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Pulling Back the Veil on Stem Cell Research: The Generation of Induced Pluripotent Stem Cells as an Alternative to Embryonic Stem Cell Research

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PULLING BACK THE VEIL ON STEM CELL RESEARCH: THE GENERATION OF INDUCED PLURIPOTENT STEM CELLS AS AN ALTERNATIVE TO EMBRYONIC STEM CELL RESEARCH

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Abstract:

Stem cell research has captured the imagination of many in society, and although it holds hope for repairing the human body, curing disease, and alleviating human suffering, the effects of this pioneering technology present profound ethical and legal implications. While a general consensus exists that the use of adult stem cells is ethically and morally just, embryonic stem (ES) cell research has generated significant debate in both the scientific and legal communities. Decades of legislation and policymaking have led to the creation of a set of human subject research principles that are now well recognized and rooted in the inherent worth and dignity of the individual. ES cell research presents a new challenge regarding the moral status of the human embryo that has led to further debate on the scope and application of these principles.

Rapid changes in science and technology, such as the development of ES cell research methods, have created the need for research codes, principles, and laws designed to protect individuals who have increasingly been used as subjects for research in recent years. The use of human subjects for research has also introduced new ethical challenges which these laws attempt to address and have shaped the current legislation for ES cell research as well. Scientists, ethicists, and policymakers currently are engaging in discussions to determine whether the bioethics of ES cell research are consistent with the standard bioethical principles and laws for human subjects and whether legal inconsistencies exist in regards to federal funding for this type of research. It is also important to study state laws to illustrate similarities and differences as well as compare United States laws to the laws of other countries governing ES cell research.

Finally, the use of additional methods of research must be explored that will serve as viable alternatives void of ethical considerations. The most recent ground-breaking technology to be introduced into the stem cell research community is induced pluripotent stem (iPS) cell

research. iPS cells are adult cells that have been genetically reprogrammed to an embryonic stem cell-like state thus expressing hallmark characteristics of embryonic stem cells. The purpose of this study was to conduct research in order to develop methods to allow for the successful generation of iPS cells useful in clinical settings. In addition, these cells have been further analyzed in order to determine whether they are indistinguishable from embryonic stem cells thereby raising the question as to whether iPS cell research will obviate the need for ES cell research.

This Study by: Sara Ternes

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has been approved as meeting the thesis or project requirement for the Designation University Honors with Distinction and the requirements for the Bachelor of Science Biology-Honors Research Degree

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TABLE OF CONTENTS

FIGURES

TABLES

LITERATURE REVIEW

History of the Principles, Laws, and Ethical Guidelines for Human Subject Research

Principles, ethical guidelines, and policies for the oversight of human subject research were initiated in the 20th century, and the Nuremberg Code is often marked as the origin of protections for human research subjects. In addition, new policy often occurs in response to tragedy or public outcry over an apparent injustice, and the Nuremberg Code represents a clear example of this as well.

December 9, 1946 marked the beginning of criminal proceedings opened by an American military tribunal in the case of USA vs. Karl Brandt et. al., against 23 leading German physicians and administrators for their participation in war crimes and crimes against humanity. These German physicians planned and enacted the "Euthanasia" Program, the systematic killing of individuals they deemed to be "unworthy of life." The victims of the Program included the mentally handicapped, the institutionalized, mentally ill, and the physically impaired. Furthermore, during World War II, these same German physicians conducted pseudoscientific medical experiments which utilized thousands of concentration camp prisoners without disclosure of the experiment and prior consent of the individual was not obtained. Most died or were permanently crippled as a result of the experimentation. The majority of the victims were Jews, Poles, Russians, and Gypsies. Experiments conducted on the victims included but were not limited to high altitude experiments, freezing experiments, malaria experiments, lost (mustard) gas experiments, sulfanilamide experiments, bone, muscle, and nerve regeneration and bone transplantation experiments, sea-water experiments, epidemic jaundice experiments, sterilization experiments, spotted fever experiments, experiments with poison, and incendiary bomb experiments. After nearly 140 days of proceedings, a verdict was pronounced on August

20, 1947 in which 16 of the German physicians were found guilty, seven of which were sentenced to death and executed on June 2, 1948.[1]

The Nuremberg Code appearing in the judges' verdict from the Doctor's Trial is a set of 10 principles, based upon the oath of Hippocrates. The principles were formulated in an attempt to establish substantive standards and procedural guidelines for permissible medical experimentation with humans.[2] Among the 10 principles are three key features that became the basis for subsequent legislation and today's human protection laws: 1) The subject's voluntary and informed consent is required 2) Risks and harms to the subject should be minimized 3) The results of the study must be valuable to society.[3]

The guidelines enumerated in the Code were visionary in their depth and scope for their time, but still left much room for interpretation for future medical practice and human experimentation. It left unanswered questions on how to judge the risk to experimental subjects against the benefits to society. Furthermore, by centering exclusively on war crimes and not on their broader context, the Nuremberg Code solely set boundaries for permissible experiments and addressed the question of biomedical research on subjects within Germany during the war. The Code failed as a broader legal doctrine to protect individuals against potential harm induced by scientific practices at large, not only as humans as subjects of medical experiments, but also as consumers and beneficiaries of the outcomes of science.[4]

The Declaration of Helsinki (DoH) was first adopted at the 1964 World Medical Association (WMA) General Assembly in Helsinki in response to the atrocities revealed during the Nuremberg Trials and the Nuremberg Code created as a result. The WMA's initiative was to further the reach of the Nuremberg Code and establish a set of universal ethical principles for medical clinicians and researchers. The purpose of the DoH itself was to provide guidance to

physicians engaged in clinical research while focusing on the responsibilities of researchers for the protection of human research subjects. The basic principles that the document outlines include the use of research involving human subjects based upon previously performed animal and laboratory experimentation, formulation of a protocol transmitted to a committee for approval, and research conducted only by scientifically qualified persons. In addition, the responsibility of the research subject rests with the qualified research individual, and the concern of interest of the subject must always take precedence over the interest of science and society.[5]

The Declaration also seeks to attend to limitations identified in the Nuremberg Code by considerably advancing the detail of ethical conduct in research. It is clearly stated that the advancement of medical science and promotion of public health, although recognized as important objectives of medical research are at all times subordinate to the well-being of individual research subjects. Furthermore, the stringent guidelines of informed consent are reexamined as the DoH addresses research with subjects incapable of making their own decisions or consent. This reflects the recognition that research extends beyond the sacrifice of vulnerable subjects that framed the Nuremberg Code, to include research, such as with mentally disabled people and with infants and children, that is ethical to undertake and may be unethically discriminatory to deny.[6]

In the wake of revelations that serious abuses of research ethics were relatively commonplace despite the wide acceptance of the Declaration, the WMA sought to make certain guidelines explicit that had only been implicit in previous versions of the DoH through a series of six separate revisions spanning the course of three decades. The most extensive revision in 1975, and arguably the single most important development, was the significant elaboration on the duty of the individual to be given primacy over society. The Declaration states: "In research

on man, the interest of science and society should never take precedence over considerations related to the well-being of the subject." (paragraph III.4, 1975)[7] To ensure this guideline was adhered to, new requirements were added including the advance review of projects by independent committees and adherence to the principles of the DoH as a condition for publication of the results of the research.[8]

Given the extensive nature of the 1975 revision, changes adopted in 1983, 1989, and 1996 hardly warrant the revision term, but are rather trivial amendments that focus on textual alterations. In contrast, the new version of the Declaration released in 2000 includes significant revisions and expansion of the document. For the first time, although emphasis on the primacy of the individual was retained, increased attention was brought to the needs of public health in order to bring benefits to populations as well as the individual. The subtle shift in the balance of responsibilities was denoted in the DoH with the removal of the distinction between therapeutic and non-therapeutic research that had been a hallmark of the document since it was first released in 1964. The previous distinction was based on the premise that the vast majority of medical research is therapeutic or intended to benefit the research subject, as illustrated in an excerpt from the 1996 version of the DoH: "The physician can combine medical research with professional care, the objective being the acquisition of new medical knowledge, only to the extent that medical research is justified by its potential diagnostic or therapeutic value for the patient." (paragraph II.6, 1996).[9] The 2000 version, on the other hand, introduced an entirely new concept of the responsibility of researchers to provide benefits to populations as well: "Medical research is only justified if there is a reasonable likelihood that the populations in which the research is carried out stand to benefit from the results of the research." (paragraph 19, 2000)[10] The final revision of 2008 contains important new requirements related to the

registration of clinical trials reporting their results. Medical research has a clear public purpose and the concept of transparency addressed in this revision is a crucial requirement to ensure that this public purpose is respected. A firm commitment to transparency will aid in building trust in medical research, reflects an ethical commitment to the respect for research subjects, and is a core component of promoting the physical well-being of research participants and patients who will benefit from the results of the research.[11]

The DoH has been subject to much criticism in recent years, particularly in regards to the shift towards promoting the benefits of public health over the benefits and integrity of the individual. While addressed in the Declaration, there is still room for interpretation regarding the delicate balance of risk versus benefits. The most significant drawback to both the Nuremberg Code and the Declaration of Helsinki lies in the fact that both are strictly a collaboration of research and ethics principles. Neither document carries the force of law nor is the document a legally binding instrument of international law. Regardless of this fact, the DoH has been referred to as "the most widely accepted guidance worldwide on medical research involving human subjects." It is described as "the cornerstone of biomedical research for the last 30 years and the largely unquestioned anchor for ethical decision-making in clinical trials."[12]

Despite the clear ethical guidelines laid out in the Nuremberg Code and the Declaration of Helsinki, 1972 revealed the longest non-therapeutic experiment in the history of medicine that continues to stand as a prime example of medical arrogance, unethical behavior, and racism in research. The Tuskegee Syphilis Study was conducted from 1932 to 1972 by the U.S Public Health Service. The research project consisted of 600 low-income African American males, 400 of whom were infected with syphilis and monitored for 40 years. Free medical examinations were offered, but the subjects were never told about their disease. A proven cure became

available in the 1950's, but participants were denied access until 1973, when the study was officially terminated by the U.S Department of Health, Education, and Welfare after its existence was publicized and became a political embarrassment.[13]

Due to public outrage and publicity from the Tuskegee Syphilis Study, the National Research Act of 1974 was created and passed into law. The act established a temporary National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research. The charge of this commission was to investigate ethical principles involved in biomedical and behavioral research, including psychosurgery and living fetus research, and to develop ethical guidelines for developing such research. In addition, the Act also established a permanent National Advisory Council for the Protection of Subjects of Biomedical and Behavioral Research and required institutional review boards to approve all studies involving human subjects.[14]

One of the responsibilities of the Commission was to identify the basic ethical principles that should underlie the conduct of biomedical and behavioral research involving human subjects, and develop guidelines to be followed to ensure that such research is conducted in accordance with those principles. The Belmont Report, released in 1979, attempts to summarize the principles identified by the Commission, and represents a statement of the basic ethical principles that should assist in resolving ethical dilemmas that surround the conduct of research with human subjects. The three basic principles that were identified to be particularly relevant to ethics involving human subject research include: respect of persons, beneficence, and justice.[15]

The principle of respect for persons incorporates two ethical convictions and two separate moral requirements. First, individuals should be treated as autonomous agents, and second, persons with diminished autonomy should be entitled to protection. Therefore, moral requirements require the acknowledgement of the individual's autonomy and the requirement to

protect those with diminished autonomy. The extent of protection afforded should depend upon the risk of harm and likelihood of benefit to the research subject. Additionally, judgment of individuals who lack autonomy should be periodically reevaluated and will vary depending on the situation.[16]

Treating people in an ethical manner, not only by respecting their decisions and protecting them from harm, but also making efforts to secure their well-being, falls under the principle of beneficence. In regards to human research, beneficence goes beyond covering acts of kindness or charity, but in this sense is an obligation. Expressions of beneficent actions in this sense are the obligation to not harm the individual and maximize possible benefits and minimize possible harms. The obligations of beneficence affect both the investigator and society because they extend to both research projects and society at large. In the case of particular projects, investigators and members of their institutions are obliged to give forethought to the maximization of benefits and the reduction of risk that might occur from the research being conducted. In the case of scientific research in general, members of the larger society are obliged to recognize the longer term benefits and risks that may result from the improvement of knowledge and from the development of novel medical, psychotherapeutic, and social procedures.[17]

The principle of justice seeks to answer the question of 'who ought to receive the benefits of research and bear its burdens.' The question of justice is in the sense of fairness of distribution and what is deserved. Injustice occurs when some benefit to which a person is entitled is denied without good reason or when a burden is imposed unjustifiably. Another way of conceiving the principle of justice is that equals ought to be treated equally. Whenever research by public funds leads to the development of therapeutic devices and procedures, justice

demands that these advancements do not just provide advantages only to those who can afford them. Also, such research should not unduly involve people from groups that are unlikely to be among the beneficiaries of subsequent applications of the research.[18]

The most current piece of legislation put in place that directly addresses the ethics of ES cell research is the Dickey-Wicker Amendment. In 1993, former President Bill Clinton initiated the National Institutes of Health (NIH) Revitalization Act which eliminated the guidelines put in place by the 1979 NIH stating that no application or proposal involving human in vitro fertilization by the Department could be carried out until the application or proposal had been reviewed by the Ethical Advisory Board. This act paved the way for federal funding to study human fertilization without the need for review by an Ethical Advisory Board. When the act was introduced to Congress in 1996, representatives Jay Dickey and Roger Wicker authored a rider bill attached to the appropriations bill for the Department of Health and Human Services. The rider articulated that none of the funds appropriated by the National Institute of Health should be used to support any activity involving the creation of a human embryo or embryos for research purposes or research in which a human embryo is destroyed, discarded, or knowingly subjected to risk of injury or death greater than that allowed for research on fetuses in utero. Furthermore, the term 'human embryo' or 'embryo' was defined to include any organism not protected as a human subject under the Federal Policy for the Protection of Human Subjects derived by fertilization, parthenogenesis, cloning, or any other means from one or more human gametes or human diploid cells.[19]

Evolution of the Legislation and Policy Governing Embryonic Stem Cell Research

The history of ES cell research and the policy and legislation governing this work goes back to the mid1970's. It is essential to understand evolution of policy that led to the current

legislation. Shortly after the ruling of Roe vs. Wade, discussions began about the ethics of conducting research on human fetal tissue. The National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research was established in order to investigate research involving the fetus and the circumstances such research could be conducted or funded by the federal government. Most research on live human fetuses was banned in the United States prior to the establishment of these regulations. The Commission concluded that a live fetus was not to be used as a research subject unless the research was intended to benefit the fetus or its mother and it would not pose a risk to the fetus. Research was also not permitted if it terminated the heartbeat or respiration of the fetus. Strict guidelines were also put in place under which research on living fetuses or their tissues would be allowed. An institutional review board had to grant approval, those harvesting tissue could not have any part in the timing, method or procedures used to terminate a pregnancy, the termination procedure could not entail greater than minimal risk to the fetus or pregnant woman, termination of a pregnancy could not include any inducement, both the woman and man (parents) must give consent for the termination of pregnancy, and there could not be an artificial maintenance of the vital signs of living, nonviable fetuses. The recommendations of the Commission led to the lifting of the Department of Health, Education, and Welfare (HEW)'s funding ban in July of 1975 and the publication of regulations for fetal research.[20] The 1970's also marked an attempted reinvention of human embryology. In 1979, Clifford Grobstein, a developmental biologist, introduced the term "pre-embryo" to science, politics, and ethics. A "pre-embryo" is defined as a human embryo or fertilized ovum in the first 14 days after fertilization before implantation in the uterus has occurred. Grobstein held that since identical twins may occur up to 14 days following fertilization, only a "genetic individual" is present that has "potential" for life, not a "developmental individual", and that

therefore an embryo, a "person", is not present.[21] Grobstein and other biologists believed there was a qualitative difference between an embryo less than 14 days old and all subsequent stages of development.

