Alternatives to Embryonic Stem Cells and Cloning: A Brief Scientific Overview

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Advances in recent years have begun to elucidate the distinct mechanisms that maintain embryonic stem cells (ESCs) undifferentiated, self-renewing, and pluripotent. One of the “grails” of therapeutic stem cell biology is the ability to confer these special properties of the embryonic stem cell onto an easily accessible, differentiated cell from the adult (such as a skin or blood cell) without the creation of an embryo as a necessary intermediate step. Such a technology would not only provide an ethically acceptable alternative to research cloning, but it would also offer a method to interrogate the biological basis of “stemness,” the constellation of gene expression and protein signaling that underlie self-renewal and pluripotency.

A landmark study published in 2006 and many subsequent reports demonstrate that the reactivation of a handful of particular genes can “reprogram” a differentiated cell from a variety of rodent and human tissues into a cell with several properties of embryonic stem cells, including self renewal and pluripotency.⁴ These reports demonstrate that much of the “grail” has now been found, albeit with some important limitations. A number of studies have successfully demonstrated the viability of theoretical proposals previously offered by President Bush’s Council on Bioethics to generate alternative sources of pluripotent cells, at least in the experimental setting.⁵ These promising

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1. Kazutoshi Takahashi & Shinya Yamanaka, Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors, 126 CELL 663 (2006); see also Takashi Aoi et al., Generation of Pluripotent Stem Cells from Adult Mouse Liver and Stomach Cells, 321 SCIENCE 699 (2008); Alexander Meissner, Marius Wernig & Rudolph Jaenisch, Direct Reprogramming of Genetically Unmodified Fibroblasts into Pluripotent Stem Cells, 25 NATURE BIOTECHNOLOGY 1177 (2007); Keisuke Okita et al., Generation of Mouse Induced Pluripotent Stem Cells Without Viral Vectors, 322 SCIENCE 949 (2008); Kazutoshi Takahashi et al., Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors, 131 CELL 861 (2007); James A. Thomson et al., Embryonic Stem Cell Lines Derived from Human Blastocysts, 282 SCIENCE 1145 (1998); Marius Wernig et al., A Drug-Inducible Transgenic System for Direct Reprogramming of Multiple Somatic Cell Types, 26 NATURE BIOTECHNOLOGY 916 (2008).

advances stand in stark contrast to the earlier revelation that reports of highly efficient derivation of several new human ESC lines through research cloning by South Korean researchers were false. Nevertheless, it remains clear that clever and innovative efforts to generate pluripotent stem cells through research cloning as well as through alternative methods continue unabated.

In this Article, I discuss the recent development of “alternative” methods—that is, techniques that do not involve research cloning—to derive pluripotent stem cells, most prominently among them, induced pluripotent stem (iPS) cells. Here, easily obtainable differentiated cells may be genetically manipulated to revert the cell to a stem cell state, from which clinically desirable cell types can be derived. Similarly, a “parthenote” (derived entirely from one parent) that does not have the potential to develop into a person might be a source of cell lines with potential comparable to that of embryonic stem cell lines. Ironically, this is what was proven to be the origin of the so-called “cloned” human embryonic stem (ES) cell lines claimed by South Korean researchers in 2005.

This overview will focus primarily on the scientific developments and challenges of alternative sources of stem cells. In Part I, I will first review basic facts of cell differentiation, reprogramming, and the epigenetic state. In Part II, I will discuss recent work in adult stem cells (ASCs), including ASCs derived from reproductive tissues. Part III will discuss the more ethically complex procedures of extracting embryonic stem cells from “dead” embryos, “living” embryos, and biological artifacts. Part IV investigates the possibility of using existing stem cell lines for further research, but modulating host immune responses and rejection when tissues derived from those lines are introduced into potential patients. Finally, Part V will address the most cutting-edge and scientifically promising alternatives of dedifferentiation and transdifferentiation, both of which involve reprogramming specialized cells.

I. REPROGRAMMING AND THE EPIGENETIC STATE

Before outlining developments in “reprogramming,” it is important to review the basic processes of cell differentiation and reprogramming. Every somatic cell in the body harbors identical genetic information: each cell contains the same DNA, which encodes the same genes. Although each cell contains the same genes, the unique pattern of gene expression specifies each cell’s unique identity, and differential gene expression is responsible for the diverse array of specialized

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4. See, e.g., Takahashi et al., supra note 1.
5. See Woo S. Hwang et al., Patient-Specific Embryonic Stem Cells Derived from Human SCNT Blastocysts, 308 SCIENCE 1777 (2005); Kitai Kim et al., Recombination Signatures Distinguish Embryonic Stem Cells Derived by Parthenogenesis and Somatic Cell Nuclear Transfer, 1 CELL STEM CELL 346 (2007).
cell types that constitute the organism. Gene expression is regulated by chemical modifications to DNA and DNA-associated proteins called histones, which are proteins around which DNA is “wrapped.” Methylation is a chemical modification to specific nucleic acids on DNA that “silences” certain genes by preventing proteins called transcription factors from accessing crucial activating sequences of a gene. Transcription factors enable expression of a particular gene on the relaxed segment of DNA. Acetylation, phosphorylation, methylation, and other chemical modifications of histones play central roles in regulating gene expression. Importantly, these modifications of DNA and histones do not alter the sequence of DNA.

Specific patterns of these DNA and histone modifications define the epigenetic state of the cell, namely the cumulative chemical modifications that determine the unique constellation of gene expression, and consequently, cell type. Embryonic stem cells appear to have a distinctive epigenetic state, especially with regard to patterns of histone methylation. Namely, large stretches of DNA are marked by a type of histone methylation associated with gene repression. Interestingly, within these regions are smaller domains in which genes harbor a type of histone methylation associated with gene expression. Many of the genes that encode developmentally regulated transcription factors display such “bivalent domains” and are expressed at low levels. One theory is that such domains allow silencing of tissue-specific transcription factor expression while simultaneously being “poised for activation” during subsequent differentiation.

