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Vritti R. Goel
Scripps College

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A proposal to test the effects of factor ECAT1 on pluripotency, from reprogramming to differentiation of human somatic cells

A Thesis Presented

by

Vritti Goel

To:

To the Keck Science Department of

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Abstract

The field of stem cell research has been growing more because of the interest in using stem cells to cure diseases and heal injuries. Human embryonic stem cells, because of the controversy surrounding them—and subsequently the difficulties in acquiring samples of the existing aging cell lines—can only be used in limited capacities. While the development of induced pluripotent stem cells in the last decade has allowed the field to progress closer to medical treatments, the low efficiency of reprogramming a somatic cell to a pluripotent state, and the vast molecular and genomic differences between human embryonic stem cells and human induced pluripotent stem cells is still an issue. Therefore, the goal is to discover methods, chemicals, and factors that can reduce these differences and increase the efficiency of inducing pluripotency.

This proposal aims to look at the effects of the protein ECAT1 in inducing pluripotency in human somatic cells. Little is known about ECAT1, otherwise known as Embryonic Stem Cell-Associated Transcript 1, beyond its presence in human embryonic stem cells and oocytes and its absence in differentiated cells. While originally considered by scientists during the development of the reprogramming technique, ECAT1's effects have not been tested in humans. Therefore, a series of experiments will be performed in which ECAT1 will be used in conjunction with OSKM to induce pluripotency in adult human dermal fibroblasts, which will then be differentiated into spinal motor neurons. The three stages of this proposal--inducing pluripotency, comparing pluripotencies in the reprogrammed cells and embryonic stem cells, and differentiating the stem cells--should answer questions about ECAT1 and the reprogramming process. It is predicted that ECAT1 should reduce the genomic and molecular differences between embryonic stem cells and induced pluripotent stem cells. ECAT1's presence should also increase the efficiency of reprogramming as well as successful differentiation to other cell types.

Introduction

The search for cures to diseases such as cancer, Parkinson's, sickle cell anemia, and diabetes has been long and difficult. Similarly, a search for solutions to spinal cord injury and other debilitating injuries has proven slow and relied on mechanical technology. The use of stem cells as a solution has been gaining popularity since their discovery.

The Discovery of the Stem Cell

The discovery of the stem cell arose through observations of anomalous teratocarcinomas. Scientists discovered that tumor cells have origins in embryonic cells, and that cells taken from a tumor, when injected elsewhere, could produce a variety of different cell-types.[1-3] These cells were termed to be stem cells, and research progressed to using cell feeder layers to establish what are now known as embryonic carcinoma cells. Discoveries in mice preceded discoveries of human embryonic stem cells, beginning with the search for a cure for leukemia—the first experiment using stem cells was of bone marrow transplantation in irradiated mice, which led to the formation of multilineage colonies and to the idea that stem cells could be used as a medicinal technique.[1,2]

Scientists began work using hematopoietic stem cells—stem cells that give rise to all cells of the blood system[2]—and continued to develop the extremely difficult bone marrow transplant procedure using these cells. At the same time, research dealt with the properties of non-hematopoietic stem cells, specifically focusing on whether carcinomas were the only source.

To aid in this endeavor, the effort focused on enzymes and factors that characterized stem cells. In the 1970's, one such enzyme, alkaline phosphatase, was shown

to play a major role in maintaining the main characteristic of pluripotency—the ability of a cell to differentiate into most cell types—in a stem cell. In particular, alkaline phosphatase was instrumental in uncovering the identities of other pluripotency-related enzymes such as SSEA-3, SSEA-4, and NANOG.[4] The enzymes and markers were all responsible for the ability of embryonic stem cells to be grown indefinitely in the pluripotent state under optimal conditions, without differentiating.[3]

Then, in the 1980's, the first noncarcinomal embryonic stem cells were established from mice. This eventually led to the generation of ES cell lines from humans in the late 1990's.[1]

Debating Embryonic Stem Cells

With the potential applications for human embryonic stem cells (hESCs) much publicized, debate still swirled around the idea of harvesting cells from a human embryo. Arguments over whether a human embryo was considered a human life and whether harvesting an embryo's cells was considered stealing murder led to the passing of legislation in the United States. While experimentation on human embryos faced major barriers in terms of federal funding—President Bill Clinton's administration prohibited the use of funds used in research that led to the destruction of an embryo regardless of its source—stem cells faced no such restrictions.

