Basics of Stem Cell Biology as Applied to the Brain

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Abstract   Stem cell technology can allow us to produce human neuronal cell types outside the body, but what exactly are stem cells, and what challenges are associated with their use? Stem cells are a kind of cell that has the capacity to self-renew to produce additional stem cells by mitosis, and also to differentiate into other—more mature—cell types. Stem cells are usually categorized as multipotent (able to give rise to multiple cells within a lineage), pluripotent (able to give rise to all cell types in an adult) and totipotent (able to give rise to all embryonic and adult lineages). Multipotent adult stem cells are found throughout the body, and they include neural stem cells. The challenge in utilizing adult stem cells for disease research is obtaining cells that are genetically matched to people with disease phenotypes, and being able to differentiate them into the appropriate cell types of interest. As adult neural stem cells reside in the brain, their isolation would require considerably invasive and dangerous procedures. In contrast, pluripotent stem cells are easy to obtain, due to the paradigm-shifting work on direct reprogramming of human skin fibroblasts into induced pluripotent stem cells. This work has enabled us to produce neurons that are genetically matched to individual patients. While we are able to isolate pluripotent stem cells from patients in a minimally invasive manner, we do not yet fully understand how to direct these cells to many of the medically important neuroendocrine fates. Progress in this direction continues to be made, on multiple fronts, and it involves using small molecules and proteins to mimic developmentally important signals, as well as building on advances in "reprogramming" to directly convert one cell type into another by forced expression of sets of transcription factors. An additional challenge involves providing these cells with the appropriate environment to induce their normal behavior

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outside the body. Despite these challenges, the promise of producing human neuroendocrine cell types in vitro gives opportunities for unique insights and is therefore worthwhile.

Introduction

By the beginning of the twentieth century, humanity knew that the basic unit of the brain was the neuron. We also knew that a person was born with all the neurons she would ever have, as these neurons could not—under any conditions—regenerate. This understanding left patients with diseases resulting from neuronal death caused by injury or autoimmunity with few options. Over the course of the twentieth century, this dogma has been overturned, driven by two advances: (1) the discovery of neural stem cells, and (2) reprogramming technology that allows us to make neurons that are genetically matched to individual people outside the body. While the opportunities are clear, considerable technical challenges remain before they can be fulfilled in the clinic.

The Basic Biology of Stem Cells

A stem cell is defined as any cell type with two fundamental capacities (1) self-renewal and (2) differentiation. Self-renewal refers to a cell's capacity to divide and make other cells with the same properties. Differentiation refers to its ability to make other cell types, performing other biological functions.

For instance, hematopoietic stem cells are found in the bone marrow, where they generate progenitor cells that give rise to the cells of the immune system and red blood cells.

Not all stem cells have the same "potency," the capacity to give rise to similar cell types. Broadly speaking, they are characterized as totipotent, pluripotent and multipotent. The hematopoetic stem cells mentioned earlier are a multipotent cell type: they are able to give rise to many kinds of cells, but only of the blood lineage.

In basic embryology, blood originates from the mesoderm, the middle layer of an embryo, which forms as the embryo undergoes a process called "gastrulation" shortly after fertilization. Gastrulation subdivides the cells in the group into three broad layers: endoderm, which gives rise to the cells of many internal organs, mesoderm, which gives rise to the muscles and the blood, and ectoderm, which gives rise to the nervous system and epithelial layers. These three lineages are referred to as the embryonic germ layers.

For mammals, even before gastrulation occurs, the tissues of the embryo are classified into two other broad categories: extra-embryonic and embryonic.

Extra-embryonic tissues are "outside the embryo," referring primarily to the cells of the amniotic sac and the placenta: organs that are essential for embryonic development but are discarded after birth.

To be classified as "multipotent," stem cells must make at least two different lineages, usually from the same embryonic germ layer. In contrast, pluripotent stem cells can make multiple lineages from all three embryonic germ layers but not from extra-embryonic tissue. Finally, totipotent stem cells can make all three embryonic germ layers and the extra-embryonic tissue. The only known indisputably totipotent cell is the zygote.

