HUMAN THERAPEUTIC CLONING WAS FINALLY ACHIEVED THIS YEAR: DOES ANYONE CARE?

An Interactive Qualifying Project Report

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ABSTRACT

The overall goal of this project was to document and evaluate the new breakthrough technology of human therapeutic cloning which was finally achieved this year, and to assess the ethical problems associated it. We performed a review of the current research literature and conducted interviews with academic researchers and bioethicists. Our findings indicate that the use of human eggs is the most important ethical consideration in this process, and the use of eggs could become rate-limiting if the process is used to treat human diseases. The problem is further hindered because the eggs need to be fresh and young, and because most states do not have their own specific laws governing egg harvesting, so we recommend that individual states consider changing their laws to allow for donor compensation to increase egg donations. Because the epigenetic status of a cell's DNA can affect its ability to differentiate and several labs reported DNA mutations in their cells, we recommend that all types of pluripotent cell lines be frequently monitored for DNA mutations, epigenetic modifications, differentiation potential, tumor potential, and ability to treat a disease, before AND after expansion for clinical safety. The technology of human therapeutic cloning has advanced a long way in a very short period of time, and the methods are constantly improving. We identified and recommend a best-practice methodology of directly fusing the enucleated egg with the diploid skin cell (to avoid loss of key reprogramming factors), using lower amounts of Sendai virus (to help fuse the cells) in a calcium-free medium, using kinase and protein translation inhibitors, and using histone de-acetylase inhibitors, which help block early egg activation and allow the cloned human embryos to survive to the blastula stage (from which the therapeutic ESCs are isolated). This best-practice technology should be applied by all labs attempting to clone human embryos.

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EXECUTIVE SUMMARY

Initially proposed in 1999, human therapeutic cloning has been a major goal of the stem cell community for 15 years. This process involves the transfer of a patient's skin cell nucleus into an enucleated *in vitro* fertilized (IVF) zygote (or the direct fusion of skin cell and egg), culture of the zygote to the blastula stage, isolation of embryonic stem cells genetically identical to the patient, and the use of those cells to regrow a diseased organ in the patient. Human therapeutic cloning was finally achieved in 2013 using fetal and infant skin cells, and was further improved in 2014 in a much broader application using adult patient skin cells. The overall goal of this project was to document and evaluate this new breakthrough technology, and to assess the ethical problems associated it. The specific objectives were to 1) develop a comprehensive assessment of the scientific experiments that lead to the development of human therapeutic cloning and its potential applications, 2) characterize what key scientific stakeholders believe are the strengths and weaknesses of the existing data and their ethical concerns, 3) evaluate the obtained evidence for remaining problems, and 4) recommend potential solutions to any remaining problems.

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 a set of semi-structured, in-depth interviews with various academic researchers in the stem cell field who have achieved human therapeutic cloning, or who have significantly contributed to the development of the cloning technology, to determine their range of opinions and the strengths and weaknesses of this new cloning technology. The stakeholders included academic experts on human therapeutic cloning, experts on general cloning technology, scientists in biotechnology companies doing

therapeutic cloning, and a few bioethicists. After performing the Literature Review and interviews, the group synthesized all of the information collected to ascertain the strength of the evidence for and against human therapeutic cloning, and created recommendations for further research.

Based on the research performed for this project, our group made several conclusions and recommendations. The findings are shown in more detail in the Conclusion section of the report, and are briefly summarized here. With respect to the ethics of human therapeutic cloning, our findings indicate that the use of human eggs is the single most important ethical. If human therapeutic cloning will be used in the future to treat diseases, the number of human eggs required will increase significantly relative to those currently used for research. Several interviewees had concerns about whether a high number of eggs will be available, especially given that some scientists think the eggs need to be fresh and young, and given that that most states do not have their own specific laws governing egg harvesting. One interviewee who performs human egg experiments strongly felt that donors should receive money as an incentive to compensate them for the surgical risk and pain of the procedure, and the inconvenience of two weeks of hormonal injections. Without the compensation, the number of eggs might remain rate-limiting. So, we recommend that individual states consider changing their laws to allow for donor compensation. Although some bioethicists tended to not respond to our inquiries, or they were not aware of (or were not willing to comment on) human therapeutic cloning advances, we compensated for this by expanding our interview selections, and by getting comments from the scientists directly related to ethical issues.

With respect to using cloned nuclear transfer embryonic stem cells (NT-ESCs) versus induced pluripotent stem cells (iPSCs) for therapies, our research indicates that several researchers have directly compared the epigenetic status of the three types of pluripotent cells.

The epigenetic status can affect their ability to differentiate into the desired cell type, so could affect their usefulness for therapy. All the scientists studying epigenetics focused on DNA methylation patterns, and some agreed with our recommendation that the studies should be expanded to include histone acetylation as another indicator of epigenetic status. Some scientists concluded that NT-ESCs have an epigenetic pattern that is closer to the gold standard ESCs prepared by *in vitro* fertilization (IVF-ESCs) than iPSCs. So, this could hinder the use of iPSCs in therapy, and favor the use of IVF-ESCs or NT-ESCs. Other interviewees indicated the epigenetic status of the iPSCs changed to be more gold standard-like the longer they are cultured, so we recommend repeating this interesting finding. Other scientists who had not yet analyzed epigenetics agreed with us that it is worth doing, and had plans in their own labs to do so. Several scientists indicated it is too early to determine which type of pluripotent stem cell is best in therapy until direct therapy comparisons are done. And several interviewees identified DNA mutations in the pluripotent cells which they said could lead to cancer. So, we recommend that all pluripotent cell lines (NT-ESCs, IVF-ESCs, iPSCs) should frequently be monitored for DNA mutations, epigenetic modifications, differentiation potential, tumor potential, and ability to treat a disease, before AND after expansion for clinical safety. These comparisons can all be done now that isolating all three types of pluripotent cells is possible.

With respect to cloning technology and its complexity, our analysis of the current research and our interaction with key scientists showed that the technology of human therapeutic cloning has advanced a long way in a very short period of time, and the methods are constantly improving with higher efficiencies. One key advance is the new method of directly fusing the enucleated egg with the diploid skin cell, instead of microinjecting the skin cell nucleus. Isolating the skin cell nucleus and preparing it for microinjection likely leaves behind key reprogramming factors, and this is avoided by directly fusing the skin cell and egg. This point

should be proven with further research. Other recent protocol improvements included using lower amounts of Sendai virus (to help fuse the cells) in a calcium-free medium to prevent the calcium from activating the egg, using kinase and protein translation inhibitors to block early egg activation, and using histone de-acetylase inhibitors (to block early transcription from the embryo genome). These improvements have allowed the cloned human embryos to survive to the blastula stage (from which the ESCs are isolated), and appear to be the best-practice methodology which we recommend should be applied in the future by all labs attempting to clone.

AUTHORSHIP

Author	Areas Covered
Jiaxun Xie	Reproductive v. Therapeutic Cloning Introduction to Cloning Problems Introduction to Pluripotent Stem Cell Types Human Egg Ethics
Adam McNally	Early Human SCNT Experiments Problems with Early Cloning Problems with Stem Cell Epigenetics Methods for Cloning Successes
Nicholas Scrivanich	Human Therapeutic Cloning Cloned Embryonic Stem Cell Lines Problems with Stem Cell Epigenetics Methods for Cloning Successes

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PROJECT GOALS

Initially proposed in 1999, human therapeutic cloning has been a major goal of the stem cell community for 15 years. This process involves the transfer of a patient's skin cell nucleus into an enucleated *in vitro* fertilized (IVF) zygote (or the direct fusion of skin cell and egg), culture of the zygote to the blastula stage, isolation of embryonic stem cells genetically identical to the patient, and the use of those cells to regrow a diseased organ in the patient. Human therapeutic cloning was finally achieved in 2013 using *fetal* and *infant* skin cells, and was further improved in 2014 in a much broader application using *adult* patient skin cells. The overall goal of this project is to document and evaluate this breakthrough technology, and to assess the ethical problems associated it given the recent development of other new cell therapy alternatives that do not involve cloning.

The specific objectives are to:

1. **Develop** a comprehensive assessment of the scientific experiments that lead to the development of human therapeutic cloning and its potential applications.
2. **Characterize** what key scientific stakeholders believe are the strengths and weaknesses of the existing data and their ethical concerns.
3. **Evaluate** the obtained evidence for remaining problems.
4. **Recommend** potential solutions to any remaining problems.

LITERATURE REVIEW

Introduction (Jiaxun Xie)

This year has turned out to be a major year for human therapeutic cloning (HTC). Within 13 days of each other, results were published from two different labs (Chung et al., 2014; Yamada et al., 2014) as the first to achieve human therapeutic cloning using *adult* skin cell nuclei. Since its original proposal in 1999 (Lanza et al., 1999), HTC represents the culmination of over 15 years of stem cell research, with the hope of being able to use a patient's own skin cell and a donor's egg to create an embryo from which therapeutic embryonic stem cells (ESCs) could be isolated that are genetically identical to the patient (so hopefully would not be rejected by the patient during transplant). This dream, opens the door for using a patient's own stem cells for treating a variety of diseases from diabetes, to Parkinson's disease, to spinal cord injuries, to metabolic disorders, and other diseases.

But in spite of this landmark cloning discovery, some individuals are against cloning in general. And others argue that new procedures that do not involve cloning, such as reprogramming adult cells to make induced pluripotent stem cells (iPSCs), can produce ESCs that are as potent as cloned ESCs. Others argue that the methods used to reprogram iPSCs damages their DNA or causes tumors making them unsuitable for human use.

The purpose of this project is to document and evaluate this new technology of human therapeutic cloning, and to assess some of the remaining technical and ethical problems associated with its use. In this section we will explain what human therapeutic cloning is, and generally how it is done, explain the difference between reproductive cloning and therapeutic cloning, touch on some of the problems associated with each process, and describe what

embryonic stem cells are and how they are derived from the cloning process.