The "pre-embryo" term has now been completely discredited by all human embryologists, dismissing the term as "discarded" and "inaccurate." In addition, it has been rejected by the Nomenclature Committee of the American Association of Anatomists for inclusion in the official lexicon of the anatomical terminology and is not used in any official textbook of human embryology. Both the Human Embryo Research Panel and the National Bioethics Advisory Commission also rejected the term "pre-embryo", and in turn defined an "embryo" as a developing organism from the time of fertilization.[22] The scientific evidence indicates that the moment the sperm makes contact with the oocyte, human development is an integrated continuum in which one stage follows another throughout all of life until death, and therefore, the developing human being is both a 'genetic' and 'developmental' individual from the first moment of its existence.[23] Grobstein and the Reverend Richard McCormick formed an arbitrary Ethics Committee of the American Fertility Society(AFS)and designed statements that removed the moral status and protection of the human embryo up to 14 days post-fertilization. Grobstein has subsequently admitted that the term was conceived in order to reduce the "status" of the early human embryo, which he declared to be a "pre-person."[24] Despite the rejection of the term by leading human embryologists, it is still used as a defense for some to justify funding destructive human embryo experiments and paved the way for funding for ES cell research.

In 1980, President Ronald Reagan chose not to renew the Ethics Advisory Board's (EAB) charter that recommended federally funded investigations into the safety of *in vitro* fertilization that used human embryos developed *in vitro* for no longer than 14 days post-fertilization. The

EAB was allowed to go out of existence and no new appointees were named. A *de facto* moratorium halted governmentally subsidized non-therapeutic IVF-bred human embryos and IVF research.[25] Despite the absence of federal funding for the use of human embryos, research progressed steadily throughout much of the 1980's under the guidelines outlined above.

Controversy once again erupted in 1987 when NIH scientists requested to fund research involving fetal brain tissue transplantation on Parkinson's disease patients that had been pre-approved by the internal NIH review board. This request was in response to a report in the *New England Journal of Medicine* that Mexican researchers had transplanted fetal neural tissue into the brains of two Parkinson's patients and the condition of both patients had improved substantially. Advice was sought from the Department of Health and Human Services (HHS) regarding a research protocol to transplant fetal tissue into patients with Parkinson's disease.[26] The approval of the protocol was withheld until an advisory committee could be established to evaluate the legal, ethical, and social implications of fetal tissue transplantation. The result was a temporary moratorium declared on all federal funded research involving transplantation of tissue from induced abortions into humans.[27]

In 1988, with the fetal tissue research debate still alive, a Human Fetal Tissue Transplantation Research Panel convened to deliberate fetal tissue research funding. The panel was composed of individuals representing a variety of viewpoints on the use of fetal tissue and abortion in general. In a 19-2 vote, the panel concluded that "it [was] of moral relevance that human fetal tissue has been obtained from induced abortion," and that fetal tissue transplantation research was "acceptable public policy." The panel's decision was accepted by the NIH advisory committee and then sent to the HHS with a request to lift the moratorium. Despite the vote, in 1989 the HHS Secretary Louis Sullivan elected to override the vote and extend the

moratorium indefinitely as the administration concluded that permitting fetal tissue transplantation research would increase the incidence of abortion. They argued that "additional rationalization of directly advancing the cause of human therapeutics cannot help but tilt some already vulnerable women toward a decision to have an abortion."[28] Two separate bills were introduced to Congress in an attempt to lift the ban on research, but both bills were vetoed by President George H.W. Bush and Congress failed to override the veto.

In 1993, the Clinton administration introduced a significant shift in national biomedical policy. In response to lawsuits filed against former Secretary Sullivan regarding research on fetal tissue research, the new HHS Secretary Donna Shalala removed the ban on federal funding for human fetal tissue research.[29] In February 1993, Secretary Shalala officially rescinded the moratorium in accordance with the Clinton administration's executive order, and temporary guidelines were established by the NIH for research involving human fetal tissue transplantation.

In the meantime, legislation was proposed and introduced in Congress, and on June 10, 1993, President Bill Clinton signed the National Institute of Health Revitalization Act into law. The act gave the NIH direct authority to fund human embryo research for the first time, and also overturned the Ethics Advisory Board approval requirement and allowed the NIH to appoint a Human Embryo Research Panel. The panel was charged to "provide advice as to those areas acceptable for federal funding." It is important to note that not a single human embryologist was among the members appointed to the panel. The Panel met for the first time in January1994 and made numerous recommendations for research which are outlined below: 1) it allowed for the creation or manufacture of human embryos as research objects with no intent of implantation, 2) removal of ovaries from women who were brain-dead was allowed so that ova could be recovered for laboratory fertilization and manipulation, 3) testing a wide array of drugs on the

developing human embryo, 4) human parthenogenesis, the formation of an embryo without fertilization by sperm was allowed, 5) scientists could use human embryos to create specific cell lines, 6) cells separated from human embryos to duplicate individuals or to freeze and save as potential "spares" 7) couple perform tests on human embryos for developing new lines of contraception 8) could allow for the fusion of cells or DNA fragments with those of the human embryo with the expectation of further development.[30] President Bill Clinton rejected some of the recommendations in response to thousands of letters from the public urging him to reverse his previous decision. He directed the NIH not to allocate funds to experiments that would create new embryos specifically for research. As described previously, the Dickey-Wicker Amendment introduced as a part of the Act sought to enforce President Clinton's strict guidelines against the use of creating embryos and furthered the restrictions to ban the use of federal funds for any experiment in which a human embryo is either created or destroyed. Congress has actively renewed this ban in each subsequent year and thus all human embryo research has been relegated to the private sector.

1998 was a significant year for those in the ES cell research community and once again ignited the debate regarding human embryo research. James Thomson, a professor at the University of Wisconsin School of Medical and Public Health and the founder and Chief Scientific Officer of Cellular Dynamics, was the first to isolate cells from the inner cell mass of early human embryos and from those cells developed the first embryonic stem cells. The research was published in the journal, *Science,* and later was featured as the "scientific breakthrough of the year."[31] Almost simultaneously, John Gearhart, Director of Research and of the Developmental Genetics Division of the department of Gynecology and Obstetrics, successfully derived germ cells from cells in fetal gonadal tissue, also referred to as primordial

germ cells. Both Thomson and Gearhart were able to develop pluripotent stem cell lines from both sources to be used for basic research on the human body and drug discovery and testing.[32]

Based on Thomson's and Gearhart's successes, the NIH, with input from the National Bioethics Advisory Commission, developed guidelines on the types of ES cell research that would be eligible for federal funding based on Harriet Rabb's legal opinion that embryonic stem cells "are not a human embryo within the statutory definition," and thus the Dickey-Wicker Amendment which would forbid federal funding of them would not apply. The second Clinton Administration's guidelines published in August of 2000 stipulated that human embryonic stem cells must be derived from private funds from frozen embryos from fertility clinics, must have been created for fertility treatment purposes, and be in excess of the donor's clinical needs. Consent must also be obtained from the donor in order to use the embryos. The guidelines also outlawed the federal funding of stem cells derived from embryos created by Somatic Cell Nuclear Transfer (SCNT), even if the derivation of cell lines occurred with the use of private funds.[33] Due to the aforementioned successes, President Clinton softened his previous views on ES cell research and strongly endorsed the new guidelines, saying that this research promised "potentially staggering benefits."

With the beginning of the George W. Bush Administration in January of 2001, it marked yet another momentous shift in ES politics and legislation. Bush's first action in office was to order a review of the current NIH guidelines by the Secretary of Health and Human Services Tommy Thompson. During his first few months in office, President Bush delayed his decisions on the guidelines and repeatedly rebuffed attempts of the media to generate discussion about the issue. Despite heavy pressure of members of Congress on both sides of the aisle to support ES cell research, President Bush decided against funding for this research. In August 2001, he

formally announced that cell research would go forward but only on cell lines already in existence, therefore prohibiting any and all future federal funding of any research using stem cell lines derived after August 9, 2001. President Bush decided that for cell lines already in existence, research, and in turn federal funding, would be permissible because "the life-and-death decision has already been made." In addition, the stem cells must have been derived from an embryo that was created for reproductive purposes and was no longer needed, and lastly, informed consent must have been obtained for the donation of the embryo and that donation must not have involved financial inducements.[34] It was determined that 24-25 cell lines existed that were established embryonic stem cell lines and 64 "derivations" that were in development in accordance with the established guidelines making them eligible for use by federally funded researchers.[35]

Numerous attempts were made by members of Congress to reverse the ban on funding for ES cell research. In February 2005, the Stem Cell Research Enhancement Act was introduced to Congress that would allow federal funding on stem cell research involving embryos that were "leftover" (spare). The bill passed in both the House of Representatives and the Senate, President Bush vetoed the bill, saying that it "crossed a moral boundary that our decent society needs to respect." The bill was reintroduced to Congress 2 years later, but once again, it garnered the same results as 2005.

With the election of President Barack Obama in 2008, ES cell research policy experienced its most recent shift and represents the current legislation. On March 9, 2009, President Obama, through an executive order, overturned the Bush policy and expanded the number of human embryonic stem cell lines eligible for federal funding. The executive order stated "the Secretary of Health and Human Services, through the Director of the NIH, may

support and conduct responsible, scientifically worthy human stem cell research, including human embryonic stem cell research, to the extent permitted by law."[36] The order did not lay out specific ethical guidelines, rather, it provided 120 days for the development of such guidelines by the NIH.

The new NIH guidelines were published in April 2009, and allowed funding for research on stem cells derived from donated embryos that were leftover from fertility clinics provided that a number of conditions be met, including the voluntary informed consent of donors. The NIH also promised to continue to fund alternative forms of research including adult stem cells and induced pluripotent stem cells but prohibited federal funds to be granted for research on embryos or on stem cells derived by research cloning techniques or parthenogenesis. As in the previous guidelines, current requirements include strict informed consent provisions: 1) donors are not allowed to receive money or other incentives for the use of their embryos, 2) the decision to donate must be void of the influence of researchers, as well as separate from the decision to seek fertility treatments, 3) researchers must provide documentation for requirements including the donor's awareness of all options for the use of embryos, 4) documentation that the donor's understanding of what is occurring to the embryos used for research, and finally that 5) the donor must not be able to directly use the stem cells for any particular individual's medical care.[37] These guidelines vastly expanded federal funding for ES cell research, once again opening up the moral quandary of destroying human embryos to benefit others.

The most recent debate in legislation over ES cell research that has captured media headlines for nearly a year, involves interpretation of the Dickey-Wicker Amendment, one of the most heavily debated amendments in government since its passage back in 1996. On August 23, 2010, U.S District Judge Royce C. Lamberth issued a preliminary injunction which barred the

NIH from funding human embryonic stem cell research. His ruling was based on the conclusion that ES cell research was "research in which a human embryo or embryos are destroyed," and thus, federal funding for this research is prohibited by the Dickey-Wicker Amendment as it is not possible to disentangle ES cell research from the derivation of stem cells even if private funding is utilized.

The injunction was issued in response to a lawsuit brought by scientists James Sherley of the Boston Biomedical Research Institute and Theresa Dreisher of AVM Biotechnology in Seattle. They argued that funding of ES cell research would cause them "irreparable injury" by increasing competition and stealing funding away from adult stem cell research. The plantiffs also argued that federal funding for ES cell research was also prohibited under the Dickey-Wicker Amendment as human embryos are "knowingly subjected to risk of injury or death greater than that allowed for research on fetuses *in utero*." They continued that funding for ES cell research also endangered embryos by creating a demand for additional stem cell lines and therefore increased the chances that a particular embryo might be used as a source of stem cells. They also contended that when the NIH adopted new funding rules for ES cell research, it violated the Administrative Procedure Act, which was responsible for governing rule-making by federal agencies. The basic purposes of the act were to require agencies to inform the public of the organization itself and its procedures and laws, to provide for public participation in the rulemaking process of agencies, to establish uniform standards for rulemaking conduct and adjudication, and to define the scope of judicial review. The claim was made that the NIH violated this law by ignoring and prejudging public comments submitted in opposition to the unlawful actions of the agency. The NIH received approximately 49,000 comments prior to the issuance of the guidelines and over 30,000 of the comments opposed the use of federal money

for ES cell research. Despite the overwhelming lack of support for the proposed guidelines, the NIH disregarded the comments calling for no ES cell research funding, considering them "unresponsive" and implemented the guidelines regardless.[38, 39]

The government appealed the injunction, asking Judge Lamberth to suspend it, but when he refused, the U.S Appeals Court for the D.C Circuit halted the injunction upon request from the Obama Justice Department. On April 29, 2011 Judge Lamberth's preliminary injunction was formally revoked by a 2-1 decision of the U.S. Court of Appeals for the District of Columbia Circuit. The court based its ruling on its opinion that ES cell research is not prohibited under the portion of the Dickey-Wicker Amendment that bars federal funding of research "in which a human embryo or embryos are destroyed." It also ruled that the government would be harmed by granting an injunction to a significantly greater degree than the plaintiffs would be by not granting one. They also argued that if the injunction was granted, it would place unreasonable burdens on human embryonic stem cell researchers.[40] The court of appeals decision resolved only one of the issues raised by the plaintiffs, and thus, the case shifted back to hands of Judge Lamberth to rule on the two remaining issues. Responding to the decision of the Appeals Court, Judge Lamberth dismissed the Sherley vs. Sebelius lawsuit, saying that the decision discriminates against researchers who do not use embryonic stem cells. Since the Court Circuit determined that research was ambiguous in the Dickey-Wicker Amendment, Lamberth said his court "has become a grudging partner in a bout of linguistic jujitsu, such is life for an antepenultimate court." Lamberth said he was bound to follow the appeals court decision and had no choice but to dismiss the lawsuit.[41, 42]

Embryonic Stem Cell Research at the State Level

Despite the ever-changing ES cell research funding legislation on the federal level, state governments have discovered ways to skirt restrictive national legislative policies, and many have passed legislation that has propagated ESCR funding on the state level. In 2004, New Jersey became the first state to appropriate funding to advance ESCR. New Jersey law both permits and supports ES cell research, as well as cloning of human embryonic stem cells for research and therapeutic purposes.[43] California followed suit shortly after by passing Proposition 71 in November of 2004 to fund both embryonic and adult stem cell research. The measure authorized the issuance of bonds in the amount of $3 billion beginning in 2005, spanning over a 10 year period. It established a state constitutional right to pursue stem cell research including the use of somatic cell nuclear transfer (SCNT) or research and therapeutic cloning, but still prohibited funding of human reproductive cloning research. The California Institute of Regenerative Medicine (CIRM) is responsible for the administration of the state stem cell research program.[44] There has been significant backlash from the taxpayers in recent years to continue funding as ES cell research continues to yield fruitless results. Commenting on CIRM on behalf of the LA Times, reporter Michael Hiltzik had the following to say: "It goes without saying that it hasn't found those cures, though not for want of spending. Nor has it managed to temper public expectations pumped up by the original initiative campaign, or resolved the persistent questions about whether its grant-making process is subject to adequate public oversight or free of conflicts of interest."[45] Connecticut also renounced support for Bush's restrictions on ES cell research by signing legislation in June of 2005 to provide $10 million a year to fund stem cell research. In 2005, Illinois governor Rod Blagojevich bypassed State Legislature twice to pass a law to allocate state funds for ESCR. His executive order created the