Reprogramming refers to the process by which a differentiated cell converts to another type of cell. The mechanisms underlying reprogramming thus involve dramatic changes in the epigenetic state of the cell, enabling a unique pattern of gene expression that defines the reprogrammed cell. Examples of reprogramming include conversion of a differentiated egg cell into all embryonic and extra-embryonic (e.g., placenta) cell types following fertilization by sperm. Other means of reprogramming include induced pluripotency, parthenogenesis, cell fusion, chemical inductions, and the addition of specific subcompartments of one cell to another cell (e.g., the transfer of nuclei by somatic cell nuclear transfer, or the transfer of cytoplasm by ooplasmic transfer). Not surprisingly, these processes alter the epigenetic state of the cell.

8. See id.
II. ADULT STEM CELLS

To date, human adult stem cells (hASCs) are the most thoroughly researched alternative to human embryonic stem cells. Tissues generated from autologous (genetically identical) and allogeneic human embryonic stem cells (hESCs) are obviously not the only sources of stem cell transplants. There has been an explosion of clinical and preclinical studies demonstrating the ability of both hESCs and adult stem cells to repair degenerating and neoplastic tissue, as in multiple sclerosis, spinal cord injury, diabetes, heart disease, and cancer. Tandem scientific developments in the field of hASCs—the derivation of which do not require an embryo source—will likely affect the fate of research cloning.

Tandem scientific developments in the field of hASCs—the derivation of which do not require an embryo source—will likely affect the fate of research cloning. Many opponents of hESC research believe that hASCs demonstrate all the clinically useful properties of the former without the ethically contentious process of stem cell extraction from the embryo. Therefore, many believe that hESC research should be banned or supplanted by hASC studies. Accordingly, hESC opponents have hailed each animal and human adult stem cell study that demonstrates potential therapeutic applications as evidence for the utility of hASCs over hESCs. Such assertions are often made despite clear differences in the differentiation, engraftment, and growth factor requirements among stem cell lines of particular sources. These differences highlighted to date imply medically optimal and appropriate uses for both types of stem cells under various clinical circumstances.

The category of adult stem cells is really an umbrella designation that includes all stem cells isolated from non-embryo or non-fetal sources. Generally hASCs have a more limited differentiation potential than hESCs. For instance, neural stem cells can develop into cell types that comprise the brain and spinal

12. See, e.g., Stefan Janssens et al., Autologous Bone Marrow-Derived Stem-Cell Transfer in Patients with ST-Segment Elevation Myocardial Infarction: Double-Blind, Randomised Controlled Trial, 367 LANCET 113 (2006); Amit N. Patel et al., Surgical Treatment for Congestive Heart Failure with Autologous Adult Stem Cell Transplantation: A Prospective Randomized Study, 130 J. THORACIC & CARDIOVASCULAR SURGERY 1631 (2005); Deepak Srivastava & Kathryn N. Ivey, Potential of Stem-Cell-Based Therapies for Heart Disease, 441 NATURE 1097, 1097-98 (2006).
cord and rarely, if ever, non-neural tissues. Hematopoietic (blood-forming) stem cells differentiate into all blood tissues and cells of the immune system. This limited specialization capacity is termed multipotency. In contrast, hESCs are pluripotent—that is, they have the capacity to differentiate into all somatic tissues. The actual age of the donor does not matter: hASCs isolated from a newborn, a teenager, or a sixty-year-old adult are all considered to be adult stem cells. Each tissue-specific hASC is generally isolated from a specific region of that tissue. For example, neural stem cells from a variety of species can be extracted from a certain region of the adult brain, termed the subventricular zone.

Adult stem cells isolated from patients offer autologous tissues for transplantation; because they are from the patient, no immunosuppressive drugs are required. However, with the exception of autologous and allogeneic hematopoietic stem cells, few other adult stem cells have been characterized well enough to permit their routine transplantation. Human mesenchymal stem cells (hMSCs), a type of non-hematopoietic adult bone marrow stem cell, have been evaluated in clinical trials as support for hematopoietic stem cell transplants for blood cancers as well as bone fractures. Some populations of hMSCs have been shown to engraft allogeneically (when the donor cells are not genetically matched to the recipient); according to evidence obtained from a fetal lamb model, there is little immune rejection. Research groups have reported a wide differentiation spectrum, including clinically relevant cell types such as cardiomyocytes (heart muscle cells) and chondrocytes (cartilage-forming cells). Therefore, hMSCs may offer an alternative to some types of hESC transplantation, especially if generation of autologous tissues from hESCs via research cloning proves too expensive. Moreover, even if cardiomyocytes


17. See, e.g., Sudeepta Aggarwal & Mark F. Pittenger, "Human Mesenchymal Stem Cells Modulate Allogeneic Immune Cell Responses," 105 BLOOD 1815, 1815 (2005) ("[I]n vitro experiments demonstrated that clonal human MSCs are able to differentiate into various lineages including osteoblasts, chondrocytes, and adipocytes. In vitro and in vivo studies have also indicated the capability of MSCs to differentiate into muscle, neural precursors, cardiomyocytes, and possibly other cell types."); Hiroshi Kawada et al., "Nonhematopoietic Mesenchymal Stem Cells Can Be Mobilized and Differentiate into Cardiomyocytes After Myocardial Infarction," 104 BLOOD 3581 (2004); Mark F. Pittinger et al., "Multilineage Potential of Adult Human Mesenchymal Stem Cells," 284 SCIENCE 143 (1999).
derived from allogeneic lines of hESCs can be transplanted, the risks of long-term immunosuppression may favor an hMSC-based approach.

Neural cells derived from fetal and adult neural stem cells or hESCs may also have medical application without immunosuppression because the brain is largely an “immune privileged” site. That is, the brain does not reject transplanted cells, unlike most of the body. This property has enabled cell transplantation in clinical trials for patients with stroke and Parkinson’s disease, some of which have precluded the need for immunosuppressive drug regimens.\(^{18}\) The direct reprogramming of adult stem or differentiated cells to an ES-like state without a totipotent embryo intermediate would be the least ethically contentious alternative to hESCs and a potential source of unlimited, genetically matched cells for therapeutic use. Fortunately, this has now become a reality.