Somatic Cell Nulear Transfer (SCNT)

SCNT (**Figure-1**) is a technique for creating a viable embryo from the diploid nucleus of a somatic (body) cell transferred to an egg whose haploid nucleus is usually removed. As its SCNT name implies, the somatic cell (usually a skin cell) nucleus is usually *transferred* by microinjection into the oocyte. However with more recent advances, the skin cell is directly fused with the egg cell using Sendai virus in a process that signficantly increases embryo viability.

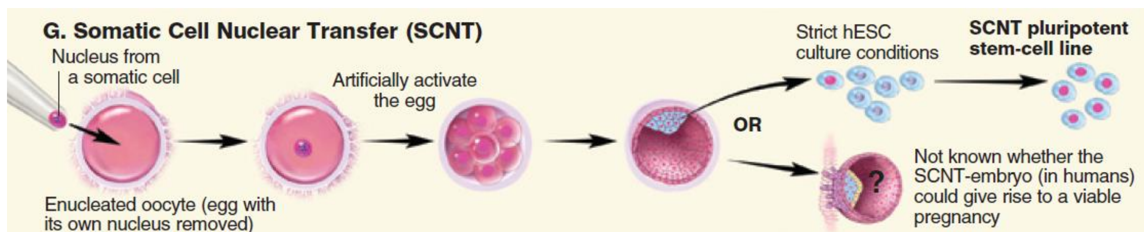

Figure-1: Diagram of the Cloning Process Using Somatic Cell Nuclear Transfer. During SCNT, a nucleus is taken from a somatic cell (usually a skin cell) and microinjected into an enucleated oocyte (diagram left). The egg is then stimulated to divide, usually by using electric current (diagram center). If the embryo survives 5-6 days, it forms a hollow ball or blastocyst from which embryonic stem cells (ESCs) can be isolated to make an ESC line (diagram right). The derived nuclear transfer ESCs (NT-ESCs) are genetically identical to the patient providing the skin cell nucleus. A key recent alterations to this process includes simply fusing the oocyte and skin cell directly using Sendai virus instead of using microinjection. (Redrawn from Yu and Thomson, 2006)

Human oocytes (eggs) are collected from a donor who has been previously injected with hormones to stimulate ovulation and the production of more than one oocyte during the menstrual cycle. In the U.S., oocytes can only be harvested for *reproductive* purposes in a fertility clinic, and then later (with donor consent) the *excess* oocytes not used for reproduction can be used for research purposes. The nucleus is removed from the donated oocyte and replaced

with the nucleus from a somatic cell, a differentiated adult cell taken from elsewhere in the body (usually a skin keratinocyte). The zygote with the newly-transferred nucleus now has the same DNA, or genetic material, as the donor somatic cell. The embryo is simulated to divide using an electric current. If the cultured embryo survives 5-6 days (only recently possible with cloned human embryos), it forms a blastocyst (diagram center), a hollow ball consisting of the outer layer of cells (the trophoblast) and the inner cell mass (ICM). Cells of the inner cell mass are embryonic stem cells (ESCs). ESCs isolated from cloned embryos prepared by nuclear transfer are sometimes abbreviated as NT-ESCs, and can in theory be used for regenerative therapies.

Reproductive vs. Therapeutic Cloning

A blastula produced by SCNT can either be implanted into the uterus of a foster mother in a process termed *reproductive cloning*, or it can be used to isolate embryonic stem cells for treating diseases in a process called *therapeutic cloning* (**Figure-2**). Reproductive cloning is only legal in the U.S. when making cloned *animals* and can not be applied to humans. This is also the case for all other countries that have bothered to legislate the cloning process. Reproductive cloning has been used for decades now, especially when combined with genetic engineering techniques, for making transgenic animals whose genomes are modified to contain a transgene not normally found in that organism. Transgenic animals include new disease models for mimicing human disease, livestock that secrete human pharmaceuticals into their milk, pigs whose organs are mostly histocompatible with humans for transplants, and scientific models that help identify the function of newly identified genes.

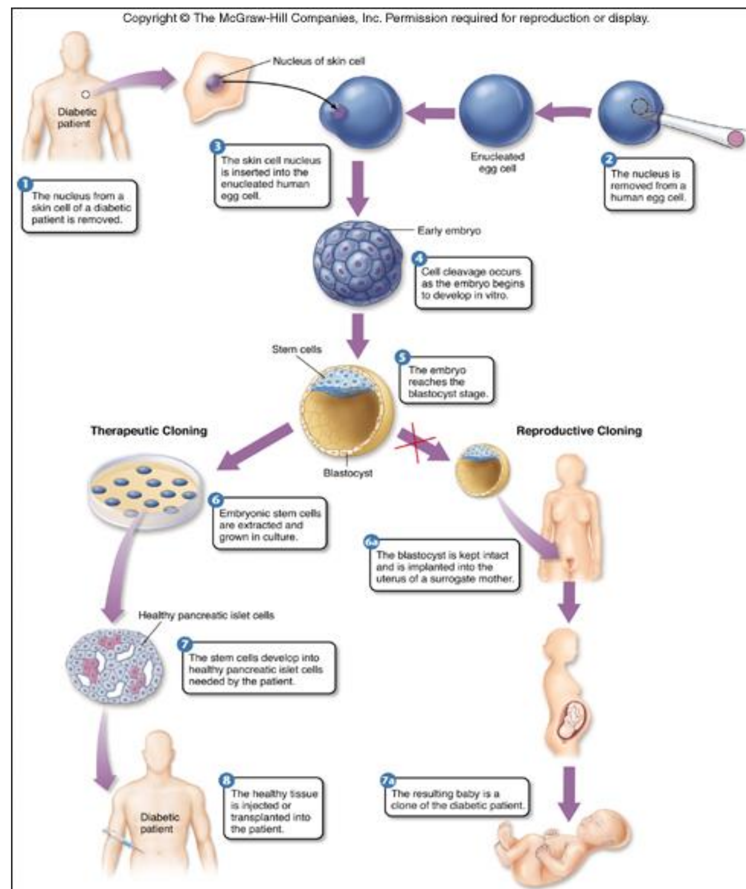

Figure-2: Diagram of Reproductive Versus Therapeutic Cloning. A cloned blastocyst (diagram center) is prepared by somatic cell nuclear transfer. The cloned blastocyst can then either be implanted into the uterus of a foster mother in reproductive cloning (diagram lower right) or embryonic stem cells can be isolated from the blastocyst to create a therapeutic cell line in therapeutic cloning (diagram lower left). (Jasper, 2013, with permission)

Different than reproductive cloning, the goal of *therapeutic* cloning is to harvest stem cells that can be used to potentially treat disease, rather than creating cloned human beings. During therapeutic cloning, the nuclear transfer embronic stem cells (NT-ESCs) are isolated from the cloned blastocyst (if it has survived 5-6 days), and the NT-ESCs are cultured to make an immortal NT-ESC line. The goal of therapeutic cloning is to prepare a NT-ESC line that is genetically identical to the patient from which the skin cell was taken. These isogenic NT-ESCs, in theory, would not be rejected by the patient's immune system when they are implanted into the

patient.

Human *therapeutic* cloning is legal in the U.S., but has only recently been achieved: in 2013 with fetal and infant skin cells (Tachibana et al., 2013), and in 2014 with adult skin cells (Chung et al., 2013; Yamada et al., 2014). Cloned ESCs have not yet been used to treat a human disease, however *non-cloned* ESCs were approved in 2010 in two different clinical trials: 1) for treating spinal cord injury (Geron Corporation, Menlo Park, CA) (Geron, 2014), and 2) macular degeneration (a specific type of blindness) (Begley, 2011; Cyranoski, 2013). Geron stopped their clinical trials in 2011 when they ran out of money (Baker, 2011), but a new biotech company Asterias Biotherapeutics (Menlo Park, CA) is now resurrecting the spinal cord trials (Hayden, 2014).

Stem Cells

The desired outcome of therapeutic cloning is the production of an embryonic stem cell line that is genetically identically to the patient. So, the topic of this IQP involves stem cells and we introduce it here. Within our bodies, stem cells are long lived cells that help replace aged or damaged tissues. These cells originate from an initial pool of stem cells formed shortly after fertilization (the inner cell mass of the 5-6 day old blastocyst). Each stem cell can either divide to produce two more stem cells, or can differentiate to become another type of cell with a more specialized function. This allows stem cells to serve as an internal repair system in many tissues. Stem cells can sometimes undergo long periods of inactivity, but under specific physiological or experimental conditions, they can be induced to become mature, specialized cells that make up our tissues and organs.

Stem Cell Types

There are three main types of stem cells: embryonic stem cells (ESCs), adult stem cells (ASCs), and induced pluripotent stem cells (iPSCs). ESCs are present in the inner cell mass of a blastocyst. These cells are pluripotent, and can form any type of cell in the adult organism. In laboratories, blastocysts prepared by *in vitro* fertilization (IVF) procedures can be used with donor consent for isolating ESCs to be used for therapies. The embryos used for this purpose must be produced in a reproductive clinic, and only the *excess* embryos not used for reproduction can be used for research purposes. Thompson et al. (1998) were the first to isolate and grow ESC lines from human blastocysts, and this advance opened the door for human stem cell treatments. Thompson et al. grew IVF embryos in culture for 5-6 days to make a blastocyst, and then human embryonic stem cells were transferring cells from this pre-implantation-stage embryo into a plastic culture dish containing culture medium (nutrient) coated with a feeder layer of mouse embryonic skin cells. The mouse cells provide a scaffold for cell attachment and also release growth factors. After repeating the feeding for several passages, the cells that do not differentiate are considered ESCs. If the plated ESCs survive long term, they are continuously cultured to make an immortal ESC line to be used for therapy. ESC lines prepared from random IVF embryos are not genetically identical to a patient, while those prepared from *cloned* embryos are genetically identical to the patient who provided the donor skin cell.

Adult stem cells (ASCs) are rare undifferentiated cells found among differentiated cells in a tissue or organ. The primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are found. Adult stem cells are multi-potent; they can give rise to a few types of cells specific to the tissue they reside, but they are not pluripotent. Far more rarely, ASCs can trans-differentiate into cell types seen in organs or tissues other than those expected from the cells' normal developmental lineage. Because they are rare, ASCs are hard to identify

and isolate. Moreover, their capacity to divide is more limited than ESCs, making the generation of large quantities of ASCs in culture difficult.

