npg

RESEARCH HIGHLIGHT

Induced neural stem cells: a new tool for studying neural development and neurological disorders

Guang-Hui Liu[1, *], Fei Yi[2, *], Keiichiro Suzuki[2], Jing Qu[1, 2], Juan Carlos Izpisua Belmonte[2, 3]

[1]National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China; [2]Gene Expression Laboratory, Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA; [3]Center for Regenerative Medicine in Barcelona, Dr. Aiguader 88, 08003 Barcelona, Spain

Recent advances in the generation of multipotent and expandable induced neural stem cells are exciting. They not only hold great promises for potential clinical applications but may also open up a new era for neural stem cell research in the near future.

Cell-based therapy for treating neurodegenerative diseases is a primary goal in regenerative medicine research that attracts tremendous attention. Advances in the direct lineage conversion of somatic cells into induced neurons (iNs) *in vitro* provides an interesting alternative to induced pluripotent stem cell (iPSC) based disease modeling. However, the innate limitations of iNs may restrict their applications in the clinic. Very recent publications describe the direct conversion of mouse somatic cells into induced neural stem cells (iNSCs) [1-6]. Together with other reports [7, 8], they constitute the basis for a rising concept that priming and retaining reprogrammed cells into an expandable neural progenitor stage may render them to be more amenable and with great

potential for both disease modeling and possible therapeutic treatment for neurological diseases.

Whereas the general concept is similar among these studies, they differ in methodology (Table 1). In 2011, Kim *et al*. [1] first reported direct reprogramming of mouse fibroblasts to neural progenitors. Using a doxycycline (dox)-inducible secondary mouse embryonic fibroblast (MEF) system, the authors found that transient induction of four Yamanaka factors reprogrammed fibroblasts into iNSCs under neural inductive conditions. These iNSCs homogenously expressed the neural stem cell (NSC) marker Pax6 and neural rosette marker PLZF. Moreover, they efficiently differentiated into mature neurons and astrocytes *in vitro*. However, the iNSCs generated by Kim *et al*. exhibited limited proliferation potential, which likely resulted from an unstable intermediate state induced by the four "Yamanaka factors". Since then, multiple studies have been carried out aiming to improve the methodology for expandable iNSCs. Based on a screening that was successful in generating of iNs, Sheng *et al*. [4] conducted a reprogramming study using a selected pool of Sox2/c-Myc/Klf4 together with another 6 neural inductive factors. The authors demonstrated that 9 factors sufficiently converted mesoderm-origin mouse sertoli cells

into iNSCs colonies. These iNSCs were multipotent *in vitro*, and could be efficiently expanded as either a monolayer or neural spheres. Interestingly, none of the 9 factors, except for Sox2, was dispensable for the conversion. Remarkably, when transplanted into the mouse brain, the iNSCs successfully grafted and committed to neural lineages *in vivo*. Importantly, on the issue of iNSCs safety, no signs of tumorigenesis were observed post transplantation *in vivo*. Later, the same strategy was successfully applied to mouse tail tip fibroblasts and generated iNSCs with neurogenesis potency both *in vitro* and *in vivo,* which further confirmed the feasibility of this method [6]. Meanwhile, Wernig and colleagues also reported a similar screening of 11 factors using a Sox2-EGFP reporter MEF system, and found that as few as two factors, FoxG1 and Sox2, were able to generate self-renewing, bipotent iNSCs [3]. When Brn2 was added to the combination, tripotent iNSCs, which gave rise to functional neurons, astrocytes as well as oligodendrocytes, were obtained [3]. Most recently, appearing side by side, Han *et al*. [2] and Thier *et al*. [5] further investigated the methodology of direct conversion of mouse fibroblasts into expandable, multipotent iNSCs. Han *et al*. [2] reported that the combination of Brn4+Sox2+Klf4+c-Myc, with the

*These two authors contributed equally to this work.
Correspondence: Guang-Hui Liu[a], Juan Carlos Izpisua Belmonte[b]
[a]E-mail: ghliu@ibp.ac.cn
[b]E-mail: belmonte@salk.edu, izpisua@cmrb.eu