A. Pluripotent Cells Derived from Reproductive Tissues

A recent report described the generation of pluripotent, ES-like cells from the neonatal mouse testis.\(^ {19}\) As noted above, the ASC designation refers to stem cells present any time after birth. In this case, neonatal mouse testes were cultured in ESC-promoting cell culture conditions, and both \textit{in vitro} and \textit{in vivo} assays demonstrated that the resulting stem cells could contribute to all somatic tissues and were therefore pluripotent. It is not surprising that these germ cells are pluripotent, as certain germ cell tumors can contain tissues from all germ layers (such as neurons, teeth, and hair!). While the derivation of pluripotent cells from germ cells of wild-type older mice was not successful, germ cells from transgenic mice lacking a certain cell cycle gene could generate pluripotent ES-like cells. The authors suggest that modification of culture conditions or \textit{in vitro} genetic manipulation of cells from the mature adult may make this process more efficient.

If it is possible to generate similar pluripotent cells from human reproductive tissues, such cells may be an ethically acceptable alternative to research cloning because no embryo is created. For instance, a child may undergo a testicular biopsy, from which germ cell-derived ES-like cell lines could be generated. Should the child need the specific tissues for future therapy, appropriate cells may be differentiated from the pluripotent line and grafted into the patient. Such a strategy is not without caveats. For example, the biopsy of testicular or other reproductive tissues carries certain risks, and it is unknown at this time how much tissue would be required to generate a pluripotent cell line. Also, even if

\(^{18}\) Curt R. Freed et al., \textit{Transplantation of Embryonic Dopamine Neurons for Severe Parkinson’s Disease}, 344 \textit{NEW ENG. J. MED.} 710 (2001); D. Kondziolka et al., \textit{Transplantation of Cultured Human Neuronal Cells for Patients with Stroke}, 55 \textit{NEUROLOGY} 565 (2000).

\(^{19}\) Mito Kanatsu-Shinohara et al., \textit{Generation of Pluripotent Stem Cells from Neonatal Mouse Testis}, 119 \textit{CELL} 1001 (2004).
such tissues were grafted autologously, some degree of immune incompatibility due to the unique methylation patterns of tissues from germ cell-derived pluripotent cells will remain. However, autologous transplantation assays in animals could quickly provide some answers.

III. EXTRACTION OF EMBRYONIC STEM CELLS FROM "DEAD" AND "LIVING" EMBRYOS AND FROM BIOLOGICAL ARTIFACTS

Given that objections to hESC research often turn on the death of the embryo, researchers have sought alternative sources of hESCs that are more ethically acceptable, such as "organismically dead" embryos, "living" embryos destined for implantation, and biological artifacts. However, these sources do not always avoid the ethical arguments about the destruction of embryos.

A. "Dead" Embryos

Tissue donation from human cadavers remains an important clinical strategy for patients with some types of severe organ dysfunction. Provided proper consent, the use of organs from those declared dead is considered ethically acceptable. The utility of such a donation relies on the fact that while a person can be declared dead, certain organs and tissues may still be functioning at a level sufficient for successful transplantation into a patient. Similarly, some consider that an embryo can be "organismically dead," but can still contain functioning, individual cells. One definition for an organismic death of an embryo is cessation of "continued and integrated cellular division, growth, and differentiation." When this happens, as is the case for many embryos derived via in vitro fertilization (IVF), the embryo would not develop any further in vitro and would not be viable following uterine transfer. Most IVF embryos are cultured to the 2-10 cell stage (2-3 days old) or up to the blastocyst stage (5-6 days old), and then transferred into the uterus. At the 2-8 cell stage, each component cell, called a blastomere, is totipotent. However, by 5-6 days following blastocyst formation, the inner cell mass—composed of the cells that are usually extracted to derive hESC lines—has formed and no individual cell is capable of full embryonic development. In other words, there are no longer any totipotent cells present.

20. This theory is primarily attributed to Donald Landry and Howard Zucker. See Donald W. Landry & Howard A. Zucker, Embryonic Death and the Creation of Human Embryonic Stem Cells, 114 J. CLINICAL INVESTIGATION 1184, 1185 (2004).

21. See G. Cauffman et al., Markers that Define Stemness in ESC are Unable To Identify the Totipotent Cells in Human Preimplantation Embryos, 24 HUMAN REPROD. 63, 64 (2009) ("The initial loss of totipotency occurs during preimplantation development and becomes apparent for the first time when two distinct cell lineages in the blastocyst segregate forming the inner cell mass . . . .").
In the case of organismic death of the early embryo (2-8 cell stage), for example, one may consider a case in which six of eight cells appear to have irreversibly ceased division. As highlighted by some members of the President’s Council on Bioethics, one could potentially remove the remaining one or two functioning blastomeres from this “dead” embryo, and further culture them in vitro to a stage from which hESCs can be isolated. It is important to note that a dividing, totipotent blastomere is potentially equivalent to a living embryo. Therefore, this strategy may be no more ethically acceptable than the current method of deriving hESCs from a living embryo. However, if an IVF embryo is cultured further to the blastocyst stage and subsequently ceases coordinated division, it is possible that some of the inner cells—which may still be dividing but are not totipotent—may be isolated to derive hESC lines.

Embryos that have ceased coordinated division are organismically dead and are frequently genetically abnormal: many contain abnormal numbers or sets of chromosomes (DNA-protein structures). From a therapeutic point of view, hESC lines derived from genetically deranged embryos may not be pluripotent. Alternatively, they may be otherwise undesirable for clinical use because of a propensity to form tumors or an inability to differentiate properly or survive. However, abnormalities that cause specific diseases, such as certain cancers and Down syndrome, may be useful to researchers who study the genetic pathways underlying such disorders.

