

Analysis of the Progress of in vivo Trans differentiation of Glial Cells into Neurons

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Abstract. In the majority of instances, neuronal damage or loss cannot be healed or restored. However, over the past few years, an increasing number of researchers have been attempting to directly transdifferentiate astrocytes into neurons in both in vitro and in vivo situations. Since Shinya Yamanaka published the first work on induced pluripotent stem cells in 2006, the area of cell transdifferentiation has been consistently increasing for more than ten years. The NeuroD1 studies have been carried out on multiple occasions by a number of researchers, who have demonstrated that these tests demonstrate the potential for the drug to trigger in situ conversion of astrocytes into neuronal cells. This is despite the fact that Chunli Zhang's team has also put out some additional theories that call into question how transdifferentiation is mediated by NeuroD1, PTBP1, and other pathways. This article will provide an in-depth analysis of the techniques for reprogramming astrocytes into neurons, as well as a discussion of the most contentious topics surrounding this research area.

Keywords: Reprogramming, transdifferentiation, PTBP1, NeuroD1.

1. Introduction

The majority of the cells that make up the central nervous system (CNS) are glial cells and neurons. In mature animals, the neurons that make up the central nervous system are practically incapable of regeneration, in contrast to the peripheral tissues and organs. In neurodegenerative disorders (e.g., Alzheimer's disease, Parkinson's disease, Huntington's disease, and stroke), neurons die in vast numbers. Because the dead neurons are unable to recover, this results in severe and permanent impairment to the brain function. Glial cells, in contrast to neuronal cells, have the capacity to renew to some degree. In order to accomplish in situ neuronal regeneration, researchers have suggested manipulating a protein using viral tools in order to stimulate glial cells to go through reprogramming (also known as conversion) and develop into neurons. As a result, in the therapy of neurodegenerative disorders, one category of cells that can regenerate, known as glial cells, are used to take the place of lost cells that cannot regenerate, known as neurons.

One of the areas of medicine that still has a substantial amount of room for improvement is the treatment of neurodegenerative illnesses. It is presently impossible to cure neurodegenerative disorders like Parkinson's disease, Alzheimer's disease, or Huntington's chorea, and there are very few treatments that can even significantly reduce the disease's course. Because of the acceleration of the aging process, neurological illnesses have become an increasingly significant burden on both the medical community and society.

In recent years, a huge number of researchers have scaled the "dangerous peak" of neurodegenerative disease therapy, and a significant number of studies have discovered that a significant number of neurons are destroyed throughout the progression of neurodegenerative disorders. Therefore, researchers in the scientific community have posed the following question: "Could the regeneration of neurons offer the hope for healing neurodegenerative diseases?"

In most cases, damaged or lost neurons are unable to be repaired or regenerated. However, in the last several years, an increasing number of researchers have been attempting to transdifferentiate astrocytes directly into neurons in both vitro and in vivo settings. Since 2010, direct trans differentiation of astrocytes to neurons in vitro and in vivo has been consistently verified by different labs across the globe. The strategy that has been adopted by the majority of researchers is

overexpression of neural transcription factors using viral vectors. This has also made cellular trans differentiation a popular study subject, and the field of cellular trans differentiation has been evolving for more than ten years, since Shinya Yamanaka published iPSC for the first time in 2006.

In 1957, developmental scientist Waddington made the analogy that each embryonic stem cell was similar to a marble sitting atop a hill, and that the growth of a normal cell was similar to a marble sliding down a hill into a valley. As it rolls down the hill and eventually stops in one of the valleys, the marble makes a decision between the several forks in the route that it may take. The location of the valley in which the marble ultimately stops is referred to as the "fate" of the cell. It was initially revealed in 1987 by the group led by Davis that mouse embryonic fibroblasts may be converted into myoblasts by the process of overexpressing a particular transcription factor known as MyoD [1]. Direct reprogramming, also known as transdifferentiating, is the process of changing one kind of mature somatic cell into another kind of mature somatic cell without first going through the pluripotent stem cell or progenitor cell stage. This technique can also be referred to as transdifferentiating. Numerous studies have shown the direct reprogramming of cells between diverse cell types. For example, astrocytes may be reprogrammed into brain cells, cardiac fibroblasts can become cardiomyocytes, and embryonic fibroblasts can become hepatocytes [2-4].