Illinois Regenerative Medicine Institute (IRMI) and transferred $10 million to provide grants to medical research for embryonic and adult stem cell research. In addition, the Illinois legislature passed a bill in 2007 permitting the IRMI to conduct research on stem cells from any source.[46]

The state of Iowa has taken its own stance on funding for stem cell research as well. In March of 2007, Governor Culver signed a bill into law that overturned the state's previous ban on human cloning, and allowed scientists to utilize embryonic stem cells from human fetuses that are cloned and destroyed. The bill repealed a 2002 ban that limited the use of embryonic stem cells in research by specifically prohibiting human cloning and the creation of stem cells through SCNT. Ironically, the measure was passed through the Senate in a 26-24 vote because a pro-life legislator inadvertently voted 'Yes" and Representative Brian Quirk, a democrat who promised to oppose the bill, changed his mind last minute after a phone call from Sheryl Crow, a staunch supporter of ES cell research. A number of Iowa voters and pro-life groups have expressed dissatisfaction with the vote. Kim Lehman, the former president of Iowa Right to Life and current board member of the John Paul II Stem Cell Research Institute in Coralville, Iowa, condemned the vote saying the new law "will allow scientists to begin cloning humans for research [and] by doing this Iowa is turning humans into a commodity for science."[47]

Many states have restricted research on aborted fetuses and embryos, but research is occasionally permitted with consent from the donor. Nearly half of the states also restrict the sale of fetuses and embryos. As of 2008, Louisiana is the only state that specifically prohibits research on *in vitro* fertilized (IVF) embryos. Illinois and Michigan also have laws prohibiting research on live embryos. Arkansas, Indiana, Michigan, North Dakota, and South Dakota prohibit research on cloned embryos. Virginia's law may restrict this research as well, but because the definition of a human being has not been defined in its law, the statute leaves room

for interpretation. Finally, California, Connecticut, Illinois, Iowa, Massachusetts, Missouri, New Jersey, New York, and Rhode Island have statutes that prohibit human cloning for the purpose of initiating pregnancy, also known as reproductive cloning, but allow cloning for research and therapeutic purposes.[48]

Embryonic Stem Cell Research at the International Level

In addition to individual state laws, it is also important to compare United States legislation on ES cell research to laws in other countries around the world governing ES cell research. It is easiest to compare policies across the globe by grouping each country's legislation into one of three defining categories: permissive, flexible, or restrictive. Permissive policy refers to legislation allowing for various embryonic stem cell techniques for research including therapeutic and reproductive cloning including SCNT. In addition to the United States, a number of other countries allow for extensive ES cell research including: Australia, Belgium, China, India, Israel, Japan, Singapore, South Korea, Sweden, and the United Kingdom. In contrast, flexible policy permits stem cell derivations from fertility clinic donations only, excluding SCNT, and often under strict restrictions. Countries that govern under these policies include: Brazil, Canada, France, Iran, South Africa, Spain, The Netherlands, and Taiwan. Countries with a restrictive policy include Austria, Germany, Ireland, Italy, Norway, and Poland. Restrictive policies range from the outright prohibition of human embryo research, to permitting research on embryonic stem cell lines only, to permitting research on a limited number of previously established stem cell lines. Additionally, other countries, such as Turkey, have no specific guidelines and regulations that have so far been defined by legal or governmental institutions regarding the legality of ES cell research.[49]

Despite the United States increasing lenience and promotion of ES cell research at the federal and state levels, other areas around the world such as Europe, are installing policies and legislation to limit the use and reach of human embryo research. On March 10, 2011 the European Court of Justice issued a preliminary opinion stating that procedures established using human embryonic stem cell lines were not patentable. The decision of the European Court, issued by Judge Yves Bot, was in response to a request by the German Supreme Court for clarification of the legal definition of human embryos in relation to their patentability. The request was brought to attention by a German court case challenging the patent of a technique to generate nerve cells from established human embryonic stem cell lines. The case was filed by the Amsterdam activist based organization, Greenpeace, which argued that patenting procedures derived from embryonic stem cell lines was unethical because the lines are derived from human embryos. Judge Bot's preliminary opinion will now go before the Court's Grand Chamber composed of 13 judges. If the Grand Chamber votes in favor of the preliminary opinion, it will put significant restrictions on the work of European ES cell research.[50]

Discussion of the Legal Inconsistencies of Funding Embryonic Stem Cell Research

The nation and the government alike have become increasingly enamored with the use of human embryos for research in recent decades under the belief that the stem cells derived from these embryos will provide the basis for medical miracles and have potential to save lives and mitigate human suffering. The question that must be addressed before allowing such research to continue is whether ES cell research satisfies the laws governing human subject research. Based on analysis of the policies and laws that have been established over time and the current legislation in place today, it is evident that inconsistencies exist between policy and the funding of ES research. Many believe that the US government has chosen to ignore the moral concerns

and inconsistencies of ES cell research, and has been successfully able to masquerade them from the general public with a promise to "make scientific decisions based on fact and not ideology." The government also promises that participation in this research will allow for America to lead the world in discoveries it may one day yield and advance the progress of all humanity.

ES cell research is in direct violation of the 3 basic research principles: respect for persons, beneficence, and justice. The first principle, respect for persons, demands that the embryo be treated as an autonomous agent. The law was designed specifically to protect the most vulnerable of society, but the human embryo is not only being denied its right as an autonomous agent, and not given the full protection of law, but is also being denied its status as a human individual. The second principle of beneficence, which calls for protecting the individual from harm and ensuring its well-being, is out rightly disregarded in ES cell research. The ultimate harm associated with this research lies with the embryo. There are no safeguards in place to protect the human embryo from harm, nor any benefits provided to ensure the well-being of the embryo. Instead of being treated as an end in him or herself, the embryo is merely treated as a means to other ends. Finally, the principle of justice employs the provision that equals ought to be treated equally. Aside from the fact that embryos are being denied their status of moral equality, ES cell research imposes an unfair burden of the research on the human embryo and provides benefits solely to another sector of society as the embryo is destroyed in order to ensure benefits for others. In this way, the embryo is treated solely as an object or raw material to be used for public good.

Despite the acute inconsistencies between ES cell research and the well-established principles guiding human subject research, reports of current advances and the promise of future success in culturing embryonic stem cells has compelled the government to promote a particular

research agenda and prompted calls for federal funding of this research. In order to gain support from the public, lawmakers sought to find a way to separate ES cell research from the guiding bioethical principles of research. To change the perception of the general public, the government called into question the moral status of the embryo by asking whether cells that are so microscopic constitute a human life, in an attempt to dehumanize the embryo. Recent biological scientific findings have highlighted this misunderstanding of basic biology and embryology, as the findings have confirmed that in the zygote resulting from fertilization, the biological identity of a new human individual is already constituted.[51] Regardless of its widespread rejection, the government still employs the faulty logic behind the "pre-embryo" term to justify using the human embryo in the first two weeks of life, when scientifically, human life is to be viewed as a continuum from a one-cell stage onward.[51a]

Some individuals still, nonetheless, deny the moral status of the embryo as a person by claiming that such a status cannot be attached to an individual void of mental and physical capabilities. The ability in question is an either/or proposition, so that in order to divide "persons from "nonpersons" some arbitrary level of functioning must be stipulated. Or, it creates degrees of abilities in which case "personhood" must possess degrees as well. Cognitive ability is often cited as a criterion for personhood, but standards that are designed to exclude the unborn may also exclude some individuals already born, including people who are brain-dead, in a coma, or are Alzheimer's patients. This argument ultimately creates a slippery slope in which one will begin to cast doubt on the personhood of a far greater class of human beings including newborns and mentally retarded adults.[52]

A further example that illustrates the flawed reasoning employed in order to disconnect ourselves from our unborn counterparts is the Bald Eagle Protection Act passed in 1940. This

stringent federal law protects not only the bald eagle, but the national bird's eggs as well. If one was to happen upon these eggs in the wilderness it would be illegal for the individual to destroy or tamper with them. The penalties for these acts would be equivalent to those administered for the act of shooting the bird out of the air. By the force of law, the scientific truth has been acknowledged that the eagle's egg, that is to say the embryonic eagle inside that egg, is the same bird that is hailed as our national symbol of freedom and spirit. If bald eagles are valuable for the pragmatic reasons of conservation it is fitting to protect them in all stages of their existence. The same logic holds for humans for intrinsic reasons. It is disconcerting that the government and general public are able to understand the importance of protecting the earliest stages of animal life but do not see the same importance in protecting human life, thereby dissociating humans from their humble embryonic origins.[53]

'Linguistic gymnastics' have also been used regarding federal funding for ES cell research through manipulation of existing federal laws to coincide with the government's agenda. This tactic was employed on the Dickey-Wicker Amendment and single-handedly allowed for the passage of Obama's federal policy which allowed for the federal funding of ES cell research to proceed unabated. The Dickey-Wicker Amendment expressly states that no funds made available may be used for the creation of a human embryo or embryos for research purposes or research in which the 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*. It also provided a definition for the human embryo term. Harriet Rabb, a lawyer for the Department of Health and Human Services, released a legal opinion which outlined her personal interpretation of the law seeking to skirt its prohibitory nature. She concluded that because human embryonic stem cells are not a human embryo within the statutory definition, the Dickey-

Wicker Amendment does not apply to them. With this interpretation, the NIH was free to provide federal funding to experiments involving these stem cells as well as stem cell lines derived from private funding sources.[54] Not all members of Congress agree with this tactic as Kansas senator Sam Brownback described Rabb's opinion as a bit of "legal sophistry." Judge Lamberth, responsible for ruling on the lawsuit involving the Dickey-Wicker Amendment, made the following statement:

This prohibition encompasses 'all research' in which an embryo is destroyed, not just the 'piece of research' in which the embryo is destroyed. Had Congress intended to limit the Dickey-Wicker to only those discrete acts that result in the destruction of an embryo, like the derivation of ESCs, or to research on the embryo itself, Congress could have written the statute that way. Congress, however, has not written the statute that way, and this Court is bound to apply the law as it is written.[55]

He went on to say "If one step or 'piece of research' of an ES research project results in the destruction of an embryo, the entire project is precluded from receiving federal funding." [56]

Lamberth's comments reveal yet another ethical concern associated with funding for ES cell research: the issue of complicity. During the early 1990's federal government debated on the use of fetal tissue from induced abortions and whether to allow for federal funding for the use of this tissue in human transplantation. Since 1993, federal funding has been granted for the use of fetal tissue from natural and induced abortions under stipulated conditions that were created in an attempt to separate the act of the abortion from the intentions of the researchers. Tissue could be harvested only after the embryo or fetus had died, the woman's required consent for harvesting the tissue could only be obtained after she had made the decision to get an abortion, and the timing and method of the abortion could never be altered to specifically accommodate

the needs of the researcher. Through the implementation of these conditions, Congress has made assurances to the public that the researcher who obtains tissue from an abortive fetus does not necessarily hold the same view of the moral status of the embryo. This falsely perceived lack of complicity has allowed Congress to pass laws in favor of fetal tissue research.

The same logic as described above has been used by those participating in the destructive harvesting of stem cells from living embryos in an attempt to gain federal funding for their research as well. Inconsistencies also exist with those that harvest ES cells from living embryos for research purposes. In this situation, those who harvest and/or use these cells are directly complicit in the destruction of the human embryo. If ES cell research were governed by the same conditions that have allowed for federal funding for research utilizing fetal tissue from abortions, cell harvesting must be done only after death, and researchers' needs must have no influence on the timing and method used in the destruction of the fetus. Therefore, under these conditions, ES cell research would not be performed at all: the stem cells are extracted from living embryos, and the method of destruction and when the extraction of these cells occurs is determined entirely by the needs of the researcher. Whether or not private funds are used to subsidize the work deriving stem cells from the embryo, it cannot be disputed that the act is an integral part of the research. Based on these conditions, it is evident that any means of funding from the federal government is directly promoting the destruction of human embryonic life, recognized by the National Bioethics Advisory Commission in 1999.

> *Whereas researchers using fetal tissue are not responsible for the death of the fetus, researchers using stem cells derived from embryos will typically be implicated in the destruction of the embryo. This is true whether or not researchers participate in the derivation of embryonic stem cells. As long as embryos are destroyed as a part of the*

research enterprise, researchers using embryonic stem cells (and those who fund them) will generally be directly or indirectly complicit in the demise of embryos.[57]

The NIH guidelines of 2009 specify that no federal funding may be used to create embryos specifically for the purpose of research, allocating funds solely to "spare" embryos from IVF clinics. These guidelines reveal inconsistencies in themselves. It is a commonly held belief by many that the use of embryos specially created from research purposes treat a nascent human life as a mere instrument for others' use. But, if creating embryos solely in order to perform destructive experiments is morally unconscionable, this must be due to the fact that the act of destroying embryos itself is morally wrong. If those who hold this belief would examine more closely their moral revulsion for this act, they would find that it is rooted in an argument against destroying embryos regardless of their origins.[58]

A final inconsistency of federal funding for ES cell research lies with the general opinion of the public regarding the use of federal tax dollars to fund this research. According to a 2010 national Rasmussen telephone survey, the majority of Americans (57%) believe that funding for ESCR should be left to the private sector. This percentage increased when respondents were informed of alternative methods for obtaining stem that promise no harm to the donor. Additionally, the overwhelming majority of the same respondents voiced disapproval of human cloning, as 76% of those surveyed did not support the use of human cloning in order to create human embryos to be destroyed for research purposes. Opinions of taxpaying citizens, potential recipients of results of this research, are essential when discussing the use of public resources such as federal tax dollars to fund these activities.

This argument is sure to raise a counterargument from supporters of ES cell research who will contend that a number of programs and activities are supported by the federal government

which have not received absolute support from the public or those individuals in the government. *Often cited as a prime example, is fighting in a war.. A significant segment of the American* population does not support war efforts and thus are opposed to the idea of the government using their tax dollars to fund the cause, although the government by the force of law may both declare and fund war nonetheless. ES cell research supporters argue that this research represents the same scenario and thus should be entitled to government support. When looking at the legal considerations surrounding funding for ES cell research and funding for war as well, it not only refutes the proposed argument outlined above, but also collectively represents one of the most compelling arguments against funding for ES cell research. Article 1 Section 8 Clause 11 of the United States Constitution not only vests in Congress the power to declare war but also to collect tax dollars from the country's citizens in order to provide for defense and welfare of the United States.. Support for war efforts has been embedded in American policy since the country was founded, much in contrast to legislation surrounding ES cell research. In fact, only two pieces of legislation address funding and the use of human embryos in research in general. Both the National Research Act of 1974 and the Dickey-Wicker Amendment set guidelines not only forbidding the use of human embryos for research but also the use of federal funding for any such research that utilizes human embryos. In addition, funding is in direct conflict with the Bill of Rights which established a government where the rights of the individual always supersede the rights of the state. By proposing support for ES cell research through federal funding, the state has the ability to decide the best use of an individual for its own purposes, putting the interest of the state over the individual for the first time in the history of American Law.