B. “Living” Embryos Intended for Implantation

Extraction of blastomeres from a “living” embryo is already performed through a clinical procedure known as preimplantation genetic diagnosis (PGD). PGD is generally undertaken in conjunction with IVF techniques in order to test for specific genetic abnormalities of the embryo prior to uterine transfer. PGD is conducted during the 2-8 cell stage when the embryo is made up of equivalent, totipotent blastomeres. Interestingly, at this stage, the embryo can compensate for the loss of a blastomere and remain viable for full-term development. One or two blastomeres from several IVF-generated early embryos are removed and tested for a genetic disease. At this early stage, extraction of a single blastomere is equivalent to “twinning” the embryo, because the cell to be biopsied, now separate from the original embryo, may have the potential to develop full-term if implanted into the uterus.

It is conceivable that an additional blastomere may be removed during PGD to derive ESC lines. In 2006, two studies by Lanza and colleagues reported the

23. See id. at 24-25.
generation of ESC lines from murine and human single cell blastomeres. In the murine study, single blastomeres were extracted from developing embryos to derive ESC lines; some of the remaining biopsied embryos, if implanted, could develop into viable mice. In the human study, some ninety-one single cells were extracted from sixteen spare IVF embryos, and two hESC lines were derived.

There are several caveats to the human study. First, cell extraction may delay implantation of the embryo, which can endanger its development. Lanza and colleagues note that in PGD, a single blastomere is taken from the embryo. In their study, multiple blastomeres were taken from single embryos and blastomeres from the same embryos were cultured together; the biopsied human embryos were destroyed without implantation. If this procedure were adapted for clinical use, the authors envision that a single blastomere would be removed and allowed to divide in culture, so that separate cells derived from a single blastomere could be used for PGD and for the generation of hESCs. (The alternative, extracting more than one blastomere from the human embryo, likely presents unacceptable risks for the viability of the biopsied embryo.) If only one blastomere were removed, it would be necessary to delay the transfer of the embryo into the uterus until the extracted blastomere divided sufficiently to permit cell extraction for both PGD and hESC derivation. This delay may compromise the success of subsequent embryo transfer and embryonic development due to perturbation of parental imprinting (an epigenetic state dependent on proper expression of maternal and paternal genes). Second, instead of culturing multiple blastomeres from the same embryo (which fosters cell-to-cell contact and may improve *in vitro* proliferation, survival and development), Lanza et al. propose that the extracted blastomere and biopsied embryo should be cultured together *in vitro*. This practice would again prolong the time the embryo would be in culture before uterine transfer. Despite these limitations, these studies offer an interesting alternative to research cloning by allowing the generation of genetically matched hESC lines for some children.

While these alternatives to research cloning may be potentially helpful for future cell therapy of children conceived via IVF and PGD, the long term risks of this method of blastomere extraction are currently unknown. Removal of an additional blastomere for derivation of a hESC line may present further risks. Finally, if a hESC line is generated from a "normal" blastomere, the derivation of hESC lines would necessarily involve the destruction of a "twinned" embryo equivalent: the totipotent blastomere, which would not develop into a child. This approach would not avoid the ethical concerns discussed above. A blastomere with severe genetic aberrations such that the resulting embryo from which the blastomere was extracted could not develop to full term, could be a valuable

source of hESCs used for studying genetic diseases. However, it is not obvious that such genetic abnormalities can be identified during PGD.

In short, extraction of a dividing, totipotent blastomere from an organismically dead embryo or one from a living embryo intended for uterine transfer does not avoid the destruction of an embryo equivalent.

C. Altered Nuclear Transfer Resulting in Biological Artifacts

Another approach, called altered nuclear transfer (ANT), advanced by William Hulburt, a member of the President’s Council on Bioethics, posits that inactivation of specific genes required for full viability but not for generation of ESCs would result in a “biological artifact” from which pluripotent cells could be derived. Since, like a parthenote, the “biological artifact” cannot develop into an embryo, some may view this as a more ethically acceptable source of hESCs than derivation of cells from a potentially viable fertilized or cloned embryo. In both ANT and the derivation of cells from a potentially viable embryo, the somatic nuclei may further be altered, such as to represent a specific genetic disorder, and the hESC lines derived from them could then be used to study pathogenesis of a genetic disease and evaluate new drugs to treat such a disorder.

Indeed, an elegant study has validated the feasibility of this approach through conditional inactivation of the gene Cdx2 in mouse embryonic stem cells. Cdx2 is essential for generation of extra-embryonic tissue called trophoectoderm, which is the outer cell layer of the embryo that implants into the placental wall. Conditional repression of Cdx2 rendered mouse embryos unable to implant, but the embryos still generate ESCs. The gene can then be subsequently reactivated, allowing differentiation into intestinal cells, whose specification also requires Cdx2 expression.

As the Cdx2 study demonstrates, there remain several concerns in translating ANT processes to humans. Cdx2 may be expressed differently in humans than in mice, and so it remains a possibility that Cdx2-inactivated human embryos may still have the ability to implant. Moreover, conditional inactivation was achieved by transfer of a retroviral vector which, if integrated in the vicinity of a cancer-growth gene (oncogene), may initiate tumorigenesis. While this work represents an important proof-of-principle demonstration of ANT, conditional activation of a gene required for placental implantation would render an otherwise healthy embryo into an abnormal one; in essence, it would create a genetically hobbled embryo. Therefore, ANT remains ethically problematic.