In 2001, President George W. Bush passed the currently adhered-to legislation limiting research to embryonic stem cell lines established only before 2001. In 2006, during his second term as president, George W. Bush signed into law the Stem Cell Therapeutic Research Act of 2005, which restricted funding for adult stem cell therapies and treatments

but not embryonic stem cell research. President Barack Obama in 2009 passed an executive order that finally gave federal funding to the study of the established embryonic stem cell lines.[5-7]

The abilities of embryonic stem cells (ESCs)—totipotency and continuous and rapid regeneration without differentiating into one of the germ layers—have proved both advantageous and harmful. Because of the close relationship with tumors, scientists realized that stem cells, if not transplanted into an adult correctly, could induce tumors and lead to cancer.[8] Additionally, they discovered that while ESCs are totipotent, their predisposed differentiation potentials could be seen by an adult body's cells as foreign, therefore causing the body to reject the cells.[3]

Stem cell characteristics

Stem cells are unique because of their ability to differentiate into any number of different cell-types, depending on the type of stem cell.[3] They can be totipotent, pluripotent, or multipotent, etc. (Table 1). Pluripotency of stem cells specifically means the ability of the cell to differentiate into all 3 germ layers—ectoderm, endoderm, and mesoderm.[6,7]

Stem cells self-renew rapidly, making them ideal for regenerating undamaged cells with the correct genetic makeup in areas where cells have been greatly damaged.[3] However, this ability has the great potential to cause not only tumor growth, but malignant tumor growth.[8] These two characteristics are crucial to stem cell medicine, therefore scientists have been focusing on finding pluripotent or totipotent cells that can proliferate like ESCs. Because of the limitations and safety risks of using embryonic cells in adult bodies,

the scientific community decided that using one's own cells was the best way to reduce the risk of immune responses to outside genetic information.

Table 1. States of potency within cells[6,7]

Potency	Definition	Examples of cells
Totipotent	The ability to divide into and produce all possible differentiated cells, including extraembryonic tissues	Spores Zygotes
Pluripotent	The ability to divide into and produce all cells of the three germ layers (endoderm, ectoderm, mesoderm)	Embryonic stem cells Induced pluripotent stem cells Some adult stem cells
Multipotent	The ability to divide into and produce cells in a closely related family of cells of multiple lineages	Hematopoietic stem cells
Oligopotent	The ability to divide into and produce few cells of limited types of cell lineages	Lymphoid stem cells Myeloid stem cells
Unipotent	The ability to divide into and produce only one cell type through self-renewal properties of stem cells	Muscle stem cells

Alternative techniques

A technique called somatic cell nuclear transfer (SCNT) was developed in which ESC nuclei were transferred into adult somatic cells whose own nuclei had been removed. The experiment proved that the genetic information and tendencies of ESC and early embryonic cells were retained in their nuclei, allowing the somatic cell to reprogram and form into a blastocyst.[9] As this technique to induce pluripotency still employed the use of embryonic cells, however, scientists kept searching for other methods to obtain ESC characteristics in normal adult somatic cells. Another technique that held promise but was quickly discarded was the cellular hybridization of somatic cell fusion with an ESC, because of its use of ESCs, something scientists did not want to rely on.[9]

The SCNT technique spawned an entirely different field of research, that of cloning, which led to the eventual cloning of 'Dolly' the sheep. However, the cloning experiments also demonstrated and helped confirm earlier theories that cells differentiated due to epigenetic changes in the cells, and that reversing those epigenetic changes—usually by affecting histone wrapping and through methylation and acetylation—could cause a cell to dedifferentiate, or return to an undifferentiated state.[10]

Inducing Pluripotency: the 2006 experiment[11]

It was not until 2006, when Kazutoshi Takahashi and Shinya Yamanaka published their seminal paper and proved the possibility of inducing pluripotency without narrowed in on a set of transcriptional factors present in ESCs that could be inserted into somatic cells of any stage—they used mouse embryonic fibroblasts and adult tail-tip fibroblasts—to induce a pluripotent state. Their findings, which set the stage for stem cell research until today, found 4 transcription factors—Oct3/4, Sox2, Klf4, and c-Myc, together known as "OSKM"—that were necessary to create induced pluripotent stem cells (iPSCs).[10-15]

