STEM CELL PLASTICITY: FACT OR FICTION

Can Adult Stem Cell Plasticity Create Potential Replacements for Embryonic Stem Cells

An Interactive Qualifying Project Report

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ABSTRACT

The purpose of this project was to determine whether adult stem cell plasticity might serve as a replacement therapy for using the ethically controversial embryonic stem cells (ESCs). This topic was investigated through an extensive literature review supplemented with interviews of key stakeholders. Our data indicate that researchers disagree considerably on whether plasticity (trans-differentiation outside a normal developmental pathway) exists, although we found strong evidence for it in some systems. We also identified several potential reasons for the discrepancies among various researchers, including the silencing of reporter genes used to detect host genes (giving false negatives), not using the sensitive and powerful Cre-Lox system to tag donor cells regardless of how they later differentiate, differences in the developmental ages of the donor and host animals leading to a decreased survival of donor cells, and differences in the purity and composition of the donated stem cells which strongly affects their potency. We identified a best practice methodology using the Cre-Lox system for demonstrating plasticity without undergoing donor-host cell fusion, and conclude that more research should be done directly comparing the effectiveness of ESCs to trans-differentiation therapies prior to deciding whether one cell type can replace the other.

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EXECUTIVE SUMMARY

Regenerative medicine uses stem cells to potentially replace damaged tissues in some diseases. These cells can expand to provide a large number for therapy, or can differentiate into more specialized tissues. There are two main types of stem cells: 1) embryonic stem cells (ESCs), and 2) adult stem cells (ASCs). ESCs are isolated from the inner cell mass of a 5-day old blastocyst embryo prepared by *in vitro* fertilization (IVF) technology. These embryos are surplus embryos prepared at a fertility clinic, and must be used with donor consent. Because the embryo is destroyed when isolating the inner cell mass, the process is ethically controversial and is opposed by individuals who believe that life begins at conception, or by individuals who argue that no life form should be harmed. In addition to their ethical concerns, ESCs have received inconsistent federal funding in the U.S. depending on which political administration is in power. President Bush prevented federal funding for deriving new ESC-lines during his presidency while President Obama allows new ESC-lines to be created but with several stipulations. Due to the ethical concerns and inconsistent funding for ESCs, scientists are constantly seeking replacement cells for therapies.

ASCs are isolated from tissues other than embryos. These cells include hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), neural stem cells (NSCs), epithelial stem cells, etc. ASCs typically function to replace aging or dying cells in adult tissues. Isolating them does not destroy an embryo, so they are less controversial. Unfortunately, ASCs typically as less potent than ESCs. *Potency* is the ability of a stem cell to become other types of cells. The higher the potency, the greater the number of tissues the stem cell can become. ESCs are *pluripotent* and can become any cell in the adult body, so ESCs are of very high interest in

regenerative medicine. ASCs are typically less potent, do not grow as well as ESCs, and are rare and hard to isolate, so using ASCs to replace ESCs in therapies is a challenge.

Some evidence exists that some types of ASCs, such as HSCs, MSCs, or bone marrow cells in general, when injected into a host can help heal tissues other than their normal differentiation pathway. For example, some evidence exists that HSCs (which normally form blood cells) can help heal heart muscle in heart attack patients. To accomplish this, the injected HSCs would need to differentiate *outside* their normal developmental lineage (*trans-differentiate*) to form cardiomyocytes. This process is also termed by some scientists as *plasticity*. For this IQP, we chose to investigate stem cell plasticity as a potential replacement for ESCs. If the process works well, the HSCs or MSCs from a patient could be expanded and injected back into the same patient (autologous transplantation) to trans-differentiate into heart tissue for example without using ESCs. Our research quickly taught us that not all scientists agree that trans-differentiation exists *in vivo*, and that scientists do not even agree on the definition of plasticity.

We first performed a Literature Review, documenting the need to find replacement cells for ESCs, and identifying several key controversies associated with adult stem cell plasticity. Then, we performed a series of interviews with key scientists on both sides of the various arguments to help evaluate the issues. One of the first things we learned about stem cell plasticity is that few researchers agree on what it is. Stem cell plasticity lacks a standard definition, and each researcher interprets the term in different ways. Some scientists use the term very broadly to indicate potency, the ability of a stem cell to become other types of cells, regardless of how they achieve the differentiation. This broad definition would include trans-differentiation plus all normal cell developmental pathways. However, other scientists use the term to indicate

differentiation outside the normal developmental lineages, and this latter more defined definition was adopted by our team for this IQP.

Experimentally determining whether a cell has undergone differentiation outside its normal lineage is not an easy task. Our Literature Review determined that most scientists use a donor-host combination to help solve the problem. In this approach, usually HSCs, MSCs, or bone marrow cells containing a mixture of the two, are isolated from a host animal and injected into a recipient disease model animal. For example, HSCs (that normally form blood cells) might be injected into a mouse model for stroke to determine whether they can differentiate into neurons or glial cells. The donor and recipient cells are usually tagged genetically to distinguish them from each other. In addition to determining whether the injection functionally improves the symptoms, our research indicates it is important to rule out fusion of the host and donor cells, which would not be a direct conversion of the donor cell into the specialized tissue. It is also important when not seeing any evidence of host-cell survival, to make sure the reporter gene has not become silenced in the donor cell (which would give a false negative finding).

A significant problem we encountered in this project was that even within one specific disease model (diabetes, stroke, myocardial infarction, liver disease), some scientists found evidence supporting plasticity while others did not, so much of our research and interviews focused on attempting to resolve this discrepancy. We determined that these discrepancies might result from: 1) the silencing of reporter genes used to detect host genes over time (giving false negatives), 2) not using the sensitive and powerful Cre-Lox system for tagging fused cells or for tagging cells of the original hematopoietic lineage), 3) differences in the developmental ages of the donor and host animals leading to a decreased survival of donor cells in the host animals, 4) differences in the purity and composition of the donor stem cells (the cruder the stem cell batch,

the more likely it will have highly potent stem cells, so with different degrees of purity the results will vary), and 5) differences in the micro-niche colonized by the injected stem cells in a given experiment.

Our findings indicate that stem cell trans-differentiation very likely exists in some systems, but it is normally rare, and in some cases represents cell fusion not trans-differentiation. Our best evidence for trans-differentiation is for the treatment of diabetic mouse models with HSCs where we identified seven studies arguing for plasticity (migration of the injected HSCs into the pancreas followed by differentiation of the cells into insulin-producing cells), and no studies arguing against it. Several other diabetes researchers observed improved normoglycemia in mice injected with HSCs, but do not believe in trans-differentiation, however those researchers did not provide any evidence against it themselves.

Our research also identified a best-practice methodology which we believe should be applied in the future by all labs attempting to demonstrate plasticity without cell fusion. The best practice is demonstrated by Ianus et al. (2003) and by Alvarez-Dolado et al. (2003). In different applications, these scientists used a Cre-Lox system to tag donor cells regardless of what those cells later differentiated into in the host mouse. For example, the donor male mouse can be engineered to contain a Cre-recombinase gene under the control of a promoter specific for the donor cells (i.e. CD45 for HSCs). The donor mouse also contains a floxed-stop-promoter-GFP reporter that switches on in cells of the original hematopoietic lineage (HSCs). Once the stop codon is removed from the GFP promoter, the GFP gene is switched on permanently, so the donor cells are GFP+ regardless of how they later differentiate. The Cre-Lox system is simultaneously also used to visualize cell fusion of donor and host cells if it occurs. The host mouse is engineered to be floxed-promoter-LacZ, so if they fuse with any donor cells (Cre

positive) LacZ will be switched on, allowing the cells to stain blue with X-gal. In the host tissues (i.e. pancreas for a diabetes mouse model) the presence of GFP+ cells containing Y-chromosome markers (original donor cells) that are LacZ-negative would be evidence of trans-differentiation without cell fusion.

Although several scientists that we interviewed did not believe cells are capable of undergoing major reprogramming *in vivo*, the fact that some cells are well proven to be capable of major reprogramming by artificial means implies that the reprogramming is at least theoretically possible *in vivo*, even if it is rare. Our research showed that major cell reprogramming into vastly different phenotypes indeed can occur under special conditions, such as the Nobel Prize winning experiments of John Gurdon (reprogramming skin nuclei injected into enucleated eggs) and of Yamanaka (reprogramming skin cells into pluripotent cells by transfection with genes encoding transcription factors).

In order to determine whether trans-differentiation therapies can replace embryonic stem cell therapies, the two treatments must be directly compared to each other. We conclude that few current studies have been designed to directly compare functional recovery between these two stem cell types, so we make a final recommendation that such important studies should be done prior to determining whether one stem cell type can replace the other.

AUTHORSHIP

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Xuan He	Stem cell definition Plasticity definition Plasticity in liver treatments
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PROJECT GOALS

Due to a relatively low number of embryonic stem (ES) cell lines, and ethical and funding issues associated with the destruction of a human embryo, stem cell researchers need a replacement for ES cells for performing cell therapies. The overall goal of this project is to document and evaluate the scientific evidence that adult stem cell plasticity exists, and if so, determine whether cells prepared by this type of trans-differentiation can serve as potential replacements for ES cells in some types of therapies.

The first objective is to **develop** a comprehensive assessment of the scientific evidence for and against the existence of stem cell plasticity, including its discovery, various methods of trans-differentiation, and diseases being treated. The second objective is to **characterize** what key scientific stakeholders believe are the strengths and weaknesses of the existing data that this process exists, and their suggestions for potential solutions. The third objective is to **evaluate** the evidence for and against the existence of stem cell plasticity. The fourth objective is to **recommend** potential solutions for resolving any issues associated with proving that stem cell plasticity exists, including rigorous methods for ruling out cell fusion, and make recommendations for resolving any remaining issues of using iPS cells for therapy.

LITERATURE REVIEW

Stem Cell Introduction

Stem Cell Definition

Stem cells are a type of cells with two unique properties: 1) self -renewal and 2) the ability to become other cell types (Stem Cell Basics, 2002; What Are Stem Cells, 2004). After cell division, a stem cell can either remain as a stem cell, or it can differentiate into a more specialized cell under specific biological conditions. Based on their unique properties, stem cells are thought to have tremendous values in regenerative medicine and are the subject of much current research (Stem Cell Basics, 2005).

Stem Cell Potencies

One of the most critical properties of stem cells is their ability to develop into other specific cell types, which refers to their *potency*. The higher the potency, the more kinds of cells they can become. Potencies are roughly classified into five categories: totipotency, pluripotency, multipotency, oligopotency, and unipotency (Stem Cell Basics, 2005). Newly fertilized eggs (zygotes) through the eight-cell stage are considered *totipotent*, and can differentiate into all cell types in an organism plus extra-embryonic tissues such as the placenta. As the zygote develops for about 5 days, it forms a blastocyst or hollow ball, with some cells specializing to form the inner cell mass and others forming the outer cell layer or trophoblast. The cells of inner cell mass of the blastocyst contain embryonic stem cells (ESCs) and are considered *pluripotent*. Pluripotent cells can form all cells of the adult organism including any of the three germ layers: endoderm, mesoderm, or ectoderm.

Following pluripotency, stem cells enter the stage of *multipotency*. At this stage, the stem cells have the potential to differentiate into multiple cell types, but cannot form all types of cells. Hematopoietic stem cells (HSCs) and mesenchymal stem cell (MSCs) are examples of multipotent stem cells. As the cells continue growing, their potency is further limited to oligopotency and unipotency. *Oligopotent* cells can only differentiate into a few cell types. For instance, a lymphoid cell can give rise to B cells and T cells but not cells from other tissue lineages. *Unipotent* cells can give rise to only one specific cell type (What Are Stem Cells, 2004; Stem Cell Basics, 2005).

Stem Cell Types

In general, stem cells can be broadly categorized into two main types: 1) embryonic stem cells (ESCs), and 2) non-embryonic or adult stem cells (ASCs) (Types of Stem Cells, 2012). In addition, researchers have manually reprogrammed adult or somatic cells into stem-cell-like cells by inserting the genes encoding several reprogramming transcription factors. These artificially made stem cells are called "induced pluripotent stem cells" (iPSCs), and much research is focused on their true potencies and uses in therapies.

- *Embryonic Stem Cells (ESCs)*

Embryonic stem cells are harvested from the inner cell mass of blastocysts 5-10 days after *in vitro* fertilization (IVF) (What are Embryonic Stem Cells, 2010; Mandal, 2013). The blastocyst is a hollow microscopic ball of cells comprised of three structures: the trophoblast (the outer layer of cells that surrounds the blastocyst), the blastocoel (the hollow cavity inside the blastocyst), and the inner cell mass (approximately 30 cells at one end of the blastocoel). ESCs

are considered to be pluripotent because they give rise to any of more than 200 different cell types in the body (Types of Stem Cells, 2012). Due to their pluripotency, ESCs are a promising source for regenerative medicine and tissue replacement. As long as they are cultured under appropriate conditions, they remain undifferentiated and can be expanded.

When ESCs are allowed to clump together to form embryoid bodies, they begin to spontaneously differentiate. To direct ESCs to differentiate into a specific desired cell type, researchers change the chemical composition of the culture medium or modify the cells by inserting specific genes (What are Embryonic Stem Cells, 2010; Mandal, 2013).

In spite of their strong potency and uses in regenerative medicine ESCs have problems, including the potential for immune-rejection from the host, problems with differentiation, ethical issues, and funding issues (discussed below) (Swaminathan, 2008; Types of Stem Cells, 2012).

- *Adult Stem Cells (ASCs):*

Adult stem cells represent any type of stem cell that is not an embryonic stem cell. These cells are typically isolated from adult tissues or from umbilical cord blood (What Are Adult Stem Cells, 2012). These cells are also called somatic stem cells, in reference to their adult tissue origins. Their roles are generally thought to maintain and repair tissues in the body. ASCs are typically thought to be capable of differentiating into only the cell types of the tissue in which they are found, however several experiments have shown that some ASCs may be able to differentiate into cell types found in organs or tissues other than their usual tissue of origin. This phenomenon is called trans-differentiation (What are Adult Stem Cells, 2012). For example, some scientists argue that bone marrow stem cells that normally form the cellular components of blood can trans-differentiate into cardiac muscle cells for treating heart attack patients. Currently,

numerous types of adult stem cells have been identified in many organs and tissues. Some ASCs are able to form many different kinds of tissues, while others can form just a few types of cells (Adult Stem Cell 101, 2013).

Overall, although ASCs may provide regenerative cells for regenerative medicine, they are rare in mature tissues, so their isolation still represents a challenge. In addition, some types are hard to grow in culture (What are adult stem cells, 2012). Below are discussed some of most common types of ASCs.