25. See President’s Council on Bioethics, supra note 22, at 36-37.
In order to use ANT and other "reprogramming" techniques to derive hESC or human iPS lines without creating or destroying the embryo, a deeper understanding of mechanisms of reprogramming and regulation of the epigenetic state will be essential. Additional research will be necessary to use these cells in human therapy. Ideally, cells would be stably reprogrammed to the desired fate by expression of particular genes, \textit{in vitro}, and then be used for clinical therapy. However and it is unclear whether human parthenotes would be considered an ethnically acceptable source of hESC lines. Alternatives to ANT, such as cell fusion-dependent processes of dedifferentiation and transdifferentiation, may not be desirable because fusion is a rare event; even if fusion is successful, the resulting cells contain two nuclei, although the expulsion of the extra nucleus may be a temporary technical obstacle. Another alternative, fusion-\textit{independent} transdifferentiation of cells following transplantation is still largely unexplored. Molecules such as 5-azacytidine and other DNA methylation- and histone acetylation-modifying molecules have proven useful in combating leukemia by "reprogramming" cancer cells to express cellular death genes;\textsuperscript{28} however, these molecules are also toxic. A promising study, discussed below, by Yamanaka and colleagues demonstrates that reprogramming may be possible via expression of relatively few specific genes; however, more research is needed.\textsuperscript{29}

\textbf{IV. IMMUNOLOGIC ACCEPTANCE AND REJECTION}

As an alternative to generating new hESC lines, recent research suggests that it may be possible to manipulate the genes that mediate the immune response in order to generate immune-compatible tissue derived from existing approved stem cell lines. The generation of immunologically compatible, autologous tissues from hESC lines remains the primary advantage of research cloning. The generation of hESCs through a non-cloning procedure, such as using embryos created through \textit{in vitro} fertilization, does not produce genetically-matched cells. As a result, transplantation would require the patient to endure a regimen of immunosuppressive drugs. Besides the known danger of tumor formation, recent evidence has suggested that transplantation of allogeneic (non-immune-compatible) undifferentiated hESCs may also result in rejection because undifferentiated hESCs express low levels of immunogenic molecules.\textsuperscript{30} Even if appropriately differentiated allogeneic hESCs are transplanted, the required immunosuppressive drugs may give rise to many adverse effects, including

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\footnotetext[29]{Takahashi & Yamanaka, \textit{supra} note 1.}
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increased risks for cancer and infection. Autologous tissues derived from hESCs produced through research cloning, which elicit little or no immune reaction, offer a superior avenue for therapy, although the potential for tumor formation from undifferentiated cells remains.

Although research cloning remains contentious, scientists may already be able to exploit the therapeutic advantages of well-studied, non-autologous hESC lines (such as those derived from supernumerary embryos from IVF clinics). A study at Stanford University in 2002 demonstrated that small numbers of kidney transplant recipients who are irradiated and then given bone marrow stem cells isolated from the original kidney donor can be successfully weaned from immunosuppressive drugs.\(^3\) This is because the donor bone marrow stem cells reconstitute the irradiated recipient’s blood and immune system, resulting in the development of donor-derived immune cells that do not reject the donor kidney.

An added advantage of this method is that the donor need not be immunologically compatible—the recipient would not necessarily have to rely on donors who have a similar immune type (such as siblings). Using this method, future tissues developed from well-studied but immunologically-mismatched hESC lines—such as the ones currently available—can be transplanted with decreased risk of immune rejection and ultimately be weaned from harsh immunosuppressive regimens. For example, following a severe heart attack, injection of cardiomyocytes derived from allogeneic hESCs into the area of infarction may offer future therapy to improve function and overall survival. In this case, following grafting of the cardiomyocytes, the patient would undergo immunosuppressive therapy to reduce the risk of immediate rejection. Following stabilization, the patient may be irradiated and then given hematopoietic stem cells derived from the same line of hESCs that generated the grafted cardiomyocytes. Transplanted hematopoietic stem cells would engraft and generate immune cells that tolerate the graft because the cells would be isogenic (of the same genotype) to the cardiomyocyte graft. Gradually, the patient would be weaned off the immunosuppressive drugs and would avoid long-term adverse effects.

However, irradiation and bone marrow stem cell transplantation carry very serious risks, including a high proportion of life-threatening infections and rejection of the hematopoietic stem cell transplant. Whether induction of immune tolerance to the graft through this method or generation of autologous tissues through research cloning on a per-patient-basis will provide the preferred method will depend on the life-threatening risk of the former versus the expense of the latter. However, the recent, comparably facile, derivation of several iPS cell lines offers an alternative method by which immune-matched cell grafts can more

safely and economically be used for therapy. This method would offer substantially lower life-threatening risks than combined irradiation and bone marrow transplantation to induce immune tolerance of grafted tissues from allogeneic hESC lines.

A. Overcoming Immune Rejection

Genetic modification of determinant genes that mediate immune response, parthenogenesis, and the transfer or addition of other cell-free extracts that contain the molecular factors capable of reprogramming the differentiated cell may offer alternative means to generate stem cell-derived, immune-compatible tissue. The major histocompatibility complex (MHC) refers to the cell surface proteins that determine whether the cell will be accepted or rejected by the immune system. Through a genetic manipulation called homologous recombination, MHC proteins may be modified or deleted such that the altered cells do not provoke an immune response in the recipient following transplantation. It would seem that this is not without new problems.

Female patients who donate the eggs from which parthenogenetic hESCs are generated may be able to receive tissue transplants derived from these cells without immune rejection. This is because parthenogenetic cells are largely endowed with the same genetic information as the female host. Still, prior recombination of MHC genes in meiosis (specialized division of the egg) may produce parthenogenetic tissue derivatives not immunologically matched to the host, resulting in a mild degree of immune rejection.

The addition of foreign mitochondrial DNA present in the transplanted donor ooplasm via ooplasmic transfer may also trigger some degree of immune rejection. Transplantation of cell extracts (rather than whole ooplasm) that reprogram the host cell into therapeutic cell types may circumvent this risk.