2. Nerve Cell Transformation in vivo

In the year 2020, Professor Xiangdong Fu published in Nature his discovery of a way to repair destroyed neurons in situ for the treatment of neurodegenerative illnesses [5]. In another one of his articles, which was published in the cell journal, he observed that an RNA binding protein, polypyrimidine sequence binding protein (PTB) with splicing action, and neuronal analog nPTB were able to transdifferentiate induced fibroblasts into functioning neurons [6]. During this stage of the induction process, PTB is responsible for the repression of miR-124. Subsequently, miR-124 is responsible for the repression of the transcriptional repressor REST. REST, in turn, is responsible for the repression of a number of neuronal transcription factors, including miR-124, creating a repression loop. Because of their great quantity, their ability to proliferate in response to damage, and their high degree of flexibility, astrocytes are an excellent candidate for stem cell programming. Through the process of infecting isolated cortical astrocytes with a virus carrying another virus that interferes with PTB production, the researchers were able to transform astrocytes into neurons. Single-cell sequencing also showed that throughout the transformation process, genes that are particular to astrocytes were lost, while genes that are specific to neurons were gained. Further investigations using electrophysiological techniques uncovered the fact that the altered neurons had electrically active features. Experiments conducted outside of living animals showed that inhibiting PTB activity converted astrocytes into neurons. In order to provide more evidence that PTB plays a function in the transformation of astrocytes into neurons. In order to bring about a decrease in the amount of PTB expression in the substantia nigra of mice, the researchers injected a virus into this area of the brain. After injection of the virus, it was discovered that 20% of the astrocytes in the mice produced neurons, and one of the percentages climbed to 80% 10 weeks after the injection. This was discovered after the animals were given the virus. After a period of 12 weeks after injection, a startling 30% to 35% of astrocytes had transformed into several kinds of dopamine neurons. Based on these findings, it seems that astrocytes are capable of forming a variety of dopamine neurons after PTB has been inhibited. Is there a particular kind of tissue that this "transformation" of astrocytes occurs in? When the researchers eliminated PTBs in the cortex and striatum using the same method, they found that the total neuronal conversion rate in these three areas of the brain was not significantly different from one another. Primarily dopamine neurons were generated in the area of the midbrain, and these neurons were the ones that were transformed into neurons in the cortex. Based on these findings, one possible interpretation is that the transformation process of astrocytes leads in the creation of certain kinds of neurons in locations that are optimal for the growth of these neurons. Getting back to the fundamentals, will the modification of astrocytes lead to considerable results? The researchers found

that by inhibiting PTB in the striatum of a mouse model for Parkinson's disease, they were able to restore the number of destroyed dopamine neurons to 65% of the original amount, as well as alleviate motor impairments. Dopamine neuron number can be restored; however, this does not always indicate that function can be. After that, a method called in vivo recording of dopamine neuron activity found that the dopamine neurons generated as a result of the remodeling of the lesion site had the ability to release dopamine and had specific neural functions. Based on these findings, it seems that the alteration of astrocytes may bring about not only changes in the quantity of dopamine neurons but also changes in their functional properties. Overall, Xiangdong Fu's team has developed a method that is straightforward and quick to convert a large number of astrocytes in the brain into neurons. This method can restore the lost dopamine neurons and alleviate the movement disorder in mice that have been modeled after people with Parkinson's disease. Further research is required to determine whether human midbrain or striatal astrocytes can also be converted into functional dopamine neurons, and whether the converted cell types and their target brain regions persist over time and in a stable manner. Only then will it be possible to determine whether or not this approach can be implemented in humans. More crucially, the existence of such knockdown PTBs does not rule out the possibility of tissue injury at this point in time; yet, the results of this article are persuasive despite this fact.

3. Controversies Still Exist

The research team led by Professor Magdalena Gotz at the Max Planck Institute for Neurobiology in Germany was the first to explain the phenomena of glial cell reprogramming. They published their findings in 2002, stating that PAX6 triggered the reprogramming of astrocytes into neurons [7]. Subsequently, a number of studies concentrated on this area of research, such as those conducted by the group led by Chun-Li Zhang at the University of Texas Southwestern Medical Center (SOX2, 2013), the group led by Gong Chen at Penn State University (NeuroD1, 2014) [8,9]. Every one of these approaches involves modifying specific components in order to turn astrocytes into neurons. Even under circumstances of depletion and repopulation, astrocytes regenerate at a rate of about 0.7% per day on average [10]. Despite the fact that astrocytes are capable of regenerating, their regeneration capacity is rather low. Therefore, researchers have questioned whether or not different kinds of glial cells that have a high ability for regeneration may be transformed into neurons by the process of induction.