- *Hematopoietic Stem Cells (HSCs)*

HSCs are isolated from bone marrow, peripheral blood, or from umbilical cord blood. Their main role is to generate more than 10 distinct mature types of blood and immune cells (Seita and Weissman, 2010). HSCs have been used since the late 1950's (Thomas et al., 1957) in human bone marrow transplants to treat blood cancers such as leukemia, lymphoma, or hereditary blood disorders such as plastic anemia, β-thalassemia, and Blackfan-Diamond syndrome (Hematopoietic Stem Cells, 2011). Because they have been used in human patients for decades, HSCs are the most characterized type of stem cell. Interestingly, several researchers have also investigated an exciting application of HSCs in cancer treatment where HSCs are engineered to attack solid tumors such as in metastatic kidney cancer (Hematopoietic Stem Cells, 2011).

Despite more than 50 years of investigation on HSCs, researchers still have difficulties identifying HSCs from other bone marrow cells. The most common approach relies on specific markers appearing on the HSC surface (Spangrude et al., 1988). In 1992, Irving Weissman and his collaborators proposed the following markers for identifying mouse and human HSCs:

Mouse: $CD34^{low/-}$, $SCA\text{-}1^{+}$, $Thy1^{+/low}$, $CD38^{+}$, $c\text{-}kit^{+}$, lin^{-}

Human: $CD34^{+}$, $CD59^{+}$, $Thy1^{+}$, $CD38^{low/-}$, $c\text{-}kit^{-/low}$, lin^{-}

However, some scientists believe these markers are too restrictive, and do not accurately represent true HSCs (Hematopoietic Stem Cells, 2011).

- *Mesenchymal Stem Cells (MSCs)*

In addition to HSCs, bone marrow also contains another type of stem cell known as MSCs or bone marrow stromal cells. However, these cells are also found in other tissues such as cord blood, peripheral blood, fallopian tubes, fetal liver, and lung. MSCs were first isolated in Russia in the 1960's (Friedenstein, 1976). They are progenitors of connective tissue lineages including bone, muscle, skin, cartilage, and adipocytes, and provide the stromal support system for HSCs in the marrow (Bianco et al., 2008). In addition to these orthodox differentiation pathways, MSCs have been reported to undergo unorthodox differentiation, forming neural and myogenic cells (Bianco et al., 2008). Therefore, both the orthodox and the unorthodox plasticity of MSCs and their isolation from adult tissue instead of embryos indicates their potential therapeutic value for repairing cardiovascular tissues, bone, and cartilage, and treating lung fibrosis and spinal cord injury (Barry and Murphy, 2004).

- *Neural Stem Cells (NSCs)*

In the mid-1980's, neuroscientists still believed it was impossible to renew neurons in the adult human brain and spinal cord. But in the late 1980's, scientists found that some parts of the adult human brain are capable of generating new neurons under certain conditions (Temple, 1989). The new neurons originate from "neural stem cells" present in the adult brain, and are

similar to those in a developing fetus that initially give rise to the brain and spinal cord (Rebuilding the Nervous System with Stem Cells, 2009). NSCs have been found to differentiate into many types of cells in the brain including neurons (the main message carriers in the nervous system), and neural-support cells oligodendrocytes and astrocytes (Rebuilding the Nervous System with Stem Cells, 2009). This discovery gives hope for treating neurodegenerative diseases such as Parkinson's disease and amyotrophic lateral sclerosis, and brain and spinal cord injuries caused by stroke or trauma (Rebuilding the Nervous System….2009).

- *Cardiac Stem Cells (CSCs)*

Although researchers used to think that cardiac tissue was unable to regenerate, it is now known that the heart contains a small population of endogenous stem cells capable of generating cardiomyocytes and coronary vessels. In 2003, researchers isolated and expanded c-*kit*-positive human cardiac stem cells (hCSCs) (Beltrami et al., 2003). These c-*kit*-positive cells possess the basic characteristics of stem cells: when locally injected in the infarcted myocardium of immune-deficient mice or rats, human CSCs differentiate into myocytes, coronary resistance arterioles, and capillaries, forming a chimeric heart that contributes to improved performance (Bearzi et al., 2007). CSCs have also been isolated with the Isl+ marker on their surface (Laugwitz et al., 2005). Therefore, hCSCs might provide therapies for patients affected by heart failure.

- Induced Pluripotent Stem Cells (iPSCs)

Induced pluripotent stem cells, are adult somatic cells (such as skin) that have been genetically reprogrammed to an ESC–like state by treatment with genes encoding reprogramming transcription factors. iPSCs were first induced in mice in 2006 (Takahashi and

Yamanaka, 2006) and in humans in 2007 (Takahashi et al., 2007). Although the potency of iPSCs remains the subject of much research, some scientists claim iPSCs are pluripotent (like ESCs) and can produce all three germ layers. If so, they would be as valuable as ESCs for regenerative medicine while not having the problems associated with ESCs (discussed below) (What are Induced Pluripotent Stem Cells, 2009). In addition, they would be immunologically matched to the skin cell donor (patient) so might not be immune-rejected like ESCs. The discovery of iPSCs also provides scientists with useful tools for studying drug development and disease modeling. However, iPSCs are sometimes reprogrammed using viruses to deliver the transcription factor genes, and the viruses can cause cancer in the cell lines. Therefore, researchers are devising non-viral delivery strategies to reprogram iPSCs (What are Induced Pluripotent Stem Cells, 2009).

Examples of Stem Cell Medical Uses

Based on their regenerative properties, stem cells have been tested in a variety of animal disease models and in a few types of human patients. In animal models, stem cells have been tested for treating leukemia, diabetes, heart attacks, stroke, spinal cord injuries, Parkinson's disease, lung cancer, and macular degeneration. The animal models have been tested with both embryonic stem cells and a variety of adult stem cells.

Human leukemia patients have been treated with bone marrow stem cells since the late 1950's (Thomas et al., 1957), and with over 50 years of research experience these cells have become the best characterized type of stem cell. With respect to ESCs, Geron received FDA approval to test their use in patients with spinal cord injuries, but terminated their trials in 2011 due to insufficient corporate funds (Baker, 2011; Kaiser, 2011). This leaves biotech company

Advanced Cell Technology, formerly of Worcester (MA) one of the only approved clinical trials for ESCs, in this case to treat macular degeneration (Lok, 2012).

Several studies have been performed with adult stem cells to treat human patients with heart attacks and stroke. Most of the students were performed with bone marrow cells, but in some cases they used purified HSCs or MSCs. For example, in clinical trials Britten et al. (2003) applied either bone marrow cells (BMCs) or circulating blood (CPC) into the infarcted artery of patients with acute myocardial infarction (AMI). Four months post-treatment, their results indicated that the heart global ejection fraction had increased significantly, and the end-systolic volume and the infarct size decreased significantly. In 2006, Schachinger et al. performed a similar experiment on 204 patients with AMI receiving an intracoronary infusion of BMCs or a placebo medium randomly. Four months post-therapy, the BMC group showed greater improvements in the heart global left ventricular ejection fraction. These clinical BMC transplantations show the feasibility of using BMCs to improve the myocardial infarction condition, and reduce the clinical end point of death.

MSCs have also been used in heart attack and stroke patients. Chen et al. (2004) and Bang et al. (2005) used MSCs in heart attack patients and stroke patients, respectively. Chen et al. (2004) randomly assigned 69 patients with AMI to receive bone marrow stromal cells (BMSCs, also called MSCs) or saline injections. Three months post-therapy, they observed that the patients receiving BMSCs had greater improvement in myocardial conditions—the left ventricular ejection fraction increased, the perfusion defects decreased, and the left ventricular end-diastolic volume and end-systolic volume decreased significantly. In Bang et al.'s study, the five stroke patients who received an intravenous infusion of MSCs showed improved neurological deficits and neurological function one year after transplantation.

In the few clinical trials performed so far, bone marrow cells have displayed the best contribution to functional recovery, showing excellent potential in regenerative medicine.

Problems Using Embryonic Stem Cells

Because ESCs are very potent (pluripotent), and are relatively easy to identify (blastocyst inner cell mass), isolate (micro-suction pipette), and grow (using James Thompson's feeder layer cells), many scientists believe they represent our best hope for treating specific human diseases. But ESCs have serious problems associated with their use. Their isolation destroys the blastocyst embryo, which has ethical issues. And because of the ethical issues, the federal funding of ESC research has varied considerably depending on the political administration.

ESC Ethical Issues

The destruction of human embryos when isolating hESCs has created much public debate on this topic. As mentioned in the ESC section, ESCs are isolated from the inner cell mass of 5-10 day old blastocyst embryos prepared by in vitro fertilization (IVF). The isolation of the cells destroys the embryo which has the potential for life, so the ethical debate focuses on the status of the 5-10 day old human embryo. The human embryo debate is not new. IVF technology was first developed in the early 1960's in rabbits and was later applied to humans. The world's first "test tube baby" Louise Brown was born in 1978 (BBC News, 1978). The development of IVF technology, primarily by Robert Edwards in Britain (see Edwards, 2001, for a historical review), resulted in Edwards receiving the 2010 Nobel Prize in Medicine or Physiology. The IVF procedure is not perfect, so doctors prepare extra embryos for implantation if the initial attempt

fails and follow-up procedures are necessary. Once the family has enough children, questions remain on what should be done with the *surplus* embryos. Should they be used in other women to make children? Should they be used for research (to derive new ES cells)? Should they be destroyed?

Generally, the ethical debate focuses on two fundamental moral principles: the duty to prevent or alleviate suffering, and the duty to respect the value of human life (Hug, 2006). However, we cannot fulfill both of these duties in the case of ESCs, because the embryo is destroyed (dis-value of human life) in order to attempt to alleviate suffering (using the ESCs to treat diseases). So, the problem is *which* duty should be given more priority in this ethical dilemma. Three main positions exist within this debate:

- *An embryo has full moral status after fertilization of the egg*

Individuals supporting this position argue that life begins at conception, and consider embryos worthy of respect and protection because embryos are potential persons. For example, the Roman Catholic, Orthodox and conservative Protestant Churches believe the embryo has the status of a human from conception, thus no embryo research should be permitted (Farley, 1999; Hug, 2006). Pope John Paul II commented against embryo research in 2001 (Pope John Paul II, 2001), and Pope Benedict XVI also addressed this issue in 2008 (Pope Benedict XVI, 2008).

- *An embryo has a moral status that begins with deserving protection and increases as the fertilized egg becomes more human-like*

Others believe that fertilized human eggs before implantation do not own any of the psychological, physiological, emotional or intellectual properties of personhood, but acquire

those properties over time. And IVF embryos are special in that unless they are implanted into a uterus, they only have the *potential* for life Thus, these groups focus on personhood being achieved *later* than fertilization, and full potential being reached only after implantation. Judaism (Gilbert, 2010) and Islam (Siddiqi, 2002) argue that an embryo does not have full human status before 40 days post-fertilization and focus on the goal of ESCs research to cure diseases and save lives, so both of these religions allow some research on embryos (Hug, 2006).

Some individuals in this group point out that people react differently to the loss of an embryo compared to a death of an infant, and we tend to make judgments of how great the loss is based on the stage of the lost life. Therefore, they consider an embryo before implantation has less moral status than a human fetus or a baby, and the protection should only increase when the embryos become more human-like.

- *An embryo has no moral status at all*

People in this category consider an embryo the same as other tissues or body parts, as embryos do not develop enough to survive independently. In this case, the only respect due to a blastocyst is the respect shown in general to the other people's property. As examples, Buddhism (Keown, 2004) and Hinduism (Bahnot, 2008) prohibit any harm on sentient beings who are able to feel. Because blastocysts have no nervous system and are not sentient beings, both of these religions do not see the destruction of 5-day old embryos in ESC research as morally wrong (Hug, 2006).

ESC Inconsistent Funding Issues

One of the most serious problems associated with ESC research is its inconsistent funding by the federal government. Federal funding levels in the U.S. vary considerably depending on which administration is in office.

Embryo research was first considered seriously under President Bill Clinton, who in 1993 the year of his inauguration designed the NIH Revitalization Act (1993) and formed a National Bioethics Advisory Commission make recommendations for performing embryo and stem cell research (Clinton, 1994). Based on their recommendations, Clinton was about to propose a bill to Congress allowing embryo research, but in 1995 the Republican-led Congress passed the Dickey-Wicker Amendment banning all embryo research (Kiessling, 2010). This ban remained in effect until Bush took office in 2001.

In 2001 in his first year as President, President George W. Bush made an executive order to ban federal funding for deriving any new ESC lines after August 21, 2001, the date of the order (Human Embryonic Stem Cell Policy, 2001). Although under this order federal money could be used to support ESC lines derived prior to that date, subsequent research showed that most of the approximately 60 ESC lines were genetically identical or were defective, so scientists complained they did not have a sufficient number of ESC lines for research purposes (Holden and Vogel, 2002). Several attempts were made by Congress to over-ride the executive order, but President Bush vetoed each attempt. Interviewed for his decision on ESC research, Bush replied: "This bill would support the taking of innocent human life in the hope of finding medical benefits for others. It crosses a moral boundary that our decent society needs to respect. So I vetoed it" (Bash and Walsh, 2006).

On March 9, 2009, newly elected President Barack Obama implemented an executive order to reverse Bush's ban on embryo research (Associated Press, 2009; President Barak Obama, 2009). Obama charged NIH with creating new guidelines for stem cell research, and he adopted those guidelines in his order. The order currently allows new ESC lines to be created from surplus reproductive IVF embryos with donor consent, and opens up many more ESC lines for researchers to use. Obama encountered a potential roadblock on August 23, 2010, when a U.S. district judge granted a preliminary injunction to stop the federal funding of ESC research because it violates the Dickey-Wicker amendment which he said was still in effect. The ruling by Judge Royce C. Lamberth was a blow to the Obama administration (CNN Library 2013), but on April 29, 2011, a federal appeals court announced that it would set aside that ruling. The uncertainty brought grant reviews at the nation's largest funding agency, the National Institutes of Health (NIH), to a halt (Singer 2011). The lower court appealed the decision to the Supreme Court, but on January 7, 2013, the Supreme Court declined to hear the appeal, allowing embryo research to continue. A three-judge appeals court panel unanimously agreed with a lower court judge's dismissal of the case (Baynes, 2013). Chief Judge David B. Sentelle said: "Unless they have established some `extraordinary circumstance,' the law of the case is established and we will not revisit the issue" (Holland, 2012). Dr. Francis Collins, Director of the NIH, said after the decision, "NIH will continue to move forward, conducting and funding research in this very promising area of science. The ruling affirms our commitment to the patients afflicted by diseases that may one day be treatable using the results of this research" (Holland, 2012).

As these examples show, ESC funding over the past 20 years has been inconsistent. The funding may decrease in the future if a President is elected who is against this type of research, making a strong argument for finding stem cell alternatives.