Induced pluripotent stem cells (iPSCs) are adult somatic cells (usually skin fibroblasts) reprogrammed to a pluripotent state by treating with specific transcription factors (or the genes encoding them). This process was first achieved in 2006 with mouse skin cells (Takahashi and Yamanaka, 2006) and one year later with human skin cells (Takahashi et al., 2007). Although the exact level of potency may vary from experiment to experiment, most scientists believe that iPSCs are as potent as ESCs isolated from IVF embryos. And because iPSCs can be prepared without cloning, some individuals believe they should be used in human therapy experiments in place of NT-ESCs. However, both of these types of stem cell have problems (see below).

Problems Associated with Therapeutic Cloning and iPSCs

One of the main purposes of this IQP is to review the published literature and to interview scientists to help determine whether iPSCs might be able to take place of NT-ESCs in human therapies. The outcome of this ongoing debate will help decide in the future which types of cells are used for regenerative medicine to treat a variety of diseases. Our literature review will help us identify key problems that we should focus on for our interviews.

With respect to cloned NT-ESCs, some individuals are against the idea of human cloning in any form, therapeutic or reproductive. So, these individuals are generally against the use of NT-ESCs because the process involves cloning. Other individuals worry about whether the injected NT-ESCs (or regular IVF ESCs) will form tumors at the injection site. However, scientists recently developed procedures for removing cancer causing cells from stem cell samples, including treating with antibodies against surface proteins found exclusively on cancer cells, or pre-differentiating the stem cells prior to injection. Other individuals worry about

whether we will be able to obtain enough donated human oocytes to be able to fully develop the technique, and whether the donors should be paid (the donation procedure is mildly painful).

With respect to iPSCs, some cell batches have been shown to cause tumors at the injection site. So, as discussed above, scientists are developing procedures for identifying and removing the cancer causing cells from iPSC batches. Other scientists are not convinced that iPSCs are as potent as ESCs (or NT-ESCs), which could be a problem when treating specific diseases (especially if scientists cannot differentiate iPSCs as fully as ECSs). Some others argue that the methods used to reprogram iPSCs damages the DNA, which would be a serious problem as the cells are expanded for therapy. Others argue that the iPSC reprogramming does not rid the host DNA of its epigenetic markers (like DNA methylation or histone acetylation), and so these cells are not capable of full reprogramming because their DNA is altered.

We will use our Literature Review to more fully explore the problems associated with the use of NT-ESCs and iPSCs, and use interviews with key stakeholders to help us evaluate the remaining problems to make recommendations for future research.

Early Human SCNT Embryo Development (Adam McNally)

Although the past two years have witnessed major successes with human therapeutic cloning (discussed in the next section), those successes built upon an earlier body of research on human embryos that began in 1970's with the advent of human *in vitro* fertilization (IVF). The world's first test tube baby Louise Brown was born in 1978, and the field of human embryo research expanded with the availability of surplus IVF embryos donated for research purposes. The field expanded further with the ability to grow IVF embryos to the blastocyst stage, and the isolation of human embryonic stem cells (ESCs) from the inner cell mass of blastocysts (Edwards,

2001). The purpose of this section is to discuss some of the key human embryo experiments that led to the recent successes with human therapeutic cloning.

As medicine and science advances, so do the abilities of doctors and the range of medical procedures possible. Surgeries involving heart, liver, lung, and pancreas transplants are now possible, but the need for donor organs and tissues continues to grow while the supply does not meet the demand. Along with the shortage of available donors, there is also the risk of immune incompatibility when the donors' tissues or organs are not accepted by the patients' immune system. If human therapeutic cloning could be achieved, the tissues derived from the cloned ESCs would be genetically identical to the patient, so theoretically the tissues would be histo-compatible with the patient and not be rejected.

The possibility of human therapeutic cloning was first discussed in 1999 by scientists at Advanced Cell Technology (Lanza et al., 1999). "Advances in cloning have resulted in therapies with the potential to eliminate immune responses associated with the transplantation of these various tissues, and thus the requirement for immunosuppressive drugs that carry the risk of a wide variety of serious complications, including cancer, infection, renal failure, and osteoporosis" (Lanza et al., 1999). Due to its potential medical applicability, the idea of human therapeutic cloning has come to the forefront of science. As discussed previously, this process involves injecting a nucleus from a patient's skin cell into an enucleated egg, growing the embryo to the blastocyst stage, isolating the ESCs, culturing the ESCs and differentiating them to the desired tissue, and placing those cells back into the same patient (Lanza et al., 1999). In its earliest stages, our "understanding of oocyte maturation was still incomplete, and protocols for *in vitro* maturation of human embryos were unreliable and needed to be optimized" (Lanza et al., 1999). Back in the early 1980's, when the idea of therapeutic cloning was still young, scientists struggled with being able to grow cloned human embryos to the blastocyst stage, but this slowly improved. And human

ESCs were first isolated and grown in 1998, opening the door for further experimentation (Thomson et al., 1998). With breakthroughs in animal cloning using fetal and adult somatic cells (discussed in the previous section), the prospect of human cloning remained promising.

In the 1990's several studies focused on the effect of oocyte donor age and the recipient age on the efficiency of IVF procedures. It was "found that the age of the oocyte donor is of paramount importance in predicting pregnancy outcome, while donor gravidity, parity, and the number of oocytes retrieved are insignificant once controlled for donor age" (Cohen et al., 1999). The researchers found "a positive effect of younger donor age on IVF outcome" for their SCNT procedures (Cohn et al., 1999). Other scientists in early cloning experiments focused on the developmental problems associated with cloned embryos, refining the procedures to allow better survival.

The world's first claim of a successful human therapeutic cloning was made in 2004 by Woo Suk Hwang, formerly of the Seoul National University in his lab's article entitled "Evidence of a pluripotent human embryonic stem cell line derived from a cloned blastocyst" (Hwang et al., 2004). The experiment was performed on fresh oocytes and cumulus cells donated by a healthy woman for the purpose of doing SCNT research. The results yielded 30 cultured SCNT-derived blastocysts from which 20 inner cell mass (ICM) samples were isolated by immune-surgical removal of the outer surrounding trophoblast layer. Of the 20 cultured ICM samples, one produced a human embryonic stem cell (ESC) line (SCNT-hES-1). The authors claimed the SCNT-hES-1 cell line contained typical ESC surface markers, could differentiate into derivatives of all three main embryonic germ layers, and after continuous proliferation for more than 70 passages maintained normal chromosomal karyotypes (chromosome types and numbers) and were genetically identical to the somatic nuclear donor cells. The authors concluded that although it was feasible to generate human ESCs from a somatic cell, the field needs a more reliable and

efficient method (Hwang et al., 2004). Initially (prior to withdrawal of the paper for fraud), this first claim of SCNT in humans was considered a huge breakthrough in the field, because it "raised the possibility of performing human SCNT to generate potentially unlimited sources of undifferentiated cells for use in research, with potential applications in tissue repair and transplantation medicine" (Hwang et al., 2004).

In 2005, Hwang's lab published another paper entitled "Patient-specific embryonic stem cells derived from human SCNT blastocysts" (Hwang et al., 2005). In this experiment the authors claimed that "eleven hESC lines were established by somatic cell nuclear transfer of skin cells from patients with disease or injury into donated oocytes" (Hwang et al., 2005). These human nuclear transfer ESC lines (NT-hESCs) were established with relatively good efficiency, were pluripotent, chromosomally normal, matched the patient's DNA, and appeared to match the major histocompatibility of the patient, which should facilitate engraftment. However, the methods were still unpolished so reliability was still a lingering issue, and to be used for human therapy the SCNT needs to be done without the use of an animal feeder layer. Worse, in a huge setback for the stem cell field, both of Hwang's 2004 and 2005 papers were retracted in 2006 for data fabrication (Cyranoski, 2006).

In 2005, another article was published that finally produced a cloned human blastocyst, but not an ESC line (Stojkovic et al., 2005). This experiment was performed at the Center for Stem Cell Biology and Developmental Genetics at the University of Newcastle, and aimed to identify "the source of human oocytes with the best potential for the development to blastocysts after enucleation and fusion with the nucleus of a heterologous donor cell" (Stojkovic et al., 2005). Until then, previous studies regarding SCNT had only used fresh oocytes recovered from female volunteers to derive human NT embryos. This experiment compared the use of various types of oocytes including "oocytes that failed IVF, oocytes that were retrieved during a follicle reduction

procedure after ovulation induction, oocytes recovered during an IVF treatment but not inseminated due to unexpected azoospermia in the partner, and oocytes recovered after routine ovarian cystectomy" (Stojkovic et al., 2005). Their data indicated that only the "oocytes recovered during a follicle reduction showed strong developmental potential after enucleation and NT", thus proving that human "heterologous NT can be successfully achieved if performed in oocytes immediately after *in vitro* maturation and recovery" (Stojkovic et al., 2005). Although the scientists were not able to derive ESC lines from their NT-blastocysts, this experiment laid the groundwork for the use of adult somatic cells as donor cells for the nucleus, and provided a better understanding of oocyte physiology, cell cycles, mitotic spindles, and gene expression during early human development.

In 2007, scientists at the Infertility Center at Ghent University Hospital (Belgium) published an article that compared three different groups of human oocytes for NT experiments: *in vitro* matured germinal vesicle oocytes (IVM), *in vivo* matured oocytes, and failed fertilized oocytes following routine intra-cytoplasmic sperm injection (ICSI) (Heindryckx et al., 2007). Although their success rates were highest for the *in vivo* matured oocytes, their study showed "for the first time that IVM oocytes can be used as recipients for human SCNT" (Heindryckx et al., 2007). Of 22 treated NT-IVM oocytes, 2 human morulae were formed.

In 2008, another relevant human SCNT experiment was the first to produce cloned blastocysts from adult fibroblast cell fusion, although no ESC lines were derived (French et al., 2008). A total of 29 oocytes were obtained by trans-vaginal aspiration from young female donors, were enucleated (either by extrusion or needle aspiration), and were fused with normal diploid adult male fibroblast cells. The embryos developed to the pronuclear stage (66%), early cleavage stage (47%), or blastula stage (23%). One cloned blastocyst was confirmed by nuclear DNA analysis to match the male fibroblast cell donor, and by mitochondrial DNA analysis to match the

oocyte donor. This study demonstrated "for the first time, that SCNT can produce human blastocyst–stage embryos using cells obtained from differentiated adult cells" (French et al., 2008).