Using 24 candidate genes known to be particular to ESCs, Takahashi and Yamanaka used retroviral transduction to insert those genes into the fibroblasts and systematically eliminated factors until a set of 10 were left. From there, the researchers eliminated more factors until only 4 were left. A series of experiments confirmed that these specific factors were essential, that fewer factors did not improve the already low efficiency of inducing pluripotency. The results, repeated with adult fibroblasts, confirmed that any cell type could undergo this method and become an iPSC. When compared to mouse ESCs, their new induced pluripotent stem cells were very similar in morphology and phenotype, which

proved that their method not only worked, but that their findings of the 4 transcription factors were correct as well.[11]

Oct3/4, Sox2, Klf4, and c-Myc, while researched extensively before 2006, have all been the subject of further study, this time to discover the mechanisms of inducing pluripotency. Each factor has a specific role in maintaining pluripotency, making each factor important. Working in an interdependent capacity, the factors go through an autoregulatory feedback look that enables for the maintenance of the ESC pluripotent state.[12] However, studies have confirmed that optimal efficiency for inducing pluripotency is obtained through different concentrations of the factors being expressed.[13] This is due, research has shown, to the different roles each factor plays in cells.

Oct3/4

Oct3/4 is a factor involved in the maintenance of the self-renewal of pluripotent cells and works closely with Sox2 and NANOG, two other transcription factors associated with ESCs.[10,14-17] Oct3/4, otherwise known as Oct3, Oct4, or POU5F1, is primarily expressed in germline cells and is specifically involved in the formation of the inner cell mass of blastocysts. A tightly regulated factor, Oct3/4 is required in large amounts when inducing a cell to the pluripotent state and has the capacity to induce pluripotency on its own, but at low efficiency. Without Oct3/4, however, a cell will not become an iPSC.[12-16]

Sox2

Sox2, a close cofactor of Oct3/4, is a member of the Sox gene family that exists in the epiblast of elongating blastocysts.[16] While only a small amount of Sox2 is required for the pluripotent state,[13] and while it is even sometimes completely dispensable to

reprogramming,[15] its presence works with Oct3/4 to ensure the cell stays pluripotent. Sox2 may also be involved in differentiation at a later stage, as certain amounts of Sox2 in a cell can lead to trophoblast formation.[18]

Klf4

Klf4, a Kruppel-like zinc finger transcription factor, is involved in cell growth regulation and differentiation. Klf4 is directly connected to tumor suppressor protein p53, which is also involved in regulating the cell cycle. Like Sox2, only small amounts of Klf4 contribute to increased efficiency of cell reprogramming. One of the main functions of Klf4 is to suppress p53—which would otherwise prevent NANOG from being activated—and lead to differentiation.[14-16,18]

c-Myc

The last of the factors, c-Myc, is perhaps the most dispensable. This basic helix-loop-helix leucine zipper transcription factor has roles in cell growth, differentiation, and proliferation. It can block differentiation or block proliferation, depending on the cell type and depending on other factors around it. By far the gene with the most binding sites, c-Myc works with the LIF (leukemia inhibitory factor) signaling pathway that helps maintain pluripotency. When Klf4 activates p21, a target for p53, c-Myc can sometimes step in and silence p21, therefore keeping the cell in a proliferating state. While not entirely required, large quantities of c-Myc prevent iPSCs from apoptosis.[14-16,18]

While other factors have since been found that can induce pluripotency, OSKM are still considered the main and most efficient transcription factors for inducing pluripotency

in a cell. These factors also transcend organisms—they are found in murine embryonic stem cells as well as human embryonic stem cells (hESCs).

Inducing Pluripotency: the 2007 human experiment[19]

The ability to induce pluripotency in human somatic cells was proved in 2007 by Takahashi and Yamanaka, just a year after their groundbreaking discovery that reprogramming could be induced in mice.[11] The two scientists reproduced their 2006 experiment and proved that human iPSCs could be formed from the transduction of OSKM into human fibroblasts.[19] Similar to mouse iPSCs, human iPSCs were morphologically and phenotypically similar to hESCs. Some other genes were also found to be in both human and murine stem cells (Fig. 1A,B), solidifying the theory that stem cells of any organism are similar in genotype. A major difference between human iPSCs and murine iPSCs can be found in the expression of these genes, however. The expression of certain genes in human iPSCs mirrors those expressed in hESCs, while the expression of genes in murine iPSCs differs slightly from mouse ESCs—some factors are less expressed in the iPSCs than in the ESCs, and some genes, while known to be present in ESCs, are not expressed at all in iPSCs (Fig. 1A), for example the gene ECAT1, the only gene not expressed to some extent in iPS-MEF4-7. This difference in expression could account for the slight differences observed in iPSCs.

