

Converting Human Skin Cells to Neurons: A New Tool to Study and Treat Brain Disorders?

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DOI 10.1016/j.stem.2011.08.004

Recent publications in *Cell Stem Cell* (Son et al., 2011; Ambasudhan et al., 2011), *PNAS* (Pfisterer et al., 2011), and *Nature* (Caiazzo et al., 2011; Pang et al., 2011; Yoo et al., 2011) report that functional neurons can be directly generated from human fibroblast cells without going through the pluripotent state.

In 2006, Takahashi and Yamanaka reported their groundbreaking work showing that forced expression of four transcription factors (Oct4, Sox2, Klf4, and c-Myc) could revert fully differentiated somatic tissues to embryonic stem cell (ESC)-like pluripotent cells, so called “induced pluripotent stem cells (iPSCs)” (Takahashi and Yamanaka, 2006). Their work, and that of others after them, opened the door to the generation of patient-specific cells for use in disease mechanism studies, drug screening, and future personalized medicine. At the same time, this cellular alchemy emphasized that somatic cells retain broad cellular plasticity that can enable fate changes, under appropriate circumstances, and raised the question of whether directed transdifferentiation might be a more tenable goal than previously imagined, for instance, beyond germ layer boundaries. In this light, Wernig and his colleagues demonstrated that three neuronal lineage-inducing transcription factors (Brn2, Ascl1, and Myt1l; BAM) could efficiently convert mesodermal mouse fibroblasts into induced neuronal cells (iNs) (Vierbuchen et al., 2010). The resulting ectodermal neurons displayed authentic functions such as expression of neuron-specific genes, firing of action potentials, and formation of functional synapses. However, strangely, the majority of iN cells resembled cortical neurons and exhibited excitatory traits. In contrast, GABAergic neurons were sparse and other neurotransmitter types (like DA neurons) were undetectable, suggesting that additional factors may be necessary for conversion to diverse neuronal subtypes. Furthermore, it is unclear whether the same recipe can convert human cells into iNs.

Now, six independent groups demonstrate that varied combinations of factors can indeed convert human fibroblasts not only into human iNs (hiNs), but also into specific neuronal subtypes such as dopamine (hiDAs) and motor neurons (hiMNs) (Table 1). First, Pfisterer et al. (2011) tested the same BAM factors and found that both embryonic and postnatal human fibroblasts can be converted into hiNs with reported efficiencies of approximately 16% and 4%, respectively. While these hiNs remained immature at 23 days after conversion, they exhibited the electrophysiological properties of mature neurons after 30–32 days in culture. Similar to mouse iNs induced by the same three factors (Vierbuchen et al., 2010), these hiNs were predominantly glutamatergic and rarely GABAergic, but no other phenotypes such as DA neurons were apparent. Thus, the authors extended their approach to include a panel of lineage-specific genes involved in midbrain DA neuron development, and found that the addition of two factors (Lmx1a and FoxA2) to the BAM factors was optimal for generating hiDAs (~10% of hiNs). These hiDAs expressed other midbrain markers and generated spontaneous action potentials, both indicative of midbrain DA neurons. Wernig and colleagues also extended their original findings and tested the same BAM factors in human cells. They observed that human fetal fibroblasts were converted into immature neuronal cells within 20 days of transgene expression (Pang et al., 2011). Subsequently, they screened 20 additional factors, focusing on general neuronal induction rather than DA-lineage induction, and found that the addition of NeuroD1 (BAMN) improved the genera-

tion of Tuj1+ neuronal cells. Interestingly, the BAMN factors induced hiN generation from fetal and postnatal fibroblasts with similar efficiencies (2%–4% of cells plated). These BAMN-iNs showed mature neuronal morphology, expressed pan-neuronal genes, generated action potentials, and formed synaptic contacts. Furthermore, Fluidigm dynamic RT-PCR analysis of single hiNs showed that approximately 10% (5 out of 54 cells) were positive for tyrosine hydroxylase (TH), indicating that they might be dopaminergic. Caiazzo et al. (2011) designed a strategy to more directly promote the DA neuron fate by using combinations of inducible lentiviruses expressing the BAM cocktail with 11 DA-inducing factors to transduce mouse embryonic fibroblasts from TH-GFP transgenic mice, resulting in bright GFP+ reprogrammed cells. By varying the factors used, the authors found that the combination of Mash1/Nurr1/Lmx1a induces GFP+ cells with up to 18% efficiency. Remarkably, the majority of induced Tuj1+ cells (85%) were GFP+/TH+ and exhibited functional DA neuronal phenotypes, such as expression of midbrain DA neuron markers, electrophysiological properties, and controlled DA uptake. Furthermore, the same recipe efficiently converted human fetal fibroblasts to Tuj1+ and TH+ neuronal cells. Finally, this procedure was used to derive hiNs and hiDAs from the fibroblasts of Parkinson’s disease (PD) patients and healthy adult donors. These hiDAs coexpressed multiple midbrain DA markers, showed typical electrophysiological properties, and showed depolarization-induced DA release. Together, these studies demonstrate that it is indeed possible to generate functional