In 2009, scientists at Advanced Cell Technology (then based on Worcester, MA) investigated whether animal oocytes could be used to reprogram human somatic cells (Chung et al., 2009). They investigated human-human, human-bovine, and human-rabbit embryos, comparing gene expression patterns to determine whether "human stem cells can be successfully generated using interspecies somatic cell nuclear transfer (iSCNT)" (Chung et al., 2009). The results showed that all 3 types of embryos appeared morphologically similar and they developed to the morula stage at the same rate, but the pattern of genomic reprogramming was dramatically different between the various embryos. The "bovine and rabbit oocytes do not support appropriate embryonic genome reprogramming of human somatic cell nuclei, and call into question the ability of animal ooplasm to generate patient specific human stem cells" (Chung et al., 2009).

In addition to the technical advances of human SCNT, because the process involves human eggs, strong ethical issues arise. In 2007, in response to the increasing use of human eggs for research, the American Society for Reproductive Medicine (ASRM) published a report from their ethics committee raising the question of whether human egg donors should be compensated, and if so, at what level (Ethics Committee, 2007). The report details two ethical questions that financial compensation raises: "[1] do recruitment practices incorporating remuneration sufficiently protect the interests of oocyte donors, and [2] does financial compensation devalue human life by treating oocytes as property or commodities" (Ethics Committee, 2007). After numerous ethical considerations were discussed in the report, it was concluded that a specific incentive structure should be put into place with regards to payment and oocyte sharing. "Monetary compensation should reflect the time, inconvenience, and physical and emotional demands associated with the oocyte donation process" (Ethics Committee, 2007). With respect to oocyte sharing, a general

approach should be to “reduce the donor’s total IVF costs by about half, in exchange for a donation for research of half the oocytes retrieved” (Ethics Committee, 2007).

Another article published in 2009 also discusses ethical issues and payment regarding egg donors in stem cell research (Klitzman and Sauer, 2009). The article discusses issues of donor exploitation from compensation, undervaluing the physical and psychological risks of donation, and undue inducement to donate for poor individuals. The public was surveyed on stem cell related questions to gain a general consensus on the public point of view. The results indicated that women were most willing to donate oocytes for research when they felt they were fairly compensated. The report also concluded that medical care should be provided as necessary following any procedure, and that donor recruitment processes should be highly monitored and “the overseeing of egg providing must be vigilant” (Klitzman and Sauer, 2009).

Other key advances that helped open the door for using human ESCs in therapy were the development of protocols for differentiating human ESCs into specialized cells that could heal diseased tissues. For example, Assady et al. (2001) developed a procedure for differentiating human ESCs into insulin-producing cells, while Kroon et al. (2008) were able to use those cells to treat a mouse model of diabetes. And Perrier et al. (2004) were able to derive dopamine-producing cells from human ESCs, while Kriks et al. (2011) used the cells to treat rodent models of Parkinson’s disease.

All of the above articles highlight the progress made in early human SCNT embryo research that helped lead to the breakthrough articles of 2013 and 2014 deriving human ESC lines from cloned embryos using adult patient skin cells (discussed in the next section). Throughout the early years of its development, SNCT research suffered from several major obstacles that delayed progress, including: 1) protocol inefficiency (which necessitates using a large number of oocytes), 2) the inability to grow the SCNT embryos to the blastocyst stage, 3) the inability to derive human

ESC lines from the cloned blastocysts, and 4) the inability to differentiate the derived ESC lines into usable tissues for therapy (Heindryckx et al., 2007). These major hurtles appear to have finally been solved now. In recognition of the large body of work on SCNT and cell reprogramming, in 2012 John B. Gurdon (Oxford University) and Shinya Yamanaka (Kyoto University) were jointly awarded The 2012 Nobel Prize in Medicine or Physiology for discovering "that mature, specialized cells can be reprogrammed to become immature cells capable of developing into all tissues of the body" (Nobelprize.org, 2012). Both men made significant contributions to cell reprogramming. In 1962, John B. Gurdon discovered "that the specialization of cell is reversible" by replacing an "immature cell nucleus in an egg cell of a frog with the nucleus from a mature intestinal cell" (Gurdon, 1962). In 2006, Shinya Yamanaka discovered "how intact mature cells in mice could be reprogrammed to become immature stem cells" (Takahashi and Yamanaka, 2006). These developments further came to the public eye in 2013 and 2014 with the first successful human therapeutic cloning using adult skin cells (discussed in the next section).

Human Therapeutic Cloning of NT-ESC Lines (Nicholas Scrivanich)

Originally proposed in 1999 (Lanza et al., 1999), human therapeutic cloning (HTC) has been a major goal of the stem cell community for over 15 years. Major successes were finally achieved this past couple of years, opening the door to the use of therapeutic cloning to potentially treat patients. The previous section of the Lit Review described some of the earliest human somatic cell nuclear transfer (SCNT) experiments some of which were fraudulent, and others achieved only short periods of embryo development. Initially the cloned human embryos survived only 1-2 days, then they achieved morula status (about 3 days), and finally blastocyst

status (5-6 days). But what finally opened the door for potential therapeutic use of the cells was the successful isolation of human embryonic stem cells (ESCs) from the cloned embryos. In 2013, the successes used *fetal* and *infant* donor skin cells, and in 2014 *adult* patient skin cells. The purpose of this subsection of the Lit Review is to discuss in detail these recent landmark cloning experiments, identifying potential problems that should be addressed in interviews for the IQP.

Triploid Embryos

The world's first "successful" human therapeutic cloning was achieved in 2011 in Dieter Egli's lab at the New York Stem Cell Foundation (Noggle et al., 2011). These scientists examined various ways of reprogramming somatic nuclei to a pluripotent state using human oocytes. But unlike most of the previous animal experiments, they left the haploid nucleus *inside* the oocyte, creating a cloned *triploid* embryo when they added the diploid skin nucleus during SCNT. Triploid embryos are unsuitable for human therapies, but can be used to study biological development.

They started the study by finding human oocyte donors between the ages of 22-33 years old from the Center for Women's Reproductive Care (CWRC) at Columbia University. After initially meeting the requirement for egg donation for reproductive purposes, these women donated their extra oocytes for research purposes. All of the women in this study had a college education or better. None of the women in the study were financially disadvantaged and they all had full time jobs, which lessened the ethical worries about providing financial incentives for egg donation. During a 19 month period, 16 out of 252 women agreed to donate their oocytes. These women were told what was going to happen with their oocytes by talking with their physician. Sixteen women donated 270 mature oocytes at meiosis stage-2 (MII) (range of 2-26,

and an average of 16.9 oocytes per donor cycle). Each participant was paid $8,000 for their donation.

Their results showed that removing the haploid (1N) oocyte nucleus prior to microinjection with a diploid (2N) skin nucleus *arrested* the diploid embryo development at late cleavage stages (2-3 days), presumably due to the inability of the oocyte to activate critical embryonic genes from the somatic donor cell nucleus. Thus, the removal of the oocyte nucleus negatively impacted the subsequent embryo's ability to induce reprogramming. These defects happened even though they used high quality young oocytes taken from women without a history of infertility.

Alternatively, when the scientists left in the haploid oocyte nucleus and then microinjected the diploid skin nucleus to create *triploid* cells (1N + 2N), this allowed full blastocyst formation (5-6 days) and successful ES cell isolation. The scientists concluded that removal of the oocyte nucleus was a critical step that prevented long-term survival of the embryo. In the interview portion of our project, we hope to clarify from these scientists why other later experiments using *diploid* embryos allowed ES cell isolation with no apparent abnormalities.

SCNT Success Using Fetal and Infant Skin Nuclei

As mentioned above, the *triploid* ES cells isolated from the triploid embryos of Noggle et al. (2011) could not be used for human therapy. But this setback proved moot in 2013 with the first production of human nuclear transfer ES cells (NT-ESCs) from *diploid* cloned embryos (Tachibana et al., 2013). This landmark experiment was done in Shoukhrat Mitalipov's lab at the Oregon National Primate Research Center using *fetal* and *infant* skin cells to provide the donor nuclei. The scientists identified two key steps (a premature exit from meiosis in human

oocytes and a suboptimal activation) as key factors responsible for the earlier cloning failures. Their development of more optimal SCNT procedures allowed the production of diploid cloned embryos from which viable NT-ESC lines were derived. The procedures were so efficient, the NT-ESC lines could be derived from as few as 2 oocytes. Importantly, the NT-ESC lines showed gene expression profiles apparently equivalent to normal ESCs derived from fertilized embryos, and they showed normal differentiation potential, so in theory the NT-ESCs should be suitable for therapies (for very young patients providing the skin cells).

Mitalipov and his team developed a protocol pieced together from several other earlier findings. They used Sendai virus to efficiently unite the enucleated egg and diploid skin cells (using no microinjection), and gave the fused cell an electrical shock to activate development. After the first several attempts failed, they added *caffeine* to the process which protected the egg from premature activation. The techniques had previous been used in different combinations with monkey cells, but not human cells. The whole process including evaluating the derived NT-ESCs took several years because most of the time was spent navigating through US regulations on embryo research (Vogel, 2014). The researchers were able to prove their NT-ESCs could widely differentiate. Their first NT-ESC line was created using fetal skin cells, the others were from an 8 month old patient with rare metabolic disorder Leigh syndrome. One NT-ESC line took 15 oocytes, while another took only five, an overall efficiency that is considered excellent. Tachibana et al.'s production of *diploid* NT-ESCs from cloned embryos in 2013 was considered a major innovation, and provided hope that the protocol could be later refined to include adult somatic cells. Their countless hours finally paid off for these scientists with this huge breakthrough. The breakthrough was extended in a spectacular way one year later in 2014 with the production of human NT-ESCs from human cloned embryos (see below).

