Going Uphill: Inducing Pluripotency.

By Ben Laffin

From the idealized epigenetic landscape of C.H. Waddington to the modern use of epigenetic regulatory factors, the ability to revert a cell to a pluripotent state has been a dream of biologists for nearly seventy years. In that time we have come to discover a great deal about histone regulation, chromatin remodeling, and the factors that control it and how to control them. Early activities in inducing pluripotency involved the painstaking removal of nuclear information and replacement from a donor cell, creating largely unhealthy, vaguely multipotent lines. Eventually entire mammals could be reproduced from an adult genome. In more modern examples, factors responsible for the maintenance of multipotency in embryonic cell lines are able to be manipulated and combined in the presence of an adult genome so as to induce it into an earlier structural and chemical conformation. In this way, cells that had reached senescence may be able to be reactivated, in the activity of other lines; the power of such a tool in medical and laboratory settings would be incredible.

Introduction to Pluripotency

More than a decade before even the identification of DNA as the genetic material passed through generations, and the eventual solving of its molecular structure, C.H. Waddington would describe the way in which a developing cell would find itself at a crossroads, at which without genetic changes would have 'two or more alternative modes of reaction open to it'. This, he would say, was not due to genes having multiple modalities, but by some higher mechanism that guided their use, or nonuse, (2). In later works Waddington coined the term 'epigenetics'. In a combination of the study of early development, epigenesis and the study of genetics, Waddington brought focus to a problem that many biologists of his time had lost hope in solving; by what mechanisms do changes in the genome of an organism lead to a change in its phenotype, (1).

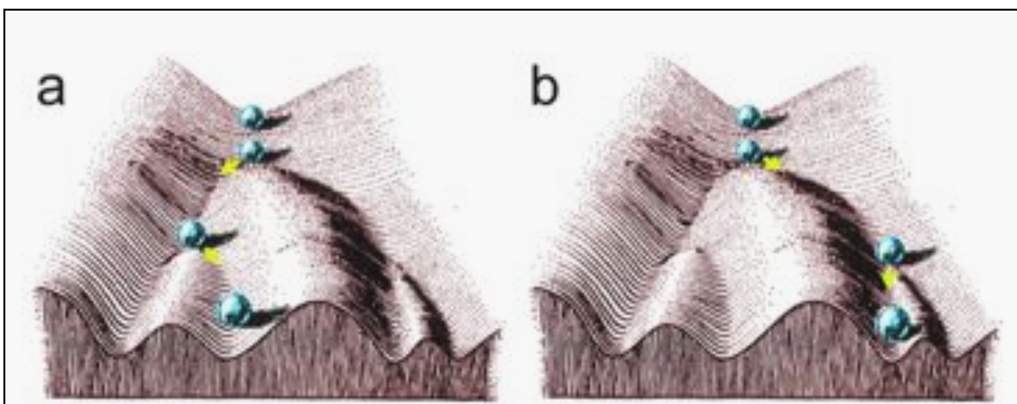
In a culmination of these ideas, an image of cell fate and determination formed. Waddington's epigenetic landscape was born to describe the way in which, like a marble rolling down a grooved hill, cells throughout development reach checkpoints of potentiation, at which they can fall down one developmental path or another. After each 'choice' a cell will lose its ability to return to the previous state of potential, with the forgone path now completely lost to it, (3).

At the time of its conception, it was believed that this deterministic visualization of cell development was immutable, but modern advances have allowed not only the utilization of Waddington's landscape to guide the determination of cells as they develop, (4). But also to revert a fully determined cell to an earlier state, essentially going against developmental gravity, allowing it to be reprogrammed into a cell of a different variety. Several methods of cell reprogramming have been displayed, particularly nuclear transfer and careful application of epigenetic factors responsible for chromatin organization, (6,5).

