do you think that ethical issues associated with ES cells increase the need to identify ES cell replacements?

Questions for Academic Bio-Ethicists:

1. Are you familiar with iPS cells? If not, we will explain this briefly to them.
2. If iPS cells are pluripotent, are they a potential replacement for ES cells?
3. If iPS cells are later shown to be totipotent, would you still view them as a potential replacement for ES cells?
4. Do you think that more research should be done on iPS cells prior to using them for human therapies?

INTERVIEW PREAMBLE

We are a group of students from the Worcester Polytechnic Institute in Massachusetts, and for our research project we are conducting a series of interviews to investigate problems associated with stem cell plasticity and the use of cells derived by trans-differentiation for therapy.

Your participation in this interview is completely voluntary, and you may withdraw at any time. During this interview, we would like to record our conversation for later analysis. We will also be taking notes during the interview on key points. Is this okay with you?

Can we also have your permission to quote any comments or perspectives expressed during the interview? This information will be used for research purposes only, and we will give you an opportunity to review any materials we use prior to the completion of our final report. If the subject does not agree to be quoted, we will respond as follows: "Since you would not like to be quoted during this interview, we will make sure your responses are anonymous. No names or identifying information will appear in any of the project reports or publications."

Your participation and assistance is greatly appreciated, and we thank you for taking the time to meet with us. If you are interested, we would be happy to provide you with a copy of our results at the conclusion of our study.

Appendix, Continued: Interview Table

Contacting Group Member	Potential Interviewee	Date of Initial Contact	Date of Second Contact	Interview Date or Termination Date
Luisanna	<p>Name: Tanja Dominko Address: Associate Professor, Dept. Biology and Biotechnology, WPI, Worcester, MA. Email: tdominko@wpi.edu Phone: (508) 831-4108 Expertise: Works with Induced Replication-Competent (iRC) cells that reprogram with hypoxia and FGF-2, using no viruses.</p>	Called 6-15-13. Yes responded		Interviewed in person on 6-25-13
Luisanna	<p>Name: George Daley Address: Children's Hospital Boston, Karp Building, 7th Floor, One Blackfan Circle, Boston, MA 02115. Email: Daley.Lab@childrens.harvard.edu Phone: (617) 919-2015 Expertise: Works with iPS cell lines.</p>	Phoned 6-15-13	Emailed 6-27-13	Interviewed by email 7-25-13
Luisanna	<p>Name: Fen-Biao Gao Address: Professor, Department of Neurology, University of Massachusetts Medical School, Worcester, MA 01605. Email: Fen-Biao.Gao@umassmed.edu Phone : (508) 856-8504 Expertise: General questions on stem cells.</p>	Emailed 6-11-13	Emailed 6-15-13	Interviewed by email 6-27-13
Luisanna	<p>Name: Rudolf Jaenisch Address: Professor, Department of Biology, MIT. Founding member of the Whitehead Institute. Email: Jaenisch@wi.mit.edu Expertise: A world leader in transgenesis, cloning, and stem cells.</p>	Emailed 6-27-13	Emailed 7-1-13	Terminated 7-24-13
Luisanna	<p>Name: Shinya Yamanaka Address: Professor and Director of the Center for iPS Cell Research and Application (CiRA), Kyoto University. Email: yamanaka@frontier.kyoto-u.ac.jp Expertise: Father of the iPS field. He was the first to develop mouse iPS cells in 2006, then was the first to develop human iPS cells in 2007.</p>	Emailed 7-8-13	Emailed 7-16-13	Terminated 7-24-13