32. See infra Section V.B.

33. See, e.g., Vanessa J. Hall, Petra Stojkovic & Miodrag Stojkovic, Using Therapeutic Cloning to Fight Human Disease: A Conundrum or Reality?, 24 STEM CELLS 1628, 1633 (2006) (“It should be considered that allogeneic mitochondria present in NT-ESC or NT-ESC derived cells could be recognized by the host immune system, leading to disrupted mitochondrial membrane potential that induces the apoptotic cell signaling pathway, thus leading to cell death.”); Charlotte Kfoury, Therapeutic Cloning: Promises and Issues, 10 MCGILL J. MED. 112, 116-117 (2007) (“Immune rejection of the ntESC in cell replacement therapy is due to mitochondrial heteroplasmy as a consequence of SCNT since the nuclear donor and ooplasmic host cells are not autologous in most cases . . . Also, antigens such as Mta are encoded by the mitochondrial genome and trigger an auto-immune response targeting the hybrid (36) after transplantation.”); Robert P. Lanza, Jose B. Cibelli & Michael D. West, Prospects for the Use of Nuclear Transfer in Human Transplantation, 17 NATURE BIOTECHNOLOGY 1171, 1173 (1999) (“The mitochondrial genome of vertebrates is extremely specialized, and incompatibilities are likely between distantly related species.”).
Nonetheless, without elucidation of the molecular factors capable of reprogramming and the mechanism by which it takes place, it is unknown whether tissues derived through this procedure would elicit immune rejection.

V. REPROGRAMMING: DEDIFFERENTIATION AND TRANSDIFFERENTIATION

Finally, the most scientifically promising techniques for pursuing hESC alternatives are dedifferentiation and transdifferentiation, both of which involve reprogramming specialized cells.

A. Dedifferentiation: Fertilization and Parthenogenesis

Dedifferentiation is a specific type of reprogramming in which a specialized cell reverts to a more primitive state, such as a progenitor or stem cell. Both fertilization and parthenogenesis result in the reprogramming and dedifferentiation of a differentiated egg cell into primitive and other differentiated cells. Fertilization is the predominant means of reprogramming—and reproduction—among mammalian species. The generation of hESC lines from embryos generated via fertilization of donated human eggs and sperm requires the creation and destruction of an embryo. It is also less therapeutically attractive because such lines would not be genetically matched to any patient. Germ cells (eggs and sperm) have been generated from mouse ESCs. In vitro generation of germ cells, especially eggs, from existing hESC lines would obviate the need for donation of germ cells from human volunteers for generation of new hESC lines. However, induced pluripotent stem cell reprogramming (see below) or research cloning would still be necessary to produce genetically compatible tissue.

Parthenogenesis is the process of development of an unfertilized egg into viable offspring. In general, this process does not occur in mammalian species, but it occurs in other types of animals such as reptiles and insects. Through chemical manipulation, mouse and monkey parthenogenetic blastocysts can develop in vitro, from which pluripotent ESCs can be harvested. These ESCs have a full complement of DNA, can be extensively propagated, can differentiate into most if not all cell types, and can engraft following transplantation. Recently, unfertilized oocytes coaxed to the blastocyst stage have been used to

36. Rosario Sanchez-Pernaute et al., Long-Term Survival of Dopamine Neurons Derived from Parthenogenetic Primate Embryonic Stem Cells (Cyno-1) After Transplantation, 23 Stem Cells 914 (2005).
generate parthenogenetic hESC lines, just as parthenogenetic monkey ESCs were derived in 2002.\textsuperscript{37} Parthenogenetic human embryos are unlikely to be viable; parthenogenetic rodent embryos are not viable unless the methylation of certain genes is modified in the laboratory.\textsuperscript{38} For this reason, some may consider these as more ethically acceptable sources for generating hESC lines than potentially viable fertilized eggs and cloned embryos. An interesting consequence of this method is that parthenogenetic ESCs would, in theory, be immunologically matched only to those females who donate the eggs from which the cells were derived. Recently, parthenogenetic hESC lines were generated from oocytes of women representing different immunologic groups, which might be a step toward generating immunologically compatible parthenogenetic tissues.\textsuperscript{39}

B. Dedifferentiation with Cell-Free Extracts: Nuclear and Cytoplasmic Transfer

In somatic cell nuclear transfer (SCNT), an adult nucleus from a differentiated cell is reprogrammed to a primitive state, recapitulating embryonic development, from which pluripotent ESCs or even viable, entire organisms can be derived. In one experiment, a nucleus from a differentiated human immune cell was transferred into a frog egg. Nuclear and cytoplasmic factors from the frog egg reprogrammed the adult human nucleus to express a primitive hESC protein while extinguishing the expression of differentiated genes, suggesting that it may be possible to dedifferentiate the differentiated human nucleus into a pluripotent-like state.\textsuperscript{40} While it has been considered that the nucleus of an immature, undifferentiated cell (e.g., an ESC) is more efficient than that of a mature cell that has ceased dividing, recent evidence has suggested the opposite.\textsuperscript{41} Nuclei from a type of differentiated immune cell, a postmitotic granulocyte, have proven to be much more efficient donors for SCNT than nuclei from hematopoietic stem cells (from which granulocytes are derived). This is a positive development for research cloning; differentiated cells from the blood and immune system, skin, and other organs are, in general much more accessible (and more common) than immature cells such as stem cells, which are often rare or inaccessible for isolation from adult tissues.

\textsuperscript{37} Elena S. Revazova et al., Patient-Specific Stem Cell Lines Derived from Human Parthenogenetic Blastocysts, 9 CLONING STEM CELLS 432 (2007).
\textsuperscript{38} David A. Loebel & P.P. Tam, Genomic Imprinting: Mice Without a Father, 428 NATURE 809 (2004).
\textsuperscript{39} Elena S. Revazova et al., HLA Homozygous Stem Cell Lines Derived from Human Parthenogenetic Blastocysts, 10 CLONING STEM CELLS 11 (2008).
\textsuperscript{40} James A. Byrne et al., Nuclei of Adult Mammalian Somatic Cells Are Directly Reprogrammed to Oct-4 Stem Cell Gene Expression by Amphibian Oocytes, 13 CURRENT BIOLOGY 1206 (2003).
\textsuperscript{41} Li-Ying Sung et al., Differentiated Cells Are More Efficient than Adult Stem Cells for Cloning by Somatic Cell Nuclear Transfer, 38 NATURE GENETICS 1323 (2006).
Hitherto used exclusively as an experimental assisted reproductive technology, ooplasmic transfer involves the transfer of oocyte cytoplasm into another cell such as a damaged egg in order to repair defects in the recipient cell. The technique has resulted in the birth of over thirty children to mothers previously unable to conceive. Other "zona-free" cloning techniques effectively fuse ooplasm with a differentiated cell, which can also reprogram differentiated cells. Central to reprogramming methods, including SCNT, is the presence of reprogramming factors in the egg and ESC cytoplasm. These factors contain particular proteins that can alter the epigenetic state and patterns of gene expression in a cell, thereby reprogramming differentiated cells into a primitive state.