Currently, the majority of work in the field of inducing pluripotency has been on mouse

Figure 1: Waddington's Landscape

Waddington, C. H., 1956, Principles of Embryology, op. cit., p. 412.



models. With a future focus on human applications of these technologies, the medical contributions these advances could make are nearly boundless; particularly in diseases of cellular breakdown of tissues notorious for their inability to regenerate, (spinal cord injury, neurons in Parkinson's, Alzheimer's). While the ability to re-grow tissues is the end goal of inducing pluiripotency, many issues in its application remain, immune rejection due to lack of viable cell types is concerning, and for the moment the reprogramming of cells retains limitations depending on the source cell's lineage, (7). It is important then, to discuss the current state of Induced pluripotency, its faults, possible future directions and its potential for medical application. Further, of the many methods for inducing pluripotency, each with their own strengths and shortcomings, a clear consensus has yet to be made to focus the community as a whole down a particular path of research.

A Crash Course in Epigenetics

At the Heart of epigenetics, is the concept that the DNA sequence of a gene, or related genes is not the only source of genetic control. Consider for a moment the genome of a single cell as a library, with each section of the library representing a chromosome, and each book a gene. Consider now, a freshman Biology student, walking into the library for the first time to do some research for a paper. Unfortunately for our student, through some mix of mismanagement and misfortune, every book in this library has an identical cover, and although they are titled, the books appear to have no clear organizational pattern and are simply piled upon each other on the floor.

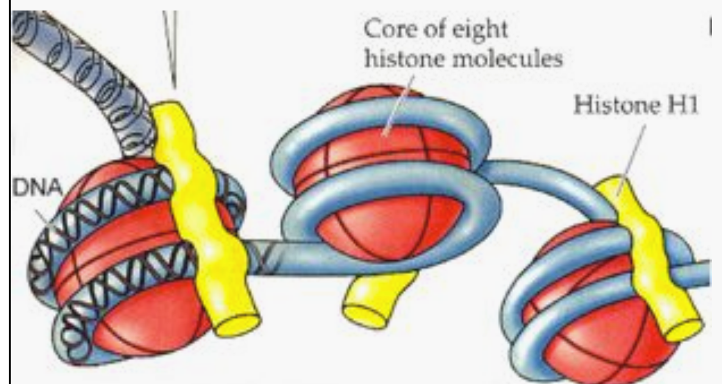
Given an unlimited amount of time and energy, this student could scan every title of every book in the entire building and try to pick out the titles of interest. But this would be exhausting, and would likely take up all of the time they needed for researching. This student faces the conundrum of the uncontrolled genome. The information the cell requires to become a healthy adult is available, but the amount of unnecessary genes is staggering and reading them all would waste many resources, and lead to its death.

Thankfully there exists a mechanism by which the information can be sorted for more efficient selection. Consider the library again, our student has rightfully complained about the awful organization in the building and has gotten an extension of their paper. As they enter the library a second time, we discover that although the books still appear jumbled about, the library staff assures them that many of the books with related topics have been bunched together in various places. Further, the books have all been shelved, giving more room to move about and skim their spines.

While this is certainly an improvement, our student shudders to find that although the books are shelved and grouped, the shelves have been placed directly back-to-back on one another, trapping the majority of books between the gigantic wooden stacks. They will surely need another extension.

Here we see a cells genome carefully organized and compacted about histone cores, (shelves). Each of these proteins consists of an octamer of smaller histone monamers. While several variants of Histones exist, their purposes can largely be generalized. Each histone octmer wraps a DNA double helix about itself 1.6 times, encompassing 146 base pairs. Together, this combination of histone and DNA creates a nucleosome. This compacting of DNA allows for the vast amount of information within a genome to fit into the small volume of a nucleus.

Figure 2. The Nucleosome



Tsankova, Nadia, et al. "Epigenetic regulation in psychiatric disorders." *Nature Reviews Neuroscience* 8.5 (2007): 355-367.