Stem Cell Plasticity as a Potential Replacement for ESCs

Due to the problems discussed above of using embryonic stem cells, scientists are constantly seeking alternative cells for therapies. This project focuses on the potential use of stem cell plasticity as a replacement therapy. In this process, cells other than ESCs are used to differentiate outside their normal pathways to replenish cells in other tissues. The existence of plasticity is extremely controversial, with many scientists arguing it does not exist, and if it does not even agreeing on what it is. Can hematopoietic stem cells that normally form blood cells be used to heal damaged heart cells in heart attack patients? If so, how do those cells heal the heart tissue; do they directly differentiate into heart muscle, or do they induce surrounding muscle cells to form more muscle?

Stem Cell Plasticity: Definition and Assay

Currently, there is no universally acknowledged definition of stem cell plasticity on record in this little understood and controversial field. Questions about what stem cell plasticity is, whether plasticity resides within the stem cell itself or is induced in surrounding cells, and what evidence can prove plasticity exists remain controversial. Due to these controversies, researchers have devised their own definitions of stem cell plasticity. Some representative definitions are discussed below.

In Theise's review article (2010), stem cell plasticity was given a very broad definition. Four different cell development pathways were described: 1) The first pathway describes the standard lineage differentiation, including the natural events of cell development and tissue maintenance. The growth of a fertilized egg to an embryo is one of the examples of this natural pathway. 2) The second pathway involves cell dedifferentiation. For instance, a somatic cell

regains the properties of stem cells which allow the cell to develop into another cell type. iPS cells are an example of this process. 3) The third pathway includes the most accepted definition of "plasticity," where "cells of one lineage become cells of another lineage, across organ or embryonic tissue barriers, by changing gene expression in response to microenvironmental cues" (Theise, 2010). Based on this definition, Theise implies the importance of a cell's environment inducing cell conversion. 4) The fourth pathway is cell fusion, the joining of the plasma membranes of two adjoining cells. Cell fusion results in a tetraploid cell with a change in gene expression caused by the merging of the cells. Much controversy exists about how adult stem cell plasticity occurs, especially between transdifferentiation and cell fusion. Both pathways have been confirmed by many researchers, but some researchers see only one type. Overall, in Theise's study, the term "plasticity" was defined as a change in cell types. So, based on this *broad* definition, stem cell plasticity can refer to normal cell differentiation within natural developmental pathways (i.e. the formation of blood cells from hematopoietic stem cells) and refers to the non-natural and rarer conversion of one stem cell type to other cell types (i.e. the formation of hepatocytes from hematopoietic stem cells) (Theise, 2010).

In contrast to Theise's comprehensive definition of plasticity, Wagers and Weissman (2004) state that "plasticity" refers to *unexpected* stem cell potency. In this view, adult stem cells are thought to be multipotent but normally tissue-specific, with adult stem cells playing a key role in tissue regeneration within a normal subset of cell lineages. Plasticity of adult stem cells, however, is the breakthrough of the normal subset barriers or trans-differentiation *outside* the normal developmental pathway to a completely different tissue. Trans-differentiation results in replacing tissue-specific markers and functional phenotypes of the original cell type with those of the new cell type (Wagers and Weissman, 2004).

For purposes of this project, we will use Wagers' definition and its focus of differentiation *outside* the normal developmental lineage into a different type of tissue. Given this definition of plasticity, the experimental proof of plasticity should be based on acquiring evidence for cross-lineage cell differentiation, and the acquisition of new gene profiles and new functional cell phenotypes of the original donor cell without evidence of fusion of the donor cell with a host cell.

Most of the documented evidence for plasticity in adult stem cells use bone marrow stem cells, which contain hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) (also called multipotent stromal cells). Under normal biological conditions, HSCs give rise to all blood cell lineages. MSCs normally generate multiple mesenchymal type lineages. Both types of adult stem cells are reported to have unexpected capacities to differentiate *across* cell lineages under specific experimental settings, although some scientists deny these cells can do this. Some studies attempt to purify the donor cells to include only HSCs or MSCs, while other studies simply use bone marrow cells regardless of what cells are included.

To demonstrate trans-differentiation potential, it is important to use a donor cell whose genome is tagged or is distinctly different than the host genome, so the survival and location of the injected cell can be mapped. Methods for tagging the genome of the donor cell include the use of fluorescent transgenes, like green fluorescence protein (GFP), or by Y-chromosome mapping. Some studies use the Cre-recombinase system for determining whether the original donor cells are the same cells expressing the therapeutic phenotype post-therapy. The acquisition of new functional characteristics by a donor cell can be assayed by transcriptional profiling techniques, such as RT-PCR or hybridization arrays, or RNA-SEQ techniques. Evidence for trans-differentiation would include a cell whose genome maps to the donor cell

while at the same time expressing mRNAs of a new cell type outside its normal lineage. And most importantly, based on the definition of plasticity chosen for this project, the evidence for plasticity should rule out cell fusion, as this process would not be the direct conversion of the donor cell outside its normal pathway (Wagers and Weissman, 2004).

Plasticity in Hepatic Cell Lineages

Evidence For Plasticity in Hepatic Lineages

One of the earliest studies showing evidence for bone marrow cell plasticity into hepatic lineages was in 1999 (Petersen et al., 1999). This research group transplanted bone marrow stem cells from male rats into female rats with induced liver injury. Several donor genetic markers were assayed including Y-chromosome, cross-strain, and cross-sex genes, and demonstrated the presence in liver of a subset of donor-derived regenerated hepatic oval cells. These hepatic oval cells then continued differentiating into mature hepatocytes. This initial study suggested that bone marrow stem cells are capable of trans-differentiating into a hepatic epithelial cell lineage (Petersen et al., 1999).

In 2000, one of the first trans-differentiation experiments using purified human hematopoietic stem cells showed they could be used to derive hepatocytes (Alison et al., 2000). Bone marrow transplants using male HSCs were performed on female patients with liver damage. Y-chromosome mapping was performed to indicate the origins of the donor HSCs. The result showed that many hepatocytes present in the transplanted female liver were Y-chromosome positive, showing they originated from the donor HSCs. Based on this result, human HSCs may

have the potential to differentiate into an epithelial hepatocyte lineage if given the proper growth environment such as damaged tissues (Alison et al., 2000).

Following these initial studies, a 2000 study was done by Lagasse's research group to show evidence for the plasticity of purified HSCs (Lagasse et al., 2000). Similar to contemporary state-of-the-art studies, they showed that purified HSCs were capable of differentiating into liver cells by transplanting bone marrow cells (initial experiment) or highly purified HSCs (second experiment) from male mice containing the wild-type gene for fumaryl-acetoacetate hydrolase (FAH) into female mice lacking this gene. Mutation of the FAH gene causes hepatorenal tryrosinemia, an inherited disease leading to severe liver and kidney failure. Using bone marrow cells, their results showed the livers of recipient mice contained donor-derived cells that covered 30-50% of the liver mass. The donor-derived repopulating hepatocytes were morphologically similar to normal hepatocytes, producing the FAH enzyme from the donor HSC gene. Several liver functional tests demonstrated a significant improvement in the recipients' livers. The research group also identified the type of bone marrow cells that gave rise to hepatocytes. Highly purified HSCs or KTLS isolated from normal adult male mice were injected intravenously into lethally irradiated adult female FAH deficient mice. The expression of the FAH enzyme and the presence of Y-chromosome markers were used to indicate the degree of hepatic engraftment in the recipient. The result showed the regenerated hepatocytes were positive for all parameters. Moreover, HSC-derived hepatocytes replaced a section of the diseased liver indicating a functional improvement in recipients' livers similar to the result with the bone marrow cells. A third experiment confirmed the results of the previous experiments and also showed that the purified HSCs (KTLS) were the only cell source in adult bone marrow to derive hepatic progenitors (Lagasse et al., 2000).

In 2004, evidence was obtained that HSC conversion to hepatocytes can occur without cell fusion (Jang et al., 2004). Based on the definition of plasticity chosen for this IQP, it is important to rule out cell fusion as the basis for the conversion. HSCs, after being co-cultured with damaged liver tissue, expressed several key liver transcription factors and cytoplasmic proteins specific for liver cells. The cell fusion process in liver tissue is difficult to assay because liver cells are often bi-nucleate, but the authors chose to assay sex chromosomes. They found that any tetraploid cells in the liver were host-derived female-female (XXXX) not donor-derived male-female (XYXX), indicating direct cell conversion occurred instead of cell fusion with the female hepatocytes. In addition to addressing cell fusion, this experiment also suggested that acute liver injury can be an important inducer of trans-differentiation (Jang et al., 2004).

In addition to the above mentioned studies with HSCs, other studies have shown evidence for the trans-differentiation of MSCs into liver cells. For example, in the presence of in vitro induced hepatocyte growth factor, bone marrow stromal cells (BMSCs), isolated from rat femurs and tibias, were capable of differentiating into hepatocyte-like cells (Wang et al., 2004). Several tests at the molecular level confirmed the hepatic functional characteristics of these BMSC-derived cells (Wang et al., 2004).

Possible Mechanisms of Plasticity and Liver Tissue

If plasticity exists, how it is accomplished (its mechanism) helps prove it exists. In general, two potential mechanisms for the engraftment of bone marrow-derived hepatic cells into liver tissue have been suggested: 1) with or 2) without the presence of liver tissue injury (Theise and Krause, 2002). Induced by various types and severities of liver damages, bone marrow cells engraft into liver through the circulation. Oval cells or other hepatic progenitor cells are present

at the engraftment site as a result of the liver injury. Under such a complex microenvironment, the marrow cells adopt the morphology of hepatic progenitor cells and continue developing into hepatocytes. To aid recruitment of the injected HSC to the wounded liver, one study reported that hepatic cells from a severe injured liver site release a type of chemokine (SDF-1) into the nearby bloodstream which attached to an SDF-1-receptor on the surface of the marrow cells (Theise and Krause, 2002).

Without acute liver injury, without the presence of oval liver cells, the marrow cells are thought to engraft in liver through a different pathway. The site and distribution of the engraftments are different from the ones during injury. In this case, some liver cells may act as short term progenitors that assist the marrow cells with engraftment and their further differentiation (Theise and Krause, 2002).

Several studies have identified specific growth factors responsible for deriving hepatic cells from adult stem cells. For instance, hepatocyte nuclear factor 4-alpha (HNF-4α), a nuclear receptor protein critical to liver development, was found to have a significant role in converting MSCS into hepatocytes. Compared with hepatoma cell lines, the hepatic cells derived from human mesenchymal stem cells expressed HNF-4α (Chen et al., 2010). To improve the efficiency of hepatic differentiation, the rat HNF-4α gene was introduced into MSCs through adenovirus delivery. As a result, the expressions of several hepatocyte nuclear factors and key hepatic markers like albumin were significantly increased (Chen et al., 2010). In addition, the P450 gene that expresses a detoxifying enzyme in the liver, was also induced by HNF-4α treatment. Overall, the differentiation level of MSCs-derived hepatic cells and further functions of these cells were efficiently enhanced by the over-expression of HNF-4α, thus HNF-4α appears to be one of the key determinants in hepatic induction from mesenchymal stem cells.

Comparison of Plasticity for Liver Tissue and ESC Therapy

Since the discovery of embryonic stem cells, with their strong pluripotency and ease of isolation and growth, ESC research has been regarded as the most promising area of regenerative medicine. Several studies have successfully used ESCs to generate hepatocytes, so these studies provide a comparison for judging whether bone marrow cells can replace ESCs for this type of therapy. One study directly compared ESCs to adult stem cells for liver therapy, identifying each of their advantages and disadvantages. ESCS are pluripotent so they have the ability to differentiate into all cell types in the body; therefore, designing ESCs to differentiate into a specific cell type (liver in this case) is more plausible than with adult stem cells. However, ESCs have been reported to form tumors in experimental settings, and the ethical issues of ESCs pose a problem. In any case, the use of ESCs or adult stem cells must produce cells that are similar enough to the target tissue to be functional and be stable, the level of differentiation must be sufficient to provide clinical improvement, and the mechanism must be more fully understood. So it might be too early to declare a winner between ESCs and adult stem cells.

In one study (Brolen et al., 2010), hepatocytes were derived from human ESCs in three stages. The first stage involved converting undifferentiated human ESCs into endoderm which can create liver cells. The second stage derived hepatic progenitor cells from the endoderm in the presence of key growth factors. In the final stage, the hepatic progenitor cells were further differentiated into mature hepatocyte-like cells that exhibited typical hepatocyte morphology and functional characteristics (Brolen et al., 2010).

In another study, scientists cultured human ESCs in 10% serum containing hepatocyte growth factors which helped convert the ESCS into hepatic lineage cells that expressed

endoderm-specific and hepatocyte-specific genes (Basma et al., 2009). These derived cells also produced functional hepatic proteins similar to those of well-differentiated human mature liver cells. Basic liver metabolic activities were also detected in these cells (Basma et al., 2009).

In a 2013 study, scientists showed that iPS cells can be used to create liver cells (Takebe et al., 2013). They co-cultivated hepatic endoderm cells induced from human iPSCs with endothelial and mesenchymal cells resulting in liver bud formation. The iPSC-derived liver buds were further transplanted into mouse models, and surprisingly were able to form vascularized and functional liver (Takebe et al., 2013). This study provides the first demonstration of functional human *organ* generation from pluripotent stem cells, which provides a breakthrough achievement in stem cell research. Someday, similar success may also be achieved with plasticity treatments.

Evidence Against Plasticity in Hepatic Tissue

A significant number of studies have failed to find evidence of adult stem cell trans-differentiation into liver cells. Based on his review of 77 studies related to derivation of hepatic cells from hematopoietic stem cells, Thorgeirsson and Grisham (2006) concluded that HSCs have little potential in hepatocyte regeneration. Thorgeirsson argues that HSC conversion into hepatic cells is a rare event, with a frequency of less than one in 10^{-4}. Without special treatment to enhance cell conversion, more than 80% of the studies examined resulted in the production of hematopoietic-derived hepatocytes with less than 0.05% of the total liver mass. Only 6% of the studies showed an engraftment rate in excess of 1.5%. However, under selective conditions such as induced liver injury, more than 50% of the studies reported a significant increase in the cell conversion rate. However, even under injury conditions, Thorgeirsson argues the conversion is

not due to trans-differentiation of hematopoietic cells; instead, he suggests the following possibilities: a) differences in post-engraftment amplification of hematopoietic cells is the cause of variation in efficiency of cell conversion; b) for those experiments performed in vitro, the unnatural culture environment might alter the nature of HSCs making them more "plastic" than occurs naturally; c) there is a lack of precision in donor cell detection methods. Overall, due to the low number of hematopoietic-derived hepatocytes, there was little chance these regenerated cells would contribute to liver repair, and compared to regular hepatocyte transplantation, hematopoietic stem cell treatment showed no advantage. In some cases the bone marrow cells responsible for the conversion were not identified, and cell fusion was not ruled out. And in some cases, liver function could improve without the injected cells fully becoming hepatocytes. Without converting into hepatocytes, HSCs can naturally contribute to liver function and repair by producing cytokines and growth factors. So, Thorgeirsson suggested further studies are required to verify the ability of HSCs to regenerate hepatocytes (Thorgeirsson and Grisham, 2006).