Preimplantation Development and Embryonic Stem (ES) Cells

In most animals, development occurs outside the body and the embryo is not physically connected to the mother. Mammals, particularly placental mammals, are an exception. However, even in placental mammals, not all development occurs in the uterus. During the first few days of its development (exact number of days varies depending on the species), the early mammalian embryo travels down the fallopian tubes into the uterus. Once inside the uterus, the embryo invades the uterine wall and establishes the organs that will support its further development—a phenomenon known as implantation. Thus, the first days of development within the fallopian tubes are called "preimplantation development."

During preimplantation development, several important developmental events occur. Of the biggest relevance to us is the first cell fate determination, or segregation of the early totipotent cells into two lineages: extra-embryonic and embryonic.

We will review these events as they occur in the mouse, the most commonly studied mammalian model of development, and then discuss differences between human and mouse development. At the first stage of development, the fertilized zygote undergoes a series of three cell divisions to produce eight cells. At these early stages, these cells are called blastomeres. The divisions that produce these blastomeres are thought to be mostly "symmetric" (to produce cells with similar properties), though blastomeres have been reported to exhibit bias toward particular developmental lineages (Tabansky et al. 2013). During these early divisions, cells do not increase in size: every division produces two daughter cells that are half the size of the mother; they are called "cleavage" divisions.

Until the eight-cell stage, these cleavage stage blastomeres have very few cell adhesion molecules, and they are separate from each other and readily distinguishable under a microscope. However, at the eight-cell stage, the molecules on the cell membrane start to bind to each other, and the boundaries of the cells become indistinguishable. This moment in development is called "compaction," and though compaction is morphologically striking, it is far from being a mere cosmetic

change. Instead, it serves a very important role: differentiating the inside of the embryo from the outside for the first time.

Immediately after compaction, most of the blastomeres are still able to give rise to embryonic and extra-embryonic lineages. However, as they continue to divide, some cells become separated from the outside. At the same time, the tight junctions between the outside cells allow the formation of a fluid-filled cavity within the embryo. The cells on the outside will now comprise the trophectoderm, which gives rise to the placenta. Inside of the fluid-filled cavity, known as the blastcoel, the cells with no contact with the outside of the embryo form a clump that adheres to the trophectodermal cells. This clump is known as the inner cell mass (ICM). It contains the pluripotent cells that will give rise to the embryo proper, as well as a newly formed cell lineage that will give rise to the amniotic sack: the primitive endoderm, or PE.

The trophectoderm is the cell lineage that will intercalate with the uterine lining and allow implantation to occur. As this process proceeds, the pluripotent lineage loses its ability to form PE, becoming another cell type known as the epiblast. The distinction between ICM and epiblast is very important for understanding the differences between mouse and human embryonic stem cells.

Derivation and Maintenance of Pluripotent Stem Cells: Differences Between Mouse and Human

Mouse ES cells have been known and used for years before human embryonic stem cells were derived (Thomson 1998).

While mouse and human ES cells indubitably share multiple features, including pluripotency and the capacity to self-renew, they do not grow under the same conditions in culture. More specifically, mouse ES cells absolutely require activation of the JAK-STAT3 signaling pathway in order to continue to proliferate, usually achieved by the addition of the Leukemia Inhibitory Factor (LIF) to the medium. In contrast, human ES cells absolutely require basic fibroblast growth factor (bFGF) and Activin A signaling, and they will lose their ability to differentiate and grow without them.

Human ES cells are not unique: ES cells isolated from most species share the features of human ES cells, but not mouse ES cells. The question then becomes, why is the mouse the outlier?

Mice have a unique property known as diapause; in times of stress or starvation, females can delay implantation of blastocysts, which persist in the oviduct until conditions improve. Most mammals do not have this ability. Diapause is mediated by LIF; in fact, defects in diapause are the main phenotype of LIF-knockout mice. These observations led researchers to conclude that conditions for culturing mouse ES cells mimic the response of the ICM to diapause, whereas the conditions for culture of ES cells from other animals do not.