Luisanna	Name: Amy Briggs Address: Former Relations Manager at UMass Stem Cell Bank, UMass Medical School. Email: Expertise: Questions on stem cell ethics.	Emailed 7-2-13	Second email 7-16-13	Terminated 7-20-13
Luisanna	Name: Maria Borowski Address: Former Relations Director at UMass Stem Cell Bank, UMass Medical School. Email: Expertise: Questions on stem cell ethics.	Emailed 7-8-13		Interviewed 7-30-13
Michelle	Name: James A. Thomson Address: Director of Regenerative Medicine, University of Wisconsin. Email: Thomson@primate.wisc.edu Expertise: Father of the human ES cell field. Discovered how to derive human ES cells. With respect to this IQP, he also developed a method for reprogramming iPS cells using only 3 factors instead of 4.	Emailed 6-18-13	Emailed 06-27-13 , 07-10-13	Terminated 7-24-13
Michelle	Name: Junying Yu Address: Assistant Scientist, Primate Research Center, University of Wisconsin at Madison. email: jyu@primate.wisc.edu Expertise: Corresponding author on article with James Thomson.	Emailed 6-18-13	Emailed 06-27-13, 7-10-13	Terminated 7-24-13
Michelle	Name: Kevin Eggan Address: Professor, Stem Cell and Regenerative Biology, Harvard University. Email: eggan@mcb.harvard.edu Diane Santos, Lab Administrator dsantos@mcb.harvard.edu Phone: (617) 496-5611, (617) 496-8659 Expertise: Author of article titled: induced pluripotent stem cells line derived from human somatic cells. Could comment on using iPS cells in patients.	Emailed 6-18-13	Emailed 06-27-13, 7-10-13 Called Diane Santos 7-30-13	Terminated 8-8-13
Michelle	Name: Laurie Boyer Address: Professor, Department of Biology, MIT. Email: lboyer@mit.edu Phone: 617-324-3335 Expertise: ES stem cell research. Can give opinion on ES vs. iPS cells.	Emailed 7-24-13	Called, left message 7-30-13	Interviewed 08-05-13
Michelle	Name: Piyush Gupta (and Assistant Laura Resteghini) Address: Assistant Professor, Department of Biology, MIT. Member, Whitehead Institute. Email: praj@wi.mit.edu resteghini@wi.mit.edu	Emailed 7-24-13	Called, left message 7-30-13	Terminated 8-8-13

	Phone: (617) 324-0086 Expertise: Subversion of normal cell mechanisms to cause cancer.			
Michelle	Name: Destin Heilman Address: Associate Professor, Department of Chemistry and Biochemistry, WPI. email: dheilman@wpi.edu Expertise: works with viruses to reprogram cells	Emailed 08-06-13		Interviewed in person 08-15-13
Michelle	Name: Raymond Page Address: Assistant Professor, Department of Biomedical Engineering, WPI. email: rpage@wpi.edu Expertise: Collaborates with Prof. Tanja Dominko, works with Induced Replication Competent (iRC) cells, induced by hypoxia and FGF-2.	Emailed 7-24-13		Terminated 8-24-13
Michelle	Name: BD Colen/Joseph Caputo Address: Director of Communications, and Communications Manager, Harvard Stem Cell Institute. email: bd_colen@harvard.edu / joseph_caputo@harvard.edu Expertise: General questions on stem cells and stem cell funding and ethics.	Emailed 7-24-13	Emailed again 08-06-13	Terminated
Michelle	Name: Keisuke Kaji Address: MRC Centre for Regenerative Medicine, Institute for Stem Cell Research, University of Edinburgh, Edinburgh EH9 3JQ, UK. Email: keisuke.kaji@ed.ac.uk Expertise: Virus-free induction of iPS cells (excision of the DNA).	Emailed 7-30-13 Out of office notification until August 6th	Emailed again 08-06-13	Terminated
Michelle	Name: Hans R. Scholer Address: Department of Cell and Developmental Biology, Max Planck Institute for Molecular Biomedicine, Rontgenstrasse 20, 48149 Munster, NRW, Germany. Email: schoeler@mpi-muenster.mpg.de Expertise: Reprogramming iPS cells with only two factors.	Emailed 7-30-13	emailed again 08-06-13	Terminated
Michelle	Name: Martin F. Pera Address: Eli and Edythe Broad Center for Regenerative Medicine and Stem Cell Research, Keck School of Medicine, University of Southern California, Los Angeles, California 90089, USA. Email: martin.pera@keck.usc.edu Expertise: New programming techniques to make iPS cells safer for therapy.	Emailed 7-30-13	emailed again 08-06-13	Terminated
Michelle	Name: Konrad Hochedlinger Address: Massachusetts General Hospital Cancer Center and Center for Regenerative	Emailed 7-30-13	Emailed again	Terminated 8-15-13

