LETTER TO THE EDITOR

Tumor cells undergoing direct lineage conversion to neurons: unnatural but useful?

Dear Editor,

In 2011, Son et al. (2011) reported that the forced expression of selected transcription factors is sufficient to convert mouse and human fibroblasts into induced motor neurons (iMNs). The authors used three factors (Ascl1, Brn2, and Myt1l) to convert fibroblasts into neuronal-like cells. After confirming that the cells had neuronal morphology, but with absence of motor neuron markers, eight candidate transcription factors were added, which participate in various stages of motor neuron specification. As expected, a significant number of motor cells emerged with known characteristics of cultured embryonic motor neurons. These cells displayed the expected electrophysiological properties and formed functional synaptic connections with myotubes. After transplanting motor neurons into the developing chick spinal cord, the authors observed that iMNs engrafted into the ventral horn of the spinal cord in locations where endogenous motor neurons were found. Importantly, Son et al. also pointed out three limitations of their study. First, with present techniques only small numbers of neurons can be generated and synapse formation is also limited. Second, iMNs are heavily dependent on the level of brain-derived neurotrophic factor (BDNF). Indeed, if there is an insufficient amount of BDNF in growth media the survival rate of iMNs is negligible. Third, and most importantly, most primary cells were collected from fetuses, which limits the clinical application of the findings reported.

In 2012, Zhao et al. (2012) showed that the combination of three transcription factors Ascl1, Brn2, and Ngn2, could efficiently convert human glioma cells to functional neurons. Similar to functional neurons, Ascl1, Brn2, and Ngn2 induced neurons (ABN iN) derived from glioma cells expressed multiple neuronal markers and voltage-gated functional membrane channel proteins, and fired action potentials. During the conversion process, the proliferation of human glioma cells was greatly suppressed both *in vitro* and *in vivo*. Further, the iN cells did not grow over time if they were successfully induced and obtained from ABN infected human glioma cells. However, it has been shown that the conversion efficiency of iN cells is higher (20–40%) from primary human glioma cells compared with iN cells from primary human fibroblasts (less than 5%) (Pang et al., 2011). The iN induction time using human glioma cells is short (less than 2 weeks), whereas the induction time using human fibroblasts is longer. These results suggest that glioma cells may maintain more active transcriptional networks and are more easily induced to become neurons. Future studies should focus on increasing induction efficiency and optimizing culture conditions for neuronal maturation.

Based on these previous findings, we studied whether melanoma cells could be induced to become neuronal-like cells and have achieved the following three objectives using lineage reprogramming technology: (1) induction of melanoma cells into neural progenitor cells by transfection of Ascl1, Brn2, Myt1l, and Ngn2 (ABMN) transcription factors: (2) simultaneously promoting the maturation of induced cells through the neuronal lineage by addition of human BDNF: (3) taking into consideration overlapping portions of the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathways between human BDNF and melanoma genes, constructing a new type of induced human neural progenitor cell from human melanomas, which secretes BDNF continuously, as a potential cellular therapy to avoid the limitations of stem cell modification. Thus, after successfully acquiring the 5-transcription factor group of cells we added the human BDNF gene into induced cells. Through RT-PCR, human BDNF was greatly expressed for 4 weeks and almost 55 times greater compared with another three groups without the BDNF gene. Compared with previous successful experiments, our approach here created a new way to format human neurons and to potentially cure traditional tumors, and laid the foundations for investigating human diseases, examining human-specific genes, and drug screening. In future experiments, we would like to investigate methods to detect and control tumorigenic quality, while retaining cell viability and inducible capability. We hope that this new kind of human-induced neural cell from human melanomas that secretes BDNF continuously will be a potential clinical cellular therapy.

The major factor limiting our understanding of many human brain disorders is arguably the lack of human neurons suitable for experiments. Thus, an ever increasing number of researchers are attempting to develop effective methods to generate neurons with region-specific phenotypes and mature functional properties. After the limitations of fibroblast-neuron generation were revealed, improvements in the strategies by which tumor cells are induced into neural progenitor cells may allow for further study of the unknown part of the PI3K/Akt pathway, while supplying new ideas for treatment of malignant tumor cells and exploring potential clinical value.