To demonstrate trans-differentiation, the donor cells must express tissue-specific markers, must show no remaining hematopoietic markers, and must exhibit morphology as well as phenotype of tissue-specific cells. Following this guideline, Wagers' group transplanted highly purified HSCs (or KTLS) containing the gene for green fluorescent protein (GFP) to track the donor cells during trans-differentiation. They saw no GFP-positive donor cells in the host liver, and concluded that no trans-differentiation occurred (Wagers et al., 2002). They acknowledged that HSCs might exhibit a wider potency when exposed to injured tissues, but concluded that even during injury there is little evidence for HSC trans-differentiation into liver (Wagers et al., 2002).

In 2005, Lian's group (2005) tested for adult stem cell plasticity using highly purified HSCs and two types of stimulation: cytokine addition and tissue injury. When treated with cytokines, several liver markers and hepatocyte transcription factors were detected in hematopoietic-derived liver cells. However, the same result was shown in their control group lacking the cytokines. Further tests indicated the HSCs were naturally capable of expressing liver-related markers both in vivo and in vitro. In addition, bone marrow cells could pick up albumin from the blood without trans-differentiation. Moreover, no difference was shown in hepatic markers whether the HSCs were cultured alone, with healthy liver, or with injured liver. Therefore, inconsistent with previous studies, neither cytokine nor tissue injury had any significant effect in inducing transdifferentiation and enhancing expression of tissue-specific markers (Lian et al., 2005).

Although several studies mentioned earlier showed evidence for bone marrow cell trans-differentiation into hepatocytes in FAH-mutant mouse models without cell fusion, not all studies agree with those studies. For instance, in one study, bone marrow cells from female FAH positive wild type mice with a specific transgenic marker were transplanted into lethally irradiated male FAH-mutant mice (Wang et al., 2003). Southern blot analysis for the transgenic marker indicated only a small fraction of positive cells, therefore, there was little possibility that trans-differentiation occurred. Additional experiments showed that a larger proportion of hepatocytes expressed the genotypes that could only result from cell fusion. This study concluded most of the regenerated hepatocytes in the FAH mouse model resulted from cell fusion between donor bone marrow cells and the host hepatocytes, with trans-differentiation being a rare event (Wang et al., 2003).

Plasticity in Myocardial Infarction Treatments

Acute myocardial infarction (MI) is a leading cause of morbidity and mortality worldwide. A myocardial infarction occurs when blood supply to the heart is interrupted, causing the irreversible loss of cardiac muscle or death (American Heart Association, 2002). Recently, the discovery of stem cell plasticity gives hope for repairing damaged myocardium without using ESCs. This section discusses the evidence for and against trans-differentiation of bone marrow derived cells into cardiomyocytes.

Evidence for Plasticity in Myocardial Infarction Treatments

In 2001, Orlic et al. demonstrated that when bone-marrow derived Lin^{-} c-kit+ hematopoietic stem cells (BM-HSCs) were injected directly into hearts in mouse heart attack models, these cells could improve cardiac performance, by increasing vascularity and regenerating cardiomyocytes by trans-differentiation into endothelial cells and cardiomyocytes, respectively. The injected cells were also able to produce cytokines and other factors that induce myogenic repair and prevent fibrosis (Herzog et al., 2003). Evidence for the direct conversion of HSCs into heart muscle was followed by tracking the fate of the transplanted cells using Y-chromosome mapping and GFP transgene markers present in the donor cells.

MSCs have also been shown to potentially trans-differentiate into cardiomyocytes. Treating these cells with the DNA demethylating agent 5-azacytidine (which switches on the expression of specific repressed genes) was shown to induce multiple new phenotypes, including cardiac phenotypes, showing the trans-differentiation capacity of MSCs in vitro (Makino et al., 1999; Hakuno et al., 2002**)**. In 2005, Fukuda and Fujita isolated and transplanted BM-MSCs into

mice finding that it was the MSCs not the HSCs in bone marrow that trans-differentiate into the cardiomyocytes. However, due to the small number of incorporated MSCs, and the use of animal serum to expand these cells in vitro, MSCs are still limited for clinical trials on humans (Yoon et al., 2005).

Possible Mechanisms for Plasticity Treatments for Myocardial Infarction

When cells undergo reprogramming, they alter their gene expression profiles. It is suggested that transplanted adult stem cells may recognize a change in microenvironments through cell surface receptors, which stimulate various transcription factors and regulatory molecules by signal transduction pathways (Avots et al., 2002). BM-HSCs and BM-MSCs stay in the BM stem cell niche under hypoxic conditions by increasing Oct4 expression and telomerase activity to maintain their stemness (Hass et al., 2011). With changes to the hypoxic conditions, stem cells increase the release of multiple paracrine mediators, which helps accelerate angiogenesis, arteriogenesis, cardiomyogenesis, reduce apoptosis, and promote the recruitment of circulating progenitors into the regenerating the damaged myocardium (Yoon et al., 2005).

MicroRNAs (miRNAs) are noncoding RNAs which bind the 3'UTR of target mRNAs to decrease their translation. Many miRNAs, such as miRNA-181, miRNA-223, and miRNA-142 are specifically expressed during hematopoietic lineage commitments (Chen et al., 2004). In BM-MSCs, miRNA-124 has been shown to regulate the in vitro synthesis of cardiomyocytes from BM-MSC (Cai et al., 2012).

Effectiveness of ESCs versus Plasticity for Treating Myocardial Infarction

ESCs have been used to treat heart attacks in mouse models. Utilizing differences in glucose and lactate metabolism, researchers are now able to isolate cardiomyocyte populations of up to 99% purity differentiated from human ESCs (Tohyama et al., 2013). And these cells can be electromechanically integrated into the host heart tissue (Shiba et al., 2012). However, the transplant of ESCs into human myocardium has not yet been performed, as teratoma formation was observed when human ESC-derived cardiomyocytes were transplanted into immunosuppressed Rhesus monkeys (Blin et al., 2010).

Myocardial infarction is one of the few diseases currently being treated in human patients by stem cells, so in this category some data is available to compare to ESC treatments. Bone marrow-derived stem cells used for autologous transplant have shown safety profiles in both animals and humans for treating myocardial infarction (Amado et al., 2005; Hare et al., 2012). The results, however, vary between clinical trials. Some trials showed significant improvement in the left ventricle ejection fraction (LVEF), while other trials reported either no improvement in LVEF upon treatment or increased LVEF in the control group. In 2010, Assmus et al. performed a double-blind and placebo-controlled clinical trial, termed the REPAIR-AMI trial, by transplanting autologous bone marrow cells back into the same patient (intracoronary delivery) following an acute myocardial infarction. Two years post-treatment, the patients showed significant improved outcomes and ventricular function. However, the TIME trial by Traverse et al. (2012) did not show any significant improvements in ventricular function after intracoronary delivery of autologous bone marrow cells in similar patients. Likewise, there was no significant improvement in the POSEIDON trial after the trans-endocardial injection of BM-MSCs in patients with ischemic cardiomyopathy (Hare et al., 2012). Differences in the cell preparation or

baseline patient statistics may explain the discrepancies in patient improvement. However, the issue needs to be investigated more systematically, and larger clinical trials are currently underway.

Evidence Against Plasticity for Myocardial Infarction

Although six studies have shown evidence for HSC or MSC plasticity when treating myocardial infarction, three other research groups argue that plasticity does not exist and are not able to reproduce any plasticity results (Wagers et al., 2002; Balsam et al., 2004; Murry et al., 2004). Wagers's group used a constitutively expressed GFP-reporter gene to tag their donor HSCs, and 4-9 months after transplant saw no evidence of GFP^+ cells in host non-hematopoietic tissues (such as heart, liver, kidney, lung etc.). They concluded that the injected HSCs did not trans-differentiate into cardiomyocytes. Similarly, Murry's research group tracked donor cells using either a cardiac restricted or a ubiquitously expressed GFP reporter gene. Although his data showed that donor cells could survive long term in the hematopoietic system, he saw no evidence of trans-differentiation into cardiac lineages. Murry's group also used another cardiomyocyte-restricted tracking system: donor BM-HSCs from a transgenic mouse line in which the cardiac-specific α-myosin heavy chain (αMHC) promoter drives the expression of a nuclear-localized β-galactosidase reporter gene. These β-gal+ cells would be readily detected if they trans-differentiated into cardiomyocytes using X-gal staining. However, 1-4 weeks post-transplant into the infarcted zone of non-transgenic recipients, no blue X-gal signals were detected in heart cells, indicating no cardiac trans-differentiation. In this case, the GFP reporter gene was also utilized to eliminate the possibility of gene silencing of the Lac-Z reporter. However, after using both tracking systems, Murry's research did not find any evidence for bone marrow-derived

cardiomyocytes.

Of the three mentioned research groups, although Balsam et al. (2004) saw no sign of plasticity, they did see cardiac improvement. Six weeks post-treatment, the cell-treated mice demonstrated a statistically significant, yet modest, improvements in cardiac output, ventricle fractional shortening, and decreased left ventricular chamber dimensions at end-diastole and end-systole. However, the infarct size was not significantly different between the two groups. Their observed improvement in cardiac output was probably limiting ventricular dilation and dysfunction for reasons that remain unexplained. These researchers concluded that HSCs cannot trans-differentiate into cardiomyocytes after myocardial infraction, and have provided an alert on this matter for the ongoing clinical studies.

Other researchers have claimed that the apparent trans-differentiation is only the result of *cell fusion* (Terada et al., 2002; Alvarez-Dolado et al., 2003). Using a Cre/lox recombination system to knock out specific genes on demand, they were able to track transplanted cells *in vivo* to argue that BM-derived cells fuse *in vivo* with several types of cells including cardiac muscle, contributing to the formation of multinucleated cells (Alvarez-Dolado et al., 2003). In this method, two lines of mice were used: 1) mice expressing Cre-recombinase (Cre) and GFP constitutively under the control of a hybrid cytomegalovirus enhancer β-actin promoter, and 2) the R26R transgenic mouse containing a LacZ reporter gene under the control of a promoter with a floxed stop signal (initially off) (Alvarez-Dolado et al., 2003). If an injected donor cell fuses with a host cell, the Cre-recombinase from the donor cell excises the floxed stop signal switching on the LacZ reporter in the fused cell. LacZ expression can be detected by the formation of a blue precipitate after X-gal staining, so the presence of blue cells in the host indicates that cell fusion has occurred. To test whether BM-HSCs contributed to fusion events *in vivo*, the Alvarez-

Dolado group used a specific configuration of the Cre-LoxP system where donor mice contained Cre under the control of a CD45 promoter, so Cre would be expressed only in cells of the hematopoietic lineage. Once Cre is expressed in those cells, it switches on GFP expression tagging the cells to follow their fate. The presence of GFP^+ β-gal^+ cells in the host heart would be evidence of cells of the hematopoietic lineage (GFP) fusing with host cells (β-gal^+). CD45-Cre donor BM-HSCs were injected into four lethally irradiated R26R LacZ recipient mice, which were examined 10 months after transplantation. GFP^+/β-gal^+ cardiomyocytes were observed in two of the four mice. These fused cells were observed to have the same morphology and alignment as other surrounding mature cardiac muscle fibers, and also express a cardiac specific protein troponin I, suggesting that BM-HSCs fuse *in vivo* with pre-existing cells in the heart to form mature cardiomyocytes. Any trans-differentiated GFP^+/ β-gal^- cells were rare (Alvarez-Dolado et al., 2003).

Plasticity in Stroke Treatments

Evidence for Plasticity in Stroke Treatments

Some researchers have shown evidence for plasticity of HSCs and MSCs when treating mouse stroke models. Stoke occurs when there is interruption in the blood supply to the brain. Stroke has a high mortality rate and causes severe disabilities worldwide. Recently, many researchers have observed improvements in motor function of rat stroke models following bone marrow cell transplantation.

Chen's and Felfly's research groups have injected bone marrow stromal cells (BMSCs) or Sca1+ bone marrow cells enriched for HSCs, respectively, into rat models with transient middle cerebral artery occlusion (tMCAO). Both research groups observed that the tMCAO rats

showed significant motor improvement (spent less time removing tape on one limb using the other paw) compared to the control group without any treatment, and showed improved survival rates (Chen et al., 2008; Felfly et al., 2010). Pavlichenko's group observed similar results using MSCs and the Morris water maze swimming test to measure memory and motor performance (Pavlichenko et al., 2008). The treated rats used less time to locate the hidden platform, and also showed improved survival rates. Thus, these authors conclude that transplantation of HSCs or MSCs can restore cognitive function of rats in a stroke model to nearly normal levels.

Mechanism

In order to explore the reason for the behavioral improvements in the rat stroke models, Chen et al. (2008) and Pavlichenko et al. (2008) isolated and cultured the MSCs from normal rat whole bone marrow. Then they used histochemical staining and fluorescent labeling methodology, respectively, to track the location of the transplanted MSCs (Chen et al., 2008; Pavlichenko et al., 2008). Felfly et al. (2010) also used a fluorescent labeled marker to detect the implanted HSCs in tMCAO mice. Significantly, the three research groups discovered that the implanted bone marrow stem cells migrated to the lesion site of the brain instead of following random blood flow, and the migration correlated with a reduction in infarct volume over time.

Chen et al. (2008) suggested BMSCs had the ability to express growth factors (such as nerve growth factor and brain-derived neurotrophic factor) that help maintain neural survival and brain function. By using the human-specific antibodies and neural cell markers, Zhao et al. (2002) were able to track their implanted hMSCs in rats and concluded they gave rise to neuronal cells after migrating to the infarct site (Zhou et al., 2002). Brazelton et al. (2000) detected the

presence of NeuN-positive and class III β-tubulin-positive neurons from 1 to 6 months after bone marrow transplantation in lethal irradiated adult mice using fluorescent markers.

In human patients, Mezey et al. (200; 2003) combined immunocytochemistry and fluorescent *in situ* hybridization histochemistry (using a probe against the Y-chromosome) to detect Y-chromosome-positive cells in female patients with neural disorders who received bone marrow from normal male donors. In their previous studies with mice, the group showed that bone marrow cells perfused intravenously migrated to the brain to improve neuronal performance, so they hypothesized the same might occur with human patients. Y-chromosome positive male donor marrow cells were found in the hippocampus and the neocortex of the patients (Mezey et al., 2000; 2003). Although the transplanted bone marrow generated only about 0.2% - 0.3%, and 0.025% - 0.05% of the neurons, respectively, in Brazelton's and Mezey's studies, this is strong evidence that bone marrow stem cells have the potential to regenerate new neurons in vivo. Although the research did not focus on whether the perfused cells directly trans-differentiated or fused with existing neurons, the data provides direct evidence that cells outside the normal neuronal lineages can locate in the brain and help create new neurons.