	<p>Medicine, 185 Cambridge Street, Boston, MA 02114, USA.</p> <p>Email: khochedlinger@helix.mgh.harvard.edu</p> <p>Expertise: Re-programming iPS cells without using viruses to deliver genes. See also Devon's questions related to DNA damage and iPS cells.</p>		08-06-13	
Michelle	<p>Name: Marius Wernig</p> <p>Address: Institute for Stem Cell Biology and Regenerative Medicine, Department of Pathology, Stanford University School of Medicine, 1050 Arastradero Road, Palo Alto, California 94304, USA.</p> <p>Email: wernig@stanford.edu</p> <p>Expertise: Re-programming iPS cells with only 3 factors instead of 4.</p>	Emailed 7-30-13	Emailed again 08-06-13	Terminated 8-15-13
Michelle	<p>Name: Douglas A. Melton</p> <p>Address: Department of Stem Cell and Regenerative Biology, Howard Hughes Medical Institute, Harvard Stem Cell Institute, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138, USA.</p> <p>Email: dmelton@harvard.edu</p> <p>Expertise: World-class developmental biologist. With respect to this IQP he re-programs pancreatic cells into beta cells using only 3 factors.</p>	Emailed 7-30-13	Emailed 08-06-13	Terminated 8-15-13
Ted	<p>Name: Dr. Tortland, MD</p> <p>Address:</p> <p>Email: ptortlandvsp@jockdoctors.com</p> <p>Expertise: Medical uses of stem cells in general.</p>	Emailed 6-13-13	Emailed Again 7-3-13	Interviewed by email 7-30-13
Ted	<p>Name: Dr. Awad, MD</p> <p>Address:</p> <p>Email:</p> <p>Expertise: Medical Devices</p>	Emailed 6-13-13		Terminated 8-15-13
Ted	<p>Name: Jim Ammen</p> <p>Address: Former Speaker of the House,</p> <p>Email:</p> <p>Expertise:</p>			Terminated 7-24-13
Ted/Andrew	<p>Name: Jim Fowler</p> <p>Address: Editor of the National Review,</p> <p>Email:</p> <p>Expertise: Funding/ what was Need for ES replacement</p>			Interviewed in person 6/14/13

Ted	<p>Name: Takashi Aoi Address: Center for iPS Cell Research and Application, (CiRA) Kyoto University. Email: aoi-g@cira.kyoto-u.ac.jp Expertise: First author on 2008 Science paper from Yamanaka lab showing that pluripotent iPS cells can be isolated from mouse liver and stomach cells. Yamanaka was corresponding author, but he did not respond to earlier email from Luisanna. Ask him questions about how strong their evidence is for pluripotency, and why some other scientists don't see pluripotent iPS cells.</p>	Emailed 7-28-13		Terminated 8-15-13
Ted	<p>Name: Kristin K. Baldwin Address: Department of Cell Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, USA. Email: kbaldwin@scripps.edu Expertise: Corresponding author on 2009 Nature paper showing that adult mice can be generated from iPS cells, arguing for strong cell potency. Ask her whether other scientists have seen such strong evidence for potency, and if not why not.</p>	Emailed 8-4-13		Terminated 8-15-13
Ted	<p>Name: Su-Chun Zhang Address: Waisman Center, School of Medicine and Public Health, University of Wisconsin, Madison, WI 53705. Email: Zhang@waisman.wisc.edu Expertise: Corresponding author on 2010 PNAS paper showing that iPS cells from neural cells using the same transcriptional network as ES cells but at reduced efficiency and increased variability. Ask why they think they observe the variability, and have they since devised any protocols for improving efficiency.</p>	Emailed 8-4-13		Terminated 8-15-13
Ted	<p>Name: Keisuke Okita Address: Center for iPS Cell Research and Application, (CiRA) Kyoto University. Email: okita-g@cira.kyoto-u.ac.jp Expertise: Dr Okita is first author on 2007 Nature paper from the Yamanaka lab showing that iPS cells can generate germline competent cells (very potent). Yamanaka was the corresponding author, but he did not respond to earlier email from Luisanna. Ask Dr. Okita about whether other scientists also show evidence for germline competence of their iPS cells (we are aware of the 2009 Nature paper from Kristin Baldwin's lab, and if not why not.</p>	Emailed 7-28-13		Terminated 8-15-13