However, this raises an important point: why is it possible to derive ES cells from other species at all? The currently favored hypothesis suggests that most ES cultures mimic the conditions that exist in the embryo a little after implantation but before the cells have begun the process of migration that will separate them into the three germ layers. At this stage, the pluripotent lineage is called the epiblast, and the cells derived from it can therefore properly be called "epiblast stem cells."

The hypothesis described above makes several predictions about the nature of human and mouse ES cells. One is that they will require different conditions and display different properties. Indeed, they do: mouse ES cells have different growth requirements, different differentiation requirements and different morphology than human ES cells.

A second prediction would be that, if differences between mouse and human ES in fact reflect different developmental states, then it should be possible to derive mouse ES cells that have a more human-like phenotype, growth factor requirement and morphology. Indeed, mouse epiblast stem cells were derived a few years ago, and they share many of the characteristics of the cell type known as human ES cells (Tesar et al. 2007). Mouse ES cells can also be converted to mouse epiblast cells, and vice versa (Greber et al. 2010).

These findings have multiple applications for stem cell research. Of these, perhaps the most urgent is that testing protocols on cheaper mouse ES cells before trying them on human ES cells is not a good idea, as mouse ES cells are fundamentally different and respond to differentiation cues in a manner highly dissimilar to human ES cells. However, it is possible to test differentiation protocols on mouse epiblast stem cells as they respond to differentiation cues in a manner quite similar to human ES cells.

How to Test Pluripotency?

The definition of stem cells is primarily functional. Therefore, any test to determine whether a stem cell is in fact a stem cell must also be functional. For pluripotent stem cells, this functionality encompasses the ability to self-renew and also to differentiate into any cell type in the body.

The first property is quite easy to test: simply assess whether stem cells continue to grow and produce more pluripotent stem cells. However, how do you test whether a cell can differentiate into anything in the body?

In mouse ES cells, there are two tests of increasing stringency. In the less stringent version of this test, pluripotent ES cells are injected back into the cavity of the blastocyst, where they aggregate with the inner cell mass and, ideally, contribute to the germ line and multiple other lineages. Usually the coat color of the "recipient" blastocyst into which the cells were injected is different than the color of the original "donor" mouse from which the stem cells were derived. The chimeric mice therefore have variegated coloring resulting from a mix of two cells of two different genotypes in their skin.

This technology is also used to make transgenic mice: stem cells are genetically modified in an appropriate way, and the chimeric mice resulting from the stem cell transfer into the blastocyst are crossed to a wildtype mouse. If the stem cells contributed to the germ line of the chimera, these animals can be expected to produce at least some progeny where every cell carries the transgene. The presence of the transgene in these progeny animals can be assessed by analyzing DNA from their skin cells.

A more stringent test of pluripotency in mice relies on the fact that the embryo has a form of quality control where only cells with two copies of the genome (one from the mother, one from the father) can contribute to the adult organism. In using this approach, people wait for the first division of the recipient embryo and then fuse the two cells back together into one cell. The embryo continues to develop to the blastocyst stage, but each of its cells now contains four copies of its genome: two from the father and two from the mother, a feature called being "tetraploid." Due to that feature, the cells in the embryo are only able to form the placenta and other extra-embryonic lineages and cannot contribute to the adult. However, if the pluripotent cells with the normal number of genomes are introduced into this embryo, they will form all the lineages of the adult. Because they are complementing the function that the tetraploid cells lost in embryonic development, this technique is known as "tetraploid embryo complementation." It is considered the gold standard of pluripotency in the mouse, but it can also be used to generate transgenic mice more quickly.

However, neither of these techniques is applicable to humans, due to both technological and ethical reasons. Therefore, the test for pluripotency in human cells must be something different and less stringent.

One simple test is to remove the bFGF—on which the human ES cells rely to stay pluripotent—from the media and to allow the cells to differentiate without trying to influence their path. This test is frequently used as a preliminary characterization of newly derived human pluripotent cell lines.