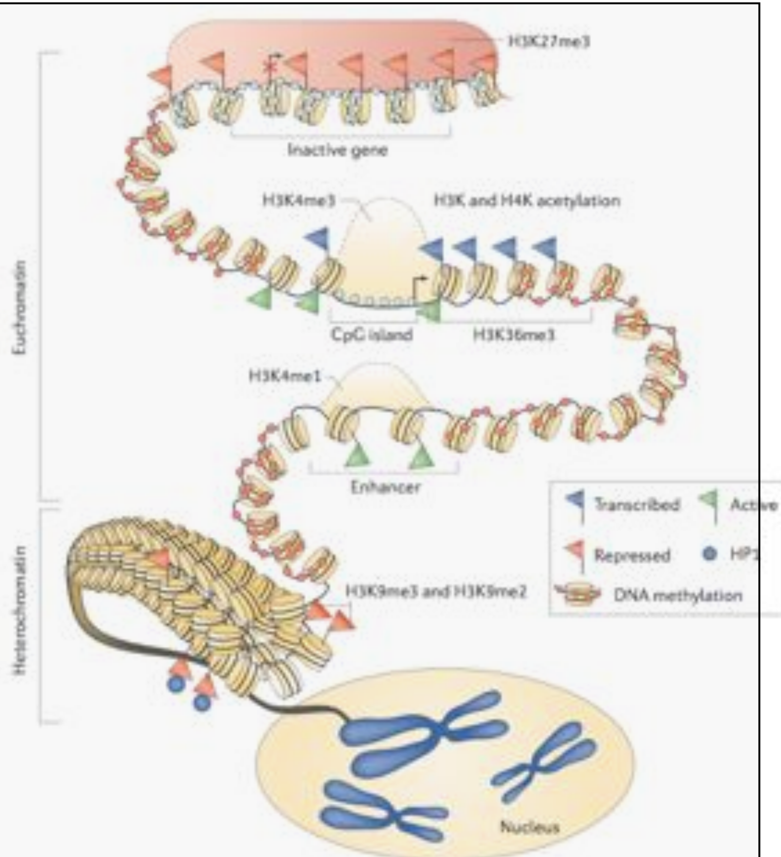


Figure 3. Histone Modifications and Chromatin States

Baylin, S.B., and Jones, P.A. (2011). A decade of exploring the cancer epigenome — biological and translational implications. Nature Reviews Cancer *11*, 726–734.

While this storage form of DNA is useful in its own right, it is clear from our library metaphor that compaction comes at the cost of accessibility. It is thereby important that the Histones have a means by which they can be adjusted, and rearranged to fit the needs of a growing cell. Fortunately the mechanism for this is rather simple; extending from each of the histone monamers is an unstructured tail domain, of signature polypeptides. While each histone core protein has its own tail sequence, the function is the same; to provide an accessible means of communicating with the nucleosome.

Through complex mechanisims of acetylation, methylation, and ubiquination, nucleosomes can be labeled for accessibility or be compacted for disuse. In response to the collections of marks on a given nucleosome DNA remodeling complexes can enact these changes accordingly. In this way DNA is typically considered to be in one of two states, Euchromatin, (open), or Heterochromatin, (closed).

At a level deeper, areas of the genome can be constitutive heterochromatin, never to be used in the course of the cells life, or be facultative heterochromatin; having a use during only certain periods of time in the cells life.

In this way a cell can have precise control over the genes to be expressed as it develops. Without this ability, every cell would require a unique genome depending on its type and function.

While other layers of control exist, the key feature to be considered when attempting to

Early Forays into Pluripotentcy

revert a cell to an earlier stage are those that mark chromatin.

While our student is set on his current career path, if in the future the field of biology should become saturated and some other interest begins to call them how can they put aside what they have learned for other endeavors? The answer is simple for the student, they begin to pick up books on other subjects and learn them as well, but for a cell it is not as straightforward.

While certain marks and chromatin changes are reversible, others are simply meant to be unchanged in the course of a cells natural life cycle. In this way, a skin cell can not be placed among bone tissue and have its function change. And a neuron lost to injury can not simply be replaced by heart tissue.

