Basics of Stem Cell Biology as Applied to the Brain

6

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Contents

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

Keywords

Blastomeres · Compaction · Diapause · Drug screening · Epiblast stem cells · Gastrulation · Hematopoietic stem cells · Inner cell mass (ICM) · Mouse embryonic stem cells · Multipotent cell · Pluripotency · Preimplantation development · Reprogramming approaches · Somatic cell nuclear transfer (SCNT) · Stem cells · Characterization · Definition · Mouse and human embryonic stem cells · Nervous system · Pluripotent · Reprogramming approaches · Teratoma · Tetraploid embryo complementation · Totipotent cells · Yamanaka method

Brief History

It is currently widely accepted that an embryo develops from a single cell, by progressive division of that cell (making two cells from one) and subsequent growth. However, that was not always accepted to be the case. In fact, before the nineteenth century, it was widely believed that the embryo was preformed but tiny at the

beginning of pregnancy and that development simply involved an increase in size. It was only as microscopy was developing in the early nineteenth century that embryos could be examined and this basic assumption could be challenged. At the same time, the theory that cells comprised the basic unit of the brain was also becoming prevalent. By the end of the nineteenth century, Santiago Ramon y Cajal using a new technique known as the Golgi stain discovered that neurons were discrete units of the brain, a theory that became the foundation for modern neuroscience (Fig. 1).

By the beginning of the twentieth century, humanity knew that the basic unit of the brain was the neuron. However, the prevailing thinking throughout the world was that the neurons could not regenerate. Working in different regions and at different times during the twentieth and early twenty-first centuries, scientists began to discover how the nervous system is shaped and how the cells of the embryo could be induced outside the body, allowing us to study the birth of neurons outside the context of the brain (Fig. 2).

The fundamental challenge of developmental biology is understanding how one cell becomes many cells. At this particular moment in time, this issue is not merely theoretic, but has medical urgency, as researchers strive to produce individual cell types from pluripotent stem cells and to study them in a dish, in order to understand diseases. In the process of making neurons from pluripotent stem cells, we discover what we don't know. But what are stem cells, and how can we use them to study development and disease?

This chapter constitutes an adaptation from our "Basics of stem cell biology as applied to the brain" (Ipsen Foundation, Springer, 2016).

Stem Cells Can Be Classified into Different Types

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 cell types other than itself, which are capable of performing other biological functions. The definition of "cell type" is a complicated issue, but here, we will refer to cell types as cells capable of performing a specific biological function within the organism (Tabansky et al. 2015).

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. For instance, the blood stem cells – called "hematopoietic stem cells" – are found in the bone marrow, where they give rise to the cells of the immune system and red blood cells. Potency here refers to the ability to give rise to multiple lineages and "multi-" refers to the multiple lineages; therefore hematopoietic stem cells are also called "multipotent." Every multipotent cell type has its own complement of cells that it can create; for instance, neural stem cells only give rise to cell types normally found within the brain, neurons and glia, whereas hematopoietic stem cells only give rise to blood cells. Thus, both kinds of cells are limited in their potency.