A more stringent test is to implant the cells into the body cavity of an immuno-compromised mouse, where they will continue to grow, giving rise to a tumor, called a teratoma, containing multiple fully differentiated lineages. After the tumor grows, it is possible to test the number of different cells that were able to develop within the mouse.

Why not just carry the whole test out in a dish, instead of implanting into a mouse? Different cell types need different environments to grow, and it is impossible to combine them all in the same preparation of cells and to allow them to survive until analysis. However, in the mouse, the supply of blood and oxygen from the body allows the teratoma to develop in a manner somewhat similar to what might happen in an embryo, but in a more disordered fashion. Since the environment is more supportive of multiple different cell types, more different kinds of cells in more mature states can be detected and the test is more stringent.

It is worthwhile mentioning that there are vast numbers of different kinds of cells within the body, and it would be a daunting task to attempt to detect them all within a teratoma. Therefore, while the teratoma can detect the ability of a cell to give rise

to all three germ layers, it cannot be used as evidence that a particular cell line can give rise to every single kind of cell in the body. Thus, a teratoma is an approximation of a test for the most stringent definition of pluripotency.

Opportunities and Challenges for Using ES Cells in Medicine

What do we do with pluripotent stem cells once we have them? Multiple uses have been proposed for these cells, including (1) studying rare cell types, (2) disease modeling, (3) drug screening and (4) transplantation therapies.

Of these, the most obvious and simple application is studying rare cell types. While mice are readily accessible and their neurons can easily be isolated from the brain and cultured in a dish, human cells are not always so easy to isolate and manipulate. This is especially true in the brain, as death is currently primarily defined by the cessation of brain function. Therefore, unlike many cells, neurons cannot be harvested from people who have opted to donate their organs to research, as the damage to the brain that is necessary to declare a person dead will also affect the cells.

To study human neurons in detail another source of cells must be found, and neurons derived from human pluripotent cells constitute one such source. Pluripotent cells from most species tend to be predisposed to make neurons, making such neurons easy to obtain. Additional protocols have been developed to ensure that particular kinds of cells—of interest to people from the investigation of diseases perspective—are preferentially made (Tabar and Studer 2014).

Growing neurons in culture can and has been used to address many questions about their basic biology and their electrophysiological properties. However, it is also true that results from experiments on cultured neurons need to be interpreted very carefully. This caution should particularly apply to human neurons when they are being studied outside the body and when differences between human and mouse are revealed by the study. The question will always arise whether the differences observed have to do with something that happens in the human brain or whether they arise from the distinct ways that human and mouse neurons adapt to the environment outside the body. Luckily, if the biochemical basis of the phenomenon is known, the neurons in culture can be compared to human postmortem brains to determine whether the phenomenon under study occurs in the body as well as in cell culture.

Disease modeling builds on the study of normal human cell types, by comparing cells that are obtained from pluripotent stem cells of patients with a particular (usually genetic) disorder with cells from patients who do not have this particular disorder. Prominent examples include amyotrophic lateral sclerosis and schizophrenia (Marchetto and Gage 2012). Disorders where a person with a particular genetic makeup is highly likely to get a disease are easier to study in culture than disorders that develop in response to environmental stimuli or involve multiple cell types, such as autoimmune diseases or Alzheimer's disease. However, when the

cells are provided with the proper environmental stimuli to induce a disease-like state, it may eventually be possible to model a wide range of diseases in culture.

Once a good disease model has been established, drug screening can begin. Drug screening in culture builds on disease modeling by treating cells with various potentially therapeutic compounds and attempting to determine which compounds can reverse or slow down the course of the disease. The simplest approach is to use cells that express some sort of fluorescent protein or that secrete a particular metabolite that indicates health and then measure how treatment with compounds can alter the amount of fluorescence (a proxy for cell number) or metabolite in the dish. Automated drug screening robots that can measure fluorescence from tens of thousands of different samples are routinely used for drug screening. In this case, it is not even necessary to know the mechanism of disease or the mechanism of action of the compound in order to isolate an effective drug; however, it is desirable to understand at least a little about the function of the drug before administering it to patients.