Also in human patients, Bang et al. (2005) treated human neural disorder patients with MSCs. The Phase I study showed that MSCs are safe to apply in larger clinical trials. In addition, since the MSC transplantation was autologous, obtaining the MSCs from the patients themselves, the immune reaction after the transplantation appeared to be avoided (Bang et al., 2005).

Borlongan et al. (2004) claimed that BMSCs were able to recover stroke impairments in rat models due to their ability to restore the damaged cerebral blood flow (CBF) and the blood brain barrier (BBB). Borlongan's research group measured CBF restoration of the rat models

before stroke surgery, during MCAO, and after BMSC transplantation, and they measured the BBB level with an Evans Blue permeability assay. They observed that the CBF level and the BBB level of the MCAO rat models reached to nearly normal levels after receiving more than 40,000 BMSCs from day 4 to day 11 after transplantation, compared to the control group with MCAO only. However, this study did not show any improvement in infarct volume reduction, perhaps due to the overall low level of new tissue regeneration.

Felfly et al. (2010) stressed that the number of injections and the number of cells are critical when treating stroke. They discovered that the tMCAO rats did not survive after only one injection of Sca1+ bone marrow cells on either day 1 or day 2 post-occlusion. Instead, the tMCAO rats survived with two consecutive Sca1+ bone marrow cell injections on *each* day 1 and day 2. The report suggested that the function of the first injection stabilized the stroke, and the second injection provided greater protection. And the number of therapeutic cells appears to be important in the treatment, which emphasizes that the cells to be used in therapy should be expanded before delivery if possible.

Comparison with ECS Therapy for Stroke

Researchers have also used ESCs to treat rat stroke models. Daadi et al. (2008) cultured human ESCs in vitro with neural growth factors EGF, bFGF and LIF, and yielded human neural stem cells (hNSCs). The cultured hNSCs were transplanted in vivo into rat stroke models, and developed into neurons (Daadi et al., 2008). The report indicated that the ESCs did not differentiate into tumor cells, which was a significant finding. However, further investigation would be required to determine whether other experiments with ESCs induce tumors as observed with other research teams.

Evidence Against Plasticity in Stroke Treatments

Some researchers argue that stem cell plasticity does not exist when treating stroke. Castro et al. (2002; 2003) tried to detect the presence of donor cells converted to neural cells after transplanting bone marrow stem cells in irradiated mice, but failed to identify any surviving donor cells. Their donor cell marker, LacZ, did not apparently express in the brain cells of the stroke mice after stem cell injection (Castro et al., 2002). Mezey et al. (2003) mentioned in their study that donor markers might not be easy to detect since they might be lost or silenced after transplantation. Thus, they suggested Castro et al. should choose more reliable markers, such as Y-chromosome mapping, to detect surviving donor cells (Mezey et al., 2003). As a reply, Castro et al. (2003) claimed that their donor marker was present in the brain after bone marrow transplantation but did not show up in any new neurons. Thus, they claimed the implanted bone marrow stem cells were incapable of trans-differentiating into neural cells.

Overall, with respect to the stroke treatments, many research groups have shown that bone marrow stem cells can migrate to non-marrow tissue and regenerate new tissues (reviewed in Borlongan et al., 2011), but their survival in the new tissues and how strongly the surviving cells contribute to the improved function is not fully understood, so more studies are needed for a conclusion.

Plasticity in Diabetes Treatments

Evidence for Plasticity in Diabetes Treatments

Diabetes is a disease where the body does not produce enough insulin or does not respond well to insulin, resulting in glucose mismanagement and hyperglycemia. In type I

diabetes, the pancreatic islet β-cells deteriorate and fail to secrete insulin. In type II diabetes, cells fail to respond to the insulin that is made. Scientists are currently using various types of stem cells to attempt to treat type I and II diabetic animal models. The treatments include ESCs, adult pancreatic stem cells, and bone marrow stem cells containing HSCs and MSCs. In the bone marrow treatments, if the marrow cells differentiate into insulin-producing β-cells, it provides evidence for trans-differentiation, as marrow cells do not normally form pancreatic tissue.

One of the first studies with diabetes showing evidence of trans-differentiation was done in 2003 (Hess et al., 2003). This study used adult bone marrow cells to treat a type I diabetes mouse model treated with streptozotocin to induce pancreatic damage. The transplanted cells localized to the pancreas, proliferated, and resulted in the formation of islet-like structures that secreted insulin and lowered glucose levels (Hess et al., 2003).

A second key 2003 study done at New York University School of Medicine also saw evidence that bone marrow could produce insulin-producing tissue *in vivo* in mice, but went further to show the treatment resulted from the direct trans-differentiation of the transplanted donor cells (Ianus et al., 2003). A Cre-lox system was used to visualize donor cells directly expressing insulin. In this system, a Cre-recombinase gene (Cre) driven by an insulin promoter was inserted in the male mouse donor genome. The donor cell genome was also engineered to contain a fluorescent tag (enhanced green fluorescent protein, eGFP) under the control of a promoter with a stop codon flanked by Lox-P sites. If a specific donor cell trans-differentiated to express insulin, the Cre-recombinase gene was switched on which removed the Lox-P sites on the GFP stop codon switching on GFP. Evidence of direct trans-differentiation of a donor cell occurs if Y-chromosome-positive cells from the male donor also express GFP. Four to six weeks

after transplantation, recipient mice showed Y-chromosome and EGFP double-positive cells in pancreatic islets, providing evidence of direct trans-differentiation. As a negative control, neither of the donor or recipient cells, from the bone marrow cell or peripheral blood cell, were positive for GFP, so none of these cells outside the pancreas trans-differentiated. The trans-differentiated GFP-positive cells purified from islets expressed insulin, glucose transporter 2 (GLUT2), and transcription factors typically found in pancreatic β cells (Ianus et al., 2003).

Another study showing evidence of trans-differentiation into β-tissue is provided by Oh et al. (2004). Similar to the previous study, this study used adult bone marrow cells to treat a type I mouse model. Western and ELISA analysis of glucose-treated cultured cells showed that insulin was produced. After transplantation of the cultured cells into hyperglycemic mice, the serum glucose levels became normal for at least 90 days. Later removal of the graft resulted in death of the animal. Electron microscopy of the graft showed that the cells had same ultrastructure as mature β-cells. So, this study showed that bone marrow stem cells can trans-differentiate into pancreatic insulin producing cells in vitro which can be used in vivo to treat diabetic mice (Oh et al., 2004).

Purified mesenchymal stem cells (MSCs) have also been used to treat diabetes mouse models. MSCs have excellent differentiation potential, and have recently become widely researched. Abdi et al. (2008) used MSCs to treat diabetic mouse models and found increased islet sizes and improved insulin production. The human MSCs cells used to treat the diabetic mice were detected in the islets as double positives for human-2-microglobulin and mouse insulin. Because MCSs have shown success in treating a large variety of diseases, including cancer, reducing the incidence of GVHD after bone marrow transplantation, myocardial infarction, amyotrophic lateral sclerosis, metachromatic leuko-dystrophy, and Hurler syndrome,

the data prove that MSCs are capable of very broad differentiation *in vivo*. However, thus far no human clinical trials have used MSCs to treat diabetic patients (Abdi et al., 2008).

Another group of scientific researchers also used MSCs to treat diabetic mouse models. Boumaza et al. (2009) found that the MSCs that migrated to the hematopoietic environment secreted immune-regulatory hormones such as IL-6, HGF, and TGF-b1, while those MSCs that migrated to the pancreas expressed insulin and glucagon, showing that the *in vivo* micro-environment is important for stem cell differentiation (Boumaza et al. 2009).

Hao et al. (2013) also confirmed the successful used of MSCs for treating diabetic mouse models. Their infusion of MSCs decreased hyperglycemia to normal levels in type 2 diabetic rats, and normoglycemia was maintained for at least 9 weeks.

However, Shin and Peterson (2012) showed little improvements in their treatment of diabetic mice with MSCs. They observed relatively few injected MSCs recruited to the pancreas in the diabetic mice. They concluded that stem cells might be impaired in diabetic mice, and identified endogenous MSCs as a potential therapeutic target in diabetes (Shin and Peterson 2012).

In addition to using adult stem cells, ESCs have also been used to treat diabetic mouse models, and their success should be compared to the use of adult stem cells when trying to ascertain potential replacement therapies. ESCs have been used in several studies for treating diabetic mice. For example, Lie et al. (2011) developed an *in vitro* protocol for differentiating ESCs into pancreatic tissue by suppressing expression of the protein Nanog. The protocol increased the expression of markers essential for pancreatic epithelium development, and the transplanted cells revealed a homogenous pancreatic exocrine-like morphology that stained positively for amylase. Several other studies have developed protocols for differentiating human

ESCs into insulin producing cells (Assady et al., 2001; Lumelsky et al., 2001; Seguev et al., 2004; D'Amour, 2006), and have used mouse ESCs to treat NOD mouse models of Type-I diabetes (Beilhack et al., 2003 and 2005; Hess et al., 2003). As discussed in the ESC section, ESCs can sometimes produce teratoma tumors. For example, Fujikawa et al. (2005) implanted islet-like tissue differentiated from ESCs into mice which negated the hyperglycemia for 3 weeks, but teratoma formation then negated the success (Fujikawa et al., 2005).

Evidence Against Plasticity in Diabetes Treatments

Although no diabetes study has directly disproved trans-differentiation, some academic researchers do not believe the diabetes plasticity data is convincing, so continue to treat diabetes by doing more traditional *islet transplantations*. For example, the laboratory of Professor Kenneth Brayman treats diabetes in patients (ClinicalTrials.gov, identifier NCT00703599) or mice by islet transplantation (not stem cells) to replace β-cells (Jahansouz et al., 2011; Chhabra and Brayman, 2013). Another islet transplantation study in 2013 from same lab restored endogenous insulin production in mice and normoglycemia. They hope for a 5 year survival of the graft. A difficult part of islet transplantation is that patients require islets from several donor pancreases to create a successful graft (Ramesh et al., 2013). In addition, Kang et al. (2005) showed that islet treatment of diabetic NOD mice can restore normoglycemia, but in humans the treatment would be complicated by the use of immune-restrictive drugs and by limited organ availability. The treatment was successful when performed early in the disease, but not when administered late after host islet destruction. For the late treatments, "despite obtaining full hematopoietic engraftment in over 50 transplanted mice, only one mouse became insulin independent, and no b-Gal positive [donor] islets were detected in any of the mice.

METHODS

This project had four objectives:

1. **Develop** a comprehensive assessment of the scientific evidence for and against the existence of stem cell plasticity, including its discovery, various methods of trans-differentiation, and diseases being treated.
2. **Characterize** what key scientific stakeholders believe are the strengths and weaknesses of the existing data that this process exists, and their suggestions for potential solutions.
3. **Evaluate** the evidence for and against the existence of stem cell plasticity.
4. **Recommend** potential solutions to resolving any issues associated with proving stem cell plasticity exists, including rigorous methods for ruling out cell fusion, and make recommendations for resolving any remaining issues of using iPS cells for therapy.

To accomplish objective-1, we performed an extensive review of the current research literature, including reputable academic journal articles, relevant books, scholarly websites, and other pertinent materials.

To accomplish objective-2, we conducted an extensive set of semi-structured, in-depth interviews with various academic stakeholders in the stem cell field who claim to have developed plasticity-type differentiation procedures, or who argue that stem cell plasticity does not exist, to determine their range of opinions on this technology and whether such cells could be used as a replacement for ES cells in therapies. The stakeholders included academic experts on stem cell plasticity (for and against), including some who are currently using such cells for treating diseases. The interviewees also included some academic ethicists to help classify such cells, and contrast their usage with ES cells. Some of the stakeholders were initially identified by referral from the project advisor, Dave Adams, but most were identified from the literature, and especially by referral from the initial interviewees (to develop a referral "snowball").

Once contact was made with a potential interviewee, a time and place was set up for the interview to be performed at the interviewee's workplace. Whenever possible, some interviews were conducted in person, but most were conducted by phone or Skype. Our first round of prospective interviewees was contacted by email and/or phone. If no response was received after one week, we used follow-up emails or phone calls. We developed our interview questions (see report **Appendix**) based on our background research. Then based on their responses to our initial questions, we tailored our subsequent questions to best obtain information from that person.

With respect to the method of the interview, if the interview was performed in person, whenever possible it involved two team members, so that one member could ask questions while the other member wrote detailed notes, and vice versa. For either email or phone interviews, we informed the interviewee about the purpose of our project, and asked whether the interviewee consented to be quoted. If necessary, we explained how we would protect their confidentiality by giving them the right to review any quotations used in the final published report, explaining that the interview is voluntary, and explaining that the interviewee may stop the interview at any time or refuse to answer any question. After the interview, we asked each interviewee for permission to follow-up with them at a later date if needed to fill in any gaps in the information. And, as mentioned above, asked them to recommend other potential stakeholders we might interview, to further increase the number of interviews with key individuals.

With respect to the total number of interviews needed for our project, we stopped interviewing when we obtained sufficient information to represent all sides of the problem, and when all unclear points had been clarified.

To accomplish objectives-3 and 4, the group synthesized all of the information collected in our literature research, interviews, and follow-up interviews to ascertain the strength of the evidence for and against stem cell plasticity, and created recommendations for further research.

RESULTS/FINDINGS

Based on the Literature Review performed for the first part of the project, we documented the need to find replacement cells for embryonic stem cells (ESCs), and identified several key controversies associated with adult stem cell plasticity as a potential ESC replacement. We then performed a series of selected interviews with scientists on both sides of the various arguments to help evaluate the issues.

ESC Ethics and Funding: The Need for an ESC Replacement

As documented in the Literature Review, ESCs are isolated from a 5-day old IVF blastocyst embryo, which destroys the embryo. The destruction of the embryo has ethical problems for individuals who believe that life begins at conception or who believe that life in any form should be preserved. Although the purpose of ESC research is to devise therapies for treating diseases to alleviate suffering, it violates the moral principle to respect human life (Hug, 2006). Our Literature Review documented that different religions have different perspectives on when embryos reach full human status after fertilization. For example, while the Roman Catholic, Orthodox, and conservative Protestant Churches believe that life begins at conception and that no embryo research should be allowed (Farley, 1999; Hug, 2006), other religions, such as Judaism (Gilbert, 2010) and Islam (Siddiqui, 2002), Buddhism (Keown, 2004) and Hinduism (Bahnot, 2008) believe that ensoulment begins later and have no major objections for embryo research so long as the research is used to attempt to save lives.