While countries should always strive for peace, this does not require pacifism. One cannot deny citizens and the government the fundamental right of armed defense. Provided

countries participate in "just war" and it is used as a last resort, it is morally justifiable to ensure individuals' protection and freedom. ES cell research, in contrast, cannot employ this same justification. . The existence of moral sentiment against harmful research on human embryos must be taken into account when judging the usefulness of the research and when deciding whether to apply federal funding to a controversial avenue of medical research. Society and the government alike must not be forced to become financially invested in an activity that is in direct conflict with the principles laid out in American legislation and policy.[59]

Significance of Moral Alternatives to Embryonic Stem Cell Research

The moral quandaries related to ES cell research and opposition to this research should not be confused with opposition to stem cell research in general. Many types of stem cell research have not only proven to be successful, but are also morally acceptable and even laudable. The most recent ground-breaking technology to enter the stem cell research community is induced pluripotent stem (iPS) cell research. iPS cells are adult cells that have been genetically reprogrammed to an embryonic cell-like state by being forced to express genes and factors that have been discovered to be essential for maintaining the defining characteristics of embryonic stem cells. Human iPS cells were discovered and first created nearly simultaneously and independently by stem cell scientists, Shinya Yamanaka and James Thomson in 2007. These iPS cells demonstrate important characteristics of pluripotent stem cells, cells that have the potential to become nearly all of the 200 cell types of the body. These defining characteristics include the expression of stem cell markers, and the capability to generate cell characteristics of all three germ layers of the inner mass of the human embryo. Both viral and non-viral methods are currently being used to introduce the necessary reprogramming factors into a variety of adult cells. This breakthrough technology has created a powerful new way to

"de-differentiate" adult cells whose developmental fate was thought to be previously determined. iPS cells are currently being used as tools of drug development and are in the process of being used for transplantation medicine to repair damaged tissue in the human body.[60]

Discovering Induced Pluripotent Stem Cells

Although the development of iPS cells and strategies for the generation of these patient-specific stem cells did not garner widespread support nor gain a foothold in the scientific community until 2006, prior to this date, extensive studies were being completed to develop strategies to generate pluripotent stem cells from somatic cells, any adult body cell other than gametes (egg and sperm). Chief among these strategies were somatic cell nuclear transfer (SCNT) to oocytes and fusion with embryonic stem cells. SCNT, also referred to as cloning, is a technique in which the nucleus of a somatic cell is transferred to the cytoplasm of an enucleated egg. Once inside the egg, the somatic nucleus is reprogrammed to become a zygote nucleus by cytoplasmic factors present in the egg. The egg is then allowed to develop to the blastocyst stage, at which time, embryonic stem cells can be cultured from the blastocyst's inner cell mass. The blastocyst may also be implanted into the uterus of a surrogate in which case the embryo develops into a fetus and is carried to term.[61] SCNT began in the 1950's when Briggs and King showed that nuclear transfer was possible in frogs by transplanting nuclei from blastula stage embryos into enucleated eggs. The embryos that resulted from this transfer developed into normal hatched tadpoles. Despite the increased difficulty of this method due to the small size of eggs, nuclear transfer was also carried out in mammals including rabbits and sheep in the late 1970's and early 1980's. A "breakthrough" for this research technique came in 1983 when scientists Campbell and Wilmut performed nuclear transfer on embryo-derived differentiated

cells and produced an adult sheep, famously known as "Dolly." Somatic cloning since has also been successful in other species including cows, mice, goats, pigs, cats, and rabbits.[62]

Results from studies highlighting nuclear transfer research showed that embryonic stem cells could be generated from cloned mouse blastocysts with an efficiency rate comparable to that of cells derived from normal embryos. Therefore, these nuclear transfer embryonic stem cells presented a possible means of avoiding immune rejection post transplantation therapy which is a hallmark setback of ES cell research. However, in 2005, a group in Korea reported the successful generation of nuclear transfer embryonic stem cells from the skin cells of individuals with spinal cord injuries and diabetes. Despite these reports, the data was later found to be fabricated and, in fact, the group was unable to generate a single nuclear transfer embryonic stem cell line from more than 2000 human eggs. In addition, this research does not skirt the requirement of the destruction of or use of human embryos for research purposes. Furthermore, cloned embryonic stem cells present an even stronger ethical quandary due to the fact that they involve the generation of human embryos exclusively for the production of embryonic stem cells. Due to these ethical objections, numerous countries have laws prohibiting cloning of humans for ethical reasons.[63]

Another strategy for reprogramming cells to a pluripotent state is fusion with embryonic stem cells. To carry out this technique, human somatic cells are combined with embryonic stem cells in the presence of a detergent-like substance that causes the two cell types to fuse. Determining whether fusion of the two cells types has been achieved by probing the fused cells for two distinctive genetic markers found in both the somatic cells and stem cells. Fusion can also be confirmed by examining the chromosomal make-up of the fused cells to determine whether they are tetraploid, meaning they contain combined chromosomes from both the somatic

cells and embryonic stem cells.[64] The first fusion experiments were conducted in 1965 when HeLa cells, cells from an immortal cell line, were fused with the Sendai virus in order to activate erythrocytes. In 1976, Miller and Rundle demonstrated that the fusion of thymocytes with embryonic carcinoma cells displayed pluripotency based on the observation that the transplantation of these cells into nude mice resulted in the formation of teratomas, consisting of tissues from each of the three germ layers. Reprogramming by fusion with mouse embryonic stem cells has also been successfully demonstrated as fused cells were observed to adopt the DNA methylation and histone modification patterns found in embryonic stem cells.[65]

Whether or not the somatic genomes are fully reprogrammed to an embryonic-like state remains to be confirmed. Based on the methylation and histone patterns, it is evident that the somatic genome is at least partially reprogrammed by fusion, but further analysis must be conducted to reveal the actual extent to which the somatic genome is reprogrammed. Unlike SCNT, rejection of these cells upon implantation is still an obstacle to be overcome because of the embryonic stem-cell-derived chromosomes that are present. In addition, little is understood about the molecular mechanism underlying the reprogramming of somatic cells with embryonic stem cells though it has been hypothesized that factors responsible for reprogramming reside either in the nucleus or cytoplasm of the cell. As with SCNT, this method of reprogramming still encapsulates the overarching moral and ethical objections that accompany the use of embryos and embryonic stem cells to carry out the research.[66]

In 2000, in an effort to devise a method that displayed increased success and efficiency and was void of ethical controversy, leading stem cell scientist Shinya Yamanaka and his laboratory began testing the idea that factors that maintain pluripotency in embryonic stem cells might induce pluripotency in somatic cells. Successful reprogramming of somatic cells by

fusion with embryonic stem cells indicated that embryonic stem cells have factors that have the ability to induce pluripotency. Therefore, Yamanaka hypothesized that it seemed probable that these pluripotency-inducing factors would also execute important roles in the maintenance of pluripotency as well. Based on this hypothesis, 24 factors were selected as initial candidates based on the observation of an important role they had in the cell or the specific expression of them seen in mouse embryonic stem cells. These 24 factors were then tested for their ability to induce pluripotency. In order to evaluate this ability, combinations of the factors were introduced into mouse embryonic fibroblasts (MEFs) through the use of a retroviral vector. The cells were also designed to carry a gene resistant to antibiotics that would be expressed only when one of the mouse embryonic stem cell genes, *Fbx015*, specific for pluripotency was turned on. Yamanaka and his team furthered their previous hypothesis stating that it should be expected that the *Fbx015* gene would be turned on if pluripotency was induced by any combination of the 24 genes.[67]

In 2006, Yamanaka demonstrated that introduction of a combination of these factors into MEFs could convert them into cells with a striking resemblance to pluripotent embryonic stem cells – a revolutionary breakthrough in the scientific community. When each of the 24 genes was introduced into the MEFs individually no colonies grew. However, when retroviruses were mixed containing combinations of the 24 factors, numerous colonies were observed. Based on further analysis, it was discovered that retrovirus-mediated introduction of only four of the 24 transcription factors (Oct-3/4, Sox2, c-Myc, and KLF4) into the MEFs was needed in order to result in the generation of induced pluripotent stem cells. These iPS cells showed high similarity when compared to embryonic stem cells in terms of their morphology, proliferation, and the presence of teratoma formation. However, these initial iPS cells that were selected for *Fbx015*

also displayed significant differences compared to embryonic stem cells as well in terms of different global gene expression patterns, and DNA methylation patterns. When these iPS cells were injected into blastocysts, the cells gave rise only to chimeric embryos but were not able to generate adult or germ line competent chimeras. This led to the conclusion that these initial iPS cells exhibited only partial reprogramming.[68]

Three separate groups since have sought out to improve the reprogramming of these iPS cells, and have generated success by substituting Nanog for *Fbx015* as the selection marker. In contrast to *Fbx015* selected iPS cells, those selected for Nanog show gene expression patterns with a significantly increased similarity to embryonic stem cells. Additionally, the Nanog selected iPS cells also contributed to the generation of adult and germ line chimeras. While both *Fbx015* and Nanog are targets for the Oct-3/4 and Sox2 genes, the data suggests that *Fbx015* is not essential for pluripotency while Nanog has a critical role in allowing the cell to develop and maintain a pluripotent state. The results generated by Yamanaka and his team upheld the original hypothesis formulated. They demonstrated that full reprogramming of somatic cells indistinguishable from their embryonic stem cell counterparts was feasible through the combination and expression of four specific factors as well as choosing the proper selection marker.[69]

Four Factors Regulating Induced Pluripotent Stem Cell Reprogramming

Oct-3/4

The most well characterized gene in regards to pluripotency is the Oct-3/4 gene, also referred to as Pou5f1. Oct-3/4 was identified as a novel protein belonging to the Octamer binding protein family, and was specifically expressed in embryonic carcinoma cells, early embryos, and germ cells. Expression of this protein was restricted to the blastomeres of

developing mouse embryos, the inner cell mass of blastocysts, germ cells, and pluripotent stem cells and is known as the master regulator of pluripotency. Expression levels of Oct-3/4 are down regulated upon differentiation of pluripotent cells and its central role, to maintain stem nature, was demonstrated in embryos that were Oct-3/4 deficient. These Oct-3/4 deficient embryos did not grow beyond the blastocyst stage and did not display the presence of pluripotent cells in their inner cell mass. In addition, suppression or overexpression of Oct-3/4 resulted in spontaneous differentiation into trophoblast lineages in both the mouse and human embryonic stem cells. This data effectively demonstrates the essential role of the Oct-3/4 protein in the maintenance of pluripotency.[70]

Oct-3/4 also maintains an essential role in the promotion of differentiation. Small increases in the protein in mouse embryonic stem cells have led to spontaneous differentiation of the cell into endoderm and mesoderm, consistent with the increase of Oct-3/4 expression observed during the initial stages of the differentiation of the endoderm from the inner cell mass of the blastocyst. Studies have also shown that Oct-3/4 also plays a key role in neural and cardiac differentiation in mouse embryonic stem cells suggesting that the level of Oct-3/4 expression is central to the cell fate in mouse embryonic stem cells.[71]

Sox2

Sox2 is part of a family of DNA binding proteins known as sex-determining region Y (SRY) related high mobility group (HMG-box) proteins. Sox proteins interact with and recognize a specific DNA binding motif on gene enhancers through the use of their HMG domains. Sox2 also interacts with numerous co-activators including Oct-3/4 which serve to maintain more stable and efficient DNA binding. Similar to Oct-3/4, Sox2 is expressed in the inner cell mass of embryos and is essential for development. In addition, both Oct-3/4 and Sox2

mark the pluripotent lineage of early embryos. However, unlike Oct-3/4, Sox2 is expressed in multipotent cells in addition to its expression in pluripotent cells. Additionally, its expression is associated with uncommitted dividing of stem cells and precursor cells and has been detected during the development of the central nervous system, the inner ear, pituitary gland, forebrain, eye, lungs, stomach, as well as adult neural cells. The expression of this gene can also be used to isolate such cells.[72]

Embryos that are without Sox2 will die at the time of implantation due to the lack of the development of the primitive ectoderm. The blastocysts of the embryos appear to be morphologically normal, but when they are cultured *in vitro*, the undifferentiated cells fail to proliferate and differentiation results. Therefore, just as with Oct-3/4, Sox2 is essential in maintaining the pluripotency of a cell. Additionally, analyses have determined that Oct-3/4 and Sox2 share a number of similar target genes in both mouse and human embryonic stem cells. This led to the observation that Sox2 deletion in embryonic stem cells is not only corrected by the cDNA of Sox2, but also Oct-3/4. These findings suggested to researchers that in addition to the functions outlined above, a primary function of Sox2 may be to maintain appropriate expression of the Oct-3/4 gene.[73]

c-Myc

c-Myc was one of the first protooncogenes to be discovered in human cancers and is required for cell growth and proliferation. In addition, c-Myc has a large number of binding sites found in the genome and it has been hypothesized that it modifies chromatin structure and also can activate expression of various microRNAs. Although it has been determined the c-Myc is not required for iPS cell induction, it has been shown to significantly enhance the efficiency and speed of the induction process. Despite these positive benefits, the gene cannot be used

clinically, as animals injected with iPS cells transduced with c-Myc developed tumors. In addition, it has also been linked to a number of cancers.[74]

KLF4

KLF4, also referred to as Kruppel-like factor 4, is a zinc finger protein that is primarily responsible for regulating cell proliferation and differentiation. It also plays an important role in regulating the molecular events that allow a cell to achieve pluripotency. Embryonic stem cells with an overexpression of the transcription factor were found to display higher levels of the Oct-3/4 gene, and therefore, were better able to more successfully display the ability to self-renew. KLF4 is also directly involved in the inactivation of a tumor suppressor gene, p53, which represses Nanog, thereby inhibiting the expression of a selection marker essential for pluripotency. The inhibition of KLF4 has been shown to suppress pluripotency and initiate differentiation. Studies done with murine epistem cell lines that actively expressed Sox2, Oct-3/4 and Nanog were not able to revert back to an embryonic stem cell-like state. It was only when KLF4 was reintroduced to the cells through ectopic expression that the cells were able to revert back to a previous state.[75]

KLF4 can also function as both an oncogene and a tumor suppressor gene. In cells in culture, the expression of KLF4 leads to the inhibition of DNA synthesis as well as the termination of cell cycle progression. Because embryos without the presence of KLF4 develop normally but newborn mice without the gene die within a short period of time and display impaired differentiation in the skin, this suggests that KLF4 has an important role in regulating the transition from proliferation to differentiation. [76] KLF4 is also associated with a number of cancers such as gastric, bladder, and pancreatic cancer. Therefore, because both up regulation and down regulation have been shown to contribute to cancers, instead of the manipulating the

gene, efforts should be placed in finding genes or compounds to substitute for the current transcription factor. Currently known substitutes that have been identified include the orphan nuclear receptor Esrrb, Kenpaullone, and butyrate.[77]

Mechanism of Conversion to Pluripotency

While scientists clearly understand the elegance that surrounds the concept of reprogramming cells to a pluripotent state, the precise mechanism in which this occur remains highly elusive.. It is understood that expression of the four transcription factors necessary for conversion is only required for two weeks in order for cells to be reprogrammed. During these two weeks, a cascade of molecular events occurs, resulting in the silencing of endogenous genes that are active, allowing cells to maintain a specialized, differentiated state. Host genes are then activated that are required in order for the cell to demonstrate pluripotency and gain the ability to self-renew. Once the reprogrammed state has been attained by the cell, it has been observed to be maintained, suggesting that epigenetic modification of DNA appears to be essential for the induction of pluripotency and maintenance of this state. Though stable, the process of conversion remains highly inefficient as the average reprogramming frequency with human somatic cells is around the order of 1 in 1000 cells. These iPS cells are also observed to emerge in culture approximately 40 days following exposure to the reprogramming transcription factors.[78]