SCNT Success Using Adult Somatic Nuclei

Some scientists worried that when using the Tachibana et al. (2013) protocol, only young (fetal and infant) nuclei would be suitable for reprogramming, which would not be useful for most *adult* patients. This limiting situation changed this year with two landmark studies using *adult* skin nuclei as donors (Chung et al., 2014; Yamada et al., 2014).

Chung and his team, in a study published in the journal *Cell Stem Cell* online April 15, 2014, were able to isolate human NT-ESC lines from blastocysts cultured from human oocytes fused with skin cells isolated from a 35 year old male and from a 75 year old male. Their procedures and culture media were similar to those described by Tachibana et al. (2013) (involving fusion of the oocyte with the skin cell using Sendai virus, not using nuclear microinjection), but included a key difference: they tested different time points of current application (to stimulate replication) following the viral treatment to induce fusion. By day 6 of culture, the team had obtained five blastocysts: two expanded from a 30 min post-fusion stimulation group, and three blastocysts expanded from a 2 hour post-fusion stimulation group. Only those embryos derived from the 2 hour group developed to become hatching blastocysts, from which the scientists were able to derive stable NT-ESC lines. The NT-ESCs could differentiate into several different cell types, demonstrating their feasibility for therapeutic use. During the interview portion of this project it will be interesting to follow up on why Chung et al. (2014) use of the Tachibana et al. (2013) methodology worked on adult cells here, but not for the original lab group. Perhaps a key change was the waiting period of 2 hours post Sendai virus treatment prior to applying the electrical current to stimulate cell division.

Thirteen days after the April 15 online release of the Chung et al. (2014) *Cell Stem Cell* paper, on April 28 another paper (Yamada et al., 2014) appeared in *Nature* online that successfully produced NT-ESCs from blastocysts cloned from somatic cells from a newborn

baby and from an *adult* 32 year old *diabetic* woman. This study was performed in Dieter Egli's lab at the New York Stem Cell Foundation Research Institute. This group tested several novel approaches for improving the survival of cloned human blastocysts. They used kinase and translation inhibitors to inhibit early activation following the use of the Sendai virus to fuse the egg and skin cells. They also used histone deacetylase (HDAC) inhibitors to prevent HDACs from removing acetyl groups from histone proteins bound to DNA, stimulating transcription from the embryo DNA. Normally, histone acetylation (by enzymes termed histone acetyltransferases) neutralizes the positive charges on histone proteins, decreasing their binding to negatively charged DNA. Decreased histone binding converts the DNA to a more open form (termed euchromatin) which is transcriptionally active. The process can be reversed using HDACs which help remove the acetyl groups, leaving the DNA in a condensed state (termed heterochromatin) which is transcriptionally inactive. Using HDAC inhibitors (as done by the researchers), blocks the HDACs, so the acetyl groups remain on the histones (or are placed there by histone acetyltransferases) and the DNA is converted to transcriptionally active euchromatin.

The scientists also tested several different concentrations of the Sendai virus used to fuse the enucleated oocyte and the diploid skin cell. High concentrations of virus increased the concentration of calcium causing early activation. So they used diluted Sendai virus in a calcium-free medium to prevent early activation.

The scientists also tested the ability of the derived NT-ESCs to treat a diabetic mouse model. When the NT-ESCs were differentiated into insulin-producing cells and injected into the diabetic mice, their blood sugar levels decreased to normoglycemia, indicating the therapy worked. But it remains to be seen whether the cells would be rejected in human patients. Their success deriving NT-ESCs from an adult diabetic patient is a landmark finding, and opens the door for using those derived ESCs to treat the same diabetic patient. The ESCs would be

genetically identical to the patient, so hopefully would not be rejected by the host. And the cells could be differentiated into insulin-producing pancreatic β-beta cells to treat the diabetes. As stated by Susan Solomon, one of the authors on the Yamada et al. (2014) paper:

> "This advancement toward potential regenerative cell replacement therapies is significant not only for **diabetes** but also for many other diseases and conditions, including **Parkinson's, macular degeneration, multiple sclerosis**, and **damaged bones**, among others. While there remain additional research hurdles to overcome before this work can reach the clinic, we are thrilled that our scientists have once again taken the lead in breaking down barriers that face the entire field." (Farrell, 2014).

Some scientists think that 2014 has been an outstanding year for human SCNT experiments, and are excited to test the derived NT-ESCs in human therapies. Some scientists think that SCNT is a better option than using iPSCs because the latter often do not become completely reprogramed or may become damaged during the reprogramming process, which could make them less stable than NT-ESCs. Others think that iPSCs are a better way to proceed, worrying there will not be enough oocytes to do the therapeutic cloning process. And should the oocyte donors be paid, and by whom? These key issues will be addressed later in the report.

METHODS

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 a set of semi-structured, in-depth interviews with various academic researchers in the stem cell field who have achieved human therapeutic cloning, or who have significantly contributed to the development of the cloning technology, to determine their range of opinions on the strengths and weaknesses of this new cloning technology and whether other techniques that do not involve cloning could accomplish the same therapeutic goal.

Who: The stakeholders included academic experts on human therapeutic cloning, but also included academic experts on cloning technology in general or on potential replacements for cloning. A few interviews were also conducted with scientists in biotechnology companies doing therapeutic cloning. The interviewees also included academic bioethicists to help discern the ethical issues of human reproductive cloning. Some of the stakeholders initially were identified by referral from the project advisor, Dave Adams, but the majority of subjects were identified from the literature as authors on key scientific papers, or by referral from the initial interviewees (to develop a referral "snowball").

Where and When: Whenever possible, interviews were conducted in person, but the majority were performed by email, phone, or Skype.

How: We developed our interview questions based on our background research. A preliminary set of questions is shown in the Appendix. Based on our background search of the interviewee and his/her responses to our initial questions, we tailored our subsequent questions to best obtain information from that person. The appendix shows the topics needed to fully cover our project.

With respect to the method of the interview, after establishing contact with an interviewee, we informed the interviewee about the purpose of our project, and asked for permission to quote them (see interview preamble in the Appendix). If the need arose for confidentiality, we protected it by either not quoting them directly, or by giving them the right to review any quotations used in the final published report, explaining that the interview is voluntary, and explaining that they may stop the interview at any time or refuse to answer any question. At the end of the interview, we sometimes asked the interviewee 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 performed for our project, we discontinued identifying new names when we had obtained sufficient information represent all sides of the problem, and when the 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 human therapeutic cloning, and created recommendations for further research.

RESULTS / FINDINGS

Introduction to Three Types of Pluripotent Stem Cells, and Comparison of Therapeutic and Reproductive Cloning (Jiaxun Xie)

The Literature Review for this section of the report focused on the process of somatic cell nuclear transfer (SCNT), compared the processes of reproductive cloning and therapeutic cloning, and provided a general background on the use of different types of pluripotent stem cells. The types of stem cells of direct interest to this project were cloned nuclear transfer embryonic stem cells (NT-ESCs) (prepared from cloned embryos), traditional ESCs (prepared from blastulas obtained by *in vitro* fertilization) (IVF-ESCs), and induced pluripotent stem cells (iPSCs) (prepared by reprogramming differentiated skin cells to a pluripotent state). The former two processes use human eggs, while the latter process does not.

The interest of many scientists in the process of human therapeutic cloning was recently revived due to the recent 2014 successes published from two labs (Chung et al., 2014; Yamada et al., 2014) who were the first to achieve human therapeutic cloning from adult skin cell nuclei (discussed later in the report). The review of the literature in this area showed that some individuals are against the use of NT-ESCs due to the availability of iPSCs that use no eggs but appear to have similar differentiation potential. Others worry that the injected NT-ESCs might form tumors at the injection site. Others doubt we will be able to obtain sufficient donated human oocytes to be able to fully develop the new technique. One of the main purposes of this IQP was to investigate these issues by reviewing the published literature and interviewing key scientists to help determine whether iPSCs might be able to take place of NT-ESCs in human therapies, and what experiments remain to prove this.

After performing the Literature Review to identify key problems in the area, we performed a series of interviews with several researchers that had directly performed therapeutic cloning or alternatively worked with iPSCs. The interviews initially focused on clarifying some of the ethical problems that therapeutic cloning may have, and the researchers' attitudes towards the recent therapeutic cloning success, while later interviews focused on the scientists worries about epigenetic alterations in all 3 types of pluripotent cells.

The first interview was performed with Dr. Robert Klitzman, a professor at the Division of Psychiatry, Law and Ethics, College of Physicians and Surgeons, Columbia University, New York State Psychiatric Institute, New York, NY 10032. Dr. Klitzman was the first author on a 2009 article published in *Reproductive BioMedicine Online,* entitled "Payment of egg donors in stem cell research in the USA" (Klitzman and Sauer, 2009). When asked whether he would be in favor of using other stem cell reprogramming processes (such as iPSCs) that do not use human eggs if those cells are proven to be as potent as cloned cells, he validated our stance that iPSCs might ethically be a nice alternative to cloned ESCs, but he does not believe it is proven yet whether iPSCs are actually as beneficial therapeutically as IVF-ESCs. So, we concluded that more research needs to be done with direct therapeutic comparisons.

The second interview was performed with Dr. Amanda A. Skillern, a scientist in the Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California, San Francisco, CA. Dr. Skillern was the first author on a 2014 paper in *Fertility and Sterility*, 101: 248-251, entitled "Oocyte Donors Comprehension as Assessed by the EDICT (Egg Donor Informed Consent Tool)" (Skillern et al., 2014). When asked whether donors of eggs to be used for research purposes should be paid, she said that she supports paying egg donors, and indeed her egg donors are paid (she is an MD with women donors as part of her practice). She said the women should be paid to be compensated for their time and for their assumption of surgical risk

and discomfort. And moreover, the egg simulation requires significant time out of the donor's schedule, with daily appointments for ultrasounds, blood draws, and 9-12 days of daily hormone injections. The minor surgical procedure to retrieve eggs involves a small but real medical risk and is uncomfortable. Dr. Skillern also reminded us that most states currently do not allow egg donors to be paid, while it is important for the egg donors to get paid otherwise we will never get enough eggs to treat a significant number of patients. She thinks it is sad that most states do not allow compensation "as there is lots of good work that can be done with [human] oocytes that would help advance science in a number of ways". "So the reality is that there is not going to be some floodgate of women willing to go through the process without compensation. There just are not too many people willing to inject themselves for 2 weeks, undergo a surgical procedure, and incur the [surgical] risk just for the good of science". With respect to iPSCs, she said that "she worked in a CIRM-sponsored stem cell lab, and would be shocked if we were ever able to engineer a fully functional iPS cell. So, I think donated oocytes are sorely needed to continue to advance science". So, she argues that some states need to change their laws to allow egg research and to allow donors to get paid.