Table 1 iNSCs generation and characterization

	Kim et al., 2011 [1]	Sheng et al., 2012 [4]	Sheng et al., 2012 [6]	Lujan et al., 2012 [3]	Han et al., 2012 [2]	Thier et al., 2012 [5]
General strategy	Dox-inducible secondary MEFs of 4 reprogramming factors	Infection of retrovirus encoding 9 TFs (including Sox2, c-Myc and Klf4) in mouse primary Sertoli cells	Infection of retrovirus encoding 9 TFs (including Sox2, c-Myc and Klf4) in mouse postnatal TTFs	Infection of dox-inducible lentivirus encoding 11 TFs in Sox2-EGFP MEFs	Infection of retrovirus encoding 11 TFs (including Sox2, c-Myc and Klf4) in MEFs	Infection of retrovirus encoding Sox2, Klf4 and c-Myc, together with dox-inducible lentiviral Oct4 in MEFs
Starting cells	MEFs	Mouse Sertoli cells	Mouse TTFs	MEFs	MEFs	MEFs
Inductive TFs	Oct4+Sox2+Klf4+c-Myc (3-6 days dox-induction)	Ascl1+Ngn2+Hes1+Id1+Pax6+Brn2+Sox2+c-Myc+Klf4 (Sox2 is dispensable)	Ascl1+Ngn2+Hes1+Id1+Pax6+Brn2+Sox2+c-Myc+Klf4 (Sox2 is dispensable)	FoxG1+Sox2+(Brn2, dispensable)	Brn4/Pou3f4+Sox2+Klf4+c-Myc+(E47/Tcf3, dispensable)	Sox2+Klf4+c-Myc plus restricted Oct4 (5 days with dox-induction)
Delivery method	Lentivirus	Retrovirus	Retrovirus	Lentivirus	Retrovirus	Retrovirus and lentivirus
Inductive medium and cytokines	Neural induction medium contains FGF2, FGF4 and EGF	N2B27 neural induction medium contains FGF2 and EGF	N2B27 neural induction medium contains FGF2 and EGF	N3 neural induction medium contains FGF2 and EGF	N2B27 neural induction medium contains FGF2 and EGF	N2 neural induction medium contains FGF2 and EGF
Emergence of iNSCs colony	Pax6+ iNSCs colonies appear 8-9 days in neural induction medium	iNSCs colonies appeared 3 days after neural induction	iNSCs colonies appeared 3 days after neural induction	Sox2-EGFP+ colonies firstly observed 13 days by FoxG1+Sox2 induction	NSC-like colonies obtained 4-5 weeks after infection	Neurosphere-like iNSCs colonies obtained 11 days after infection
Efficiency	0.5%-0.68% NSCs-like colonies	0.001%-0.002% NSCs-like colonies	0.001%-0.002% NSCs-like colonies	5.0% cells were EGFP+ at day 25 by Sox2+FoxG1 induction	0.004%-0.01% NSC-like colonies by 5F; 0.002%-0.006% NSC-like colonies by 4F	Up to 0.008% neurosphere-like iNSC colonies
Expansion capacity	Lost within 3-5 passages	> 25 passages	--	> 20 passages	> 130 passages	> 50 passages
Silence of transgene	(Lentiviral genes induced for 3-6 days only)	Retroviral Pax6 silenced in 9F iNSCs; retroviral Id1 silenced in 8F iNSCs	--	--	All transgenes silenced in 4F iNSCs; c-Myc and E47 were silenced in 5F iNSCs	All transgenes silenced in iNSCs
Electrophysiology	Recorded	Recorded	Recorded	Recorded	Recorded	Recorded
Microarray analysis	--	iNSCs closely resemble control NSCs and clearly differ from parental Sertoli cells in global gene expression	--	--	iNSCs closely resemble control NSCs and clearly differ from parental fibroblasts in global gene expression	iNSCs closely resemble control NSCs and clearly differ from parental fibroblasts in global gene expression

Table 1 iNSCs generation and characterization (continued)

	Kim et al., 2011 [1]	Sheng et al., 2012 [4]	Sheng et al., 2012 [6]	Lujan et al., 2012 [3]	Han et al., 2012 [2]	Thier et al., 2012 [5]
In vitro **multipotency**	Neurons and astrocytes	Neurons, astrocytes and oligodendrocytes	Neurons, astrocytes and oligodendrocytes	Neurons and astrocytes (FoxG1+Sox2 iNSCs); neurons, astrocytes and oligodendrocytes (FoxG1+Sox2+Brn2 iNSCs)	Neurons, astrocytes and oligodendrocytes	Neurons, astrocytes and oligodendrocytes
In vivo **transplantation**	--	iNSCs grafted and committed to mature neurons *in vivo* 4 weeks post transplantation	iNSCs grafted and committed to mature neurons *in vivo* 4 weeks post transplantation	FoxG1/Brn2 iNSCs were able to differentiate into oligodendrocytes *in vivo* 10 weeks post transplantation	iNSCs grafted and committed to all neural lineages *in vivo* 2 weeks post transplantation	iNSCs grafted and committed to neural lineages *in vivo* 2 weeks post transplantation
In vivo **tumorigenesis**	--	No sign of tumorigenesis 4-6 weeks post transplantation	No sign of tumorigenesis observed	--	iNSCs did not generate teratomas *in vivo*	--
Epigenetic commitment	Sox1 promoter showed bivalent chromatin, H3K4 robustly activated on day 8; Oct4 promoter persistently marked by H3K27	--	--	--	The second intron of Nestin was completely unmethylated in 4F and 5F iNSCs, the same as in control NSCs, but heavily methylated in fibroblasts	--