C. Dedifferentiation by Cell Fusion

In 2002, British researchers recognized that co-culture of fetal and adult central nervous system cells with ESCs resulted in fused cells that had properties of ESCs, including ESC-specific marker expression and multilineage differentiation following transplantation. The resulting fused cell contains two nuclei and cytoplasmic components from both cells. Apparently, the actual state of the fused cell resembles the more primitive ESC, rather than the differentiated cells, suggesting that the factors in the ESC nucleus and cytoplasm are dominant to those of the differentiated cells.42

In 2005, U.S. researchers described the derivation of ES-like cells from the fusion of human ESCs with human somatic fibroblasts.43 These hybrid cells display properties of ESCs including extensive self-renewal, reactivation of the pluripotent-specific gene Oct4 (by demethylation of the promoter in the fibroblast genome), and differentiation into a variety of cell types. The fusion event takes place, however, at an extremely low frequency. Moreover, the resulting fused cell contains two sets of DNA, and the ESC nucleus must be expelled. Should removal of the hESC nucleus from the fused cell be possible, the generation of patient-matched ES-like cells through a cell fusion process that does not involve the creation of an embryo may be feasible.

D. "Transdifferentiation": Cell Fusion, Cell-Free Extracts, and Epigenetic Modifiers

Transdifferentiation, another specialized form of reprogramming, refers to the process by which a cell derived from one germ layer converts to a cell from another germ layer. Early in embryonic development, cell types are segregated into three major germ layers: the ectoderm (which generates the skin and CNS),

42. Qi-Long Ying et al., Changing Potency by Spontaneous Fusion, 416 Nature 545 (2002).
43. Chad Cowan et al., Nuclear Reprogramming of Somatic Cells After Fusion with Human Embryonic Stem Cells, 309 Science 1369 (2005).
the mesoderm (which generates blood, muscle and bone), and the endoderm (which generates the respiratory and gut lining, liver and other structures). Once the germ layers are established, it is thought that a cell derived from one germ layer cannot readily convert into a cell from another germ layer.

Some early reports described the conversion of “blood into brain,” that is, the conversion of bone marrow cells (mesoderm) into neural cells (ectoderm) following transplantation of bone marrow.\(^4\) Closer inspection of such cells revealed that they harbored two nuclei and were a result of cell fusion.\(^4\) However, other experiments have since been conducted, revealing for example, that transplanted bone marrow cells could indeed “transdifferentiate” into the cells that comprise the lining of the lung (endoderm) through a fusion-independent mechanism.\(^4\) However, these events occurred at too low a frequency to be considered therapeutically beneficial. Despite a report demonstrating extensive bone marrow cell contribution to stomach tumors (an endodermal cancer),\(^4\) it is unclear whether such transdifferentiation can robustly generate dividing, clinically useful cells for therapy of disease in which specific cell types are lost.

Additional experiments have shown that other cell-free extracts, such as cytoplasm of immune system cells, contain reprogramming factors and are sufficient to convert a differentiated cell type into another differentiated-like cell.\(^4\) Specific molecules that alter DNA methylation and histone acetylation can activate specific cell-type genes to reprogram cells. One agent, 5-azacytidine, demethylates specific portions of DNA, allowing reexpression of specific genes, including those crucial to cell type identity. In one study, following 5-azacytidine treatment, neural stem cells that normally give rise to only neural lineages generated contractile cardiomyocytes.\(^4\)

**E. The “Grail”: The Derivation of iPS cells and Direct Transdifferentiation**

Since a unique pattern of gene expression defines cell identity, manipulation of specific genes and epigenetic factors may enable dedifferentiation,
transdifferentiation, and other types of reprogramming. Insights from animals engineered to lack or overexpress certain genes have offered clues as to which genes are crucial to cell type specification. There are several methods to modify gene expression; while the technical details are beyond the scope of this discussion, the approach is to either decrease or increase expression of specific genes, such as the transcription factors discussed above, in order to reprogram the cell to a desired state.

Researchers have envisaged genetic strategies to dedifferentiate somatic cells to pluripotent cells, without the generation of a totipotent embryo as a necessary intermediate—the so-called “grail” of therapeutic stem cell biology. In a landmark study published in 2006, Japanese researchers devised a method to enable this type of reprogramming, which they called “induced pluripotent stem cell” (iPS) reprogramming.\(^\text{50}\) By introducing genes known to be expressed in stem, progenitor, and dividing cells, Yamanaka and colleagues, in a combinatorial fashion, deduced which genes could reprogram differentiated murine fibroblasts into ES-like cells. Forced expression of as few as four genes, Oct4, Sox2, c-Myc, and Klf4, reprogrammed adult cells into ES-like cells. Such cells which could be propagated extensively, expressed several ESC marker genes, differentiated into a variety of tissues, and contributed to mouse embryonic development. Interestingly, while the reprogrammed ES-like cells more resembled bona fide ESCs than the parental fibroblasts, they displayed a gene expression pattern distinct from either. Indeed, the DNA methylation state of pluripotent-specific gene Oct4 in the reprogrammed ES-like cells resembled an intermediate between ESCs and fibroblasts. By identifying the genes capable of reprogramming a differentiated somatic cell, Yamanaka and colleagues offer some of the first evidence that pluripotent cells can indeed be derived from differentiated cells without the creation of an embryo.