Visit
http://www.sciencedirect.com/science/article/pii/S0092867406009767, Figure 3A

and

http://www.cell.com/fulltext/S0092-8674%2807%2901471-7, Figure 2A,

for access to Figure 1 A and B, respectively.

Figure 1. Gene expression of hES cell-marker genes. Comparison of genes tested for and expressed in both mice and humans. **A** RT-PCR analysis of ES marker genes in mouse iPS cells, ES cells, and MEFs.[11] **B** RT-PCR analysis of ES cell-marker genes in human iPS cells, ES cells from cell line H9, human embryonic carcinoma cell line NTERA-2, and HDFs.[19]

Difficulties with iPSCs

While induced pluripotent stem cells are very similar to ESCs, it has been found that iPSCs still carry traces of their original cell types and are therefore more likely to differentiate into certain cell types instead of being able to differentiate into any cell type. Supporting this finding is the realization that the genes in ESCs and iPSCs differ in gene expression.[20] The stability of iPSCs has also come under question, as they seem genetically unstable.

Other difficulties include iPSC generation efficiency: the most common method by far of retroviral transduction—used by Takahashi and Yamanaka in the initial experiments[11,19]—involves using a viral vector to introduce OSKM into a somatic cell. The properties of the virus could also induce mutagenesis in the cell and, combined with OSKM and the new pluripotent properties of the cell, lead to oncogenesis.[21] Using a retrovirus poses dangers, however, because of the body's innate immune response to a foreign substance—the host cell of the virus, upon identifying the virus, would initiate the sequence of events that includes activation of p53 and eventual cell death.[22] Additionally, the retroviral vectors integrate randomly into the host chromosome, leading to leaky expression for each cell and making the cells hetereogeneous.[9] The efficiency of retroviral transduction for cellular reprogramming is low, leading to the search for other methods to get OSKM into somatic cells.

Proposal

The characteristics of embryonic stem cells as described previously make them the ideal to strive towards in iPSC research. Therefore many studies have, to date, found epigenetic differences that could explain the genomic imprinting in iPSCs that differentiates them from ESCs. If genetic expression differs between the two, it can be assumed that there are molecular differences as well. The challenge is to find methods to reduce these differences, to make iPSCs more similar to ESCs. A method proposed in this study is adding an ESC-associated protein, which would theoretically confer more ESC characteristics on iPSCs, whether through the presence of more ESC pluripotency markers, or through a greater similarity in gene expression and methylation mapping between iPSCs and ESCs.

ECAT1

One such ESC-associated protein, whose gene was studied in Takahashi and Yamanaka's 2006 experiment, is the relatively unknown ECAT1. Also known as ES Cell-Associated Transcript 1 or c6orf221, ECAT1 belongs to the ECAT family, which describes any factors characterizing pluripotency such as Oct3/4, Sox2, and its fellow ECAT protein, ecat4, or NANOG.[23,24]

It is not known for sure whether each member of the ECAT family has the same roles in stem cells. For example, the nonessential NANOG activates transcription of pluripotency markers to increase reprogramming,[3,18] maintains pluripotency for longer in cells with it compared to cells without it and thus changing cell morphology[17], and may even activate cancer-pathways, which highlights the thin line between stem cells and tumor cells that

poses a risk in stem cell medicine[3]. ECAT1 differs in one respect: the protein contains an atypical KH domain, commonly seen in DPPA5, OOEP, and KDHC1 proteins, all of which are absent in rodents[25]. The KH domain is present in nucleic acid-binding proteins and allows for binding of RNA as well as RNA recognition[26].

Although ECAT1 is present in embryonic stem cells and oocytes, it was not found to be expressed in initial reprogramming efforts in murine cells (Fig 1A).[6] When reprogramming was attempted with human somatic cells, the presence of ECAT1 was not tested and confirmed. Since the study only tested the possibility of inducing pluripotency using OSKM, and given ECAT1's fellow factor NANOG's role in maintaining pluripotency, it could be assumed that ECAT1's role—and presence—in inducing pluripotency in human cells has yet to be extensively discussed, especially as so little is known about ECAT1.