Currently, most states do not have their own specific laws governing egg harvesting, so they function under federal guidelines mandating IVF clinic involvement (Summary of State Laws and Egg Harvesting, 2014). One state (Louisiana) specifically prohibits egg donors from being compensated for any purpose. Five states (MA, CT, MD, CA, AZ) prohibit compensation for egg donors for research purposes, but are silent for egg harvesting for IVF purposes. Four states (RI, NJ, IL, MN) prohibit the sale of eggs obtained from human fetuses. Two states (NY, ID) allow for donor compensation. Although in 2013 the California legislature approved *Assembly Bill 926* that would allow the sale of human eggs in that state, on August 13, 2013, Governor Jerry Brown vetoed the bill. Gov. Brown stated that, "Not everything in life is for sale,

nor should it be. In medical procedures of this kind, genuinely informed consent is difficult, because the long-term risks are not adequately known" (Donner, 2013).

The next interview was performed with Dr. Hossam E. Fadel, a scientist and therapeutic cloning ethics expert at 3503 Lost Tree Lane, Augusta, Georgia 30907. Dr. Fadel was the author of a 2012 article published in *Bioethics*, 26(3): 128-135, entitled "Developments in Stem Cell Research and Therapeutic Cloning: Islamic Ethical Positions, A Review" (Fadel, 2012). When asked what he believes are the main ethical problems associated with human therapeutic cloning, Dr. Fadel said that ethically the two most important concerns are 1) the use of human eggs in general, and 2) their use for research not reproduction. Comparing the ethics of human therapeutic cloning versus iPSCs, he stated that because iPSCs do not use human eggs they have fewer ethical considerations. However, "either type of procedure should first show evidence of success in clinical trials, and have informed patient consent prior to proceeding". So, Dr. Fadel validated the important point that the requirement for human eggs is the main ethical consideration when working with human therapeutic cloning, and reiterated the point seen with other researchers that we need more data on the use of pluripotent cells in therapies to determine which is best.

The next interview was performed with Dr. Mark Tomishima, a scientist in the Developmental Biology Program, Sloan-Kettering Institute, 1275 York Avenue, New York, NY 10065. Dr. Tomishima was an author on a 2008 article published in *Nature Medicine* performing therapeutic cloning on Parkinson's disease mice (Tabar et al., 2008). The researchers derived ESCs from mice, differentiated them into dopaminergic neurons, and then used them to treat the Parkinson's model. The cloned ES cells showed efficacy and a lack of immune rejection, providing "proof of principle" that therapeutic cloning can work. When asked in his opinion on what the biggest hurdle is for using therapeutic cloning in humans, he replied: "We [first] are

trying to use pluripotent stem cells to treat Parkinson's disease in non-genetically matched mice". This situation would more likely mimic what will be encountered in the clinic if non-cloned ESCs are used to treat a patient, because the IVF-ESCs would not genetically match the patient. He also argued that with respect to "perfect genetic matching" (therapeutic cloning), "It would be very hard to do that on a case-by-case basis". So he argues that we need to continue researching IVF-ESCs for treating human diseases because those cells will be more readily available for most individual patients.

The next interview was performed with Dr. Andrew P. Feinberg, Director of the Center for Epigenetics, and Professor in the Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205. Dr. Feinberg was one of the two corresponding authors on a 2010 paper published in *Nature*, 467: 285-290, entitled "Epigenetic memory in induced pluripotent stem cells" (Kim et al., 2010). The authors compared the differentiation potential and epigenetic signature (genomic methylation patterns) of several types of pluripotent stem cells (including iPSCs, NT-ESCs, and IVF-ESCs) and concluded that after reprogramming to a pluripotent state, iPSCs retain an epigenetic signature (DNA methylation) similar to their tissue of origin (usually skin) especially during the early passages (before they become adapted to culture). When asked whether he had extended his analysis to include histone acetylation patterns (another type of epigenetic modification), or think it is worth doing, he replied "Clever of you! Yes, we have done this, but have not yet published it". Thus, although most stem cell epigenetic labs are currently studying DNA methylation patterns, expanding the analysis to include histone acetylation patterns (another type of epigenetic modification) is also important.

The final interview was performed with Dr. Peter J. Rugg-Gunn, a scientist at the Babraham Institute, Cambridge CB22 3AT, UK. Dr. Rugg-Gunn was corresponding author on a 2007 paper published in *Human Molecular Genetics*, 16(2): R243-R251, entitled "Status of

genomic imprinting in human embryonic stem cells as revealed by a large cohort of independently derived and a maintained lines”. The authors analyzed 46 individual ESC lines as part of an International Stem Cell Initiative, and concluded that the lines were mostly epigenetically stable, despite differences in genetic background or method of derivation; however some lines showed loss of allele-specific expression. They identified several genes that were more variable in their methylation pattern (H19, IGF2, and MEG3) that could be used to provide a marker of epigenetic status. In the interview, Dr. Rugg-Gunn said he has not yet extended his DNA methylation marker analysis to compare cloned-ESCs with IVF-ESCs because human therapeutic cloning is too new. He pointed us to two 2013 and 2014 studies (discussed in our Literature Review), and he stated “As far as I am aware, there has not been a more thorough characterization of cloned-ECSs since those [2013 and 2014] studies”.

Overall, the interviews performed for this section of the report indicate that the use of human eggs is the most important ethical consideration when performing human therapeutic cloning. One interviewee had strong concerns about obtaining a sufficient number of eggs for human therapeutic cloning treatments without changing specific state laws to allow egg donors to be paid as an incentive to compensate for their inconvenience, risk, and pain. Another interviewee indicated that performing human therapeutic cloning would be too hard to do on a case-by-case basis, so we should continue researching iPSCs that are easier to obtain. And several scientists indicated it is too early to determine which type of pluripotent stem cell is best in therapy until direct comparisons are done. With respect to stem cell comparisons, most stem cell labs are currently studying DNA methylation patterns, and one interviewee validated our opinion that we should expand the epigenetic analysis to include histone acetylation patterns, another type of epigenetic modification.

Early Human SCNT Embryo Development (Adam McNally)

The Literature Review for this section focused on some of the early human embryo experiments that eventually led to the recent 2013 and 2014 successes with human therapeutic cloning. Human somatic cell nuclear transfer (SCNT) experiments span both the medical and scientific spectra, and are a key topic in the field of regenerative medicine and stem cells. Cloned ESCs or nuclear transfer ESCs (NT-ESCs), in theory, are genetically identical to the patient, so likely they will not be rejected. The advent of human *in vitro* fertilization (IVF) and the use of excess embryos that were donated for research when not used for reproductive purposes, allowed research into early human development and the production of blastocysts from which ESCs could be isolated. The Literature Review on this topic revealed several successes and failures of human therapeutic cloning experiments. Early experiments were not able to grow the embryos to the blastocyst stage, so they celebrated a simple embryo cell division or its survival to the morula stage (pre-blastula). Later experiments allowed embryo survival to the blastula stage, isolation of ESC-lines from the blastula, and a better understanding of human oocyte physiology. But the experiments also identified potential problems associated with NT-ESCs, including DNA mutations, inefficiency of the cloning technique, and the growing preferred usage of induced pluripotent stem cells (iPSCs) which use no eggs but which may have epigenetic problems.

To investigate these issues further, interviews were performed with several researchers that had early direct experience with human SCNT. The first interview was conducted with Professor Miodrag Stojkovic, a scientist at the Centre for Stem Cell Biology and Developmental Genetics, University of Newcastle, Newcastle upon Tyne, UK. He was the first author on the 2005 paper in *Reproductive BioMedicine Online*, entitled "Derivation of a human blastocyst after heterologous nuclear transfer to donated oocytes" (Stojkovic et al., 2005). His lab was the first

to produce a human blastocyst by SCNT, although they did not derive ESCs from the blastocyst for therapy. When asked what he believed was the key step leading to his success when the previous labs had failed, he stated that the oocytes used in the experiment had to be young and "fresh" in order to create human cloned embryos with a decent efficiency. However, this made it hard to obtain large numbers of suitable oocytes, and will continue to do so especially as human therapeutic cloning expands. So, this problem may need to be solved before moving into large numbers of human therapy experiments. When asked about future therapeutic cloning experiments, he stated that he likes the use of non-cloned cells like iPSCs for doing therapies, because they don't need eggs, and that cloning "is too complicated, and too time and money consuming". Although on the other hand he argues for continued scientific research on cloning to better "understand which oocyte factor(s) are involved in reprogramming adult somatic and donor cells".

The second interview was performed with Dr. Andrew J. French, a scientist at the Stemagen Corporation, 4150 Regents Park Row, La Jolla, CA 92037. Dr. French was the corresponding author on a 2008 paper published in *Stem Cells*, entitled "Development of Human Cloned Blastocysts Following Somatic Cell Nuclear Transfer (SCNT) with Adult Fibroblasts" (French et al., 2008). His experiment showed that cloning could create a human blastocyst; although he did not derive embryonic stem cells from the blastocyst to use in therapeutic cloning. During the interview he attributed his lab's success to: A) his lab's close association with an IVF clinic, B) his use of pre-tested media from the IVF clinic, C) access to high quality egg donors, and D) quality control (half the eggs were used in their lab while the other half of the eggs were tested in the IVF clinics). He also remarked that a key problem remaining using NT-ESCs for therapy is that we "don't yet know the effectiveness of those cells for treating disease. But scientists' newfound access to these cells will help alleviate this problem". When asked

about his opinion on induced pluripotent cells (iPSCs) he stated, “iPSCs are useful cell types for discovering stem cell treatments, but observable differences remain between iPSCs, ESCs, and NT-ESCs”. His belief was that ESCs, NT-ESCs, and iPSCs should all be experimented with because they all show different epigenetic problems, and in the end, only testing them in therapies will show which one is best.