Stem cell scientist Yamanka has sought out to propose a model to explain the dismal efficiency, as well as, reports from several groups that outlined experiments in which cells seemed to be pluripotent based on observed characteristics but were discovered to be only partially reprogrammed. Due to this fact, the cells remained dependent on the continuous transgene expression of the four reprogramming factors in order for them to retain the ability to

self-renew. Two models have been proposed to provide an explanation for the low reprogramming efficiency and the partial reprogrammed nature observed in iPS cell generation.[79]

The Elite Model

The elite model, which can be further subdivided into a predetermined elite model and an induced elite model, suggests that only a select number of cells are competent for reprogramming. In the predetermined elite model, only a small number of cells are suitable for reprogramming even prior to the transduction of the four reprogramming factors. Elite cells that have been determined to be predisposed to successful reprogramming are stem cells found in tissues and any other undifferentiated cells which are regenerative in nature.[80]

There are two key pieces of evidence that serve to contradict this proposed model. Most importantly, the efficiency of generating iPS cells has increased significantly from the efficiencies outlined in previous experiments regardless of the cell type used. The generation efficiency was increased over 10-fold from previous reports through the use of specific chemicals to assist in the induction to pluripotency as well as delaying the timing of selection for Nanog expression. Because it is highly unlikely that the 10% increase in efficiency can be attributed solely to the presence of cells comprised of 10% tissue stem cells or other undifferentiated cells, this suggests that a wide variety of somatic cells are being reprogrammed during iPS cell generation.[81]

The second piece of evidence to contradict the predetermined elite model was discovered through genetic lineage tracing analyses. The analysis data showed that iPS cells had been generated from fibroblasts, but more surprisingly, from various tissues as well, including the pancreas and the liver. These analyses also revealed that a number of the iPS cells were obtained from cells in the liver and pancreas which expressed albumin and insulin respectively. This

clearly suggests that in addition to undifferentiated cells, the four reprogramming factors have the ability to reprogram lineage-committed cells that had already undergone differentiation. This data also demonstrates that a wide range of lineage-committed cells are capable of being reprogrammed through defined factors, although it is still understood that a few select cell types may be more easily and efficiently reprogrammed than others.[82]

In the induced elite model, genes from a number of factors, in addition to the four reprogramming factors, must first be activated or inactivated through viral integration in the genome of the host cell. Similarly to the predetermined elite model, the induced elite model proposes that only cells containing specific viral integration sites are suitable to be reprogrammed. Data has been presented that suggests an alternative model for reprogramming needs to be proposed. It has been found that iPS cells created from tissues such as the liver and skin contain fewer viral integration sites than those derived from fibroblasts. In addition, further analysis revealed that iPS cells derived from a common source displayed no similar viral vector insertions. Therefore, this indicates that viral integration in specific sites in the cell is not required in order to generate viable iPS cells. Furthermore, since this model has been proposed, a number of reports have come forward that have successfully generated iPS cells with alternative methods that did not incorporate retroviruses including the use of adenoviruses and plasmids, both of which did not result in integration. Even more recently, iPS cells have been created through protein transduction and episomal expression vectors in which the episomal DNA disappeared throughout the culture of the cells. It has been determined that creating iPS with viral integration is much more efficient by enhancing reprogramming. It has also been discovered that the position of the viral integration sites is important to successful generation

which would serve to explain why only a select few of the transduced cells are fully reprogrammed leading to the generation of an iPS cell.[83]

The Stochastic Model

This model states that all cells have the potential to become an iPS cell following transduction with the four reprogramming factors. In normal development, pluripotent cells are transient, that is, they cannot maintain this state for extended periods of time, and differentiate into a variety of different cell types. On the other hand, embryonic stem cells are capable of self-renewal and have the ability to maintain a pluripotent state for an extended period of time. The four reprogramming factors introduced to somatic cells seem to function to not only allow previously differentiated cells to revert back to pluripotency, but also to retain this pluripotent state once it has been reached.[84,85]

At least two requirements have been determined to ensure complete reprogramming of cells. It is critical that the four reprogramming factors are expressed in a pattern that provides the cell with the capability to reach a pluripotent state. This requirement is not always achieved as the current reprogramming technologies cannot control the expression levels in a precise manner. The second requirement is that each cell must be blocked by an 'epigenetic bump' so that they remain in a pluripotent state even in the absence of transgene expression. This requirement, much like the first, is achieved only in a stochastic manner as each of the four factors expressed alone does not create the required 'epigenetic bump.' Other reprogramming methods such as nuclear transfer have effectively shown that both DNA methylation and modification of histones are believed to play crucial roles in the induction of cells to pluripotency.[86]

It has been determined that amount, balance, continuity, and silencing of the transgene expression of the four reprogramming factors each play an important role in the direct reprogramming of cells. Data suggests that strong transgene expression is initially required, but continued endogenous expression is not required to produce iPS cells. In addition, iPS cell generation is also dependent on the balance as an excess of both Oct-3/4 and Sox2 had negative effects on a cell's ability to maintain pluripotency. The balance between c-Myc and KLF4 was found to be essential in order to prevent apoptosis of cells and senescence that would be caused by an overexpression of either of the factors. iPS cell generation is also dependent on continuity and silencing of transgene expression as maintenance of this expression must remain for at least 14 days to ensure successful induction. But, for complete reprogramming to occur, this expression must be silenced after a certain period of time and endogenous genes must be able to express the correct factors following the initial two weeks. Cells lacking this endogenous expression and transgene silencing have been found to only be partially reprogrammed and although they resemble their embryonic stem cell counterparts, the partial reprogrammed state does not allow them to differentiate readily.[87]

Even when appropriate expression of the four factors is observed in cells, in order for the cells to maintain the pluripotent state this induces, transgene expression must occur and the endogenous loci present in each of the factors must be prompted to an activated state. DNA is a crucial requirement that allows for activation. Analysis of promoter regions on genes associated with pluripotency has shown that in multipotent or differentiated cells, such as fibroblasts and somatic cells, the genes are highly methylated, in contrast with the hypomethylation observed in both embryonic stem cells and iPS cells. None of the four necessary factors have DNA demethylation activity suggesting that demethylation is not a primary effect of reprogramming

and only occurs with multiple cell divisions. Induction of cells to a pluripotent state also is dependent on precise histone modifications. Differentiated cells contain hypoacetylated histones, particularly H4, while in embryonic and iPS cells, the promoter regions of genes associated with pluripotency have hyperactylated histones. c-Myc is the only transcription factor that has been found to contain histone modification activity and studies showing that histone deactylase inhibitors such as valproic acid significantly increase the efficiency of generating legitimate iPS cells suggests that histone acetylation is important to ensure direct reprogramming in cells.[88]

Molecular Mechanism of Induction to Pluripotency

Embryonic stem cells maintain pluripotency by regulating the four reprogramming factors through protein-protein interactions, as well as binding to specific promoter regions on common genes. These factors also induce the expression of stemness genes, but also inhibit the expression of numerous genes related to differentiation. Specifically, the inactivation of Thy-1, a differentiation gene, and the simultaneous activation of a stemness gene, SSEA-1, occur at the beginning of reprogramming and are crucial for successful iPS cell induction. Endogenous activity of the four factors, in addition to telomerase, is detectable in the cells as well as a number of other transcription factor genes. Transduction of the cell stimulates it to divide symmetrically, hence maintaining its morphological cellular shape. Although most cells die during this process, a select few remain viable and transform themselves to a shape resembling embryonic stem cells and thereby become the desired iPS cell.[89]

It has also been determined through experiment and observation that three distinct types of iPS cells exist and have been characterized by the expression of cell surface markers as well as retroviral silencing. The most notable differences between the three cell types is the amount

of methylation in the promoter region of the marker gene Nanog, as well as the differentiation potential of Oct-3/4. Of the three separate cell types, the group that displayed the most complete reprogramming was positive for SSEA-4 and TRA-1-60, both markers of pluripotency, and was distinctly negative for CD13, a fibroblast marker. In addition, the retroviral promoters in these cells were in an inactivated state. Only this cell type had the ability to form teratomas in mice containing all three germ layers. Being able to distinguish between each of the three cell types is important due to the fact that partially reprogrammed cells are morphologically very similar to legitimate iPS cells displaying a fully reprogrammed state. It has also been observed and reported that iPS cells that are allowed to be cultivated for an extended period of time will develop gene expression essentially the same as that of embryonic stem cells as opposed to the same iPS cells studied in earlier passages.[90]

As mentioned above, the expression levels and balance maintained between the each of the reprogramming factors are essential to the induction of iPS cells. In addition, gene expression is also regulated through epigenetic medications including DNA methylation and acetylation of histones. Several studies have shown that iPS induction efficiency was greatly enhanced when treated with epigenetic modification drugs including DNA methyltransferase inhibitor, histone deacteylase inhibitors, or histone methyltransferase inhibitors. The global gene methylation pattern of iPS cells and embryonic stem cells is thought to be very similar, though recent studies show that 71 or more differential methylation regions exist between the two stem cell types particularly in genes known to control developmental processes and can therefore provide some explanation of iPS cells' failure to differentiate in some cases. Gene expression profiles also highlighted differences between the two stem cell types suggesting that iPS cells may retain some degree of "memory" of the their somatic origin. Despite this fact, there is not

sufficient evidence to suggest that this memory retained in iPS cells is fatal to their role in cell based therapies though it does call for the need to develop methods that will ensure more efficient and accurate reprogramming qualities.[91]

Induced Pluripotent Stem Cell Induction Methods

Following reports of the first successful creation of human iPS cells in 2007, this phenomena has taken the scientific community by storm. Since that time, human iPS cells have been generated from a number of tissues utilizing a wide variety of approaches. iPS cells generated from dermal fibroblasts have proven to be the most common, as these cells are easily accessible and have a relatively high reprogramming efficiency. Two major obstacles currently exist in the reprogramming methods that have slowed the progress of reliable and consistent derivation of iPS cells: the reliance on viral vectors and the overall inefficiency of the reprogramming process. Alternative approaches have been devised to attempt to overcome these hurdles including the use of single or multiple transient transfections, non-integrating vectors, excisable vectors, direct protein transduction, and microRNA transduction.[92] In addition, small molecule compounds have been studied in depth as a way to not only increase reprogramming efficiency but may also be used to replace one or more of the factors currently be used in the reprogramming process. These findings have encouraged many to begin to explore screening of small-molecule libraries in order to devise methods of reprogramming purely through chemical means, making therapeutic use of reprogrammed cells not only safer, but more practical as well.[93] Both viral and non-viral methods that are currently being used to generate iPS cells are outlined below in addition to the advantages,as well as, the potential drawbacks of some methods.

Viral Methods

Retroviral Vector

The retroviral vector represents the original method used for transgene expression in order to achieve the first successful conversion of human fibroblasts to iPS cells by Takahashi and Yamanaka in 2007. The vector has since been found to infect a variety of different cell types as well. The retroviral vector, containing each of the four reprogramming factors, introduces these coding genes into the host genome through the use of an enzyme called reverse transcriptase. This method of transfection enables constant transgene expression through the entire reprogramming process. Inactivation of the promoter is observed in both embryonic stem cells as well as iPS cells created using this method, most likely due to epigenetic modifications. The expression of the retroviral transgenes continues until the cells become iPS cells and then the expression levels are observed to drop off significantly indicating retroviral silencing allowing the reprogrammed cells to activate endogenous pluripotency genes.[94]

Most patient-specific iPS cells have been established using retroviral vectors proving it to be an effective method for iPS induction in human somatic cells. These retroviral vectors have also been shown to infect nearly all mammalian cells approaching 100% efficiency. It is the intrinsic property of the vector to integrate into the genome of the host cell that allows for stable expression of each of the reprogramming factors to be achieved.[95] Despite the success using this method, iPS cells derived retrovirally contain a number of transgene integrations in their genome. These integrations have the possibility of leading to 'leaky expression' which would cause disturbances in the expression of endogenous transcription factors and could lead to decreased or failed differentiation. Another difficulty caused by transgene integration is the high tumorigenic risk that exists post-transplantation. Specifically, c-Myc has been found to initiate

transgene-derived tumor formation in mice. Efforts have been made to reduce this potential setback by adapting the transduction method, possibly through the removal of the c-Myc gene from the 'reprogramming cocktail.'[96] Despite the drawbacks of this method, it remains the most efficient of all methods currently being used. While it may not be suitable for use in transplantation-based therapies, iPS cells derived via this method will remain an invaluable resource for research based purposes including disease modeling and drug screening.

Lentiviral Vector

The lentiviral vector retains a number of similar properties to the retroviral vector method including the method of entry into the host. The lentiviral vector containing the four reprogramming factors and packaging plasmids successfully introduces these factors through the injunction of its DNA into the host cell through the reverse transcriptase enzyme in a process called transfection. This method was utilized by another leading stem cell scientist, James Thomson. Thomson and his colleagues derived iPS cells from human somatic cells nearly simultaneously but independently from Takahashi and Yamanka using the lentiviral vector, heeding the same success. In contrast to the retroviral vector used, the vector produced by Thomson contained a different reprogramming cocktail. Lin28 and Nanog were substituted for Klf4 and c-Myc respectively as the expression of the c-Myc gene causes death and differentiation of embryonic stem cells, and also suggests that combinations of factors lacking this gene are required in order to reprogram human cells.[97]

An advantage of the lentiviral vector, in addition to its high transfection and reprogramming efficiency, is that in contrast to the retroviral vector, it has the ability to transduce both dividing and non-dividing cells. Once the vector enters the cell and has been converted from RNA to DNA by the reverse transcriptase enzyme, it integrates itself into the

host chromosomes where integrase is responsible for allowing the transgenes to be taken up by the cell. Retroviral vectors, conversely, require an open nuclear envelope in order to deliver the reprogramming genes to the cell and therefore can only transduce dividing cells.[98]

Similar to the retroviral method, the use of lentiviral vectors results in genomic integration in order for stable expression of the reprogramming factors to occur. These integrations also raise the risk of 'leaky expression' and because of the transgene integration, the risk of tumor formation after transplantation still remains an issue. However, methods are now being devised that use lentiviral vectors which can be removed following the transduction of the host cell. A polycistronic "hit and run" vector has been designed that allows for transduction of the cell, but following transduction, the reprogramming sequences can efficiently be deleted from the iPS cell genome. Small remnant DNA fragments remain, but because they do not contain promoter or enhancer sequences or manipulate the coding sequences and regulatory elements in any way, the probability of insertional activation or endogenous gene inactivation is extremely low. These results provide a foundation for the use of this type of viral vector in a clinical setting in addition to its current use of studying the development and function of human tissues, and the discovery and testing of new drugs.[99]

Nonviral Methods

DNA Vectors

Despite the success of the viral methods, due to the safety issues, and therefore, limited use in clinical settings, alternative methods which promote reprogramming without integration have been actively sought. DNA vectors have been found to effectively introduce reprogramming factors to the host cell through means of transient expression. This allows for the cell to be inducted into a pluripotent state but also remain void of insertional mutations in the

cell's genome which would interfere with iPS cell derivatives' normal functions as well as residual exogenous transgene expression which may have an influence on the differentiation of iPS cells to specific lineages.[100]

The DNA vectors currently being used for iPS cell induction are plasmids, episomal plasmids, and the minicircle DNA vector. Each of the three vectors function in a similar manner as a plasmid is used to introduce the necessary reprogramming factors to the cell through transient expression without genomic integration and have the ability to undergo self-replication in the cell. Studies have determined that exogenous expression of reprogramming is only required for 14 days post-transfection. Therefore, because episomes and plasmids are lost at a rate of ~5% per cell generation due to defects in plasmid synthesis and partitioning, cells void of plasmids can easily be isolated.[101]