Proposed Experiments

With research focusing on ways to replicate ESC characteristics when inducing pluripotency in somatic cells, this could provide an opportunity to observe ECAT1 in more depth by testing its effects on inducing pluripotency. This study therefore aims to look at the effects of ECAT1 through the entire iPSC process, from reprogramming to differentiation, in three stages:

1) ECAT1 will be used with the OSKM factors to induce pluripotency in adult human fibroblasts using retroviral transduction of polycistronic vectors.

2) The newly-formed hiPSCs will be compared against hESCs for any changes suggesting increased similarities between hiPSCs and hESCs.
3) The differentiating ability of the new hiPSCs will be tested by differentiating them into spinal motor neurons and analyzed for functionality and similarities to existing motor neurons.

Using tried and true methods of previous studies researching iPSCs, this proposal offers a chance to study a relatively unknown factor—ECAT1—and also discover more about the differences between hiPSCs and hESCs.

Materials & Methods

Stage 1: Retroviral transduction-based reprogramming

Because of its popular use in stem cell research, retroviral transduction will be used as the method to induce pluripotency in adult human fibroblasts. Retroviral transduction was first used in the seminal 2006 and 2007 papers that confirmed the possibility of creating iPSCs (both murine and human). The efficiency of inducing pluripotency (Table 2) using retroviruses, which, along with lentiviruses, are known as integrating viruses, is relatively low[27]. However, non-integrating vector viral transduction—using adnoviruses and RNA viruses like the Sendai virus[28,29]—as well as nonviral methods—direct protein delivering, non-integrating episomal vectors, piggyBac transposition[30], miRNAs, nucleofection, mRNA[31], minicircle vectors, artificial chromosome vectors[32], and nanoparticles[33]—which could lower the chances of inciting an immune response from somatic cells during reprogramming[9], yield lower reprogramming efficiency. Continuing research has shown that efficiency increases with the supplementation of small chemical compounds.

Table 2. Approximate reprogramming efficiencies of human fibroblasts[27]

Vector	Method	Approximate reprogramming efficiency in human fibroblasts [a]
Virus	Retrovirus	++++
	Lentivirus	+++
	Adenovirus	+
	Sendai virus	++++
DNA	Episomal plasmid	+
	Transposon	++
	Minicircle	+
RNA	RNA	+++
Protein	Cell transparent protein	+

[a]+, <0.001%; ++, <0.01%; +++, <0.1%; ++++, >0.1%.

In accordance with an experiment performed by Zhang et al[34], human OSKM fusion genes (hOSKM) with 2A sequences separating human cDNA sequences of each reprogramming factor will be created (Figure 2A). This gene, along with another gene sequence containing human cDNA for ECAT1 only, will be constructed into a vector, pMIGR1, with high-expression MSCV (Murine Stem Cell Virus) promoter, which is especially used in research with stem cells.[35] A GFP marker will be cloned downstream of the factors, after an IRES sequence, to enable factor tracking during the experiment. Retroviral 293T cells will then be transfected with the vectors to create the retrovirus.[34] One sample of