The microglia in the central nervous system are the glial cells that regenerate themselves the greatest. Microglia are capable of regenerating an average of 20% of cells every day via the process of self-proliferation under reproductive settings, as was discovered in a previous work carried out by the group led by Peng Bo at Fudan University [11]. If it is possible to stimulate the reprogramming of microglia, then an inexhaustible source of replenishment will be discovered, which may be utilized to refill injured neurons in vast numbers. In 2019, the research team led by Kinichi Nakashima from Japan published their findings that heterologous production of NeuroD1 using lentivirus might trigger the reprogramming of microglia into neurons [12]. However, there are many differing opinions about this occurrence among the scientific community. The primary point of contention is upon its justification: (1) astrocyte-neuronal reprogramming, as discovered by previous authors, both kinds of cells are derived from the neural ectodermal lineage, differentiated from radial glia, and are closely related and may undergo transdifferentiating. Radial glia is the precursor to both astrocytes and neurons. Microglia, on the other hand, grow from myeloid cells in the yolk sac and have a completely different developmental history than macroglia do. Microglia also have a very distinct function in the immune system. If microglia-neuronal reprogramming can be achieved, this would belong to a category of transgenerational transformations that are, in theory, difficult to accomplish. (2) The previously discovered variables that are responsible for astrocyte-neuron reprogramming (such as NeuroD1) are all factors that are connected with cell fate choices made during the process of radial glia differentiation. However, NeuroD1 is not expressed in the myeloid lineage, which is where microglia are found. Additionally, it is questionable as to whether the cross-spectrum expression of

such an important pioneer factor has corresponding downstream components to support the fate decision of cells in the neuroectodermal lineage. At the same time, there is a new big disagreement about whether or not the NeuroD1-mediated astrocyte-neuron reprogramming is an experimental artifact (Cell). Re-examining the phenomena of glial cell-to-neuronal transdifferentiation in mice (using a technique called genealogical tracing) was the goal of the team led by Chun-Li Zhang [13].

Through the use of live cell imaging, stringent genealogy tracing, and pharmacology, the research team led by Peng Bo has investigated NeuroD1-mediated microglia-neuron reprogramming in a methodical manner [14]. The results of the experiments point to the possibility that the so-called "microglia-derived neurons" identified by earlier writers are really the product of experimental artifacts. In addition, the phenomena that the researchers saw provides evidence that infection with viral tool vectors is accompanied by the possibility of non-specific leaking. This was deduced from the findings of the study. A non-specific viral leakage might result in incorrect interpretation of the outcomes of an experiment. The fact that the mortality produced by NeuroD1 microglia may be prevented by the BCL2 pathway shows that NeuroD1 not only fails to trigger microglia reprogramming but also causes apoptosis of microglial cells. This explains why the previous authors and the team saw such a high proportion of "microglia turned into neurons" artifacts: microglia that effectively expressed NeuroD1 perished of apoptosis, leaving primarily non-specific leaky cells. Therefore, the findings demonstrate that the "microglia-neuron reprogramming" that was reported in the past is not a genuine phenomenon, but rather an artifact of the experiment that was caused by non-specific viral leakage.