While episomal and non-episomal plasmids are similar in the mechanism of action and efficiency of the conversion of cells to a pluripotent state, subtle differences do exist that have raised questions over which vector method is most effective. Unlike the episomal plasmid, the non-episomal plasmid ensures that no integration into the host genome will result when being introduced into the cell. Though reports have shown that nucleic acids from the episomal vectors do not integrate into the host genome, iPS cells generated by this method must be subcloned to isolate iPS cells that are void of vector DNA. Though the episomal method has been found to be valuable and efficient, it suffers from lack of simplicity and requires substantial knowledge beyond that of molecular biology.[102]

In contrast, episomal vectors, compared to their non-integrative plasmid vector counterpart, can sustain a longer and more stable expression in cells, thereby facilitating the induction of pluripotency in cells that require a prolonged presence of exogenous reprogramming

factors.[103] In addition, episomal vectors have a wide host cell range, and no repeated treatments with reprogramming factors are required. A single transfection of episomal vectors has been determined to provide the appropriate level of expression of reprogramming factors in order for the successful derivation of iPS cells to occur.[104]

Minicircle vectors are circular non-viral DNA molecules that are generated through an intramolecular recombination from a parental plasmid mediated by integrase. The primary difference between minicircle DNA and the standard non-integrating plasmid vectors is that the minicircle DNA no longer contains the bacterial origin of replication or antibiotic resistance markers. Therefore, these supercoiled molecules have higher transfection efficiency as well as a longer ectopic expression due to the reduced activation of silencing mechanisms against foreign DNA.[105] The most notable drawback to this method is the inability of the minicircle DNA to self-replicate and the low efficiency of reprogramming which was observed in each of the DNA vector methods.[106]

MicroRNA

A number of recent studies have reported that microRNAs (miRNAs) acting alone are sufficient for the derivation of iPS cells in both mice and humans. Of the miRNAs expressed at high levels in both embryonic stem cells and iPS cells, the miR302/367 cluster specifically has been shown to be a direct target of Oct-3/4 and Sox2, two transcription factors that essential for iPS cell reprogramming. Levels of this miRNA cluster are correlated with Oct-3/4 transcript levels in embryonic stem cells and embryonic development, indicating its crucial role not only in embryonic stem cell homeostasis but also of maintenance of pluripotency.[107] In addition, two studies reported efficient generation of both mouse and human iPS cells through the use of lentiviral transfection of miR302 or transfection of additional miRNAs including miR200c,

miR32, and miR369. The miRNAs produced were indistinguishable from those derived using transcription factors in terms of pluripotency marker expression and teratoma formation. However, miRNA seems to offer several advantages over methods utilizing conversion via reprogramming factors primarily because it is void of the use of oncogenic transcription factors and does not introduce genetic change into the genome of the cell.[108]

Additionally, conversion efficiencies using this method were reported to be significantly higher than previously described methods which utilized the reprogramming transcription factors. The mechanism that explains this increased efficiency using the miR302/367 cluster revolves around the nature of miRNA itself. Protein translation is not a requirement of miRNA expression and therefore leads to a quick response of protein expression based on inhibition of translation of mRNA and stability. miRNAs also target hundreds of mRNAs that are responsible for the expression of a number of proteins. Therefore, this can quickly impose dominant phenotypic changes in the identity of the cell. Analysis of these miRNA targets also may provide information on both the pluripotent gene network as well as information regarding factors that experience suppressed expression in order for iPS cell reprogramming to be efficient.[109]

Transposon

Methods have also derived in order to avoid the issue of integration of factors into the genome of the cell and unwanted exogenous expression of factors through the use of excision methods. One such method entails the uses of a transposon system that encodes for reprogramming factors specific for iPS cell generation. With the assistance of a transposase enzyme, the transduction of the plasmid-based transposon vector integrates in the genome of the host cell and generates iPS cell formation. Following the iPS colony formation, transposase is

re-expressed and recognizes the terminal repeats found on each end of the transposon vector that has been integrated into the cell's genome. Once the repeats have been identified, the transposase enzyme excises the vector from the genome. Excision ensures that a "footprint" is not left behind so the cell is able to maintain its original endogenous sequences and is void of exogenous transcription factor expression as well.[110, 111]

Although methods exist that are transient in nature and have greatly minimized the potential for insertion mutagenesis, they are limited by reduced reprogramming efficiencies. iPS cells generated using this method not only express markers characteristic of pluripotency but also have proved to be effective in terms of differentiation as well. There are four ways in which transposon-based reprogramming represents a momentous innovation that will allow for significant improvements in the efficiency of transgene delivery and iPS cell induction success. The piggyBac transposition allows for technical simplifications and reprogramming methodology more accessible through the use of plasmid DNA which can be routinely prepared as well as the use of standard commercial products for transfections. Second, the range of somatic cell types that can be used for generation of iPS cells will increase drastically as the issue of decreased susceptibility to viral infection does not present itself as an obstacle to be overcome using this method. In contrast to current viral methods which require xenobiotic conditions, delivery mediated through the using of a transposon will allow for the production of cell lines void of these conditions. Finally, it has clearly been demonstrated that transgenes can be accurately removed from a number of cell lines through the expression of a transposase enzyme. Therefore, once exogenous expression is no longer necessary for maintenance of the pluripotent state, the reprogramming factors can be removed from the iPS cells 'without a trace.' These characteristics of transposon-based induction methods will pave the ways for significant

advances towards developing methods of generating induced pluripotent stem cells that are both research based and clinically acceptable.[112]

Proteins

Despite the fact that methods have been derived that effectively remove DNA from the host cell; it still involves introducing foreign DNA to the cell nonetheless. The sequencing of iPS cells lines generated through these methods is therefore necessary to ensure that they are free of any genomic alterations. To avoid this potential drawback, the delivery of reprogramming factors in their protein form has been studied extensively as an alternative method which does not make use of DNA. iPS cell lines free of transgene use and expression were derived by fusing reprogramming factors with cell-penetrating peptides that mediate protein transduction. The cells were repeatedly exposed to these proteins and eventually transformed to a pluripotent state displaying characteristics that are hallmark to embryonic stem cells including similar morphology, expression of undifferentiated cell markers, as well as the ability to differentiate. This protein transduction method eliminates any risk associated with the presence of exogenous gene expression or the potential of modifying the host cell genome. This approach is also deemed to be much quicker and simpler and the availability of large scale recombinant protein production will allow for much broader applications of reprogramming methodology.[113]

Despite the wide-reaching potential of this transduction method, its applicability has been limited due to difficulties in successfully repeating this process with the same success outlined in the previous study. In addition, this method requires numerous rounds of treatment presenting a need for large quantities of pluripotency proteins. This method has also cited slower reprogramming kinetics and a very low reprogramming efficiency up to 1000 fold lower than that of the method with the highest reported reprogramming efficiency: the retroviral vectors.[114]

Characterization of Induced Pluripotent Stem Cells

Consistent standards have been developed since the induction of iPS cell technology for the identification and evaluation of these cells as well as standards for an assessment of their equivalence to embryonic stem cells. Assessing reprogramming and conversion to iPS cells starts with identifying morphological similarities between the two stem cell types. This includes compact colonies with distinct borders and well-defined edges. The colonies must also lack granularity and must be tightly packed. In addition, cells should be comprised largely of nucleus and have large nucleoli in comparison to a scant cytoplasm.[115]

Morphological characteristics alone are not sufficient enough to accurately distinguish a partially reprogrammed cell from a fully reprogrammed iPS cell. Therefore, a number of molecular hallmarks have been identified that give rise to the presence or absence of fully reprogrammed cells. First, a cell that is fully reprogrammed will express the four necessary genes for pluripotency in levels comparable to embryonic stem cells. They will also show a reactivation of telomerase gene expression and up regulate other specific genes, most notably, SSEA1. Positive alkaline phosphatase staining may also be used as a preliminary test, but further assessment is required, as it has been shown that partially reprogrammed cells may stain positive as well. iPS cells produced with a viral method are generated specifically by silencing the viral reprogramming genes while simultaneously activating endogenous pluripotency genes. When this occurs, the expression of specific embryonic antigens including SSEA3, TRA-1-60, TRA-1-81, DNA methyltransferase 3B and REX1 will be observed. Because the epigenetic status of reprogrammed cells is so important, the degree of reprogramming can also be measured by identifying the methylation status at the promoters of genes which are responsible for maintaining pluripotency within the cell as well as at the genes responsible for initiating and

controlling differentiation. In addition, the most crucial epigenetic reprogramming event is the reactivation of the silent X chromosome and is the hallmark of ground state pluripotency.[116]

Finally, a number of functional assays also exist which gives rise to a cell's reprogrammed state. Characterizing functional ability is first assessed based on the ability of the cell to be differentiated into embryoid bodies, a rounded collection of cells which arise when embryonic or iPS cells are cultured in suspension. If the embryoid body is formed, it can then be further analyzed for the presence of markers characteristic of each of the three germ layers. Functional assays also include the ability of pluripotent cells to develop chimaeras. Chimaerism can be measured by the ability of chimaeras to produce offspring that are exclusively all iPS cell mice. The functional 'gold standard' is currently an iPS cell's ability to form teratomas. If the cell is fully reprogrammed and pluripotent, then it will form a differentiated tumor with parts of each of the three germ layers when injected into immunodeficient mice. This assay is important as it gives rise to the potential of iPS cells to spontaneously differentiate.[117]

MATERIALS AND METHODS

Purpose of Cellular Engineering Technologies (CET) Inc.'s Research

The purpose of CET's research is to successfully reprogram a number of differentiated human somatic cells into a pluripotent state to generate both patient and disease-specific iPS cells. The goal of CET's work was to create four normal iPS cell lines from multipotent stem cells, human keratinocytes, human foreskin fibroblasts and bone marrow cells. In addition, CET has been working to demonstrate the generation of disease-specific iPS cell lines from human donors for the following diseases and disorders: Alpha 1 Protease Inhibitor, Alzheimer's disease, Amyotrophic Lateral Sclerosis (ALS), Bartter Syndrome, Cystic Fibrosis, Cystinosis, Fragile X, Gaucher's disease, Muscular Dystrophy, Niemann-Pick Disease Type 2 (NPC), Parkinson's disease, and Schizophrenia. In addition, the purpose of the research was to develop a method that would allow the iPS cells generated to be used in a clinical setting.

Experimental Procedures

Cell Culture

IRB permission was granted through the Mercy Medical Plaza in Iowa City, Iowa for the use of tissue from human donors. From this tissue, cells were isolated for experimental purposes. Human foreskin fibroblasts and Niemann Pick C Type 2 cells were obtained through these tissue donations. The remaining disease-specific cells including Alpha I Protease Inhibitor, Amyotrophic Lateral Sclerosis, Alzheimer's disease, Bartter Syndrome, Cystic Fibrosis, Cystinosis, Fragile X, Gaucher's, Muscular Dystrophy, Parkinson's disease, and Schizophrenia and human somatic cells including keratinocytes, mesenchymal stem cells, adipose cells, and cells from the amniotic membrane were purchased from the Coriell Biorepository.

These cells and subsequent pluripotent cells were cultured and maintained on feeder cells or feeder-free cultures. The feeder cells were mouse embryonic fibroblasts (MEF) in DMEM/F12 culture medium which was supplemented with 20% KnockOut serum replacer, 1 x 10^{-4} M non-essential amino acids, 2 mM L-glutamine, and basic fibroblast growth factor (bFGF). Feeder-free cell cultures were maintained on plates coated with Geltrex, a reduced growth factor basement membrane matrix with chemically defined mTeSR medium containing a bovine albumin source (Stemcell Technologies).

MEF cells were plated one day prior to the plating of target cells to ensure their viability. Geltrex plates were prepared one day prior as well in the following manner. Geltrex was diluted with pre-chilled DMEM/F12 medium. Approximately 5 mls of diluted Geltrex solution was added to cover the entire surface of the culture dish. The coated plates were then incubated at 37°C and 5% CO_2 for approximately one hour. Following incubation, the plates were placed at room temperature for an hour. The supernatant of the Geltrex coating was aspirated.

In order to plate the cells, iPS medium or mTeSR was added to a conical tube. Vials of each desired cell type were removed from the liquid nitrogen tank and put in a 37°C water bath until most, but not all of the cells were thawed. The cell suspension was then transferred to the tube prepared with medium, centrifuged, and the supernatant was discarded. The pellet of cells was then resuspended in iPS or mTeSR medium and then transferred to the dishes containing MEF feeder cells or dishes coated with Geltrex respectively. The cells were plated at a density of 1200 cells/cm^2 /dish. The cells were then incubated in a 37°C, 5% CO_2 incubator until the cells became 80-90% confluent.

Transfection and transduction methods.

Lentiviral method. 293FT cells were plated the day prior to transfection on culture dishes with 293FT medium and allowed to incubate overnight to allow for the monolayer cell density to reach approximately 70-80% confluence at the time of transfection. One hour prior to transfection, complete culture medium with serum was added to each well. Two micrograms (ug) of plasmid DNA was added to each well and contained the reprogramming factors and a GFP reporter tag (Cell Bio Labs) or a 302bcd/367 microRNA cluster and reporter tag (System Bio) was diluted in serum-free DMEM medium. A pipette was used to gently mix each solution. For each well, a specified amount of the 293FT calcium phosphate transfection reagent (Signage Laboratories), yielding a 3:1 ratio of transfection reagent (ul): DNA (ug) was diluted in the same manner. The diluted 293FT reagent was added immediately to the diluted DNA solution all at once, being careful not to mix the solutions in the reverse order. The solution was then vortexes briefly to mix followed by a 15 minute incubation at room temperature in order to allow the transfection complex to form. 293FT reagent/DNA mixture was added drop-wise onto the medium of each well and the mixture was homogenized by gently swirling the plate. The complex containing medium was removed approximately 12 hours after transfection and replaced with serum containing medium. Forty eight hours after transfection, 293FT cells were centrifuged and the supernatant containing the lentivirus (multiplicity of infection (MOI) of 5) and necessary reprogramming factors was collected.

Each target cell type previously cultured was removed from the incubator to undergo transduction. The medium was removed and replaced with the virus-containing supernatant and cells were incubated overnight at 37^0C with 5% CO_2. Approximately 24 hours after transduction, the virus-containing medium was aspirated and fresh iPS or mTeSR medium was

added to the cells. The transduction procedure was repeated 2-3 times on the same cells to ensure transgene expression was at its maximal level.

Minicircle DNA method. The procedure followed for the minicircle DNA method is identical to the steps followed for standard plasmid DNA methods. However, the minicircle vector differs from a plasmid vector in that it no longer contains the bacterial origin of replication or the antibiotic resistance markers. Therefore, delivering minicircle vectors to cells will lengthen the expression of the transgene in comparison to transient transfections using standard plasmid DNA.