The next interviewee was Dr. Jeanne F. Loring, a Professor of Developmental Neurobiology, and Director of the Center for Regenerative Medicine, Department of Chemical Physiology, The Scripps Research Institute, La Jolla, CA 92037. Dr. Loring was the senior author on a 2011 paper published in *Cell Stem Cell*, 8: 106-118, entitled “Dynamic changes in the copy number of pluripotency and cell proliferation genes in human ESCs and iPSCs during reprogramming and time in culture” (Laurent et al., 2011). The authors used high-resolution single nucleotide polymorphism (SNP) analysis of 186 pluripotent and 119 non-pluripotent stem cell lines. They found a high frequency of sub-chromosomal (individual gene) copy number variations. Especially interesting, they found several deletions in tumor suppressor genes and duplications in oncogenes, either of which could increase tumor formation. They concluded that all pluripotent cell lines should frequently be monitored at the DNA level for clinical safety prior to use in humans. In the interview she pointed out a recent 2014 mini-review she published in the *Journal of Biological Chemistry* on stem cell chromosome instability as related to clinical applications (Peterson and Loring, 2014). In that paper, she strongly recommended using high SNP genotyping to monitor all stem cell batches used for human therapies, because all types of stem cells when cultured are known to acquire mutations over time (ESCs and iPSCs). She argues that they all get the same types of DNA duplications, deletions, and mutations. When asked about whether the gene alterations could cause cancer, she stated: “Yes, it would be great

if someone would do a comprehensive study on the linkage between certain common mutations and actual tumor genesis [so we would know what to look for prior to use in therapies]. The FDA has a general rule that you need to show that your cells are safe and don't cause tumors, but they aren't really pushing for anyone showing cause [specific DNA mutation) and effect [cancer]". They all show mutations, but "the important issue is whether they matter." However, she did assert that most of the mutations are less of a big deal than most people have suggested.

The next interview was performed with Dr. Timo Otonkowski, a Professor of Medical Stem Cell Biology, Children's Hospital and Biomedicum Stem Cell Center, University of Helsinki, Helsinki Fl-00014, Finland. Dr. Otonkowski was one of the corresponding authors on a 2011 article published in *Nature*, 471: 58-62, entitled "Copy number variation and selection during reprogramming to pluripotency" (Hussein et al., 2011). The authors used high-resolution single nucleotide polymorphism (SNP) analysis to compare iPSCs, fibroblast cells, and ESCs. They found several types of copy number variations between the cell types, and the more the iPSCs were expanded, the more ESC-like the DNA became. From the interview it was learned that he thinks iPSC lines should be expanded prior to use in any therapies to eliminate any aberrant cells present in the samples at the beginning. He also stated that "it is a great challenge for therapeutic applications to obtain absolute evidence for the genetic safety of these cells, since they occasionally gain mutations in culture that aid their adaptation to the culture environment, and this is true for all expanded cells". This means that all cells grown for human therapies should be checked for genetic mutations prior to use in therapy, and they should be expanded to allow aberrant cells to be eliminated and to allow the DNA to become more ESC-like epigenetically. The cells should also be monitored for DNA alteration after expansion in case random mutations have occurred.

The next interview was conducted with Dr. Alexander Meissner, a scientist at the Broad Institute, Cambridge, MA 02142. Dr. Meissner was one of two corresponding authors on a 2011 paper published in *Cell*, 144: 439-452, entitled "Reference maps of human ES and iPS cell variation enable high-throughput characterization of pluripotent cell lines" (Bock et al., 2011). The authors indicate that substantial genetic variation has been reported for various pluripotent cell lines, which could affect their safety. They created genome-wide reference maps of DNA methylation and gene expression patterns for 20 human ESC lines and 12 iPSC lines, and made correlations with their ability to differentiate into more specialized cells (as would happen during therapy). They concluded that their assays could provide a "scorecard for quick and comprehensive characterization of pluripotent cell lines" (Bock et al., 2011). During the interview, when asked about extending his analysis to include cloned-ESC lines, he remarked that it would not be necessary, because "while SCNT remains interesting, it's no longer a likely method for routine generation of stem cells". It became clear that Dr. Meissner believes more in reprogrammed iPSCs (that do not use eggs) than in NT-ESCs.

The final interviewee was Dr. Akihiro Umezawa from the Department of Reproductive Biology, National Institute for Child Health and Development, Tokyo, Japan. Dr. Umezawa is a corresponding author on a 2011 paper published in *PLoS Genetics*, 7(5), e1002085, entitled "DNA methylation dynamics in human induced pluripotent stem cells over time" (Nishino et al., 2011). The authors determined the DNA methylation patterns of 22 human iPSC lines and 5 human ESC lines, and concluded that the two cell types initially differed in their patterns, but the iPSCs became more ESC-like over time (the iPSCs lost their original methylation patterns inherited from the parental cells over time). When questioned about his work and whether he had analyzed NT-ESCs, Dr. Umezawa indicated that it is worth analyzing "the DNA methylation of

[cloned] ESC lines," and not only has he done it, but he is in the process of submitting another paper on it. "We finished it and are preparing a draft now. I hope you will also enjoy reading it once it gets published".

Overall, the interviews performed for this portion of the project validated the preliminary findings of the Literature Review and brought up different opinions on using ESCs and iPSCs for human therapies. Both Dr. Stojkovic and Dr. Meissner favored work with iPSCs over ESC cells because of the ease of reprogramming and the avoidance of human eggs, while Dr. Meissner went so far as to say that working with NT-ESCs is almost not even viable in the present iPSC environment. On the other hand, Dr. Umezawa who has recently been working exclusively on ESCs feels that further research on ESCs will prove most fruitful, and that ESCs have fewer epigenetic problems than iPSCs. Encompassing both sides, Dr. French feels that only further testing will determine whether iPSCs or ESCs are better in human therapies. Our analysis of the current research and our interaction with key scientists has shown that human therapeutic cloning has advanced a long way in a very short period of time, and the methods are constantly improving with higher efficiencies. However, many avenues of research still need to be done. If NT-ESCs are used for human therapies in large numbers, suitable human eggs may soon become rate-limiting because some scientists found that only fresh and young eggs currently work in the cloning protocol. And several interviewees recommended, and we agree, that all pluripotent cell lines (NT-ESCs, IVF-ESCs, iPSCs) should frequently be monitored for DNA mutations, epigenetic modifications, differentiation potential, and tumor potential, before AND after expansion for clinical safety. And in the end, we do not know the effectiveness of any of these pluripotent cells for treating human diseases, so research should continue in all areas.

Human Therapeutic Cloning of ES Cell Lines (Nicholas Scrivanich)

The Literature Review performed for this section of the report showed that years 2013 and 2014 were outstanding for human therapeutic cloning experiments. Scientists finally succeeded at preparing human embryonic stem cells (hESCs) by microinjecting a skin cell nucleus from an adult human patient into an enucleated egg, growing the embryo *in vitro* to the blastocyst stage, and isolating the cloned ESCs from the blastocyst inner cell mass. This process creates nuclear transfer ESCs (NT-ESCs) that are genetically identical to the patient, and which in theory can be used to treat the same patient without immune-rejection. The NT-ESCs could also be prepared by simply fusing the skin cell directly with the human egg using Sendai virus to aid the fusion. At the end of their landmark papers, the scientists concluded how excited they are to test the NT-ESCs in human therapies in the future.

However, our review also pointed us to some potential problems of using NT-ESCs for therapies, including potential epigenetic modifications (DNA methylation, histone acetylation) that might prevent full cell reprogramming, and logistical and ethical problems associated with using human eggs in the procedures. Some scientists thought that using induced pluripotent stem cells (iPSCs) instead of NT-ESCs might be better. iPSCs can be prepared from adult skin cells by directly reprogramming them with specific genes that initiates the reprogramming process, and the iPSC process does not use human eggs. Other scientists worried that iPSCs show even greater epigenetic problems than NT-ESCs which could hinder their use in therapies.

To help resolve some of these issues, we performed a series of interviews with scientists who have performed the NT-ESC process themselves, and who have characterized these cells. The first interview was performed with Dr. Shoukhrat Mitalipov of the Division of Reproductive and Developmental Sciences, Oregon National Primate Research Center, Oregon Health and Science University, 505 NW 185th Avenue, Beaverton, OR 97006. Dr. Mitalipov is senior

author on a 2013 paper published in *Cell* (Tachibana et al., 2013) that was the first to achieve human therapeutic cloning without producing undesired triploid cells that earlier protocols had produced (triploid cells are produced when diploid nuclei are injected into non-enucleated eggs containing haploid nuclei). Mitalipov's group succeeded while using fetal and infant skin donor cells, but not with adult skin cells. When asked why he was able to achieve his human therapeutic cloning success, Dr. Mitalipov responded that his success resulted from finally being able to grow the cloned embryos to the blastula stage from which the ESCs could be isolated. "Yes, we succeeded in human SCNT because we were able to support growth of embryos to blastocysts". And when commenting on other scientist's worries about whether NT-ESCs can be used for human therapies and how robust he thinks the cells are, he stated "So far, it seems that human SCNT-derived stem cells are much better [than iPSCs] in terms of quality." So, he believes that we need to continue researching NT-ESCs not just iPSCs for human therapies.