Of all these approaches to using stem cells for medicine, perhaps the most daunting and fraught with potential side effects is transplantation of stem cells and cells derived from them back into a patient. Ideally, the cells would be perfectly genetically matched to the patient, negating the necessity for immunosuppressive drugs, which are necessary for conventional organ transplantation. This approach can be risky because cells tend to accumulate abnormalities in culture, potentially causing some of them to become tumorigenic; also, if the pluripotent cells are insufficiently differentiated, their inherent tumorigenicity (see above) also becomes a problem. However, recent phase 1 clinical trials have at least suggested that stem cells could potentially cause functional improvements—with few adverse effects— over the course of several years (Schwartz et al. 2012); whether this will hold true for larger cohorts and longer term trials remains to be determined.

Obtaining Cells Genetically Matched to Patients: Reprogramming, Cloning, and Induced Pluripotent Stem Cells

In animals, pluripotent stem cells can be derived from embryos quite easily, but human preimplantation embryos, while sometimes used in research in very specific circumstances, are not widely available. In addition, the cells used for modeling disease, drug screening and transplantation need to be genetically identical to the patient, necessitating that the cells be derived from the person and not from their offspring.

Given that a patient is an adult and therefore does not have any more embryonic cells, some applications require that the cells be induced to revert back to an embryonic-like state, or "reprogrammed." While here reprogramming refers to the conversion to an embryonic-like state, the term can also indicate a direct

interconversion of two different cell types into each other: for instance, a muscle cell into a neuron. It generally refers to the types of interconversion that do not occur under natural circumstances. In contrast, the term "differentiate" refers to making a more adult cell type from a more embryonic cell type (or from a multipotent stem cell), thereby replicating a process that normally occurs in nature.

Historically, there have been three methods for obtaining pluripotent stem cells from patients: cell fusion, somatic cell nuclear transfer and direct reprogramming. Of these, cell fusion is the simplest technique. The cytoplasms of the cells are induced to combine together to form one cell (the nuclei can also combine into a single tetraploid nucleus). Interestingly, if cells of different type are fused, they do not produce an intermediate kind of cell. Instead, one of the cell types is "dominant" over the other, and the resulting cell will have multiple nuclei but will otherwise be functionally very similar, if not identical, to the dominant cell type. It so happens that pluripotent cells are dominant over every other kind of cell, allowing reprogramming by cell fusion.

However, the complication of this method is that, while it may theoretically be possible to enucleate one of the cells or to remove one of the nuclei after fusion, no practical method for doing so on a large scale has yet found wide acceptance. Thus, most products of cell fusion are tetraploid (with the associated problems) and, to make pluripotent stem cells genetically matched to a patient, you would have to start with pluripotent cells from that patient, which obviates the usefulness of the whole endeavor.

An alternate method of reprogramming cells to a pluripotent state first came into prominence in 1996, when people were able to produce an adult sheep from a skin cell isolated from another sheep. In this approach, called "somatic cell nuclear transfer" (SCNT) and referred to colloquially as "cloning," an egg cell has its nucleus removed and replaced with a nucleus from a donor cell. In a way, SCNT is simply a special case of cell fusion of an enucleated totipotent zygote with a differentiated cell. The egg cell then goes on to develop as though it is an embryo, producing a blastocyst from which stem cells can be derived and also, potentially, an adult animal. Blastocysts have been produced by SCNT from multiple animals, including, quite recently, humans (Chung et al. 2014). However, logistical and ethical considerations involved with obtaining human eggs and making embryos preclude this research from being applicable on a large scale to medicine. It may eventually be possible to make cells resembling human eggs in culture from pluripotent cells, but there are currently no established protocols for this approach.

Currently the most popular method of reprogramming for drug screening and disease modeling relies on the delivery of four transcription factors (genes that regulate expression of other genes) to adult cells in order to convert them into an embryonic-like state (Takahashi and Yamanaka 2006). Named after Shinya Yamanaka, who originally discovered this approach, they are also sometimes known as the "Yamanaka factors."