Each target cell type to be transfected was plated on either MEF feeders or Geltrex coated plates 24 hours prior to transfection so the monolayer cell density would be 70-80% confluent. Culture medium containing serum and antibiotics was added to each well 1 hour prior to transfection. For each well, depending on its size, minicircle DNA containing the necessary reprogramming factors and a GFP reporter tag (Cell Bio Labs) was diluted in serum-free DMEM medium with high glucose. The amount of DNA used was 2 ug of purified plasmid DNA per 50,000 cells. The solution was then mixed by gentle vortexing. Next, for each well, a specified amount of the PolyJet reagent (a 3:1 PolyJet reagent: minicircle DNA) used to ensure effective and reproducible transfection with less cytotoxicity was diluted in the same serum-free medium containing high glucose and was vortexed. The diluted PolyJet reagent was immediately added to the plasmid DNA solution all at once being careful not to mix the solutions in the reverse order. The solution was vortexed briefly and was incubated for 15 minutes at room temperature to allow the lipid-DNA complex to form. A specified amount of the mixture was then added drop-wise onto the medium in each well and the mixture was homogenized by gently swirling the plate. The complex-containing medium was removed and replaced with fresh serum-

containing medium 12-18 hours post transfection. The transfection efficiency was monitored 24-48 hours post transfection and the process was repeated in order to ensure maximal expression of the transgenes.

MicroRNA (miRNA) method. Each desired cell type was transfected with a miRNA vector (Cell Bio Labs) using the lentiviral transfection method. The procedure followed is outlined above under lentiviral method with the following substitutions: the virus containing supernatant contains the miRNA cluster instead of the four reprogramming factors.

PiggyBac (PB) transposon method. A PB vector containing the desired components to induce pluripotency was obtained. A PB contains a clone of the appropriate plasmid DNA, minicircle DNA or miRNA and is combined with a transposon vector. This transposon vector is a genetic element that efficiently transposes between vectors and chromosomes using a "cut and paste" mechanism.[118]

Each target cell type was plated and grown as described above in the cell culture procedure. The cells were grown until they were 60-80% confluent. The PB transposon vector (System Bio), the PB transposase vector (System Bio), and a transfection reagent (System Bio) were combined with serum-free DMEM medium. The solution was mixed by brief vortexing.. The solution was then incubated at room temperature for 15 minutes to allow the DNA complexes to form between the vectors. The complex, containing 2 ug of plasmid DNA per 50,000 cells was then added drop-wise to the target cells and swirled in order to disperse. It was expected that the transposase activity would terminate after 72 hours but the transposon vector would be integrated in to the target cell genome. The cells were evaluated for positive integration 3 days after transfection.

Table 1 presents a summary of the methods and the factors used to reprogram each cell type in order to form iPS colonies.

Table 1 –Components used for Reprogramming Human Somatic Cell to iPS Cells

Vector Type		Cell Types	Reprogramming Factors
Integrating	Lentiviral	Human Foreskin Fibroblasts, mesenchymal stem cells, adipose cells, multipotent stem cells, keratinocytes, bone marrow cells, 12 disease specific cell types	c-myc, KLF-4, Oct 4, and Sox 2 302 bcd and 367 microRNA
Excisable	PiggyBac Transposon	Human Foreskin Fibroblasts, mesenchymal stem cells, adipose cells, multipotent stem cells, keratinocytes, bone marrow cells, 12 disease specific cell types	c-myc, KLF-4, Oct 4, and Sox 2
Non-integrating	Minicircle DNA	Human Foreskin Fibroblasts	Lin28, NANOG, Oct 4, and Sox 2

Maintenance of iPS cells

Approximately 1 week after transduction each transduced cell type was harvested by trysinization and replated on MEF feeder layers or Geltrex coated dishes with iPS medium or mTeSR medium respectively. The feeder cell medium was changed daily and the mTeSR medium was changed every other day. The cells were monitored daily and replated to prevent overgrowth on the plate that could lead to cell death. Approximately 30 days after transduction (time was dependent on cell type and method used), colonies were selected and transferred to

new dishes in iPS cell medium. The colonies were mechanically dissociated by pipetting up and down in a repeated fashion. The suspension of cells was then transferred to a feeder layer. This transfer represented the first passage.

Characterization of iPS cells

Alkaline phosphatase (AP) detection was used in order to characterize reprogrammed cells and detect iPS colonies. This staining was done using an AP Staining kit (Stemgen). Material preparation was the first step in the staining process. The buffer, PBST, was prepared by adding 1X phosphate buffered saline (PBS) and Tween 20 to a conical tube which was then mixed well and stored at room temperature. The AP substrate solution was prepared by mixing Solutions A and B provided in the kit together in a conical tube. The mixture was allowed to incubate at room temperature for 2 minutes and then mixture C, also provided in the kit, was added.

In order to characterize cells through AP staining, the culture medium was aspirated; each well of cells was then washed with 1X PBS. The fix solution was then added to the washed cells and incubated at room temperature for 5 minutes paying close attention not to over-fix the cells. The fix solution was then aspirated and the cells were washed with PBST. The PBST was removed and the freshly prepared AP substrate solution was added. The cells were incubated in the dark (wrapped in foil) at room temperature for 15 minutes. The color change was closely monitored and the reaction was stopped when a bright color was observed under a light microscope. To stop the reaction, the AP substrate solution was aspirated and the wells were washed twice with 1X PBS. PBS was added to each well to prevent drying. The cells were then observed for detection of a red or purple stain signaling AP expression.

RESULTS

My project was to develop both patient and disease iPS cells lines from a variety of human tissue cell types and disease-specific cell types. At the time of this writing, CET is still in the process of developing their first normal and disease-specific iPS cell lines. However, the results collected from this and previous attempts provided important observations and conclusions that have allowed the company to revise their techniques and protocols and utilize new, more efficient methods for successful cloning.

Table 2 summarizes the results obtained for a select number of target cell types in the preceding experiments.

Table 2 – Efficiency of iPS cell Reprogramming Methods

Vector Type		Target Cell Type	iPS Reprogramming Factors	293 LTV/Phoenix Transfection Efficiency (%)	GFP Signal	Alkaline Phosphatase Staining (%)	Reprogramming Efficiency (%)
Integrating	Retroviral (Control)	Human Foreskin Fibroblasts	Sox-2, Oct-4, Klf4, c-Myc	>99%	Yes in primary iPS colonies	20% positive colonies	0.5%
	Lentiviral	Human Foreskin Fibroblasts	Sox-2, Oct-4, Klf4, c-Myc	>95%	Yes in primary iPS colonies	10% positive colonies	1%
		Human Foreskin Keratinocytes	Sox-2, Oct-4, Klf4, c-Myc	>95%	Yes in primary iPS colonies	15% positive colonies	3%
		Human Multipotent Stem Cells	Sox-2, Oct-4, Klf4, c-Myc	>95%	Yes in primary iPS colonies	10% positive colonies	1%
		Human Multipotent Stem Cells	302 bcd and 367 microRNA	>95%	Yes in primary iPS colonies	5% positive colonies	1%
		Niemann Pick C Type 2	Sox-2, Oct-4, Klf4, c-Myc	>95%	Yes in primary iPS colonies	5% positive colonies	1%
		Cystic Fibrosis	Sox-2, Oct-4, Klf4, c-Myc	>95%	Yes in primary iPS colonies	2% positive colonies	1%
Excisable	PiggyBac Transposon	Human Foreskin Fibroblasts	Sox-2, Oct-4, Klf4, c-Myc	Not Applicable	Yes in primary iPS colonies	2% positive colonies	0.25%
		Human Multipotent Stem Cells	Sox-2, Oct-4, Klf4, c-Myc	Not Applicable	Yes in primary iPS colonies	2% positive colonies	0.25%
		Niemann Pick C Type 2	Sox-2, Oct-4, Klf4, c-Myc	Not Applicable	Yes in primary iPS colonies	2% positive colonies	0.25%
Non - Integrating	Minicircle DNA	Human Foreskin Fibroblasts	Lin-28, NANOG, Oct-4, Sox-2	Not Applicable	Not applicable	0% positive colonies	0.1%

Results from the table indicate that in comparison to the retroviral control, the lentiviral method was most efficient in the transfection process as well as reprogramming efficiency. Human foreskin fibroblasts were observed to have the highest reprogramming efficiency though the multipotent stem cells and disease specific Niemann Pick and Cystic Fibrosis cells displayed favorable reprogramming efficiency in comparison to other cell types. Bone marrow cells and

disease cell types such as Bartter's syndrome, Amyotrophic Lateral Sclerosis, and muscular dystrophy (not shown in the table) were on the opposite end of the spectrum displaying extremely low reprogramming efficiencies when using the lentiviral method.

It was also determined from the results of this method that Yamanaka's factors (Oct-4, Sox-2, KLF4, and c-myc produced more successful results in both transfection and reprogramming efficiencies than the microRNA cluster used for a number of cell types as well. Despite the collectively low reprogramming efficiencies with this method, granular iPS colonies were observed in the multipotent stem cells using the four reprogramming factors. Upon further passaging, it was determined that these cells were not fully reprogrammed. The other target cell types displayed some iPS cell morphology; however the colonies were granular and determined to not be in a reprogrammed state.

The PiggyBac transposon method resulted in a much lower transfection efficiency, 30%, as well as lower reprogramming efficiencies than the lentiviral method. However, positive alkaline phosphatase staining was observed, indicating the presence of reprogrammed cells. As with the lentiviral method, multipotent stem cells were observed to have formed visible iPS colonies, but did not remain viable upon further passages indicating a partially reprogrammed state. Granular colonies and GFP signal was observed in the other target cell types although no reprogrammed iPS colonies were observed.

The minicircle DNA method was used only to transfect and reprogram human foreskin fibroblast target cells. Due to the extremely low reprogramming efficiency and the lack of any positive alkaline phosphatase staining, it was determined that this method was collectively inefficient and therefore the methodology was discontinued.

Alkaline phosphatase staining was performed on a number of different cell types transfected through each of the methods. As illustrated by Table 2, a red color was observed in a number of the target cells transfected. However, despite the positive AP results, the observable morphological characteristics such as tight, round borders, flat colonies that were tightly packed, lack of granularity and lack of the GFP signal in the virally transfected method was not seen and thus these cells were not determined to be true iPS colonies. Although the first few passages of the colonies were successful, pluripotent characteristics were not maintained in subsequent passage of cells signaling a lack of true iPS character. It was determined that these cell types displaying positive AP results were partially reprogrammed but still retained a number of their differentiated somatic characteristics.

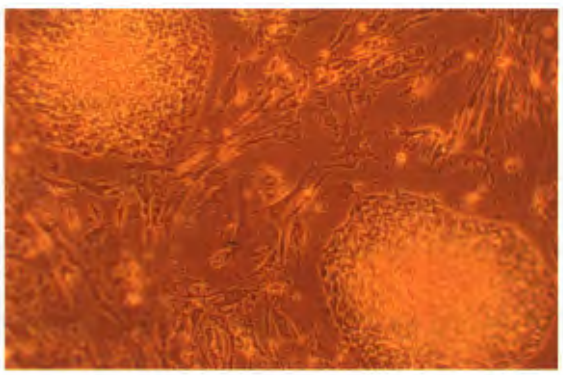

Figure 1. Multipotent stem cells partially reprogrammed into iPS colonies using lentiviral method viewed under phase contrast.

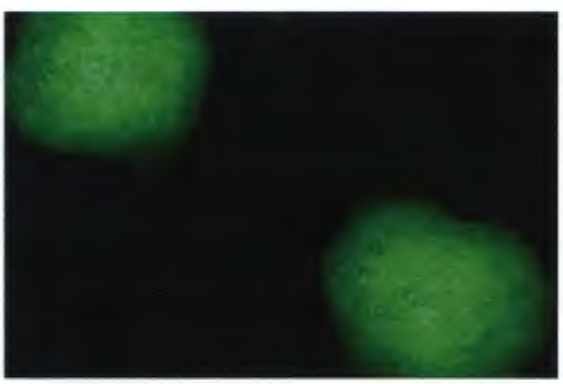

Figure 2. Multipotent stem cells partially reprogrammed into iPS colonies using lentiviral method viewed under fluorescence.

DISCUSSION

The purpose of CET's work was to develop novel methods that would be useful in improving upon the efficiency of reprogramming human somatic cells to a pluripotent state. Specifically this was to be accomplished through the derivation of four normal iPS cell lines as well as 12 disease specific iPS cell lines. While no true, sustainable iPS colonies were formed using any of the methods described above, the research was useful in identifying steps in the reprogramming process that are necessary to be altered in order to ensure that future methods developed will produce viable iPS cell lines as well as the ability to derive these cell lines efficiently and in large quantities.

The results of the experiments indicated that the transfection efficiency was low for each of the target cell types transfected, and this therefore can have an impact on the efficiency of reprogramming these cells to an iPS cell state. Cells that are allowed to grow to be 100% confluent do not have the ability to divide due to the spatial limitations imposed upon them, so in order to improve the transfection efficiency of all cell types, it would be advantageous to transfect the cells before they are allowed to reach a 100% confluency. This has been done after my work and preliminary work suggests that lower level of confluency does improve transfection. Lower confluency would allow for the presentation of reprogramming factors or miRNA clusters when the cells are at the peak of growth and division allowing for maximal uptake of the factors and depending on the method used and allow for more efficient integration into the host genome.

In addition to the low transfection efficiency, reprogramming of each of the target cell types proved to be equally inefficient as well. Among the reasons for this inefficiency may be due to the toxicity of the transfection reagent to the cells and the continued exogenous expression

of reprogramming factors following induction to a pluripotent state. In order to improve upon the reprogramming efficiency, the addition of small molecule compounds such as valproic acid and sodium butyrate could be added to the cell culture medium for the lentiviral vector and PiggyBac transposon method using the miRNA clusters. These compounds are histone deacetylase inhibitors that could allow for an increase in the reprogramming kinetics of the cell, thereby producing higher reprogramming efficiency. Valproic acid is thought to control the rate-determining step of the reprogramming process and may also be used to replace on or more of the factors used for the reprogramming process. Sodium butyrate enhances the efficiency through chromatin modifications, a key step in developing pluripotent cells. However, it has been observed that valproic acid and sodium butyrate can have a toxic effect on a variety of cell type. Although, if the concentrations of these small molecules are altered and introduced to the same cell types, there may be an observable increase in the reprogramming efficiency as well as the reprogramming kinetics. Another methodology to improve transfection efficiency that could be used is to supplement the reprogramming factors with additional small molecule compounds such as antibiotics. Currently, formulations for cell media that contain compounds such as valproic acid and sodium butyrate and antibiotic selection are currently being researched to determine their effectiveness.[119a]

In an effort to increase the viability of the target cells once they have been transfected, methods are now moving away from a feeder based system in favor of maintaining the cells on a protein mixture instead. There are a number of reasons why in theory this will improve upon the ability to derive iPS cell lines. First, because the feeder cells are typically MEFs, the differences in cell characteristics can add stress to the target cells therefore reducing the probability that they will successfully reprogram. In addition, the use of feeder cells from mice will not allow iPS

cells produced in this manner to be therapeutically relevant. Finally, these cells are difficult to remove and confound data, making it difficult to determine which cells have effectively been reprogrammed to a pluripotent state.

Several other possibilities exist that would explain the low efficiency of iPS cell derivation. Namely, the levels of factors required for producing iPS cells are thought to have narrow ranges. Therefore, only a small portion of cells exist that are in a state with the appropriate levels of reprogramming factors and therefore will express ES cell-like characteristics. Second, the role of chromosomal alterations is being studied in detail to understand their effects on the reprogramming process and how these changes inhibit the transduction of the cells or propels them forward to a pluripotent state.