As many a dog-loving biologist has noted, one simply cannot teach an old cell new tricks. More than a playful observation, this idea identifies a key step in the journey to the fully reprogrammable cell, the initial use of the youngest cells available, embryonic stem cells.

The earliest attempts at creating a truly pluripotent cell line emerged from the breakthrough success in culturing mouse embryonic stem cells, that until that point were largely inaccessible due to the difficulty with which they were cultured in vitro. In 1981 M.J. Evans considered previous failures at culturing these cell lines and attempted a new method by which they could be gathered.

Three main concerns had to be overcome in order for success; the first of which being that the pluripotent stages of development exist purely within the embryo, but the optimal stage of embryonic development for their growth was unknown. Secondly, they existed only in very small numbers; and thirdly, upon culturing these cells they rapidly differentiate into non-pluripotent stages.

By using antigenic markers associated with embryonic carcinomas, Evans was able to identify the embryonic stage of most rapid stem cell expansion to those of the early post-implantation stage. While this was useful knowledge it presented yet another problem; how would one isolate an embryo precisely at this stage?

Closely observing the mouse mothers, the precise moments before implantation would occur, the early embryos were halted in development using certain hormonal treatments, removed and re-planted on media that would allow implantation to continue. Then after a brief growth period, the stem cells were isolated and plated as individual colonies. While this in itself was a clear success, others had in the past grown colonies of stem cells briefly, only to have them fall into carcinogenic growth patterns. Therefore it was important to verify the genetic health of their colonies using karyotype comparisons to embryonic carcinomas. Through this it was then made clear that Evans truly had successfully isolated and cultured embryonic stem cells, (4).

This early success in stem cell study was tempered only in the eventual fate of the cultured cells. Over the course of several cell cycles these colonies began to form cystic growths, bearing antigenic surfaces of epithelial tissues; and when introduced into hosts, quickly became carcinogenic. In this, Evans had isolated pluripotent cells, but lacked the necessary tools to use them. Further, the concept of reversing a cells fate still lay far beyond the horizon.

Nuclear Transfer and Clone Sheep

While the ability to transfer nuclei between cells was a decades old technology in the late 1990's, the use of adult somatic cells as donors of the genetic material had yet to be observed.

In an attempt to draw conclusions about the fluid nature of genetic development, I. Wilmut and K. Campbel of Edinburgh, using enucleated oocytes and donor nuclei from embryonic lines, fetal fibroblasts and adult somatic cells, created the first properly developed mammal from adult somatic genetic material.

In this, oocytes from adult ewes were made to enter quiescence and their nuclei removed. The genetic material of the donor lines was then introduced and the egg implanted. In their study, the rate of embryonic loss in the litters was ten fold higher than observed in natural mating, and upon birth a high degree of perinatal death was also observed. In these losses no gross anatomical malformation was seen, and by all observations made were without obvious cause. In the end however, a single specimen did appear to be a truly healthy individual.

The Lamb produced by introduction of adult somatic genome was not only the healthiest subject, but also the first healthy nuclear clone of an adult mammal. More than another wooly

Figure 4. A wooly genetic marvel, (Dolly and mom), (6).

animal, Dolly was an important milestone in our understanding of epigenetics. It was becoming clear that the notion of irreversible genetic development was at least somewhat false. Although mentioned primarily as a point of interest in their groundbreaking work, Wilmut and Campbel began to bring light to an all important notion; the cytoplasmic environment of a genome is the true key to its expression, that through development into an adult, no material is lost and in the proper surroundings can be returned into a state that can act as a progenitor to all other cell types, (6).