Since the publication of this seminal paper, a flurry of reports have confirmed and extended these observations. iPS cells have since been derived from human somatic cells by Yamanaka’s team using the same factors as with mice, while another American team has used a slightly different combination of transcription factors to achieve a similar result.\(^\text{51}\) Adult neural, stomach, and liver cells have been reprogrammed to iPS cells.\(^\text{52}\) By treating mouse and human fibroblast cells with epigenetic modifiers (agents that modulate DNA and histones by promoting or inhibiting methylation and acetylation), successful conversion to iPS cells can be achieved without ectopic expression of oncogenes.

\(^{50}\) Takahashi & Yamanaka, supra note 1.

\(^{51}\) Takahashi et al., supra note 1; Jungying Yu et al., *Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells*, 318 SCIENCE 1917 (2007).

\(^{52}\) Aoi et al., supra note 1; Jeong Beom Kim et al., *Pluripotent Stem Cells Induced from Adult Neural Stem Cells by Reprogramming with Two Factors*, 454 NATURE 646 (2008).
such as c-Myc or Klf4.\textsuperscript{53} Recently, Yamanaka and colleagues have demonstrated that transfection (a technique whereby a virus encoding an oncogene like c-Myc does not permanently integrate into the cell genome) of fibroblasts can yield iPS cells.\textsuperscript{54} The stage is set whereby transient application of reprogramming factors, such as cytokines that induce expression of specific transcription factors or chemical epigenetic modifying agents, can be used to convert easily isolable adult cells to iPS cells without prolonged expression of tumorigenic genes.

The use of murine and human iPS derivatives for therapy in animal models, along with our increased understanding of the susceptibility of certain cell types to environmental insults, hints at future applications of iPS technology. A recent study reprogrammed skin from mice with the sickle cell anemia mutation. The skin from these mice was reprogrammed into iPS cells using Oct4, Sox2, Klf4 and c-Myc, as above. The resulting iPS cells were then electroporated with the normal hemoglobin gene, pushed toward the hematopoietic lineage, and autologously transplanted into the sickle cell mice. The transplanted cells engrafted and reconstituted the blood system and improved red cell morphology, mass, and urine concentration defects (also seen in human sickle cell patients).\textsuperscript{55}

Another report has derived patient-specific iPS cell lines from the skin of patients living with amyotrophic lateral sclerosis (ALS). Large numbers of motor neurons could be generated from these iPS cells, allowing the production of immune-matched cells for autologous transplantation as well as the study of pathophysiologic processes from crucial cells lost in specific diseases.\textsuperscript{56}

In order to generate clinically useful cell types, is a “dedifferentiation” step actually required? That is, must one reprogram a differentiated cell to a “dedifferentiated” embryonic/iPS intermediate state before producing another differentiated cell type? As discussed in the “transdifferentiation” section, cell-free extracts and chemical inhibitors of DNA methylation have converted one differentiated cell type into another. However, it is difficult to know if a more primitive intermediate cell was generated.

A recent study has demonstrated direct reprogramming from one differentiated cell type to another \textit{in vivo}.\textsuperscript{57} Viruses encoding cell-type specific

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\textsuperscript{53} Danwei Huangfu et al., \textit{Induction of Pluripotent Stem Cells from Primary Human Fibroblasts with Only Oct4 and Sox2}, 26 Nature Biotechnology 1269 (2008); Yan Shi et al., \textit{Induction of Pluripotent Stem Cells from Mouse Embryonic Fibroblasts by Oct4 and Klf4 with Small-Molecule Compounds}, 3 Cell Stem Cell 568 (2008).

\textsuperscript{54} Okita et al., \textit{supra} note 1.

\textsuperscript{55} Jacob Hanna et al., \textit{Treatment of Sickle Cell Anemia Mouse Model with iPS Cells Generated from Autologous Skin}, 318 Science 1920 (2007).

\textsuperscript{56} John T. Dimos et al., \textit{Induced Pluripotent Stem Cells Generated from Patients with ALS Can Be Differentiated into Motor Neurons}, 321 Science 1218 (2008).

\textsuperscript{57} Qiao Zhou et al., \textit{In Vivo Reprogramming of Adult Pancreatic Exocrine Cells to Beta-Cells}, 455 Nature 627 (2008).}
transcription factors were injected into the adult mouse pancreas, and have been shown to convert pancreatic exocrine cells into pancreatic endocrine cells in the adult pancreas. While these two populations of cells are from the same germ layer (endoderm), they have separate metabolic functions and produce separate products. Amylase-producing exocrine cells, when forced to express Ngn3, Pdx1, and Mafa—three transcription factors important for development of endocrine pancreas—were induced into insulin-producing, endocrine cells in vivo. After injection of the virus, there was no increase in pancreatic cell division, suggesting that a dividing, stem-like intermediate cell is not required to achieve this conversion. Moreover, injection of the virus encoding the three transcription factors into diabetic mice demonstrated reduction in blood sugar levels.  

**CONCLUSION**

Research cloning and the cutting-edge “alternative” technologies discussed above exemplify the creativity of the researchers who push the boundaries of science and medicine in order to better understand the biological world and seek powerful new treatments for intractable diseases. Currently, most researchers still hope for expanded public funding of research using additional, newer embryonic stem cell lines, from both iPS cells and traditional sources. Despite formidable challenges, exciting progress has been made, most notably the derivation of iPS cells from easily isolable tissues such as adult fibroblasts and skin. The rapid pace of science and medicine suggests there may well be a day, sooner than the very distant future, when a simple skin biopsy will provide an unlimited number of immune-matched cells for any patient. Together, these studies have heralded the new era of stem cell biology. As a prominent stem cell biologist has concluded, “the controversial issues (ethical and technical) specific to human therapeutic [research] cloning may well be left behind along with the procedure itself, a refreshing change for the field, indeed.”

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58. *Id.*