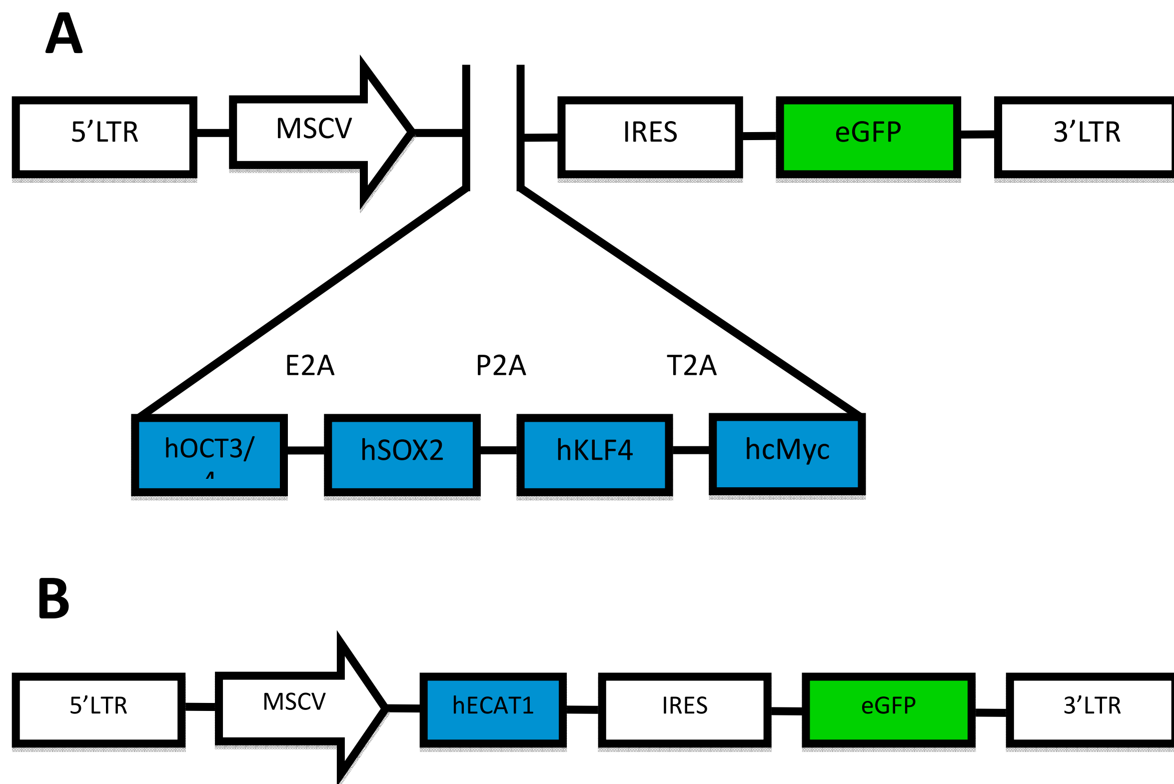

Figure 2. pMIGR1 vector designs for retroviral transduction-based cell reprogramming. MSCV: Murine Stem Cell Virus promoter. IRES: internal ribosomal entry site. **A** Polycistronic vector with OSKM factors and inserted 2A sequences between[35] **B** ECAT1-containing retroviral vector. ECAT1's close connections with Oct3/4 and Sox2 suggest same promoter would work.

retrovirus will have only the OKSM sequence vector, while another sample will have both the OSKM sequence vector and the ECAT1 sequence vector.

Confirming pluripotency

To test the ability to reprogram, human dermal fibroblasts will be infected with the retroviral T293 cells. Adult human fibroblasts have been extensively used in stem cell studies[37,38], making them the ideal candidates to test the effects of ECAT1. The cells will be plated and grown in medium containing sodium butyrate, SB431542, and PD0325901,[34] which have been shown to increas the efficiency of reprogramming somatic cells, and which have supplemented the findings of researchers that certain chemicals and other growth factors can increase reprogramming efficiency.[9]

A series of tests will confirm progress of reprogramming. To confirm transduction of the factors into the fibroblasts, the cells will be studied for colony growth, GFP expression, as well as cell morphology. The main success of this stage will be shown through formation of cells, indicating that the retrovirus integrated into the cells instead of inciting an immune response and cell death. The hiPSCs will be tested against human adult dermal fibroblasts as well as a set of 6 hESC lines (Table 3), which have been used as comparisons to iPSC lines in different experiments[38]. The multiple hESC lines should provide more information to be able to compare the hiPSCs to the different variables within hESC lines themselves. RT-PCR analysis of the cells should molecularly confirm presence and expression of the reprogramming factors in the cells, and comparison of factor levels in hiPSCS and hESCs will further confirm successful reprogramming.

Table 3. Cells used in comparing pluripotency and other cell characteristics[38]

Cell Type	Variation	Sample
Somatic cell	HDF	Control
hiPS	OSKM	Control
hiPS	OSKM + ECAT1	Test
hESC	HuES-3[39] HuES-6[39] HuES-9[39] HuES-13[39] HuES-3 hb9:GFP[40] RUES1[41]	Control

Stage 2: hiPSCs vs hESCs

The major component of this research proposal is studying the comparison between the induced pluripotent stem cells and human embryonic stem cells, to see how similar the two cell types are. Ideally, the newly-formed hiPSCs mirror hESCs exactly, in morphology, cell marker expression, gene expression, and ability to differentiate into absolutely any of over 200 different cell types[12]. Induced pluripotent stem cells retain an ESC-like rounded shape, especially in the late passages of the cell[20,42] and can therefore be one of the first indications of pluripotency. In fact, the cell becomes is more stem cell-like during the late passages than in the early passages, suggesting that the cell is constantly changing during the reprogramming.[20] Therefore, rounded cell morphology in late passage hiPSCs should indicate pluripotency of the reprogrammed cells. The major comparisons will be done in the forms of two studies: a molecular study and a genomic study.