Andrew	<p>Name: Nancy Kavanaugh Company: Cell Signaling Expertise: Works with cell rejection and understands complications with funding, but seems to think biggest concern does not lie in the funding.</p>	Emailed 7/24/13		Interviewed 7/30/13
Andrew	<p>Name: Masumi Abe Address: Transcriptome Research Group, National Institute of Radiological Sciences, Chiba 263-8555, Japan. Email: abemasum@nirs.go.jp Expertise: His group in 2013 observed a negligible immunogenicity of iPS cells in mice compared to ES cells. They showed equal transplantation success. Earlier 2011 studies by Zhao et al. showed different results. Why the difference?</p>	Emailed 7/27/13 8/3/13		Terminated 8-15-13
Andrew	<p>Name: Yang Xu Address: Section of Molecular Biology, Division of Biological Sciences, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0322, USA. Email: yangxu@ucsd.edu Expertise: His group in 2011 showed that mouse iPS cells injected into B6 mice were rapidly rejected by the recipients. This contrasts starkly with a follow-up study by Araki et al., 2013, who showed strong engraftment and minimal immunogenicity. Why the stark difference in results?</p>	Emailed 7/27/13 8/3/13		Terminated 8-15-13
Andrew	<p>Name: Tim M. Townes Address: Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Schools of Medicine and Dentistry, Birmingham, AL 35294, USA. Email: ttownes@uab.edu Expertise: His group in 2007 showed that mouse iPS cells can be used to treat a mouse model of sickle cell anemia. The therapeutic cells were not immuno-rejected by the recipient mice. This contrasts with the 2011 Zhao et al. study showing poor engraftment and strong immunogenicity. Why the difference?</p>	Emailed 7/27/13 8/3/13		Terminated 8-15-13

Alex	<p>Name: Francois Baylis Address: Professor, Department of Philosophy, Dalhousie University, 6135 University Avenue, PO Box 15000, Halifax, Nova Scotia, Canada B3H 4R2. Email: Francoise.Baylis@Dal.ca Phone: (902) 494-2873 Expertise: Well-known expert on the ethics of ES and iPS cells. What does she feel are the main obstacles of using iPS cells as replacements for ES cells in human therapies?</p>	Emailed 7/30/13		Interviewed 7/30/13
Alex	<p>Name: Cynthia B. Cohen Address: Research Affiliate, Kennedy Institute of Ethics, Healy Hall, 4th Floor, Georgetown University, Washington, DC 20057. Email: Unknown Phone: (202) 687-6766 (the institute, not her private office) Expertise: Well known expert on bioethics and stem cells. What does she feel are the main obstacles of using iPS cells as replacements for ES cells in human therapies?</p>			Terminated 8-15-13
Alex	<p>Name: Sally Lehrman Address: Markkula Center for Applied Ethics, Arts and Sciences Building 214, Santa Clara University, Santa Clara CA 95053. Email: slehrman@scu.edu Phone: (408) 551-3000, x4256 Expertise: An ethicist who brought up some interesting points about who and how we will control use of the donor skin cells for making iPS cells.</p>	Emailed: 7/30/13	Emailed: 8/7/13	Terminated 8-15-13