In order to have a better understanding of the ethical concerns associated with various types of stem cell research, we conducted an interview with Dr. Jason T. Eberl, an academic bio-

ethicist who occupies the Semler Endowed Chair for Medical Ethics at Marian University, College of Osteopathic Medicine in Indianapolis, Indiana. Dr. Eberl indicated that unlike ESC therapies, ASCs therapies usually receive acceptance from the public, including the opponents of human ESCs (Eberl, personal communication, 7-23-13). In fact, most of the stakeholders knowledgeable in stem cell research think that ASC research will eventually lead to beneficial therapeutic outcomes. Dr. Eberl also pointed out a prominent example for individuals against ESC research but who support ASC research is the second conference held by The Vatican in April 2013, who emphasized the use of different ASC therapies for different diseases. In addition to supporting ASC therapies in general, Dr. Eberl also mentioned several other therapies such as altered nuclear transfer with oocyte-assisted programming, iPSC therapies, and chimeric embryos as great alternatives for ESC therapies. Referring to the safety and efficiency of stem cell clinical trials, Dr. Eberl claimed there is always some degree of risk in any clinical trial, so the most important thing moving forward is to make sure that applicants are informed of both known and unknown risks before agreeing to participate in the trials.

Our Literature Review also documented how the federal *funding* for ESC research has varied considerably over the past three U.S. presidencies. President Clinton was generally in favor of embryo research and instituted the NIH Revitalization Act (1993) recommending that embryo research be funded under special circumstances. But before he was able to institute any funding, in 1995 Congress enacted the Dickey-Wicker Amendment that prohibited federal spending on embryo research. In 2001, newly elected President Bush enacted his Human Embryonic Stem Cell Policy (2001) which allowed federal spending only on ESC lines derived prior to August of 2001, and that ban remained in effect until President Obama took office in January of 2009. Obama currently allows federal funding for embryo research (Bayes, 2013),

but only on those embryos prepared by IVF in reproductive clinics, no longer needed for reproduction, and only with donor consent. This inconsistent funding not only applies to the U.S. but to all five of the top stem cell research countries, the U.S., Japan, Germany, UK, and France (Couffignal-Szymzcak, 2009). In an interview with *Science* magazine, Janet D. Rowley, the Deputy Dean for Research at the Pritzker School of Medicine of the University of Chicago, commented on why more progress has not been achieved for ESC therapies by saying "It's fair to ask why we lack convincing data on the use of embryonic stem cells to treat diabetes, Parkinson's disease, and other medical problems. The answer is hardly surprising: U.S. scientists have been prevented [during periods of funding bans] from working on these very critical problems….in federally funded projects" (Rowley et al., 2002).

Adult stem cell (ASC) therapies, which are not associated with the destruction of human embryos and are not subjected to the same inconsistent funding as ESCs, appear as promising replacement for ESC therapies. However, our research has determined that ASCs also have a few problems. They typically do not grow as well as ESCs, they are rare cells in the body so are hard to to identify and isolate, and their differentiation potential is typically lower than ESCs. So, research progress on ASCs has also been relatively slow. The type of ASC therapy investigated in this project as a potential replacement for ESCs involves stem cell *plasticity*.

Definition of Plasticity

Initially, our attention was drawn to the topic of stem cell plasticity from several studies (discussed in the Literature Review) showing that adult stem cells such as hematopoietic stem cells (HSCs) or mesenchymal stem cells (MSCs) (both typically isolated from bone marrow cells) sometimes appear to benefit an organ outside their normal developmental lineages, such as HSCs

benefiting human heart attack patients. Such treatments if they actually work might serve as alternatives to using ESCs to treat the same disease.

But one of the first things we learned about stem cell plasticity is that few researchers agree on what it is. Stem cell plasticity lacks a standard definition; each researcher interprets the term in different ways. To illustrate the confusion, Saul J. Sharkis from Johns Hopkins University defined stem cell "plasticity" as being stem cell *potency,* the ability of a stem cell to form multiple cell types (Sharkis, personal communication, 6-18-13). This definition would include trans-differentiation and all normal cell developmental pathways. However, Diane Krause from Yale University School of Medicine stated, "stem cell plasticity is a problematic term; although the term mostly refers to the potential of a cell to differentiate into a lineage that it "should not," ironically, no one has a clear understanding of what a cell should do or should not do *in vivo* (Krause, personal communication, 7-2-13). Our Literature search indicated that scientists are loosely divided into two camps: 1) those with a very broad interpretation of the term that focuses on a change in the molecular status of a cell, and 2) those with a more narrow interpretation more pertinent to our project that it applies to cells differentiating in a pathway different than usual biological development. As an example of the broad definition group, Neil David Theise, of the Albert Einstein College of Medicine (NY) believes that plasticity is one of the innate properties of all cells, and refers to their ability to change their gene expression profiles over time (Theise, personal communication, 7-10-13). These changes can result from normal cell development, from cell fusion, or from cell trans-differentiation outside a normal developmental pathway. For purposes of our project, we chose the narrower version of the definition, trans-differentiation outside the normal developmental pathway. As an example of this stance, Hady Felfly from University of Miami, who studies the use of HSCs to treat mouse

stroke models, when interviewed claimed that stem cell plasticity is "the ability of a stem cell to differentiate across tissue boundaries, that is, a stem cell resident in a particular tissue gives rise to a cell type of a different tissue" (Felfly, personal communication, 6-15-13). However, this more narrow definition of plasticity referring to differentiation "outside the normal developmental pathway" implies that we actually *know* what normal development is. So, for purposes of this project we will indicate when the evidence for plasticity is strong and when it is weak.

Detecting Plasticity

Experimentally determining whether a cell has undergone differentiation outside its normal lineage is not an easy task. Our Literature Review determined that most scientists use a donor-host combination to help solve the problem. In this approach, usually HSCs, MSCs, or bone marrow cells containing a mixture of the two, are isolated from a host animal and injected into a recipient animal that is a model for a disease. For example, HSCs (that normally form blood cells) might be injected into a mouse model for stroke to determine whether they can differentiate into neurons or glial cells. The donor and recipient cells are usually tagged genetically to distinguish them from each other. In the stroke example, valid evidence for trans-differentiation would consist of demonstrating clearly that an injected HSC tagged with a GFP reporter migrates to the recipient's brain and in that environment differentiates into a cell expressing a variety of neuronal markers and helps improve neural performance. Our results indicate that it is important to make sure the evidence rules out the possibility of cell fusion, i.e. the injected cell itself should express the neural markers not a host cell the injected cell has fused with (Wagers and Weissman, 2004). It is also important when staining cells with antibodies

against tissue-specific markers to make sure it is the injected cell itself that expresses the new markers not cells above and below the focal plane. It is also important when not seeing any evidence of host-cell survival, to make sure the reporter gene has not become silenced in the donor cell (which would give a false negative finding).

Based on our interviews and Literature Review, we have identified what we believe is the best practice methodology for detecting trans-differentiation. This method uses the Cre-recombinase / Lox-P system for tagging fused donor-host cells. **Figure-1** shows how the Cre-Lox system typically works (we will subsequently show how to modify the general system for detecting fused cells). In the first figure, the mouse on the upper left expresses Cre-recombinase (Cre) under a promoter that is always on. This mouse is crossed with the one in the upper right containing a target gene flanked by Lox-P restriction sites. Genes flanked by Lox-P sites are termed "floxed". The floxed target gene is upstream from a GFP reporter. In the offspring mice actively expressing Cre (lower left) in the same cells that contain the floxed target gene, the Cre cuts the Lox-P sites excising the targeted gene for deletion, switching on GFP expression.

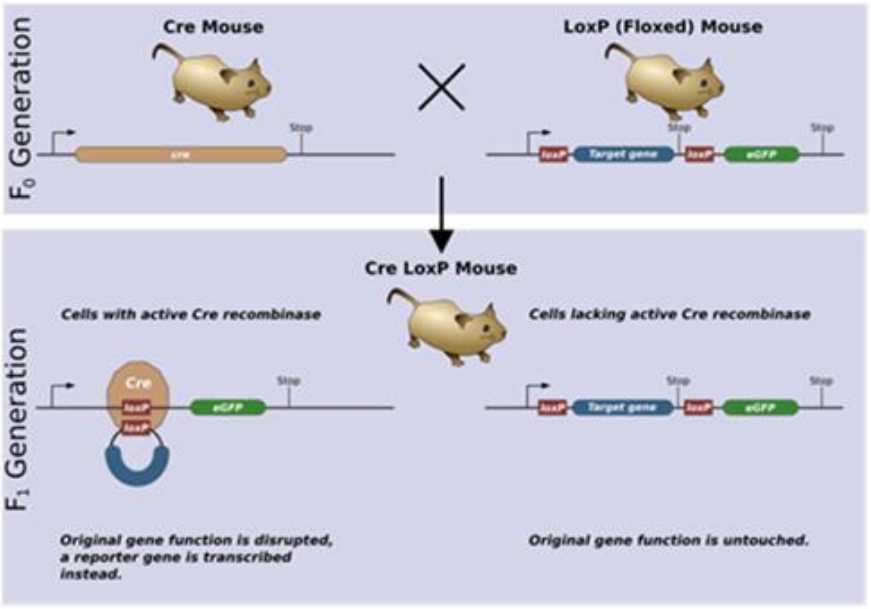

Figure-1: Diagram of the Cre-Recombinase / Lox-P System for Tagging Cells. (Zepper Wikipedia.com)

Ianus et al. (2003) modified the Cre-LoxP system to demonstrate that donor bone marrow cells trans-differentiate into pancreatic islet beta cells without cell fusion (**Figure-2**). The donor mice contained the Cre gene under the control of an insulin promoter that would be switched on if the cells became pancreatic. The donor cells also contained eGFP reporter under the control of a floxed stop codon (**Figure-2a**). Male donor bone marrow cells were transplanted into lethally irradiated female mice (bone marrow transplant) to rescue the hematopoietic system (**Figure-2b**). Their results showed that the host pancreas contained cells glowing for GFP and containing male Y-chromosome markers (of donor bone marrow origin).

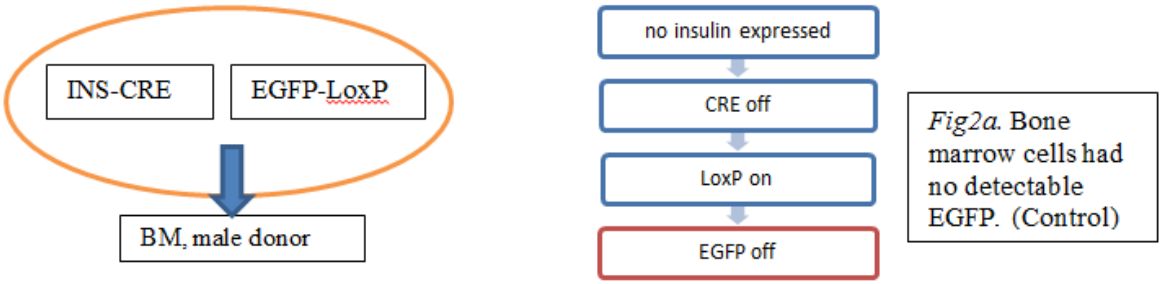

Fig2a. Bone marrow cells had no detectable EGFP. (Control)

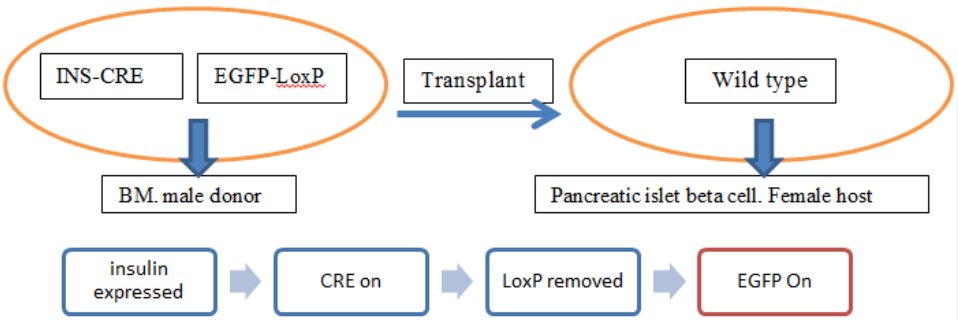

Fig2b. Transplanted bone marrow cells were EGFP positive indicating possible trans-differentiation.

To eliminate the chance of cell fusion, the experiment was modified to that the male donors contained Cre under the control of an insulin promoter, while the female recipients contained eGFP under the control of a floxed stop codon (**Figure-2c**). In this case, GFP

fluorescence would only occur if the donor cells fused with the host cells (the Cre would be expressed in insulin-producing cells, and if fused with host the floxed stop codon in front of the GFP gene would be removed). Alvarez-Dolado et al. also used Cre-LoxP system that was reported by lacZ gene instead of EGFP, to demonstrate cell fusion (2003).

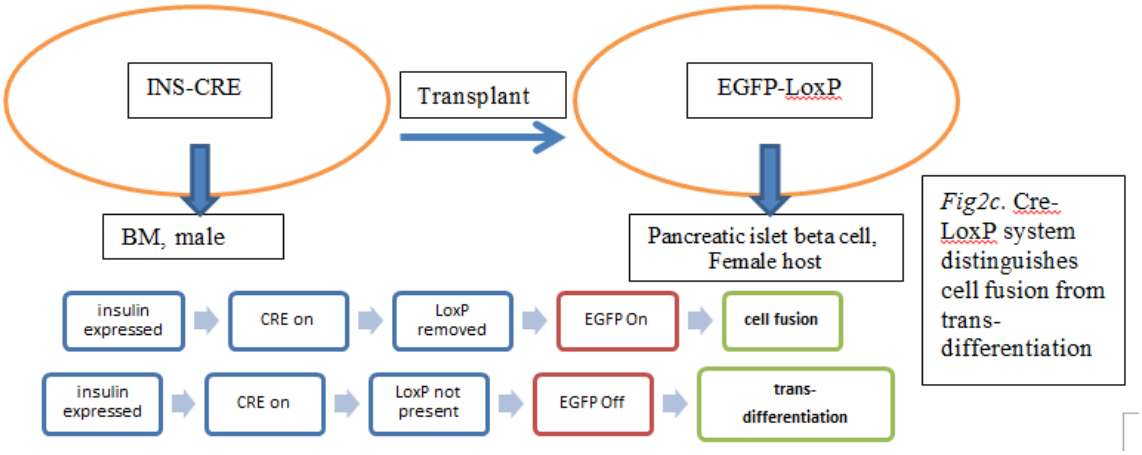

Fig2c. Cre-LoxP system distinguishes cell fusion from trans-differentiation

Evidence for Plasticity

In this project, we focused our research to determine whether plasticity exists for four different therapies: liver disease, strokes, myocardial infarction, and diabetes. For each disease, we found evidence in the literature and interviews for and against plasticity. The evidence in favor of plasticity was strongest for diabetes, where we identified 7 studies arguing for plasticity, and no studies arguing against it. The evidence for plasticity was somewhat balanced for the other three diseases analyzed: stroke (2 for, and 3 against), liver disease (5 for, and 4 against), and heart attacks (5 for, and 6 against). Several researchers demonstrated evidence for stem cell plasticity when observing bone marrow derived cells trans-differentiate into different cell types *in vivo* such as hepatocytes (Lagasse et al., 2000), cardiomyocytes (Orlic et al., 2001), β-cells

(Oh et al., 2004), neural cells (Felfly et al., 2010). Our research has identified what we believe are two best-practice methodologies for tracking trans-differentiation without cell fusion. The first is Ianus et al. (2003) performed at the New York University School of Medicine who inserted the Cre-recombinase gene driven by an insulin promoter into the male mouse donor genome. The donor mouse genome was also engineered to contain a floxed-stop-GFP gene. If an injected donor cell migrated to the pancreas and trans-differentiated to express insulin, the Cre-recombinase gene was switched on removing the Lox-P sites on the GFP stop codon switching on GFP. Evidence of direct trans-differentiation of a donor cell occurs if Y-chromosome-positive cells from the male donor also express GFP, which the authors indeed observed.