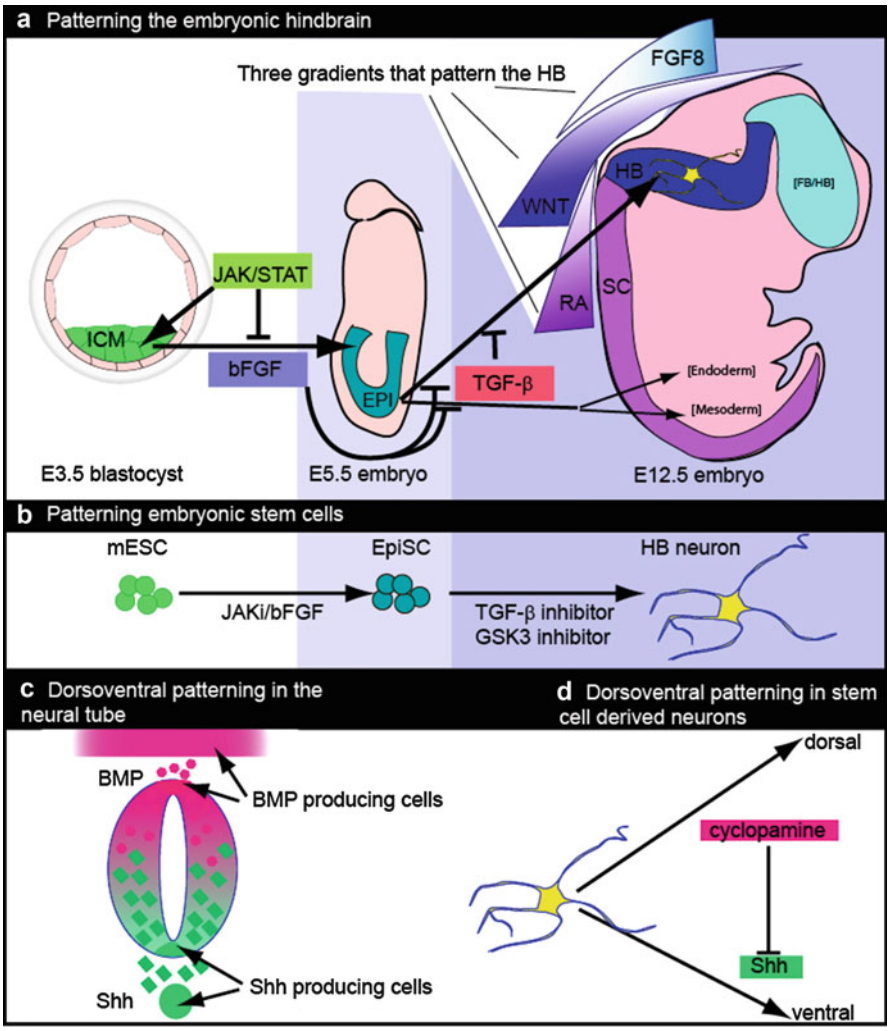

Fig. 1 Summary of rostrocaudal and dorsoventral patterning of the neural tube in embryos and in stem cells. *HB* hindbrain, *FB* forebrain, *MB* midbrain, *SC* spinal cord, *Epi* epiblast. Stages of embryos (E3.5, 5.5, and 12.5 refer to days post-fertilization). (**a**) JAK/STAT3 signaling promotes the inner cell mass (*ICM*) fate, whereas bFGF (basic fibroblast growth factor) represses ICM fate and promotes epiblast fate. Transforming growth factor β (*TGF-β*) signaling promotes endoderm and mesoderm formation at the expense of the neurectoderm. Once the neurectoderm is formed, a set of patterning factor gradients determines rostrocaudal identity. Wnt signaling caudalizes the brain, whereas retinoic acid (*RA*) caudalizes the hindbrain/spinal cord. In contrast, fibroblast growth factor 8 (*Fgf8*) promotes a midbrain fate. (**b**) It is possible to imitate the normal patterning during neural tube development by inducing differentiation of stem cells into the epiblast with bFGF and then inhibiting TGF-beta signaling with SMAD inhibitors to promote a neurectoderm fate. Rostrocaudal patterning can be promoted achieved by manipulating Wnt signaling. (**c**) Dorsoventral patterning of the neural tube depends on opposing gradients of sonic hedgehog (*Shh*) and bone morphogenic protein (*BMP*) signals. (**d**) Embryonic dorsoventral patterning can be imitated in culture with cyclopamine (an Shh antagonist) and Shh agonists