The second interview was performed with Dr. Dieter Egli of The New York Stem Cell Foundation Research Institute, New York, NY 10032. Dr. Egli is senior author on a key 2014 *Nature* paper (Yamada et al., 2014) that was the first to derive diploid pluripotent stem cells (similar to embryonic stem cells) from an *adult* patient with diabetes. When asked why he was able to succeed when previous researchers had failed, he said that he never liked microinjecting the skin nucleus into the egg [which presumably leaves key reprogramming factors behind], and instead prefers using Sendai virus to directly fuse the egg and skin cell together. He credited his recent success to three key steps not done previously that help increase human embryo survival to the blastocyst stage (from which the ESCs were isolated): 1) using lower amounts of Sendai virus than previous experiments in a calcium-free medium to prevent the calcium from activating the egg, 2) using kinase and protein translation inhibitors to block early egg activation, and 3) using histone de-acetylase inhibitors (to block early transcription from the embryo genome). He

verified that their timing of egg activation with the electric current to initiate cell division was the same timing used in Chung et al. (2014) (2 hours post Sendai fusion). He was not convinced that 30 minute activation times were really accurate. In a follow-up interview, Dr. Egli said that he thought we would need BOTH cloned ESCs and iPS cells for human therapies, as it is too soon to know which will work best.

The third interview was with Dr. Young Gie Chung of the Research Institute for Stem Cell Research, CHA Health Systems, Los Angeles, CA 90036. Dr. Chung is first author on the 2014 *Cell Stem Cell* paper (Chung et al., 2014) which was the first to show that adult dermal fibroblasts (from 35- and 75-year old donors) can be used to create embryonic stem cells by SCNT cloning. Dr. Chung clarified that the methods allowing him to succeed with human therapeutic cloning from adult dermal fibroblasts were very similar to those of the earlier 2013 Tachibana et al. paper from the Mitalipov lab that succeeded with fetal and infant donor cells. They used the Sendai virus envelope protein to aid cell fusion between the adult dermal fibroblast cell and the egg, not nuclear microinjection into the egg. And they used electric current (PIEZO actuation) to activate the fused cell to begin dividing. The only difference is they waited 2 hours after cell fusion before activating the eggs to begin dividing. “Other than the waiting time difference, our protocol was virtually same as Dr. Mitalipov's.”

The fourth interview was with Dr. Joseph Ecker of the Genomic Analysis Laboratory, The Salk Institute for Biological Sciences, La Jolla, CA 92037. Dr. Ecker is one of three corresponding authors on a July 10, 2014 article published in *Nature,* entitled “Abnormalities in human pluripotent cells due to reprogramming mechanisms” (Ma et al., 2014). The authors did a direct comparison of IVF embryonic stem cells (IVF-ESCs), cloned nuclear transfer ESCs (NT-ESCs), and induced pluripotent stem cells (iPSCs) with respect to abnormalities, and concluded that scientists should not discontinue studying cloned NT-ESCs because they have fewer

epigenetic problems than iPSCs which tend to retain the epigenetic status of the original differentiated skin cell. The DNA methylation pattern and transcription profile of the NT-ESCs looked closer to the gold standard IVF-ESCs, so they should do better in therapies, in spite of the fact that we would still need human eggs. But he reminded us that this is "only molecular data, not functional data". He still has to prove the point in therapy experiments. "Right now we have no data on whether they are more or less suitable for human therapies but our analysis showed that the epi-genome of SCNT cells is closer to ESCs (the gold standard) than are iPSCs. They have less reprograming and gene expression differences, so if gene expression is an indication of utility, then they may turn out to be more useful. But again this has not been established, it is only a prediction based on molecular (not functional) evidence".

The fifth interview was with Dr. Louise Laurent of the Department of Reproductive Medicine, University of California San Diego, La Jolla, CA 92037. Dr. Laurent is also an author on the Ma et al. (2014) paper along with Dr. Ecker. She verified that our interpretation of her data was correct, that NT-ESCs are more faithfully reprogrammed than iPSCs, "Yes that is right". And because of that, they believe the former may be better suited for human therapies.

Overall, the interviews from this section of the report indicate that most of the interviewees are concerned with the epigenetic status (DNA methylation, histone acetylation, etc.) of the pluripotent cells that might prevent their full reprogramming or their ability to differentiate into the desired cell type needed for therapy. Some scientists thought NT-ESCs were more faithfully reprogrammed than iPSCs and should be used for therapies in spite of their use of human eggs. Some interviewees had logistical (where to obtain large numbers of fresh eggs) and ethical concerns (can iPSCs replace NT-ESCs) associated with using human eggs in the NT-ESC procedures. Verification was obtained on how the scientists were able to succeed with human therapeutic cloning. A key ingredient appears to be the direct fusion of egg and skin

cell, instead of using only the skin cell nucleus which presumably leaves key reprogramming factors behind. Other specific alterations included using lower amounts of Sendai virus than previous experiments in a calcium-free medium to prevent the calcium from activating the egg, using kinase and protein translation inhibitors to block early egg activation, and using histone de-acetylase inhibitors (to block early transcription from the embryo genome).

CONCLUSIONS / RECOMMENDATIONS

Based on the research performed for this project, our group is able to make several conclusions and recommendations. With respect to the **ethics of human therapeutic cloning**, our findings indicate that the use of human eggs is the single most important ethical consideration in the entire process. If embryonic stem cell lines obtained by therapeutic cloning (nuclear transfer ESCs or NT-ESCs) are used to treat human diseases, the number of human eggs required will increase significantly relative to those currently used for research. Several interviewees had concerns about whether a high number of eggs will be available, especially since some scientists think the eggs need to be fresh and young, and considering the problem that most states do not have their own specific laws governing egg harvesting. Only two states (NY, ID) currently allow for egg donor compensation, one state (Louisiana) specifically prohibits donor compensation for any purpose, and five states (MA, CT, MD, CA, AZ) prohibit donor compensation for research purposes (but are silent for egg harvesting for IVF purposes). In 2013 the California legislature approved *Assembly Bill 926* that would allow the sale of human eggs in that state, but Governor Jerry Brown vetoed the bill on August 13, 2013. One interviewee who performs human egg experiments strongly felt that donors should receive money as an incentive to compensate the donors for the surgical risk and pain of the procedure, and the inconvenience of two weeks of hormonal injections. And without the compensation, the number of eggs will remain rate-limiting. So, we recommend that individual states consider changing their laws to allow for donor compensation.

A significant problem encountered in this project was that the bioethicists tended to not respond to our inquiries, or they were not aware of (or were not willing to comment on) human

therapeutic cloning advances. We compensated for this by expanding our interview selections, and by getting comments from the scientists directly related to ethical issues.

With respect to **using NT-ESCs versus induced pluripotent stem cells (iPSCs) for therapies**, our research indicates that several researchers have compared the epigenetic status of the three types of pluripotent cells. The epigenetic status can affect their ability to differentiate into the desired cell type, so could affect their usefulness for therapy. All the scientists studying epigenetics focused on DNA methylation patterns, and some agreed with our recommendation that the studies should be expanded to include histone acetylation as another indicator of epigenetic status. Some scientists concluded that NT-ESCs have an epigenetic pattern that is closer to IVF-ESCs than iPSCs, the latter of which more closely resemble differentiated skin cells. So, this could hinder the use of iPSCs in therapy, and favor the use of IVF-ESCs or NT-ESCs. Other interviewees indicated the epigenetic status of the iPSCs changed to be more IVF-ESC-like the longer they are cultured, so this interesting finding is worth repeating. Other scientists who had not yet analyzed epigenetics agreed with us that it is worth doing, and had plans in their own labs to do so. Several scientists indicated it is too early to determine which type of pluripotent stem cell is best in therapy until direct therapy comparisons are done. And several interviewees identified DNA mutations in the pluripotent cells which they said could lead to cancer. So we recommend that all pluripotent cell lines (NT-ESCs, IVF-ESCs, iPSCs) should frequently be monitored for DNA mutations, epigenetic modifications, differentiation potential, tumor potential, and ability to treat a disease, before AND after expansion for clinical safety. These comparisons can all be done now that isolating all three types of pluripotent cells is possible.

With respect to **cloning technology and its complexity**, our analysis of the current research and our interaction with key scientists showed that the technology of human therapeutic

cloning has advanced a long way in a very short period of time, and the methods are constantly improving with higher efficiencies. One key advance is the new method of directly fusing the enucleated egg with the diploid skin cell, instead of microinjecting the skin cell nucleus as was originally done. Isolating the skin cell nucleus and preparing it for microinjection likely leaves behind key reprogramming factors, and this is avoided by directly fusing the skin cell and egg. Other recent protocol improvements included using lower amounts of Sendai virus (to help fuse the cells) in a calcium-free medium to prevent the calcium from activating the egg, using kinase and protein translation inhibitors to block early egg activation, and using histone de-acetylase inhibitors (to block early transcription from the embryo genome). These improvements have allowed the cloned human embryos to survive to the blastula stage (from which the ESCs are isolated), and appear to be the best-practice methodology which we recommend should be applied in the future by all labs attempting to clone.

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APPENDIX

LIST OF INTRODUCTORY INTERVIEW QUESTIONS

Example Questions for Researchers Who Have Achieved Human Therapeutic Cloning:

1. How strong do you think your evidence is that you have finally achieved human therapeutic cloning?
2. Technically what did your lab do that allowed you to succeed in this highly competitive area?
3. What experiments do you think need to be done prior to using such cells for therapy?
4. Do you think that embryonic stem (ES) cells isolated from therapeutically cloned embryos are as potent as ES cells isolated from non-cloned embryos?
5. Do you think that other types of stem cells, like induced pluripotent stem (iPS) cells, can serve as a replacement for ES cells isolated from cloned embryos?
6. Do you think that iPS cells have serious problems that must be overcome prior to use in therapies?
7. Do you think that the funding for therapeutic cloning experiments will increase or decrease?

Example Questions for Academic Bio-Ethicists:

1 Are you familiar with this year's major discovery of the ability to perform human therapeutic cloning using adult patient skin cells? If not, we will explain this briefly to them.
2 What do you think are the main ethical concerns with human therapeutic cloning and the use of such cells for therapy?
3 Do you think researchers should try to find alternatives for therapeutic treatments?
4 Do you think that more research should be performed to more fully understand therapeutic cloning prior to using such cells for therapy?
5 Do you think that individual state laws should be changed to allow egg donors to be compensated?

Example Questions for General Cloning Researchers:

1. How strong do you think the evidence is that scientists have achieved human therapeutic cloning with adult skin cells?
2. What do you think were the key technical improvements that led to success?
3. What were the main problems that prevented success?

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 human therapeutic cloning and the use of those cells 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, which will be published on-line in WPI's archive of projects.

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.