4. Response to the Controversy

Fu referenced Zhang's publication in Cell and Seth Blackshaw's lab preprint in his presentation titled "Astrocyte-to-neuron conversion: evidence, interpretation, and understanding." [15]. He started by providing some background information on the two potential processes that can happen when a cell trans-differentiates from one type of cell to another: dedifferentiation, which occurs when sox2 is overexpressed and causes the cell to first become a neural stem cell, slowly differentiate into a young cell, and then become a mature neuron; and re-/trans-differentiation, which occurs when PTBP1 is expressed. Measurements such as PTBP1 knockdown, may not differentiate glial cells into neural stem cells but instead induces direct conversion of glial cells into neurons without passing through the stage of developing into young neurons, preventing the expression of the immature neuronal marker DCX. Furthermore, as no approach is flawless, there are many technical causes as well as other potential explanations. For instance, knocking off genes directly using transgenic mice may have distinct impacts on cells than knocking down gene expression. The cre-flox system itself may have a preference (hGFAP will shut down and reduce GFP; CAG promoter persistent; tamoxifen-induced cre) and transgenic mice lines may be somewhat faulty. Tamoxifen may also impact cells. mGfap-cre; R26R-YFP lineage tracing sans Tamoxifen. Episomal LSL-RFP is more sensitive to cre than chromosomal LSL-RFP, GFAP promoter activity is not equivalent, and astrocytes are diverse. In a previous Nature article, he did discover some intermediate states, such as dopamine neurons' axons mistakenly projecting to septal nuclei and slowly traveling to the striatum. Early labeled neurons are also electrophysiologically immature, and later they develop into electrophysiologically mature neurons. In prior research published in Nature, he used AAV or ASO to knock down PTBP1 rather than only employing AAV. However, he also acknowledged that leaking in endogenous neurons continues to be a significant problem, which essentially supported Zhang's study. Even if the findings of Zhang's experiment may still be interpreted in a variety of ways, the fact that he did not highlight the potential toxicity of the AAV titers may be a sign of its validity. It also reiterates several of the earlier points, such as the fact that little is known about the complexity of glial cell taxa. Further proof is needed, such firsthand observation of the transformation of glial neurons into neurons. More information is anticipated, and a robust conversation is a good approach to advance science.

This intriguing research was released by Chunli Zhang's lab [15]. Without much disagreement, glial cells can be transdifferentiated into neurons in in vitro culture. In this article, the question of whether or not glial cells may transform into neurons in an in vivo setting is discussed. It primarily questions certain earlier in vivo results, such as the overexpression of NeuroD1 in glial cells and the finding that neurons may develop from glial cells when Ptbq1 is knocked out. In order to demonstrate that large doses of AAV, which exclusively label on pre-existing neurons, do not transform glial cells into neurons, this article primarily use a genealogical tracing technique.

When employing lesser quantities of AAV, a comparable report from Gong Gong Chen's team reveals that at least part of the neurons is transformed from glial cells (no statistical graphs are provided, so it can only be determined as "at least some").

The absence of control for low AAV concentrations is the sole negative aspect. People believe that the findings are a little bit too "perfect" since there are so many AAV-tagged studies in the manuscript but hardly no glial cells labeled for genealogy tracing. Could it be that the high concentration of AAV favors AAV infection and expression? There is a chance that glial cells will be "accidentally" labeled by AAV since it is thought that AAV is not selective and may "miss expression," which allows it to infect a variety of cells. Additionally, it is important to look at how local neurons are preferred by large concentrations of AAV with various promoters and how they control gene expression.

Additionally, this study did not undertake behavioral testing in mice, it does not dispute the possibility that additional variables may one day convert glial cells into neurons; it simply questions the in vivo scenario that certain circumstances (NeuroD1/Ptbq1) cannot convert glial cells into neurons.

The possibilities include some of the following, assuming that everyone's experiments are trustworthy: 1) Glial cells may transdifferentiate into neurons, although the effectiveness varies widely depending on the environment, from 1% to 90%. 2) It is unclear if glial cells can transform into neurons; this might happen in unidentified cells with glial characteristics, such certain types of resting stem cells. 3) There is basically no trans differentiation from glial cells to neurons, a change in the nature of the neuron that has a curative impact on illness, some as-yet-undefined stem cells, quick trans differentiation of the neuron itself, or practically no trans differentiation.

Future in situ trans differentiation research will need to include genealogy tracing since this work shows that large quantities of AAV preferentially label native neurons. Reproducibility of the experiments is necessary to assure that there will be no future disputes in science. Future papers from the key laboratories involved are now being awaited.

Similar findings were found in the biorkiv paper Ptbp1 deletion does not trigger glia-to-neuron conversion in adult mouse retina and brain, which also used transgenic mice and used both gene tracking and genetic manipulation.

5. Other Current Controversies in the Field

The concept that glial cells can be transformed into neurons in in vitro cell culture, or in vivo (in vivo) situations, is not controversial. What is controversial is the method to study in vivo trans differentiation, especially the reliance on viral labeling that is not specific: for example, one wants to infect only glial cells with the GFAP promoter, but the actual situation may be labeled with already existing neurons, as if to create the illusion that glial cells become neurons.