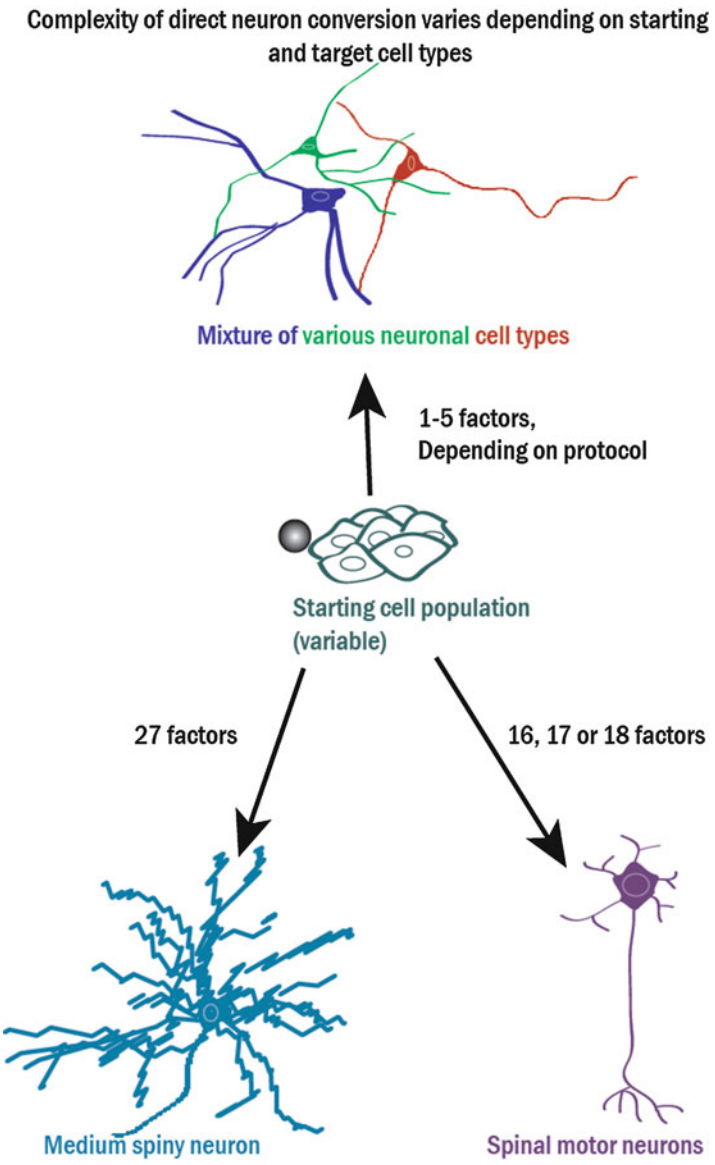

Fig. 2 The ease of inducing direct reprogramming of differentiated cells into neurons varies according to the starting cell population and the type of neurons being produced. For instance, depending on the starting cell population, making a mixture of neuronal cell types can require 1–5 factors. However, making a medium spiny neuron requires 27 factors, making the procedure much more complicated (Adapted from Tsunemoto et al. 2015)

The origins of these cells, like every other cell in the body, lies in the zygote, the cell resulting from the union of the sperm and the egg. The zygote is able to give rise to every single cell within the body of an adult, and it is therefore called "totipotent."

"Toti" is the Latin prefix meaning "everything" because this cell type can give rise to every other type of the body. Thus, the zygote is the most potent kind of cell there is and the only known totipotent cell type.

In most animals, such as birds and many species of fish, development occurs outside the body, and the embryo is not physically connected to the mother. Mammals are an exception. However, even in mammals, not all development occurs in the uterus. During the first few days of its development (the exact number of days varies depending on the species), the early mammalian embryos travel down the fallopian tubes into the uterus. Once inside the uterus, the embryo and the uterine wall intermingle their cells and establish the organs that will support further development of the embryo. These organs include the placenta, which helps nutrients from the mother cross the uterine wall to get to the fetus; the amniotic sack, which wraps around and protects the embryo; and the umbilical cord, which delivers nutrients from the placenta to the embryo. This intermingling of embryonic cells with the uterine wall is 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. We will focus on the first cell fate determination or segregation of the early totipotent cells into two lineages: extraembryonic 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; thus they are called "cleavage" divisions.

Until the 8-cell stage, these cleavage-stage blastomeres have very few of the types of proteins that allow cells to stick together, and they are separate from each other and readily distinguishable under a microscope. However, at the 8-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 extraembryonic lineages. However, as they continue to divide, some cells become separated from the outside. At the same time, the really tight adhesion between the outside cells allows 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 blastocoel, the cells with no contact to the outside of the embryo form a clump that adheres to the trophectodermal cells. This clump is known as the inner cell mass (ICM). The cells

of the ICM are pluripotent, intermediate between multipotent and totipotent. Like totipotent cells, they can give rise to all the different cell types in the adult, but they cannot give rise to the placenta and some other kinds of cells that support embryonic development. However, they retain their ability to produce the primitive endoderm (PE), the lineage that gives rise to the amniotic sack.

During implantation, the trophectoderm mixes with the uterine lining to produce the placenta. At the same time, the pluripotent lineage loses its ability to form PE, transforming from the ICM into another cell type known as the epiblast. While both the ICM and the epiblast are pluripotent, the distinction between them is very important for understanding the differences between mouse and human embryonic stem cells.

Mouse Embryonic Stem Cells and Human Embryonic Stem Cells Have Different Properties

Mouse embryonic stem (ES) cells had been known and used for years before human embryonic stem cells were derived (Thomson 1998). Both of these cell types are produced when a preimplantation blastocyst is grown in a dish under a particular set of conditions, which are not the same. Thus, they are pluripotent cells grown outside the embryo.

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 a signaling pathway known as the JAK-STAT3 signaling pathway in order to continue to proliferate. The activation of this pathway is 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, but they do not need LIF.

While it could be expected that human cells are unique or that each species has its own requirements, in practice that is not true. Embryonic stem 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 defect 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 embryo began the next stage of its development, a process called gastrulation,

which we will cover later. 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 cells in fact reflect different developmental states, then it should be possible to derive mouse ES cells that have a more humanlike 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 embryonic stem 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 (EpiSCs), as they respond to differentiation cues in a manner quite similar to human ES cells.

Pluripotency Can Be Determined Based on a Series of Functional Tests

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 their transfer into the blastocyst are crossed to a wild-type mouse. If the stem cells had contributed to the germ line (sperm or eggs) 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 second, 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 extraembryonic 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 are applicable to humans, due to both technological and ethical reasons. Therefore, the test for pluripotency for 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.

Unlike mice, where ES cells can be used to make a new mouse, the most stringent test in humans is to implant the cells into the body cavity of an immunocompromised 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 many different cell types, 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.