The original method relied on a type of genetically modified retrovirus from which the DNA encoding viral genomes was removed and replaced with DNA encoding each of the Yamanaka factors. Once the cell infected with the genetically

modified virus became pluripotent, they were able to activate the intrinsic protective mechanisms found in pluripotent cells to inactivate this particular kind of virus. Thus, once reprogrammed, these cells were again differentiated into other cell types, and Yamanaka and multiple other groups were able to test the pluripotency of these cells. The caveat is that retroviruses by themselves are carcinogenic, and their presence is undesirable for any cells being transplanted back into patients, which is why the cells reprogrammed by the Yamanaka method (called induced pluripotent stem cells) are used primarily for disease modeling and studies of diseases processes. However, multiple groups have published papers on alternative approaches to reprogramming, including pieces of DNA that do not integrate into the genome, a special kind of RNA molecule called micro-RNA and small molecules (Schlaeger et al. 2015). The Yamanaka method currently remains the most widespread technique but, going forward, it is quite likely that one of these other methods will eventually replace it.

Opportunities and Challenges of Producing Hypothalamic Neurons from Stem Cells

There is wide agreement that investigation of the function of the human hypothalamus could be enhanced by the production of hypothalamic-like neurons from ES cells. Many diseases exist in which particular subpopulations of hypothalamic neurons are absent or defective, and replicating the disease in culture for testing of drug candidates or even producing the neurons and transplanting them back into patients are obvious therapeutic opportunities. However, before neurons can be investigated or transplanted, they first need to be produced, and that is quite a daunting challenge.

In embryonic development, every cell needs to know what it has to become. It would be inappropriate, for instance, for a cell located where the skin will be to become a liver cell. However, a cell does not necessarily know where in the body it is located. To inform each cell of its precise position and eventual fate, the developing embryo relies on complex, overlapping gradients of multiple secreted proteins (patterning factors) that activate molecules on the surface of the cells that, in turn, alter the gene expression patterns of these cells. The history of the previous signals is then recorded in the DNA of the cell by chemical alterations to both the histones and DNA.

Thus, the fate determination of each cell in development depends on a variety of inputs, including the timing of exposure to gradients of patterning factors, the cell's previous developmental history, and types and concentrations of patterning factors that the cell experiences. Interactions with other cells and the local microenvironment also play a considerable role, including determining whether a given cell will survive or die. The cues and responses of the cells can be stunningly complex, and development is incompletely understood even for the best-studied cell types. Even

in the cleavage-stage embryo, where the system is quite simple, the signaling pathway that differentiates inside from outside cells was discovered only in 2009 (Nishioka et al. 2009).

In the context of this complexity, it is stunning that we are at all able to differentiate cells along particular pathways. Most stem cell differentiation protocols are far from 100 % efficient when it comes to the phenotype of the cells that they output. When contemplating that we do not actually understand most of the interactions that occur during development, and that most differentiation protocols use cell aggregates, it is quite clear that intercellular signaling within the dish is an important component of stem cell differentiation protocols.

In the hypothalamus, one published protocol indeed relied on self-patterning of mouse ES cells. In brief, cells were allowed to aggregate and develop with as few (known) disruptive chemical cues as possible (Wataya et al. 2008). The success of this protocol suggested that the hypothalamic cell fate is developmentally rather simple and relies on few cues in order to be induced. Cut off from external gradients, the cells produced hypothalamus almost by default. This approach appeared to produce a number of neurons of different types expressing markers found in the hypothalamus, so no particular peptide-secreting cell was the default fate. An alternative explanation is that, in the absence of environmental cues, cells tended towards fates that secreted the molecules necessary to induce the hypothalamus.

However, in human ES cells, this protocol is considerably less efficient, so two directed differentiation protocols have recently been published. This protocol seems to produce a mixture of hypothalamic-like neurons (particularly, neuronal subtypes found in the ventral hypothalamus; Merkle et al. 2015; Wang et al. 2015).