Specifically, controversial issues are as follows.

1) In the in vivo situation, does knocking out or knocking down PTBP1 in glial cells convert glial cells into neurons?

2) Does overexpression of NeuroD1 in glial cells in the in vivo context convert glial cells into neurons?

3) Can knocking down or down-regulating PTBP1 and overexpressing NeuroD1 cure the disease?

In the in vivo case, the vast majority of classical glial cells cannot be converted into neurons when PTBP1 is knocked out or knocked down in glial cells. In addition to the evidence from Chun-Li

Zhang's lab at the beginning (Wang et al., 2021), there is similar evidence from other labs [16-20]. In particular, one of them is a bioRxiv article from Yang Hui's lab. Previous work supporting the conversion of glial cells into neurons by knocking out or knocking down PTBP1 in glial cells has been published by both Xiangdong Fu's lab and Hui Yang's lab. However, there is still another explanation (currently weak and awaiting evidence to support it): glial cell conversion to neurons occurs mainly in a small subpopulation of glial cells - a small subpopulation that may not be easily labeled by various genealogical tracking tools.

In the somatic case, overexpression of NeuroD1 in glial cells converts glial cells into neurons, but with high and low efficiency, with different brain regions, different viruses, and different viral concentrations, cf. the series of articles from Chen Gong's lab, and the recent review article by Beverly L Davidson. It is possible that the vast majority of classical glial cells fail to convert to neurons when efficiency is very low (evidence from Chun-Li Zhang's lab (Wang et al., 2021)). It is hoped that more experimental support will be available in the future. It is worth mentioning the bioRxiv article from Chen Gong's lab, which in addition to verifying that different virus types and virus concentrations affect virus specificity; overexpression of NeuroD1 with a strong promoter can convert some glial cells into neurons; a more interesting data is the observation of how glial cells convert into neurons using imaging at different times, which could have been a decisive data, but the drawback is that the lack of precise statistics and the lack of using mice capable of genealogical tracing (perhaps this is already being done now) makes this part of the data quite weak.

Therefore, the question arises: if knocking out or knocking down PTBP1, or overexpressing NeuroD1, do not convert glial cells into neurons, how do they cure the disease? There are many possible mechanisms: for example, if these operations act directly on neurons, they can increase neuronal resistance and make neuronal survival rate increase in disease models; if these operations act directly on glial cells, glial cells may enhance neuronal survival rate in disease models by secreting some factors; maybe they still act on a small subpopulation of glial cells with stem cell properties. More experiments are needed to support this in the future.

6. Conclusion

The basic consensus on knocking down PTBP1 is that knocking down or knocking out PTBP1 in glial cells does not turn them into neurons. However, Prof. Fu speculates that there may be a small group of precursor cells in which PTBP1 acts, and he is expected to come up with strong evidence in the future.

After listening to Prof. Gao's report, similar to the news provided by Prof. Zhang, as shown in the attached figure, many strains of mice labeled with glial cells have different specificities, and the same strain of mice are said to be labeled with different specificities in different brain regions, which should be carefully identified when used.

Regarding the overexpression of NeuroD1, after listening to Prof. Chen's report, he confirmed that "overexpression of NeuroD1 in microglia does not convert them into neurons" (Prof. Peng's study), and using mice with genealogical tracing, he found that some glial cells could be converted. Because no newer and more definitive data were provided (e.g., identification of the spectral-tracked mice, then manipulation, imaging, and later verification of these cell molecular markers if they are not efficient, etc.), and in the spectral-tracking experiments the efficiency was low with more data available needed to support it in the future, the original view is retained (add: the spectrum tracking experiments show that viruses overexpressing NeuroD1 do directly mislabel a lot of neurons, leading to an inflated conversion rate, which must be carefully verified).

In the somatic case, overexpression of NeuroD1 in glial cells can convert glial cells into neurons, but there are high and low efficiencies, depending on the brain region, the virus, and the virus concentration. When the efficiency is very low probably the majority of classical glial cells cannot be converted into neurons. It is hopeful that there will be more experimental support in the future. Another direction is actually also important: whether overexpression of NeuroD1, or knocking down

PTBP1, has a neuroprotective, therapeutic effect on disease, is also expected, especially expected: more laboratories to come up with strong evidence. Because that is the ultimate goal of manipulating neural cells.

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