Abbreviations: Dox, doxycycline; MEFs, mouse embryonic fibroblasts; TFs, transcription factors; NSCs, neural stem cells; iNSCs, induced neural stem cells; TTFs, tail tip fibroblasts.

optional inclusion of E47, was sufficient to generate multipotent iNSCs, which could be maintained for more than 130 passages. Similar to Kim *et al.*'s finding, Thier *et al.* [5] reported that, by slightly manipulating expression of four Yamanaka factors (constitutively inducing Sox2, Klf4 and c-Myc while transiently priming with Oct4), neurosphere-like colonies could be obtained and expanded to multipotent iNSCs. Established iNSCs from both studies exhibited similar patterns of global gene expression when compared to normal NSCs and were able to undergo neural differentiation *in vivo* [2, 5].

There are several obvious advantages of obtaining expandable iNSCs instead of fully committed iNs or iPSC-derived NSCs. First, neurons directly converted from fibroblasts are not suitable to be used for cell transplantation due to their high heterogeneity and terminal differentiation. The iNSCs studies indicate the feasibility of generating a pure cell line potentially transplantable. Taking Han *et al.*'s study as an example, the iNSCs established are a homogenous cell population derived from a single colony [2]. Having multiple iNSCs lines would allow the opportunity to choose the best and most appropriate clones for both *in vitro* and *in vivo* studies. A pure population of iNSCs can also be more readily used for genome-wide analysis, such as DNA methylation, histone modification, microarrays, proteomic analysis, pull-down experiments, *etc.*, which, similar to iPSCs, will provide an ideal platform for basic research. Moreover, iPSCs studies indicate that long-term *in vitro* culture may cause accumulated chromosomal abnormalities [7]. Generation of iNSCs is not only faster but also bypasses the "induced pluripotency" stage, which may help to reduce risks of tumorigenesis. Furthermore, direct generation of homogenous iNSCs avoids the potential contamination of carcinogenic iPSCs.

Second, iNSCs have been proven to be capable of self-renewal. Most iNSCs

established by reported methods are expandable, which provides the possibility of generating a large amount of cells at the neural progenitor stage for subsequent differentiation or manipulation. In contrast, iNs directly converted from fibroblasts are terminally committed neurons that are not expandable [9]. On the other hand, the *in vitro* cultured patient iNSCs can be used to study the specific phenotypes of disease-affected NSCs, which are impossible to obtain from patients directly. In addition, the self-renewal activity of iNSCs enables the use of non-integrative reprogramming carriers. To date, almost all of the reported iNs are generated with lentiviral vectors, which may cause genome instability. Although viral vectors are also used for gene delivery in current iNSCs studies, it has been found that the retrovirus-mediated transgenes were frequently silenced in established iNSCs, resembling iPSCs (Table 1). The fact that iNSCs are self-renewable also provides the possibility of using episomal vectors [10], which would be diluted and ultimately eliminated as cells continuously divide during reprogramming.

Third, iNSCs have the potential to eliminate aging-related hallmarks from the original somatic cells. It has been found that reprogramming of fibroblasts into iPSCs resets cell's clock, thus generating "rejuvenated" cells endowed with several advantages for both basic research and therapeutic applications [11]. In contrast, direct conversion of fibroblasts into neurons is unable to remove the aging marks of original cells. As post-mitotic cells, neurons do not divide, and therefore they cannot dilute the toxic proteins through cell cycle progression. In this regard, the iNs directly converted from aged or diseased fibroblasts would, in principle, retain the toxic or pathogenic metabolites. For example, fibroblasts of Alzheimer's disease patient bear increased Aβ protein, which would likely be retained or even amplified

in iNs directly converted from them [12]. Since iNSCs regain the capability to quickly proliferate, the aging hallmarks would be easily removed with increased passaging, resulting in a reset "healthy" status more suitable for transplantation. It will be interesting to further examine various aging-related parameters such as telomere length, nuclear architecture, *etc*. in the generated iNSCs.

Last, most gene editing approaches used for the human genome require clonal expansion of targeted cells. Targeted gene editing technologies have recently been successfully applied in iPSCs due to their clonal expansion capability [13-16]. However, current methodologies of direct cell lineage conversion toward terminally committed cells would not be able to provide desired cell types for gene targeting. Again, due to their self-renewal capacity, iNSCs would potentially serve well as cell carriers for gene correction to eliminate and correct pathological mutations in patient cells.