Molecular comparisons

The markers of pluripotency have been identified through certain hESC surface markers as well as the increased presence of certain enzymes. Expression of alkaline

phosphatase, using an alkaline phosphatase assay, will be found. Additionally, immunostaining will be performed not only for the reprogramming factors transfected into the cells—Oct3/4, Sox2, Klf4, c-Myc, and ECAT1—but also pluripotency factors such as NANOG, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81, all of which are present in hESCs[4]. The most common immunostaining analysis method used in stem cell research is flow cytometry, however Western blotting will also be used to confirm expression of OSKM and ECAT1. These tests will be performed on the human dermal fibroblasts as well as the hESC lines, to create comparisons.

Genomic comparisons

While the analysis of cell surface markers and pluripotency factors in the newly reprogrammed hiPSC cells with ECAT1 can help, a deeper analysis of the differences in genome between the hiPSCs and hESCs can help gain a deeper understanding for ECAT1's effects on somatic cells and therefore a better understanding of its function in embryonic stem cells. There are approximately 3,947 genes out of 17,620 that are significantly different between all established hiPSC and hESC lines[20], making it the goal of this study to see if that difference can be decreased with the presence of ECAT1.

To each cell sample, a genome-scale bisulfate sequencing, set of microarray assays, and high-throughput transcript counting differentiation assay that made use of marker genes in embryoid bodies.[42]

The bisulfate sequencing will map each DNA methylation site, allowing for the comparisons of number and location of CpG dinucleotides across all cell samples. This is important because reprogramming cells to pluripotency has been shown to be

accompanied by hypomethylation and changes to primarily pluripotency-related promoters that are typical for ESCs.[43,44] Focus on genes such as DNA methyltransferase DNMT3B, antioxidant CAT, developmental regulator PAX6, and macrophage/granulocyte surface marker CD14 should give better ideas of changes, as well.

The microarrays performed on each sample (Table 3) should give expression of genes that can then be compared with each other and hopefully reveal fewer differences in gene expression. The differentiation assay would look at RNA expression of genes specifically associated with stem cells—those having to do with pluripotency, differentiation, and cell state monitoring, etc. Specifically focusing on the reprogramming pathway and genes associated with it in this assay will allow for a closer look at which reprogramming-specific genes are affected during the course of the pathway.[44] Statistical analyses can help quantify patterns of behavior as well.

Stage 3: Differentiation

The final set of experiments testing the effects of ECAT1 on pluripotency will look at differentiation of the hiPSCS and hESCs and compare the differentiated, newly-somatic cells to their originally somatic cell counterparts. For this part of the experiment, the stem cells will all be differentiated into spinal motor neurons, a type of cells that have proven difficult to extract to study in vitro when diseased. hiPSCs and hESCS will be converted into embryoid bodies, clusters of pluripotent stem cells that form in preparation for differentiation using a typical embryoid body formation assay. These embryoid bodies can then be made to differentiate into the neurons using specific growth factors and involving

the use of retinoic acid and the sonic hedgehog pathway in a common method of differentiating pluripotent stem cells to neural cells.[38]

Confirming Differentiation

Confirmation of differentiation can be found through comparing morphologies of the differentiated cells to existing motor neurons. Expression of TUJ1 and motor neuron marker ISLET ½ (ISL) further confirms the exact identity of the newly differentiated cells. Immunostaining for the presence of motor neuron-specific factors HB9, acetylcholine synthesizer ChAT, neural marker NCAM along with the neural precursor cells undoubtedly generated from differentiation because of the low generation efficiency rate, and mRNA encoding for markers like CHT1, can help confirm the accurate differentiation of the hiPSCs and hESCs. Proof of proper motor neuron function can come with Ki67 immunostaining to measure cycling rate of activity, as well as a Ca^{2+} assay that rigorously enhanced the activities of the neuron during spontaneous activity as well as forced depolarization of the membrane to open the gated Ca^{2+} channels. Immunostaining for ISL would confirm the positive identity of the cells as spinal motor neurons.[38] Electrophysiologial recordings of cells made under standard and stressful conditions could help when comparing their activity to existing spinal motor neurons, to look for similar data.