These neuronal mixtures are as close as we have gotten to producing individual subtype hypothalamic-like neurons, and while they are a good start, the complex microenvironment of that brain region creates problems for derivation of more specific cell types. A lack of information about the developmental cues guiding the specification of many hypothalamic cell types compounds this problem.

Direct Reprogramming: An Alternative Pathway to Obtaining Patient-Matched Neuron-Like Cells

The discovery that cells could be induced to acquire an embryonic-like cell fate by treatment with just four viruses to change gene expression naturally led to the question of whether specific types of neurons could be obtained in a similar manner. The answer from the field, thus far, seems to be a resounding "yes." Multiple papers have been published showing that infection of various fully differentiated cell types with viruses is able to produce cells similar to various kinds of neurons (Tsunemoto et al. 2015). However, not all protocols are as simple as the Yamanaka protocol, with some requiring 20+ different genetically modified viruses to enter the same

cell in order to be effective. Even with an efficiency of viral delivery of 95 %, such an approach would produce a conversion rate of less than 36 %, assuming every cell infected with virus is converted (which is very unlikely). In practice, the conversion rates are often in the single digits.

The unique challenge of trying to obtain neuronal cells using this method, as opposed to other cell types such as pluripotent cells or hepatocytes, is that neuronal cells do not replicate. Thus, while for most other cell types it is possible to feed them media that will allow replication of large numbers of that cell type at the expense of others, this is not the case in neurons.

An additional concern is that introducing so many different viruses into cells is likely to induce mutations, which could interfere with the normal function of the cells and alter their properties. In addition, these mutations would present a high risk of carcinogenesis when transferred into patients, making neurons obtained in this manner poor candidates for transplantation therapies. It is possible that direct conversion of neurons by other means, such as small molecules or delivery of micro-RNAs, will circumvent both the efficiency and mutagenesis concerns.

Relevance of In Vitro Cell Types to Neuronal Biology

Nearly every discussion of in vitro modeling would have to start with the recognition that in vitro models lack many of the factors found within an intact organism and that many aspects of the conditions found in vitro (for instance, high concentrations of oxygen and a lack of cell-to-cell contact in three dimensions) could interfere with cell survival and function, giving rise to artifacts once cells are studied in culture. It also cannot be denied that certain models in culture reflect aspects of conditions within organisms better than others. Every batch of cells differentiated from stem cells needs to be quality controlled to ascertain whether the cell type being cultured reflects particular aspects of biology within the intact organism.

Since the purpose of differentiating stem cells is fundamentally to make a particular kind of cell normally found within the body, it is important to produce a comprehensive and applicable definition of cell type. This task is complicated by more and more data from single-cell RNAseq and electrophysiology studies that are demonstrating considerable molecular and functional variation within cell populations that would normally be defined as being the same "type." One possibility is to define a cell type as a population of cells within a range of phenotypes that perform analogous functions within the intact organism (reviewed Tabansky et al. 2016). Such a definition would naturally exclude any in vitro cell type, as that cell type is found outside the organism, and it is philosophically impossible to rule out the possibility of an undetected difference between a cell in culture and its counterpart in the organism. Therefore, the aim should not be to faithfully replicate every aspect of an in vivo cell type in culture but, instead, to produce a number of models that reflect the interesting features of a cell type as closely as possible.

This way, each new discovery made with a single model can be subjected to multiple functional tests before being tested again in an organism. Using this strategy, false discovery rates from in vitro models should be decreased.

Outlook

In summary, pluripotent stem cells offer a promising path to understanding and treating neuroendocrine diseases. Considerable challenges remain before we are able to transplant neurons derived from these cells into patients, but studying them in culture might be more accessible. Using induced pluripotent stem cells, we can produce cells that are genetically matched to patients to model development and disease. However, in creating cells that can be used in culture, it is important to keep in mind that it may be impossible to faithfully mimic every aspect of the environment that they encounter in an organism, and thus the cells in culture may behave differently than they would in a brain. It is, therefore, useful to create multiple, redundant models of each cell type, so that false discovery can be minimized. It is likely that the field will continue to advance rapidly, and that it will produce considerable insights for neuroendocrinology.

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