In summary, though generation of iNSCs provides additional advantages over iNs towards both basic research and clinical applications, there are several important questions that remain to be answered, including 1) Is the epigenome of iNSCs stably reset to a NSC-like status? 2) Are the retroviral vectors used completely silenced in the established iNSCs and their neural derivatives? 3) Is the iNSC reprogramming process free of genomic alterations? In fact, it has been shown that NSCs derived from human embryonic stem cells have abnormal karyotypes with long-term culture *in vitro* [17]. In this regard, safety issues would need to be carefully evaluated in iNSCs and their derivatives before cell therapy applications can be considered. At this point, perhaps the most immediate challenge in this field would be the generation of expandable, multipotent human iNSCs. Recently, successful conversion from human astrocytes to

iNSCs has been reported [18]. Though this method is less likely to be applicable in a clinical setting, due to the obvious barriers of isolating patient astrocytes, an improved strategy using more readily available human somatic resources would be desirable in the near future.

Acknowledgments

References

1   Kim J, Efe JA, Zhu S, *et al*. Direct reprogramming of mouse fibroblasts to neural progenitors. *Proc Natl Acad Sci USA* 2011; **108**:7838-7843.

2   Han DW, Tapia N, Hermann A, *et al*. Direct reprogramming of fibroblasts into neural stem cells by defined factors. *Cell Stem Cell* 2012; **10**:465-472.

3   Lujan E, Chanda S, Ahlenius H, Sudhof TC, Wernig M. Direct conversion of mouse fibroblasts to self-renewing, tripotent neural precursor cells. *Proc Natl Acad Sci USA* 2012; **109**:2527-2532.

4   Sheng C, Zheng Q, Wu J, *et al*. Direct reprogramming of Sertoli cells into multipotent neural stem cells by defined factors. *Cell Res* 2012; **22**:208-218.

5   Thier M, Wörsdörfer P, Lakes YB, *et al*. Direct conversion of fibroblasts into stably expandable neural stem cells. *Cell Stem Cell* 2012; **10**:473-479.

6   Sheng C, Zheng Q, Wu J, *et al*. Generation of dopaminergic neurons directly from mouse fibroblasts and fibroblast-derived neural progenitors. *Cell Res* 2012; **22**:769-772.

7   Panopoulos AD, Ruiz S, Izpisua Belmonte JC. iPSCs: induced back to controversy. *Cell Stem Cell* 2011; **8**:347-348.

8   Zhang W, Duan S, Li Y, *et al*. Converted neural cells: induced to a cure? *Protein Cell* 2012; **3**:91-97.

9   Yang N, Ng YH, Pang ZP, Sudhof TC, Wernig M. Induced neuronal cells: how to make and define a neuron. *Cell Stem Cell* 2011; **9**:517-525.

10   Okita K, Matsumura Y, Sato Y, *et al*. A more efficient method to generate integration-free human iPS cells. *Nat Methods* 2011; **8**:409-412.

11   Liu GH, Barkho BZ, Ruiz S, *et al*. Recapitulation of premature ageing with iPSCs from Hutchinson-Gilford progeria syndrome. *Nature* 2011; **472**:221-225.

12   Qiang L, Fujita R, Yamashita T, *et al*. Directed conversion of Alzheimer's disease patient skin fibroblasts into functional neurons. *Cell* 2011; **146**:359-371.

13   Pan H, Zhang W, Liu GH. Find and replace: editing human genome in pluripotent stem cells. *Protein Cell* 2011; **2**:950-956.

14   Liu GH, Sancho-Martinez I, Izpisua Belmonte JC. Cut and paste: restoring cellular function by gene correction. *Cell Res* 2012; **22**:283-284.

15   Soldner F, Laganière J, Cheng AW, *et al*. Generation of isogenic pluripotent stem cells differing exclusively at two early onset Parkinson point mutations. *Cell* 2011; **146**:318-331.

16   Liu GH, Suzuki K, Qu J, *et al*. Targeted gene correction of laminopathy-associated LMNA mutations in patient-specific iPSCs. *Cell Stem Cell* 2011; **8**:688-694.

17   Varela C, Denis JA, Polentes J, *et al*. Recurrent genomic instability of chromosome 1q in neural derivatives of human embryonic stem cells. *J Clin Invest* 2012; **122**:569-574.

18   Corti S, Nizzardo M, Simone C, *et al*. Direct reprogramming of human astrocytes into neural stem cells and neurons. *Exp Cell Res* 2012; **318**:1528-1541.