Future Direction of CET's Research

While the goal of CET is to produce an iPS cell line of every tissue type of the human body, the more immediate goals are to differentiate iPS cells into mature heart cells, functional liver cells, motor neurons, neurons from Alzheimer's patients, lung tissue from cystic fibrosis patients, and muscle tissue from muscular dystrophy patients. At the time of this writing, after using the results from the optimization of the procedures, CET is in the process of deriving its first normal and disease-specific iPS cell lines from both viral and non-viral methods, through the use of the piggyBac transposition method as well as the utilization of novel chemical media formulations. These iPS cells are currently on their fifth successful passage and are displaying a number of characteristics of embryonic stem cells including morphological similarities, reprogramming kinetics, and positive anlkaline phosphatase staining. They have also been injected into nude mice in order to observe the formation of teratomas, which indicated fully reprogrammed iPS colonies have been produced.

Comparison of Induced Pluripotent Stem Cells and Embryonic Stem Cells

Embryonic stem cells are often referred to as the gold standard for pluripotency and the mechanism underlying this pluripotent state is meticulously being studied. Using iPS cell methodology offers another means to generate fully pluripotent cells for a number of applications including clinical based therapies. However, the simplistic nature of producing these cells have led many to question whether iPS cells reach the same state of pluripotency as embryonic stem cells. Global gene expression and genomic sequencing provided initial evidence of subtle differences in iPS and embryonic stem cells at the epigenetic level.[119] Further studies led to the conclusion that the only identification of differences in gene expression was much more pronounced in early passages of iPS cells.[120] The DNA sequence itself has revealed variation between the two cell types. While chromosomal aberrations are normal in stem cell populations that are grown *in vitro,* the aberrations occurred in different locations. Both iPS and embryonic stem cells have a tendency for insertions at chromosomes 12 and 17 while iPS cells have additional gains at chromosomes 1 and 9 while embryonic stem cells display additional insertions at chromosomes 3 and 20.[121]

Global epigenetic remodeling and the introduction of epigenetic changes can occur when cells are reprogrammed to a pluripotent state. Failure to demethylate genes involved in pluripotency is directly associated with partial reprogramming observed in some iPS cells.[122] Whole-genome profiling has shown that in most areas, iPS cell DNA methylomes resemble embryonic stem cell methylomes but that iPS cells can also show variability in their somatic memory, in which the cell retains genomic characteristics of its previous differentiated state subsequent to reaching a pluripotent state.[123] Despite this observation, it has been suggested that this occurs in a passage-dependent manner. Overall, the similarities of the DNA methylomes

among both cell types is remarkable but there are regions of differential methylation that must be studied more in depth to determine whether these differentially methylated regions (DMRs) have an impact on the pluripotent character of the cells. Studies have shown that the DMRs do not pertain to specific loci in the cell and thus do not represent consistent differences between the two stem cell types.[124] This lack of consistency therefore can suggest that the differences between the two can also be a result of technical limitations in reprogramming as opposed to inherent differences that would allow for one to distinguish embryonic stem cells from iPS cells. In addition, although studies have cited epigenetic differences, comparisons were often made using iPS cells from a number of different labs using different methodologies. A strong correlation has been identified between transcriptional signatures and specific labs for both iPS and embryonic stem cells indicating that methodologies and lab environment can affect the transcriptional profile of both stem cell types.[125]

It must also be noted that most iPS colonies are essentially clones that are derived from a single cell that has successfully been reprogrammed as opposed to embryonic stem cells which are not typically clonal in nature.[126] Cloning of ES cells has revealed some of the same genetic and epigenetic anomalies found in iPS cells that would have gone unnoticed when studying monoclonal embryonic stem cell populations. Somatic memory of some iPS cells has also been cited as a possible explanation for the dissimilarities in the characteristics of the two stem cell types, but because of the unreliable alignment that occurs between gene sets that were thought to be characteristic of a particular cell type, somatic memory has been attributed more to chance, following the stochastic model described previously and reflects the failure in reprogramming rather than innate differences in stem cells to rid the cell of its somatic memory.[127]

There is evidence to suggest subtle differences between embryonic stem cells and iPS cells particularly at the transcriptional, epigenetic, and functional levels.[128] However, it is yet to be determined which of these differences are a result of biological variation or are an unintentional consequence of the reprogramming process. Consensus has been reached that further studies must be conducted to determine not only the nature of the differences but also whether these alterations have a functional impact on potential therapeutic uses.[129] However, most scientists agree that study of both stem cell types is needed, as embryonic stem cells will serve as a control until iPS cells are completely understood. It is after this understanding has occurred that the question of whether or not iPS cells obviate the need for embryonic stem cells can be addressed. While the answers have yet to be found and there is still significant support in favor of the use of embryonic stem cells over iPS cells due to uncertainty of their similarity to embryonic cells, many advocates in favor of the use of iPS cells such as leading stem cell scientist James Thomson who has worked extensively with both stem cell types suggest the future direction of stem cell technology: "Only time will tell, but I know where I'm going....If you can't tell the difference between iPS cells and embryonic stem cells, then embryonic stem cells will turn out to be a historical anomaly."[130]

Implications of Induced Pluripotent Stem Cell Technology

In 2006 a scientific breakthrough discovery was introduced that added another dimension to the stem cell field: the discovery of iPS cells. The ability to generate these cells from a wide variety of human donors and the much improved capacity to differentiate these iPS cells into a number of disease-specific cell types promises a new paradigm in drug development and disease modeling – one that will position human disease pathophysiology as the central focus of preclinical drug discovery.[131] In addition, iPS cells have significantly advanced our

understanding of disease, as models for diseases have been derived that manifest the cellular phenotypes which has led to drug screens for complex diseases where the mechanism was previously unknown.[132] Finally, iPS cells can perhaps be the perfect candidate for regenerative medicine and relieve suffering and may provide cures for diseases which science has previously deemed unconquerable.

Current approaches to drug discovery can be laborious and time intensive. Approaches can involve screening a large compound library against single enzymes or receptors followed by prioritization of the positive acting compounds based on chemical tractability, potency, and selectivity.[133] The selected compounds are then tested in an animal model often with disappointing results. Cell based assays have also been used with similarly disappointing results. While this method can confirm cell permeability and the amount of toxicity that can be tolerated, this method cannot determine chemical groups responsible for biological activity. Induced pluripotent stem cells have proven to be an invaluable tool for drug discovery in terms of testing for toxicity in preclinical development and have taken drug discovery to a new level.[134]

Five steps have been identified that are essential to integrate in order for iPS cell technology to serve as a platform for drug discovery.[135] First, a patient group must be identified and recruited to participate along with healthy control cohort. Second, high-quality and thoroughly characterized iPS cells must be produced on a large-scale basis. Third, differentiation of patient-derived iPS cells must be effectively demonstrated and they must be able to differentiate into the required disease cell types. Fourth, the disease phenotype must be discovered, and fifth, an assay must be developed that can distinguish the disease phenotype.[136]

One of the most useful functions of iPS cells is the ability to model human disease. Currently, a number of human diseases are difficult to reproduce in animal models largely due to

the fact that animals have a restricted representation of human pathophysiology. Animal models are also limited due to genomic differences both in terms of the number of chromosomes and genetic background.. Disease modeling using iPS cells has been performed for Parkinson's disease, diabetes mellitus, and Down's syndrome as well as spinal muscular atrophy.[137] The use of iPS cells for disease modeling is becoming more widespread. In fact, a recent article in *The Scientist,* called iPS cells the "new supermodel" for understanding human disease.

> *The idea is that you can have a pluripotent stem cell line from a patient that already contains all the genetic background of the disease," says Gustavo Mostoslavsky, a stem cell researcher at the Boston University School of Medicine. Now that the generation of iPS cells is "routine," he adds, scientists can use the method to generate in vitro disease models, from which they can learn about molecular causes, as well as potential preventions and treatments. The strategy is proving particularly valuable for a a neurodegenerative diseases, in which it is not easy to safely and ethically extract affected cells of the brain. Instead, researchers can remove more accessible cells, such as those of the skin, regress them into a pluripotent state, and then re-differentiate them into neurons. Furthermore, iPS cells can be expanded in culture and/or frozen for years, providing an unlimited supply of cells from a single patient that can be used to create any cell types needed for the study of a particular disease, now or in the future.*[138]

Perhaps the most innovative and exciting application of iPS cell technology is the possibility of generating autologous cells for cell-replacement and the differentiation of cells into a number of tissues for transplantation-based therapies. The iPS cells of somatic origin have eliminated the obstacles that prevent embryonic stem cells from achieving the same applications. Because the cells are patient specific, unlike what is used with embryonic stem cell donors, there

is no risk of rejection or tumor formation once the cells are injected into the human body. Despite this, there are regulations that must be met in order to introduce iPS cells into transplantation-based medicine. The ability to use this technology in cell-based therapies relies heavily on the efficiency and accuracy of cell-lineage-specific differentiation, ensuring the purification of cells to eliminate the formation of tumors, the development of efficient non-viral conversion methods, and development of cell delivery methods that will effectively introduce cells into the correct organ.[139] Despite its many challenges, iPS cell technology has progressed at a breathtaking pace. With continued improvements in efficiency of protocols, it has potential to become the primary technology utilized for regenerative medicine. This technology can restore lost function of specific tissue, alleviate suffering, and provide cures to those with disease that had no hope of a successful recovery.

Has Induced Pluripotent Stem Cell Technology Obviated the Need for Embryonic Stem Cell Research?

Induced PS cells and their cousin adult stem cells have outperformed their embryonic stem cell counterparts, particularly through the development of disease therapies. Over 70 cures and treatments using iPS and adult stem cells have been demonstrated to be effective in the clinic.[140] To date, not a single therapy in humans has been successfully carried out using embryonic stem cells. A possible reason for this success is the prevalence of iPS and adult stem cells throughout the body that belong in the microenvironment of the adult body, making them patient-specific. Embryonic stem cells, on the other hand, located in the blastocyst of the human embryo, belong in this embryonic microenvironment, not in an adult body. Thus, transplantation of these ES cells can lead to tumors and immune system reactions.[141] Somatic cell nuclear transfer (SCNT) methodology has been used to try to get by the allogeneic tissues reactions that

occur with ES cells. In this method, the nucleus of an autologous, somatic (adult) cell is introduced into an enucleated egg cell that is stimulated to divide, until a blastocyst stage.[142] The goal is to produce stem cells that are identical to the DNA of the donor, thus prevent rejection. However, to date, this methodology has not produced a viable ES cell line using this technique and results in the creation and subsequent destruction of a cloned embryo.

Perhaps the most controversial matter of embryonic stem cell technology are the ethical considerations. Cells are derived from the blastocyst, found in a 3-5 day old embryo which is destroyed in order to collect these cells, thus destroying life in its earliest stages. This ethical consideration is not present with the use of iPS cells. These cells are derived exclusively from adult tissue and the procedures involved with the conversion of these cells are free of ethical consideration. Stem cell scientist James Thomson who isolated and discovered both ES stem cells and iPS cells has expressed his own reservations with ES cell research due to the ethical considerations, admitting, "If human embryonic stem cell research does not make you at least a little bit uncomfortable, you have not thought about it enough."[143]

Induced PS research looks to be a significant win for science and ethics, but those who have invested significant time and money into ES cell research continue to fight for support and funding for this research. From 2005- 2010, non-embryonic stem cell research (both human and non-human origin) has received significantly more funding from governmental agencies, Figure 5, than research for ES cells.[144] However, estimates for future funding for ES cell research show a significant rise with a change in administration and the revised stem cell regulations, Figure 6.[145] Estimates show increased NIH funding for ESCR by 31.5% while only an 8.5% increase for adult stem cell research.[146] If the estimates are accurate, ES cell research funding will increase adult stem cell research funding by the year 2017. The funding rate for adult stem cell

research is the lowest among all forms of stem cell research yet it is this research that has provided the only successes found in stem cell research, thus promising to redefine regenerative medicine by providing cures for a host of human diseases that ES cell research has failed to provide.

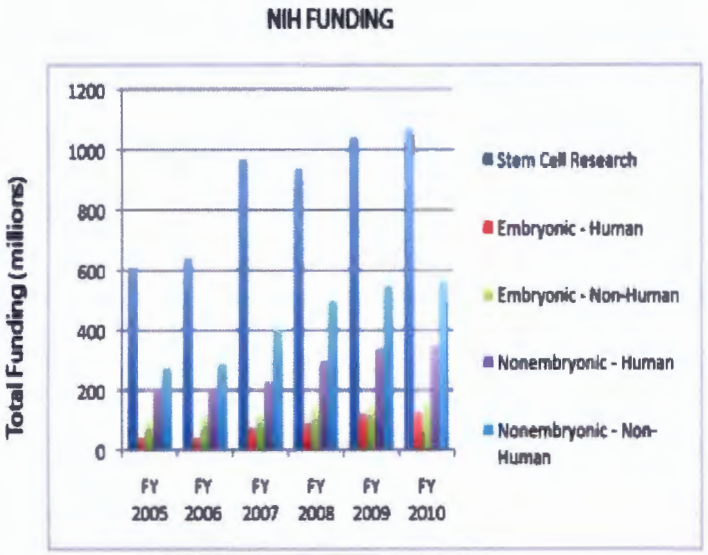

Figure 3. Funding for stem cell research from the National Institutes of Health (NIH) between 2005-2010.

2010 Congressional Research Service

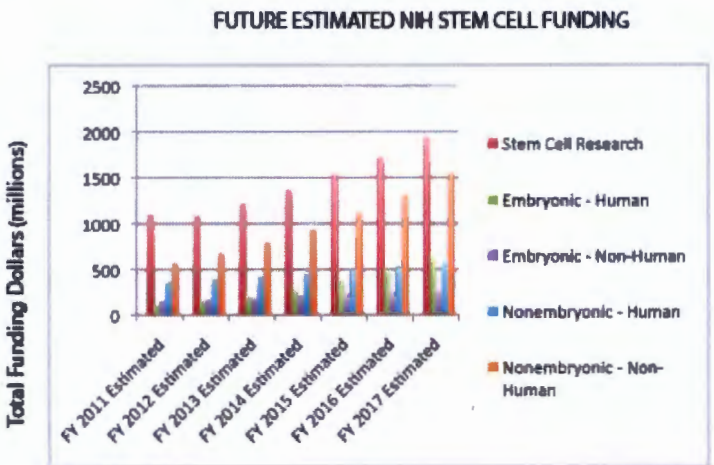

Figure 4. Estimated funding from NIH for stem cell research 2011-2017.

2010 Congressional Research Service

The Future of Stem Cell Research – my perspective

A critical moment in the national debate over stem cell research has been reached and moving into the future the debate will continue. Despite the decades of fruitless results that ES cell research has yielded, the government and much of the scientific community remain persistent on pursuing this empty avenue of research, failing to acknowledge the success and potential that other ground-breaking technologies present. When the founder of embryonic stem cell research makes a statement saying these alternative technologies marks the "beginning of the end" of his field, one should pay attention, as he operates on scientific fact as opposed to empty promises and self-interest.

ES cell research also represents an ongoing ethical battle of adhering to research principles while promoting advancements in the scientific community. The creation and evolution of these bioethical principles and laws for human subject research are designed to protect the individual and are thus rooted in the inherent worth and dignity of that individual. History has amply demonstrated the consequences of what ensues when a government becomes blinded to these inherent principles and arbitrarily defines what constitutes a human life. The law is based on precedent, and once the government allows for the continuation of ES cell research and the sacrifice of the human embryo for the greater good of the rest of society, the greatest defense for the rights of the individual will be eroded. Respect for human life at every stage must govern our treatment of all human beings in law and medical research. To the extent that it does not, we are no longer talking about authentic human progress. Until legal steps are taken to assure that the powerful and self-interested are not allowed to practice utter disregard for embryonic human life we will never be able to make the claim that we live in a civilized society.

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