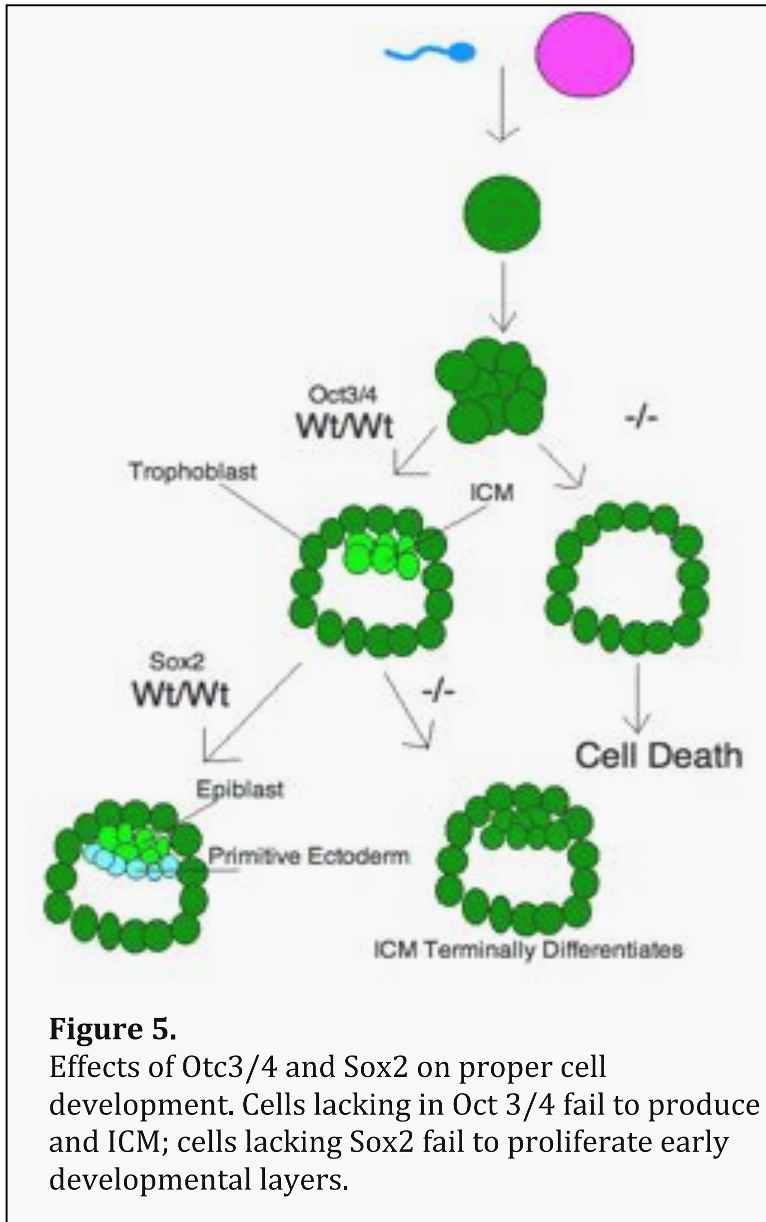


Figure 5.
Effects of Otc3/4 and Sox2 on proper cell development. Cells lacking in Oct 3/4 fail to produce and ICM; cells lacking Sox2 fail to proliferate early developmental layers.

Beyond Nuclear Transfer, Pluripotency Factors.

Although a marvel for sure, the nuclear transfer performed by Wilmut and Campbel plays a prime example of the inefficient production of pluripotency at that point. Their only means to produce stem-cell like cells was to gather pre-existing oocytes and produce an entire offspring.

Beyond cost, and resource limitations, the ethical implications in human use of these methods poses a problem that goes beyond scientific advancement. It was needed in the field to be able to produce pluripotent cells from less disputed sources, principally from any part of ones own body. From previous works, K. Takahashi and S. Yamanaka, believed they had Identified precisely the method to do so.

Because of the previous use of enucleated oocytes to produce embryonic cell lines, they proposed that certain epigenetic maintenance factors within the oocytes must be responsible for this reprogramming of the donor genomes. From their own contemporary work and older epigenetic studies, 4 key transcription factors were identified as sufficient to produce pluripotency.