Generating neurons from fibroblasts became a reality with the recent development of induced pluripotent stem cell (iPS) technology. Transcription factor-mediated conversion of fibroblasts to neurons appears to be reliable and reproducible among several labs. By reprogramming somatic cells using over-expression of transcription factors, it is possible to generate pluripotent stem cells that can be differentiated into any somatic cell type including various neuronal subtypes. By over-expressing only three transcription factors (Mash1, Nurrl, and Lmxla) in fibroblasts, Caiazzo et al. (2011) found that fibroblasts could be converted into dopaminergic neurons efficiently and

rapidly, without going through a pluripotent or a progenitor cell stage. The induced dopamine neurons expressed characteristics of functional dopamine neurons, including the expression of a marker-positive gene, and characteristic electrophysiological and biochemical properties. Future research must investigate appropriate conversation factors to definitively characterize dopamine neurons. Moreover, although these single dopaminergic neurons showed secretary function and regional specificity, it is still unclear whether these cells are capable of generating viable and operational neuronal synapses.

The need to find clinically relevant and feasible experimental options directed us to search for a new kind of cell, which could remain viable after lentivirus transfection. According to available literature and clinical requirements, primary cells undergoing lineage conversion need to meet four basic and two clinical conditions: (1) Cellular source is readily available and viable. (2) Cells must withstand repeated transfection with multiple genes, and enough must survive for use in downstream experiments. (3) After introduction, more single target pedigrees should be obtained. (4) It must be possible to simulate the original physiological environment of the cells. A criticism of stem cells is that they ignore the potential ability to create the appropriate physiological environment, despite their ability to restore patients' own cells. Experimentally transfected cells could secrete high levels of nerve growth factors for a sufficient period to reduce the dependence of exogenous addition to media, and therefore, create favorable conditions for *in vivo* use. (5) For clinical application, induced cells should not cause "malignant" tumors. (6) Finally, adult cellular sources are better than fetal cellular sources in avoiding primary clinical deficiency. With regard to the fifth condition, it is a very broad premise that primary or induced cells with "tumorigenic characteristics" are not contradicted with the other five conditions before reaching clinical application. However, cells with "tumorigenic characteristics" are consistent with the second and sixth conditions.

BDNF plays a key role in the fourth condition above. BDNF is essential for promoting the survival of neurons in the central nervous system. A variety of *in vivo* experiments have confirmed that BDNF can protect neurons from various injuries. This effect is achieved by binding of BDNF to its specific receptor TrkB. Although many intracellular signaling pathways are involved in the neuroprotective effect of BDNF, the mitogen-activated protein kinase (MAPK)/extracellular-regulated protein kinase (ERK), cAMP-response element binding (CREB) protein, and the PI3K/Akt survival pathway in neurons are essential. Pencea et al. (2001) showed that similar to its anti-glutamate-induced neuronal apoptosis effect *in vitro*, BDNF protects against excitatory amino acid neurotoxicity, mainly through the PI3K/Akt and Ras/MAPK pathways. Xia et al. (2010) also showed that BDNF can protect neurons from apoptosis, mediated through ERK and PI3K pathways.

However, only PI3K inhibitors can inhibit the survival of neurons by reducing BDNF and insulin-like growth factor 1 (IGF-1) concentration, but ERK inhibitors do not. Neurite growth is considered a critical event in neuronal development, synapse formation, and neural regeneration. Pharmaceutical control of neurite growth is of great interest clinically. Huttner et al. (1983) showed that BDNF could promote neurite outgrowth of neural stem cells (NSCs). Based on this observation, it may be hypothesized that introduction of the BDNF gene into melanoma cell-induced neural progenitor cells (MC-iNPCs) may promote MC-iNPC differentiation with respect to synapse formation. To further study neural network formation, we investigated the expression of biomarkers of MC-iNPC differentiation. Thus, the PI3K/Akt pathway plays a decisive role in the promotion of neuronal survival. However, PI3K/Akt is also the most common activation pathway in human cancer cells (Osaki et al., 2004). With regard to the first and third conditions above, skin-derived tumor cells are candidate screening targets. Malignant melanoma (MM) is a malignant skin tumor with high incidence and mortality. PI3K/Akt activation is observed in most melanoma tumor cell lines, as well as in tissue samples, at different stages of tumor progression (Huang and Houghton, 2003). It has been reported that the PI3K/Akt signaling pathway plays an essential role in preventing apoptosis and promoting survival of MM cells. In addition, PI3K/Akt is involved in the migration, adhesion, angiogenesis, and degradation of the extracellular matrix in MM (Qian et al., 2004). Most importantly, MM originates in neural crest tissue, which is similar to nerve tissue.