The second best practice methodology identified in our research is exampled by Alvarez-Dolado et al. (2003). In the donor mouse genome, they placed Cre under the control of a CD45 promoter, so Cre would be expressed only in donor cells of the original hematopoietic lineage regardless of what they differentiated into later on. The donor genome also contained a floxed-stop-GFP gene, which would be removed by Cre in originally hematopoietic cells. So, in the mixing experiment, donor cells of the original hematopoietic lineage could be identified in the various host tissues as GFP+ cells. In the host mouse, they inserted a floxed-stop-LacZ gene, so if those cells fused with donor hematopoietic cells (containing Cre) LacZ would be switched on and would be visible as a blue color with X-Gal stain. In a heart attack mouse model, they observed GFP+ cells in the heart (hematopoietic donor cells) but the cells were also blue (LacZ+) so had undergone fusion with host cells, indicating no direct trans-differentiation. This best practice methodology should, however, be used by other labs to determine whether their observed improvements in disease performance are due to cell fusion.

A significant problem we encountered was that even within one disease model, some scientists found evidence supporting plasticity while others did not, so many of our interviews focused on resolving this discrepancy. We identified several potential reasons for the discrepancies in the literature including: 1) silencing of reporter genes used to detect host genes (giving false negatives), 2) not using the sensitive and powerful Cre-Lox system, 3) differences in the developmental ages of the donor and host animals leading to a decreased survival of donor cells in the host animals, 4) the purity and composition of the donated stem cells (the cruder the stem cell batch, the more likely it is to have highly potent stem cells), and 5) the micro-niche colonized by the injected stem cell.

Hady Felfly of the University of Miami, who studies the use of HSCs to treat stroke models, pointed to the importance of stem cell micro-niches in these donor-host injection experiments. In the past several years, scientists have shown that the micro-environment immediately surrounding a stem cell strongly affects its properties. Extending those findings to the injection experiments, one experiment might show that the injected cells migrated successfully to a micro-niche that encouraged cell trans-differentiation to cure the disease, while another lab group might not see the same micro-niche incorporation. "Stem cells require specific artificial microenvironments for trans-differentiation, but the stem cells might lose some of their potentials in non-natural niches" (Felfly, personal communication, 6-15-13). And to get correct expression of a GFP reporter gene, before transplanting the donor cells into an animal, the stem cells have to go through several *in vitro* manipulation steps, such as transfecting the stem cells with a GFP expressing vector, which might change their properties. Saul Sharkis of Johns Hopkins University suggested that another reason some groups may not detect stem cell plasticity relates to the potency of the stem cells (Sharkis, personal communication, 6-18-13). He

indicated that the more primordial and potent the injected cells, the increased chance of forming other tissues and of graft survival. This could strongly be a factor when some groups work with highly purified populations of cells they believe are HSCs, while other groups inject total bone marrow cells which would include cells of a variety of potencies.

Evidence Against Plasticity

Other research labs see no evidence for plasticity. For example, our Literature Review identified the following labs against plasticity in cardiomyocytes (Wagers et al., 2001, Murry et al., 2004), neural cells (Castro et al., 2002, 2003), and pancreatic β-cells (Shin and Peterson, 2012). Dr. Daniel A. Peterson at the Chicago Medical School in North Chicago, Illinois uses MSCs to treat diabetic mouse models. His injected MSCs induced the mobilization of existing host endogenous stem cells to the injection site to heal the pancreas, but the grafted MSCs did not survive long term in the host, so they mainly appeared to affect endogenous stem cells (Peterson, personal communication, 7-26-13). His research group has not found any data supporting the trans-differentiation of MSCs into insulin-producing cells, concluding that long-term the MSCs can only form bone, cartilage, and fat tissues, not pancreas.

Likewise, Dr. David M. Harlan from the University of Massachusetts Medical School, who has been studying the Islets of Langerhans within the pancreas to understand the cause of diabetes, believes the early reported evidence for bone marrow-derived stem cell trans-differentiation is unreliable and controversial, and has not been able to reproduce the earlier findings (Harlan, personal communication, 8-2-13). After spending more than a year investigating the potential trans-differentiation of bone marrow cells into neural cells, Dr. H. David Shine from the Baylor College of Medicine and his group did not observe any evidence of

surviving donor cells that would have expressed the β-galactosidase reporter gene. Even after trying other methods, such as Mezey's recommendation to perform Y-chromosome mapping (Mezey et al., 2003), they were still unable to see any evidence for plasticity. He believes that the transplanted bone marrow cells might expedite the healing process, but themselves do not change cell fate. He regards all the claims for bone marrow cell plasticity with skepticism, believing such claims should be carefully evaluated.

Similarly, Dr. Derek Rossi from Harvard University provided reasons why stem cell plasticity is not likely to occur naturally. Dr. Rossi claims it would be extremely difficult for a cell to change its fate in vivo once it has committed to a lineage. For example, HSCs are normally only able to form blood and immune cell types in vivo based on our current knowledge of developmental biology. He thinks the reported evidence for stem cell plasticity might just be the results of cell fusion (Rossi, personal communication, 7-5-13). Secondly, he believes that stem cell plasticity, if actually occurs, would require tremendous changes in both epigenetic and transcriptional programming, which do not happen under normal physiological conditions. Our research has shown that major reprogramming indeed can occur under special conditions, such as the injection of a skin nucleus into an enucleated egg (John Gurdon's nobel prize winning experiments) and in the case of induced pluripotent cells transfected with genes encoding reprogramming transcription factors (Yamanaka nobel prize). Thirdly, Dr. Rossi suggested the technical difficulties of tracking injected cells in mice, especially when the percent surviving cells are low. For these reasons, Dr. Rossi considered stem cell plasticity mostly disproven and not an enthusiastic research area anymore.

For more than the last 10 years, several researchers have analyzed the use of HSCs for treating heart attacks, after initial studies such as Orlic et al. (2001) claimed that bone marrow-

derived stem cells could regenerate myocardium. However, some researchers including Dr. Charles Murry from the University of Washington in Seattle, Washington, and Dr. Amy Wagers from Harvard University found that the data could not be reproduced. We conducted two interviews with these researchers to attempt to understand the conclusions drawn from their studies. Dr. Murry, while believing that stem cell plasticity indeed exists under certain circumstances (such as artificial cell reprogramming or stress), did not believe that simply changing the environment of an ASC could actually trans-differentiate that cell into another cell beyond its lineage in the new environment (Murry, personal communication, 7-17-13). Dr. Murry found other reports for stem cell trans-differentiation such as Orlic et al. disproven, as such studies are irreproducible and believed the results observed from those studies could be due to poor microscopy, bad assays, or confusion between white blood cells for other cell types. Referring to the Kawada et al. paper (2004) about the potential of MSCs from bone marrow to convert into cardiomyocytes, Dr. Murry, like Dr. Peterson, did not believe that MSCs could turn into any other cell types other than bone, fat, cartilage and fibroblastic cells. Dr. Murry suggested the most convincing method to prove the existence of stem cell plasticity would have a reliable, unbiased cell tracking system to genetically track both the lineage and the phenotype of the transplanted cells in the new environment. Dr. Wagers, who has published papers refuting stem cell plasticity in different tissues, also shared the same point mentioned by Dr. Rossi, that ASCs normally only possess certain lineage-restricted differentiation potential within tissues. While acknowledging that cell fate could be changed under certain conditions, such as artificial cell reprogramming (microinjection into oocytes or iPS cells), Dr. Wagers did not believe that such interventional methods proved that stem cell plasticity occurs naturally *in vivo* (Wagers, personal communication, 7-24-13). Her research group has not seen HSCs trans-differentiate into other

lineages, or seen HSCs incorporate into solid tissues. In a few cases where her lab may have observed incorporation into solid tissues, such as in liver, Purkinje neurons, or skeletal muscle, she attributed these events to be the products of cell-cell fusion, not trans-differentiation. Dr. Wagers also discussed the inverse experiments in which non-hematopoietic tissues could regenerate blood system (Wright et al., 2001; McKinney-Freeman et al., 2002), but reasoned that the contamination of circulating HSCs in the solid tissues was the likely cause.

Diane Krause of the Yale University School of Medicine suggested that HSCs from bone marrow were not responsible for trans-differentiation into lung epithelial cells in her study (Kassmer et al., 2011) (Krause, personal communication, 7-2-13). Her group transplanted wild-type hematopoietic and non-hematopoietic bone marrow cells into irradiated surfactant-protein-C-null mice, respectively. Only the epithelial cells derived from non-hematopoietic bone marrow cells were detected in the lung of the receipt mice (Kassmer et al., 2011). For further investigation, her group discovered that very small embryonic like cells (VSELs) from the bone marrow formed most of the new epithelial cells in the lung after transplantation. Thus, her research group concluded that VSEL might be the primary source for this lung epithelial cell engraftment, but more needs to be explored.

Comparison of ESCs and Trans-Differentiation Therapies

In order to determine whether trans-differentiation experiments can replace embryonic stem cell (ESC) therapies, the two treatments must be directly compared to each other. Unfortunately our research has determined that not many studies were designed to do a direct comparison. The Literature Review summarized the ESC treatments for a few diseases in mouse

models. Mouse models remain the system where most of our ESC data lies because no ESC human clinical trial has yet concluded (two are currently underway). For example, Daadi et al. (2008) indicated that human ESCs differentiated in vitro into neurons could successfully treat stroke in mouse models. And Tohyama et al. (2013) did a similar study with differentiated ESCs to treat heart attacks in rat models. In addition, ESCs have been used to treat liver disease in mouse models (Brolen et al., 2010), and diabetes in mouse models (Lie et al., 2011). Bryon Petersen of the University of Florida indicated in his interview that there are more than 100 papers reporting the use of ESCs differentiating into beta cells to treat diabetes (Petersen, personal communication, 8-5-13). However, no clinical trials using ESCs to treat diabetes have occurred in the U.S. Dr. Petersen said that he has not heard of any here in the US, but has heard of some trials in Brazil and Argentina, but did not know the specifics of these trials. He further explained that for diabetes ESC clinical trials to take place in the US, a lot of things would need to happen to show that the cells are safe and function properly. ESCs often show problems of cancer formation. Fujikawa et al. (2005) indicated that teratoma formation was the main cause of his failures in treating diabetes type I using ESCs.

With respect to human clinical trials and trans-differentiation, our literature survey identified several studies where researchers observed cardiac functional improvements in heart attack patients treated with HSCs or MSCs. But those clinical studies were not designed to test for trans-differentiation, so they cannot be used in a functional comparison. Overall, Amado et al. (2005) and Hare et al. (2012) believe that bone morrow stem cells hold the best potential for treating diseases in animals and in humans.

CONCLUSIONS / RECOMMENDATIONS

Based on the research performed for this project, our group is able to make several conclusions or recommendations. Our findings indicate that stem cell trans-differentiation very likely exists in a few systems, but it is normally rare, and in some cases is cell fusion not trans-differentiation. Our best evidence for trans-differentiation is for the treatment of diabetic mouse models with HSCs where we identified seven studies arguing for plasticity (migration of the injected HSCs into the pancreas followed by differentiation of those cells into insulin-producing cells), and no studies arguing against it. Several other diabetes researchers observed improved normoglycemia in mice injected with HSCs, but do not believe in trans-differentiation. However those researchers did not provide any evidence against it themselves.

A significant problem we encountered in this project was that even within one specific disease model, some scientists found evidence supporting plasticity while others did not, so much of our research and interviews focused on attempting to resolve this discrepancy. We conclude that these discrepancies might result from: 1) the silencing of reporter genes used to detect host genes over time (giving false negatives), 2) not using the sensitive and powerful Cre-Lox system for tagging fused cells or for tagging cells of the original hematopoietic lineage), 3) differences in the developmental ages of the donor and host animals leading to a decreased survival of donor cells in the host animals, 4) differences in the purity and composition of the donor stem cells (the cruder the stem cell batch, the more likely it will have highly potent stem cells, so with different degrees of purity the results will vary), and 5) differences in the micro-niche colonized by the injected stem cells in a given experiment.

Our research has identified a best-practice methodology which we believe should be applied in the future by all labs attempting to demonstrate plasticity without cell fusion. The best practice is demonstrated by Ianus et al. (2003) and by Alvarez-Dolado et al. (2003). In different applications, these scientists used a Cre-Lox system to tag donor cells regardless of what those cells later differentiated into in the host mouse. For example, the donor male mouse can be engineered to contain a Cre-recombinase gene under the control of a promoter specific for the donor cells (i.e. CD45 for HSCs). The donor mouse also contains a floxed-stop-promoter-GFP reporter that switches on in cells of the original hematopoietic lineage (HSCs). Once the stop codon is removed from the GFP promoter, the GFP gene is switched on permanently, so the donor cells are GFP+ regardless of how they later differentiate. The Cre-Lox system is simultaneously also used to visualize cell fusion of donor and host cells if it occurs. The host mouse is engineered to be floxed-promoter-LacZ, so if they fuse with any donor cells (Cre positive) LacZ will be switched on, allowing the cells to stain blue with X-gal. In the host tissues (i.e. pancreas for a diabetes mouse model) the presence of GFP+ cells containing Y-chromosome markers (original donor cells) that are LacZ-negative would be evidence of trans-differentiation without cell fusion.

Although several scientists that we interviewed did not believe cells are capable of undergoing major reprogramming *in vivo*, the fact that some cells are well proven to be capable of major reprogramming by artificial means implies that the reprogramming is at least theoretically possible *in vivo*, even if it is rare. Our research has shown that major cell reprogramming into vastly different phenotypes indeed occurs under special conditions, such as the Nobel Prize winning experiments of John Gurdon (reprogramming skin nuclei injected into

enucleated eggs) and of Yamanaka (reprogramming skin cells into pluripotent cells by transfection with genes encoding transcription factors).