Oct3/4, a set of mammalian transcription factors were shown previously to be expressed solely in early embryonic and germ cells. Along with Kruppel-like factor 4 (Flf4), an embryonic self-renewal factor, Oct3/4 acts to maintain the pluripotent state of the earliest cells in embryogenesis, guiding them down proper developmental paths,(11). Cells lacking Oct3/4 fail to remain in the undifferentiated state of the Inner Cell Mass, (ICM). They instead terminally differentiate into Trophoblast cells, which would normally become part of the embryo/mother interacting tissues like the placenta. This loss of pluripotency maintenance leads to embryonic death,(8).

Working in concert with Oct3/4, the factor Sox2, acts to further develop the cells within the ICM. Sox2 acts to maintain proliferation of these multipotent cells, leading to the initial

development of the early developmental layers. Embryos lacking this factor fail to maintain their ICM, leading again to a terminal trophoblast lineage,(9). A third factor c-Myc, normally present in early stem cells and immortal tumor lines, was also identified as a potential tool. In previous studies it was found to act as the effector of several pluripotency maintenance genes, acting to stabilize long term telomerase activity and blocking differentiation specific genes from transcribing. While this factor is normally transient and rapidly degraded, blocking of its degradative signaling gene GSK3β can allow for constitutive expression of c-Myc, (10).

These factors were identified through a series of experiments in which a large candidate pool of genes were expressed and selected against until the list was shortened to the absolute minimum.

Takahashi et al. produced Mouse Embryonic Fibroblast cell lines expressing negative control cassettes, which produced resistance factors to the antibiotic G418. The gene into which the control cassette was placed was known previously to only be expressed in stem cell lines. In this way, resistance to the effects of G418 would indicate a reversion to a state of pluripotency. Through this a process of elimination and a series of global expression analyses, were used to identify the 4 factors mentioned earlier as sufficient to induce pluripotency.

The key test to these findings was, however, the ability of these induced pluripotent stem cells (IPSC's) to then be used normally within a wide array of tissue types. After their induction, the most successful colonies were implanted into adult mice. After subcutaneous injections, the colonies formed rapidly growing teratomas. However, within each growth cells appeared to have differentiated into tissues from each germ layer, displaying a wide variety of differentiation pathways available to these cells, (Figure 6).

In a proof of function in adult cells, this same process was then carried out with epithelial cells from adult mouse-tails. But in an increased test of complexity, these IPSC's were introduced directly into blastocysts of pregnant mice. In each of the embryos, the injected IPSC's were tracked using GFP tagging. It was found that the stem cells were again contributing to every area of the embryos. While they appeared to also be present in gonadal tissue, the following generation did not appear to have any IPSC originating cell lines.

These results clearly show successful creation of stem-cell line from adult tissues. However it was also clear that lot of progress was needed before this discovery could find clinical value. The rate of induction of these cells was *very* low. Enough so that Takahashi et al. noted in the same paper that the tissues from which they gathered their pre-induction cells contain small levels of stem cell activity, meaning the results could have stemmed from the