The mechanisms that drive conversion of fibroblasts into neurons have been explored. Brn2, Ascl1, and Myt1l (BAM) transcription factors induce rapid and global transcriptional changes in fibroblasts, but the mechanisms are poorly understood. Potentially, the mechanisms may include the activation of silent genes induced by conversion factors in a step-by-step manner. Exogenous factors may signal the transition between active and silent states, but the majority of markers may remain on standby at any given time point. During the early period of cell division, there may be a short period available for exogenous genes to combine into cell chromosomes. In the reprogramming process of human fibroblast-derived cells, achaete-scute homolog 1 (Ascl1) acts as a "target-specific pioneer factor" (Wapinski et al., 2013) (on-target pointer factor). Compared with the combination of Ascl1, Brn2, and Myt1l (ABM) transcription factors, there were no significant differences in the levels of neuronal nuclear antigen (NeuN), microtubule-associated protein 2 (MAP-2), and β-tubulin III between single Ascl1 and ABM groups. However, single Ascl1 is not sufficient to induce fibroblasts into mature neurons. Additionally, Brn2 or Myt1l alone cannot complete the induction process and only play a role in the subsequent maturation of neurons and in subtype differentiation. Myt1l is expressed throughout the central and peripheral nervous systems in early post-mitotic neurons.

Interestingly, the Xenopus ortholog of the Myt1l family of transcription factors, X-My1, is required for proper neuronal differentiation and synergizes with proneural basic helix-loop-helix (b-HLH) transcription factors to promote ectopic neurogenesis in the ectoderm. In fetal fibroblast cells, Ascl1 alone was sufficient to induce a neuron-like phenotype including expression of MAP2 and βIII-tubulin without a significant reduction in conversion efficiency. Moreover, neither Brn2 nor Myt1l alone resulted in the formation of a substantial number of human-derived iN cells. However, in the process of reprogramming human melanoma cell lineages we set up a special Myt1l group, because of the need for cellular screening. Cells of the single Myt1l group screened by doxycycline have a tendency to form axons. Myt1l alone was associated with high expression of several neural specific markers such as NeuN, MAP2, SYN1, and TUBB3, and expression was maintained for approximately 5 weeks. However, whether Myt1l can act as an "on-target" pioneer factor requires further investigation.

In vivo, NSCs exhibited similar differentiation potential to *in vitro* experiments, and attenuated motor dysfunction after traumatic brain injury (Riess et al., 2002). NSCs generated many cholinergic cells and a few GABAergic neurons, but astrocytes were rarely observed, similar to *in vitro* findings. Moreover, umbilical cord mesenchymal stem cell transplantation improved neurological function and self-care in patients with traumatic brain injury (Wang et al., 2013). However, it is unclear whether tumor cells undergoing direct lineage conversions can be used as a nonconventional method to treat chronic disease. In the next decade, a variety of induced pluripotent stem cells and reprogrammed cells of human origin will move further into the public domain. The question remains as to how medical professionals can use these new systems to create effective treatments against different types of diseases as early as possible for clinical application. Lineage reprogramming technology can be used to create more homogeneous cell subtypes. Reprogrammed cells based on patient samples have been used to study human neuropsychiatric, blood, and cancerous diseases. We hope to gain new knowledge and methods through basic research for the prevention and treatment of such diseases. Choosing appropriate transcription factors will allow researchers to induce an excitatory neuronal subtype with regional specificity. However, more work is required in this area to achieve clinical relevance.

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