Predicted Results

The ideal results for the three stages of this research proposal's study show ECAT1 having a positive effect on reprogramming, on maintaining pluripotency, and on differentiation. Because of ECAT1's role in hESCs as that of an RNA binder, and because of its visible detection in the cells before being silenced, when the cell begins developing and differentiating, it can be assumed that ECAT1 helps maintain pluripotency. The presence of ECAT1 should, if not actively change something in a reprogramming cell, at least influence the unwinding of parts of DNA containing more pluripotency genes. ECAT1's tendency to bind nucleic acids would come in handy here, as it could manipulate the genome

Stage 1: Pluripotency

With or without ECAT1, the presence of the OSKM factors, coupled with the chemicals sodium butyrate, SB431542, and PD0325901,[34] which allow for more effective reprogramming, should result in a yield of cells with pluripotent characters. Chief among these is morphology. Embryonic stem cells are smooth and round-shaped, whereas somatic cells tend to have more texture to their shapes. It would be easy to see a change in cell shape. Positive identification of the expression of the reprogramming factors would confirm the success of reprogramming. With ECAT1 present, it is the hope that more pluripotency factors are influenced into becoming active, thereby raising the chances of an induced pluripotent stem cell's staying alive and healthy.

Stage 2: Molecular Comparison

Embryonic stem cells have high levels of alkaline phosphatase to maintain pluripotency, in addition to having high levels of NANOG, Oct3/4, Sox2, SSEA-3, SSEA-4, TRA-1-60, TRA-1-80, and a host of other ESC markers. Immunostaining and then flow cytometry and Western Blotting should show the presence of all these in not only the OSKM-induced PSCs, but also the OSKM+ECAT-induced PSCs, as well as the established hESC lines. Little to none of these markers should be detected in the adult human fibroblasts used for reprogramming in the first place. As mentioned before, ECAT1's presence in the cell may induce a positive activation of other pluripotency genes kept tightly wrapped, and so it is the hope that ECAT1 will be visible in analyses of immunostainings, and that expression of pluripotency markers is higher.

Stage 2: Genomic Comparison

ECAT1's role of manipulating nucleic acid binding sites may prove helpful in unraveling the cell's genome to allow expression of the previously-hidden pluripotency genes. DNA methylation would be very different than before, and the approximately 4,000-difference in gene expression between hiPSCs and hESCs would shrink. Because ECAT1 has not previously been shown to be expressed naturally in cells more developed than embryonic stem cells, this may have a dampening effect on the effects of ECAT1, which still must be studied further for more information about its role in pluripotency. The counting assay would allow a look at what reprogramming-specific genes are still inactivated in the hiPSCs, which would pave the way for future studies looking into how to unlock those

genes. Hopefully, again, ECAT1 has made a difference so that less genes need to be worked on being activated.

Stage 3: Differentiation

Differentiation is a very common procedure, as the human body's cells are more likely to do that than to reprogram themselves. Hence, the differentiated cell yield should be of a good size. The presence of ECAT1 may have more of an effect in helping sort the genes to make the newly differentiated spinal motor neurons more efficient in their activity. The series of rigorous testing is meant to see if the new cells can hold up to their more established counterparts. Assuming that differentiation does not accidentally turn off any crucial genes, the new spinal motor neurons should function in exactly the same ways.

Significance & Conclusion

The field of stem cell research is growing because of interest in using stem cells to cure diseases and heal injuries. Embryonic stem cells, because of the controversy surrounding them—and subsequently the difficulties in acquiring samples of the existing aging cell lines—can only be used in limited capacities. The development and study of induced pluripotent stem cells has allowed the field to progress closer to therapeutical applications of stem cells, yet there are still many challenges. iPSCs and ESCs are still remarkable different in pluripotency character, and it has been shown on occasion that neither type of stem cell can completely differentiate into a fully-functioning somatic cell. The efforts to create stem cells that are identical to embryonic stem cells have proved and disproved a number of methods, chemicals, factors, and conditions thought to improve pluripotency in iPSCs. This proposal is one such research attempt to prove or disprove ECAT1's role in pluripotency. Since little is known about ECAT1, this research also attempts to characterize ECAT1's forced presence in iPSCs. With this study, it may just be that the key to unlocking all of an iPSC's pluripotent potential has been found. It is equally likely that ECAT1 has no effect, or even a negative effect, on reprogramming and especially differentiation. The goal of this research is to find out which of those effects is accurate, so that scientists can either continue researching ECAT1, or know there is one less component to look at in the quest to bring stem cell medicine to reality.

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