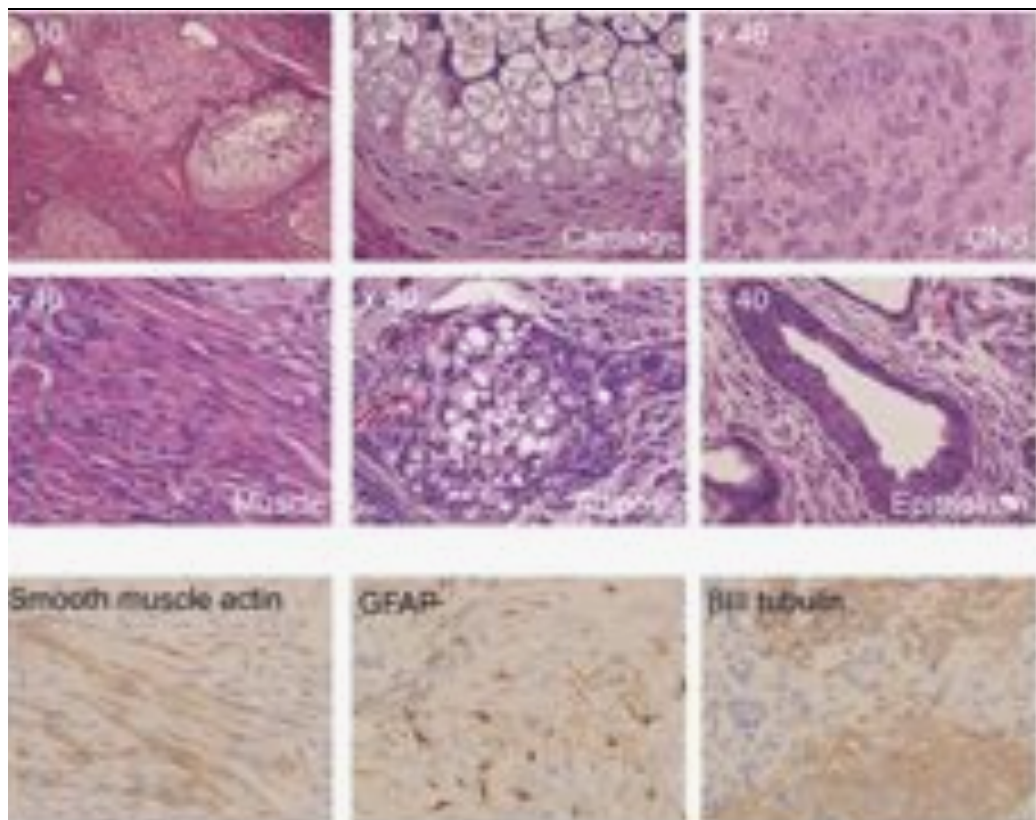


Figure 6. Histological samples from teratomas formed from subcutaneous injection of induced pluripotent stem cells. Tissues from each germ layer are present, including neural tissues,(5).

simple culturing of already-present stem cell lines. In this, the results would have to be independently corroborated before taken at their full value,(5). As well, the stem cells were only capable of developing into healthy tissues if introduced at the pre-implantation stages of embryonic development. The true goal of this technology is eventually meant to act as healthy replacement tissue in adult patients. Clearly there is room to grow.

Not one to be out done, later that same year Takahashi et al. performed the same experiment using adult human fibroblasts, to similar results,(12).

Tumorigenesis, Medicine and the Future

As the field continues its search for the perfect IPSC there are several problems that must be addressed before these cells can be properly used in medical treatments. The first and foremost is displayed in Takahashi's results. Introduction of these colonies to adult tissue inevitably leads to tumor formation. This is almost certainly due to the simplicity of the induced gene profile. More research must be done to identify control factors missed by Takahashi that may be able to prevent this uncontrolled growth.

Some promise has been seen in small molecule induction of IPCS's but the efficacy of these methods is lacking. Further, this method leads to a similar induction of stem cell factors as those use by Takahashi. Although the largest benefit in these methods is their avoidance of viral gene vectors. In medical applications, even the most carefully applied viral vectors can lead to massive immunogenic responses.

Lastly, while any cells used appear to gain large levels of pluripotency, certain cell types seem to have propensities for specific ratios of tissue formation. This is chiefly important when considering regrowth of neural tissue as certain progenitor cell lines appear to produce healthy neural tissue at different ratios,(7).

To alleviate these issues, it is likely that more time must be put into selecting proper control genes for directing the differentiation of IPSC's. As well, ethical methods of human experimentation will inevitably be necessary, as the epigenetic differences between humans and our closest evolutionary relatives is massive. As well, continued use of mouse models is expensive, and will lead to largely speculative results.

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