In order to determine whether trans-differentiation therapies can replace embryonic stem cell therapies, the two treatments must be directly compared to each other. We conclude that few current studies have been designed to directly compare functional recovery between these two stem cell types, so we make a final recommendation that such important studies should be done prior to determining whether one cell type can replace the other.

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APPENDIX

LIST OF INTERVIEW QUESTIONS

General Questions for Plasticity Stem Cell Researchers:

1. Can you tell me a little more about your current position and how you became interested in working with stem cells?
2. Which types of stem cells do you work with?
3. Which fundamental biological process are you trying to understand, or which disease are you trying to treat?
4. Open-ended question: what do you see as the strengths and weaknesses in the current evidence for stem cell plasticity?

The interviewee's response to the opening questions was tracked and used to decide which follow-up questions best applied to this particular subject. For example, if the subject brought up the issue of problems associated with low cell numbers produced by trans-differentiation, we asked whether other scientists also see this problem and how they are trying to solve it.

Questions for Plasticity Stem Cell Researchers:

1. Which **type** of stem cells do you reprogram, and why did you choose those cells?
2. What **method** do you use to perform your reprogramming?
3. Does your reprogramming use **viruses**, and if so, have you seen any evidence of integration at a harmful site in the genome?
4. Have you observed any evidence of **tumor formation** during your therapies? If so, what do you think causes that, and how might it be prevented?
5. How **efficient** is your method of reprogramming? Is it efficient enough to provide a sufficient number of cells for therapy?
6. Are your reprogrammed cells able to **graft** within the animal or patient, and how did you prove this?
7. How **potent** do you think your reprogrammed cells are? What level of potency is needed to treat the disease you are working on?
8. Have you observed any **slow growth** of your re-differentiated cells? If so, is it a problem for therapy?

Questions for Researchers Denying Plasticity Exists:

1. What is your evidence arguing that stem cell plasticity does not exist?
2. Do you disagree with other scientists claiming plasticity exists for their stem cell type?
3. What experiments do you think would conclusively prove that plasticity exists?

Questions for ES Cell Researchers:

1. Do you think that the U.S. has a sufficient number of ES cell lines for performing the thorough research studies required for using such cells for therapies?
2. In the recent past, did the periods of lack of federal funding to derive new ES cell lines negatively affect U.S. ES cell research?
3. Do you think that ES cell ethical issues increase the demand for ES cell replacements, such as those prepared by cell trans-differentiation from adult stem cells?
4. Do you think that cells prepared by trans-differentiation can serve as replacements for ES cells in therapies?

Questions for Academic Bio-Ethicists:

1 Are you familiar with stem cell plasticity? If not, we will explain this briefly to them.
2 Do you think trans-differentiated cells have fewer ethical concerns than ES cells?
3 Do you think researchers should try to find alternatives for ES cell treatments?
4 Do you think that more research should be performed to more fully understand stem cell plasticity prior to using such cells for therapy?

INTERVIEW PREAMBLE

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

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

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

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

APPENDIX CONTINUED: INTERVIEW TABLE

Group Member	Interviewee	Date of Initial Contact	Date of Second Contact	Interview Date or Termination Date
Armand	Name: **Chad Cowan** Address: Department of Stem Cell and Regenerative Biology, Harvard University, and Massachusetts General Hospital. Email: chad_cowan@harvard.edu Expertise: Diabetes and stem cells.	6-7-13	7/24/13	Terminated 7/29/13
	Name: **Mehboob Ali Hussain M.D.** Address: The Johns Hopkins Hospital, Metabolism Division. Email: mhussai4@jhmi.edu Expertise: Diabetes and stem cells.	7/18/13	7/23/13 Sent email reminder	Terminated 7/29/13
	Name: **Kenneth L Brayman** Address: Department of Surgery, University of Virginia School of Medicine, P.O. Box 800709, Charlottesville, VA 22908-0709. Email: klb9r@virginia.edu Expertise: Diabetes and stem cells.	7/18/13	7/23/13 Called to set up phone interview	**7-30-13 Interviewed by phone**
	Name: **Paolo Fiorina** Address: Children's Hospital Boston. Email: paolo.fiorina@childrens.harvard.edu Expertise: Diabetes and stem cells.	7/23/13	7/24/13 Sent questions	Terminated 7-16-13
	Name: **Daniel A. Peterson** Address: Chicago Medical School. Email: daniel.peterson@rosalindfranklin.edu Expertise Uses MSCs to treat type-2 diabetic models.	7/24/13	7/24/13 accepted interview	**Interviewed on 7-26-13 8-4-13**
	Name: **Mariusz Z. Ratajczak** Address: University of Louisville Email: mzrata01@louisville.edu Expertise: Stem Cell Biology	7/29/13	8/10/13 Follow up	8-15-13 Terminated
	Name: **John F. Tisdale** Address: National Institutes of Health Email: JohnTis@intra.niddk.nih.gov Expertise: Molecular and Clinical Hematology Branch	7/29/13	7/31/13 Does not follow the field anymore	8-1-13 Terminated

	Name: **Malcolm Alison** Address: Barts Cancer Institute, UK Email: m.alison@qmul.ac.uk Expertise: Professor of Stem Cell Biology, Centre for Tumour Biology	7/30/13	8/10/13 follow up	8-15-13 Terminated
	Name: **David M Harlan** Address: University of Massachusetts Medical School Email: david.harlan@umassmed.edu Expertise: Diabetes	7/30/13	8/1/13 called for reminder	**8-2-13** **Interviewed in person**
	Name: **Bryon Petersen** Address: University of Florida Email: bryonpetersen@ufl.edu Expertise: Diabetes and stem cells	8/4/13		**8-5-13** **Interviewed**
	Name: **Benard E Tuch** Address: Prince of Wales Hospital, Australia Email: From, Research gate Expertise: Diabetes Transplant Unit	8/4/13		**Interviewed on** **8-5-13** **8-8-13**
	Name: **HongKui Deng** (also emailed by Xuan) Address: Peking University Email: hongkui_deng@pku.edu.cn Expertise: Stem Cell Biology	8/4/13	8/11/13	8-15-13 Terminated
Chang	Name: **Eva Mezey** Address: National Institute of Neurological Disorders and Stroke, NIH, Building 36, Room 3D-10, 9000 Rockville Pike, Bethesda, MD 20892. Email: mezeye@mail.nih.gov Expertise: Stroke and stem cells.	5/28/13	6/13/13 sent reminder	**Interviewed** **6/25/13**
	Name: **Hady Felfly** Address: University of Miami Email: hadyeditorial@gmail.com Expertise: Stroke and stem cells. Uses HSCs to protect mice from stroke.	6/4/13		**Interviewed** **6/15/13**
	Name: **Harald Neumann** Address: European Neuroscience Institute Gottingen, Gottingen, Germany Email: hneuman1@uni-bonn.de Expertise: Stroke and stem cells.	6/11/13	6/19/13	Terminated 6/28/13

	Name: **Leonard I. Zon** Address: Harvard Stem Cell Institute (HSCI), Harvard Medical School. Email: zon@enders.tch.harvard.edu Expertise: Hematopoietic stem cells; nervous system.	6/11/13	6/19/13	Terminated 6/28/13
	Name: **Daniel G. Tenen** Address: HSCI and Beth Israel Deaconess Medical Center, Boston. Email: csidgt@nus.edu.sg Expertise: Hematopoietic stem cells.	6/11/13	6/19/13	Terminated 6/20/13
	Name: **Yong-San Huang** Address: Department of Veterinary Medicine, College of Veterinary Medicine, National Chung-Hsing University, Taiwan. Email: yshuang@mail.nchu.edu.tw Expertise: Stoke and hematopoietic stem cells.	6/19/13	6/28/13	Terminated 7/12/13
	Name: **George Daley** Address: Harvard Stem Cell Institute, and Boston Children's Hospital, Harvard Medical School. Email: george.daley@childrens.harvard.edu Expertise: Hematopoietic stem cells.	6/21/13	7/3/13	Terminated 7/12/13
	Name: **Derek Rossi** Address: HSCI; Immune Disease Institute, Harvard University. Email: rossi@idi.harvard.edu Expertise: Hematopoietic stem cells; denies the existence of HSC plasticity.	6/21/13	7/3/13 accepted interview request	**7/5/13 phone interview with Xuan**
	Name: **H. David Shine** Address: Department of Neurosurgery, Neuroscience, Molecular and Cellular Biology, and Center for Cell and Gene Therapy, Baylor College of Medicine. Email: hshine@bcm.edu Expertise: Denies bone marrow stem cells trans-differentiate into neural cells in vivo.	6/28/13	7-15-13 Sent questions	Terminated 8-23-13
	Name: **Cesar V. Borlongan** Address: Department of Neurosurgery and Brain Repair, University of South Florida College of Medicine. Email: cborlong@health.usf.edu Expertise: Neurosurgery	7/3/13	7/15/13	Terminated 7/23/13
Khanh	Name: **Margaret Goodell** Address: Baylor College of Medicine Email: goodell@bcm.tmc.edu Expertise: Supports plasticity for hematopoietic stem cells.	06/13/13	06/17/13	Terminated 06/24/13

	Name: **Hiroshi Kawada** Address: Tokai University School of Medicine, Japan Email: hkawada@is.icc.u-tokai.ac.jp Expertise: Supports plasticity for mesenchymal stem cells.	06/13/13	06/17/13	Terminated 06/24/13
	Name: **Charles Murry** Address: University of Washington Email: murry@u.washington.edu Expertise: Against plasticity for hematopoietic stem cells.	06/24/13	07/09/13 Sent reminder	**Interviewed 07/17/13**
	Name: **Robert Robbins** Address: Stanford University Email: robbins@stanford.edu Expertise: Against plasticity for hematopoietic stem cells.	06/25/13	07/02/13	Terminated 07/10/13
	Name: **XiYong Yu** Address: Research Center of Medical Sciences, Guangdong Provincial People's Hospital, China. Email: yuxycn@hotmail.com Expertise: Supports plasticity for mesenchymal stem cells.	07/03/13 interview after 7-20	7-23-13 Sent questions	08/15/13 Terminated
	Name: **Stefanie Dimmeler** Address: University of Frankfurt, Germany Email: dimmeler@em.uni-frankfurt.de Expertise: Supports transdifferentiation of endothelial progenitor cells in vitro.	07/01/13	07/10/13	Terminated 7-24-13
	Name: **Amy Wagers** Address: Harvard Stem Cell Institute Email: amy.wagers@joslin.harvard.edu Expertise: Against plasticity of hematopoietic stem cells.	06/27/13	07/10/13 Accepted	**Interviewed 7-24-13**
	Name: **Keiichi Fukuda** Address: Professor, School of Medicine, Keio University. Email: kfukuda@sc.itc.keio.ac.jp Expertise: Supports transdifferentiation of mesenchymal stem cells.	07/03/13 wrong email address		Terminated 7-24-13
	Name: **Sten Eirik W Jacobsen** Address: HSCL, Lund University, Sweden Email: Sten.Jacobsen@stemcell.lu.se Expertise: Supports cell fusion, not transdifferentiation for hematopoietic stem cells.	07/03/13	07/10/13	Terminated 7-24-13
	Name: **Piero Anversa** Address: Harvard Medical School Email: panversa@partners.org Expertise: Supports plasticity for bone marrow cells.	06/27/13 (accepted)	07/10/13 Sent reminder	Terminated 8-9-13

	Name: **Arturo Alvarez-Buylla** Address: UCSF Email: abuylla@itsa.ucsf.edu Expertise: Supports cell fusion of bone-marrow derived cells with Purkinje neurons, cardiomyocytes and hepatocytes.	07/09/13		Terminated 7-24-13
	Name: **Donald Bruce** Address: Edinethics Ltd., Edinburgh, UK Email: info@edinethics.co.uk Expertise: Bioethics.	07/16/13 (accepted, phone interview)	08/09/13 sent reminder	Terminated 08-14-13
	Name: **Jason T. Eberl** Address: Semler Endowed Chair for Medical Ethics, College of Osteopathic Medicine, 311C, Marian University, 3200 Cold Spring Road, Indianapolis, IN 46222-1997. And Indiana University, Center for Bioethics. Email: jeberl@iupui.edu and jeberl@marian.edu Phone: (317) 955-6601 Expertise: Bioethics	07/16/13 (accepted)		**Interviewed 07-23-13**
	Name: **Neil Chi** Address: Department of Medicine, Division of Cardiology, University of California San Diego, La Jolla, CA 92093. Email: nchi@ucsd.edu Expertise: In vivo cardiac reprogramming in zebrafish. Argues that differentiated atrial cardiomyocytes can trans-differentiate into ventricular cardiomyocytes.	07/25/13 (sent questions)	08/09/13 sent reminder	Terminated 8-23-13
Xuan	Name: **Snorri S. Thorgeirsson** Address: Center for Cancer Research National Cancer Institute. Email: snorri_thorgeirsson@nih.gov Expertise: Against plasticity.	06/17/13	_____	Terminated 08/07/13
	Name: **Neil David Theise** Address: Albert Einstein College, New York. Email: NTheise@chpnet.org Expertise: Supports stem cell plasticity.	06/12/13	06/24/13 06/26/13 07/08/13	**Interviewed 07/10/13**
	Name: **David W. Russell** Address: University of Washington Email: drussell@u.washington.edu Expertise: Supports cell fusion.	07/11/13	_____	Terminated 08/07/13
	Name: **Saul J. Sharkis**	06/12/13	06/17/13	**Interviewed**

	Address: Johns Hopkins University Email: ssharkis@jhmi.edu Expertise: Pro stem cell plasticity.			**06/18/13**
	Name: **Eric Lagasse** Address: McGowan Institute for Regenerative Medicine Email: lagasse@pitt.edu Expertise: Pro plasticity.	06/12/13	06/17/13	Terminated 06/27/13 no response
	Name: **Jacqueline Whang-Peng** Address: National Health Research Institute in Taiwan Email: Jqwpeng@nhri.org.tw Expertise: Pro plasticity.	06/17/13	_____	Terminated 07/08/13
	Name: **Diane S Krause MD, PhD** Address: Department of Laboratory Medicine, Yale University School of Medicine, New Haven, CT 06520. Email: diane.krause@yale.edu Expertise: Currently focuses on stem cell plasticity.	06/26/13 Out of office until 07/01	07/08/13 follow-up questions	**Interviewed** **07/02/13** **07/08/13**
	Name: **Hongkui Deng** (also emailed by Armand) Address: Peking University Email: hongkui_deng@pku.edu.cn Expertise: Against plasticity, pro ES cells.	07/11/13	_____	Terminated 08/07/13
	Name: **Markus Grompe, M.D** Address: Oregon Health & Science University Email: grompem@ohsu.edu Expertise: Liver and stem cells.	06/27/13	_____	Terminated 06/27/13