Stem Cells Can Be Used to Study and Repair the Nervous System in a Variety of Ways

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 necessarily be grown 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.

In order 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 for investigation of disease perspective – are preferentially made (Tabar and Studer 2014).

Growing neurons in culture can be 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, in order 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 that 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 to this 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, and 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.

Reprogramming Approaches Allow Us to Obtain Stem Cells That Are (Mostly) Genetically Matched to Patients

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" to 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 to every other kind of cells that has been fused with them, 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 in order 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 somatic cell nuclear transfer 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 where the DNA encoding viral genomes has been 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 disease processes. However, multiple groups have published papers on alternative approaches to reprogramming, including pieces of DNA that does 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, but going forward, it is quite likely that one of these methods will eventually replace it.

Several Different Kinds of Neurons Can Be Successfully Differentiated from Pluripotent Stem Cells

When stem cell differentiation from human embryonic stem cells became a possibility, people started trying to make specific cell types affected by disease. One of the first successful protocols was to make motor neurons, a cell type in the spinal cord that controls muscle movement and that is affected in several extremely fatal diseases (Haidet-Phillips et al. 2011). More recently, dopaminergic neurons affected in Parkinson's disease and cortical neurons (including neurons from patients with schizophrenia) have also been made (Kriks et al. 2011; Marchetto et al. 2011), as well as particular retinal cell types (Schwartz et al. 2012).

However, in many cases producing the neurons 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. In order 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 and the cell's previous developmental history. 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.

For instance, one published protocol for producing hypothalamic-like neurons relied on self-patterning of mouse embryonic stem cells. In this protocol, 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 the hypothalamus almost by default – by signaling to each other. 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.

However, in human ES cells, this protocol is considerably less efficient, so two directed differentiation protocols had 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). However, this protocol is very different and does not rely on self-patterning.

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.

Thus, differentiation of embryonic stem cells to various cell types both relies on our existing knowledge about development and teaches us more about developmental processes. Every time a differentiation protocol produces an unexpected result, we learn more about the signaling and microenvironmental cues that control the development of this cell type. In producing cells in the dish, we also hope to learn how the cells arise in the body.

Direct Reprogramming Is an Alternative Pathway to Obtaining Patient-Matched Neuronal-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 leads to the question of whether specific types of neurons can 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.

Once a combination of factors necessary to convert the cells to a neuronal-like cell has been establish, it may be possible to lower the number of viruses required by putting DNA encoding multiple proteins necessary for the conversion into the same

virus. This method also has some caveats, as the exact ratio of expression levels of different proteins necessary for efficient conversion is difficult to establish. Thus, while efficiency of viral delivery can be increased, we do not yet fully understand what conditions contribute the success of the conversion process, including potentially special properties of the rare starting cells that successfully undergo the reprogramming process.

An additional 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, in neurons, this is not the case.

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, and thus, neurons obtained in this manner are poor candidates for transplantation therapies. It is also possible that direct conversion of neurons by other means such as small molecules or delivery of microRNAs will circumvent both the efficiency and mutagenesis concerns. Much work remains before this approach to reprogramming could be efficient on a large scale; however, this will be a major breakthrough if and when it happens.

Defining Whether a Cell in a Dish Is Similar to a Cell in the Brain Is Quite Complex

Cells in culture (in vitro) lack many of the environmental cues 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 in order to ascertain whether the cell type being cultured reflects particular aspects of biology within the intact organism.

Given that the whole purpose of differentiating stem cells is to make a particular cell type, it is important to produce a comprehensive and applicable definition of cell type. However, the definition of cell type is continually in flux and is currently again changing due to the fact that now we can observe differences in gene expression and electrophysiological properties in neurons previously thought to be nearly identical. 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. Frontiers in Behavioral Neuroscience, in press).

If the definition of cell type is entirely functional, however, such a definition would naturally exclude any *in vitro* cell type. As that cell is found outside the organism, we cannot definitely say that it will adequately perform its function within the body, as there is always a possibility that we overlooked something that makes it

nonfunctional in the organism. It is therefore a more realistic aim to produce cells that share certain properties with cells found in the body and that behave in a manner that is sufficiently similar to allow certain aspects of disease and/or normal function to be studied. In fact, it is desirable to have multiple models of each cell type grown under different conditions; thus, if one model produces peculiar results that do not reflect conditions in the organism, we can detect it as an anomaly using a different model. It is likely that as the field continues to progress, models of cell types will become better and better, hopefully reducing the possibility of findings that are not reflective of the state of the cell within the organism.

Outlook

Due to their ability to produce human neurons for study outside the brain, pluripotent stem cells offer a promising path to understanding and treating neurological and psychiatric 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 organisms, 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 neurology and psychiatry.

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