Table 1. Conversion of Human Fibroblasts into Functional Neurons

Starting Cells	Transgenes	Method	Readout	Reference
MEF, MPF	Brn2, Ascl1, Myt1l (BAM)	i-lentivirus	iN (1.8%–7.7%)	Vierbuchen et al., 2010
HEF, HPF	BAM, NeuroD1	i-lentivirus	hiN (2%–4%)	Pang et al., 2011
HEF; HPF	BAM, FoxA2, Lmx1A	i-lentivirus	hiN (4%–16%); hiDA (~10% of hiN)	Pfisterer et al., 2011
MEF; HEF; HAF	Ascl1, Nurr1, Lmx1A	i-lentivirus	iDA (18%); hiN (10%), hiDA (6%); hiN (5%), hiDA (3%)	Caiazzo et al., 2011
HPF; HAF	miR-9/9*, miR-124, Ascl1, Myt1l, NeuroD2	lentivirus	hiN (~10%); hiN	Yoo et al., 2011
HPF	miR-124, Brn2, Myt1l	i-lentivirus	hiN (4%–8%)	Ambasudhan et al., 2011
MEF; HEF	BAM, Lhx3, Hb9, Isl1, Ngn2; BAM, Lhx3, Hb9, Isl1, Ngn2, NeuroD1	retrovirus	iMN (5%–10%); hiMN	Son et al., 2011

Abbreviations: MEF, mouse embryonic fibroblasts; MPF, mouse postnatal fibroblasts; HEF, human embryonic fibroblasts; HPF, human postnatal fibroblasts; HAF, human adult fibroblasts; i-lentivirus, inducible lentivirus; iN, induced neurons; hiN, human induced neurons; iDA, induced dopamine neurons; iMN, induced motor neuron. Readout percentages reflect reported efficiencies of conversion.

hiNs and hiDAs directly from human fibroblasts, even from adult patients' tissues.

Based on their previous studies showing that miR-9* and miR-124 critically regulate neuronal differentiation through compositional changes of SWI/SNF-like BAF chromatin-remodeling complex, Gerald Crabtree and his colleagues addressed whether expression of these miRNAs can convert human fibroblasts into neurons. Remarkably, forced expression of miR-9* and miR-124 in a single lentiviral vector could convert human fibroblasts to hiNs, which was greatly facilitated by a neurogenic transcription factor NeuroD2 (Yoo et al., 2011). Further addition of Ascl1 and Myt1l enhanced the reported efficiency up to 5% of the starting cells, although no hiDAs were detected. This recipe could also convert adult human fibroblasts into functioning neurons. In addition, Sheng Ding and his colleagues combined miR-124 and two transcription factors, Brn2 and Myt1l, to directly convert both postnatal and adult human fibroblasts into functional neurons with similar efficiencies, as evidenced by mature neuronal morphology, marker gene expression, action potential firing, and functional synapse formation (Ambasudhan et al., 2011). Notably, this method yielded many GABA and glutamatergic neurons, but DA and serotonin neurons were rare or undetectable, suggesting that lineage-specific factors may be required to guide the conversion to these cell fates.

Finally, in this issue of *Cell Stem Cell*, Kevin Eggan and colleagues report that forced expression of select transcription factors can convert mouse and human fibroblasts into functional induced motor

neurons (iMNs) (Son et al., 2011). They used a similar strategy as those described above, and tested different combinations of the BAM factors with known motor neuron specification factors, using embryonic fibroblasts from an *Hb9::GFP* mouse to monitor the conversion to motor neurons. They determined an optimal combination of seven factors (Table 1), which allowed efficient conversion of mouse embryonic fibroblasts (5%–10%) to iMNs that exhibited typical morphology, gene expression patterns, electrophysiological and synaptic properties, and even in vivo engraftment capacity, similar to that seen using embryo-derived motor neurons. In addition, these MNs showed both cell-autonomous and non-autonomous sensitivities to degenerative stimuli, strongly suggesting that these iMNs would be useful for studying motor neuron disease (e.g., ALS) mechanisms. The authors also successfully applied their seven-factor cocktail, with NeuroD1, to convert human embryonic fibroblasts into functional, cholinergic hiMNs.

Collectively, these studies further advance this fast-moving field, and demonstrate that direct cell fate conversion, or transdifferentiation, can be used to generate diverse hiNs that include hiDA, hiMN, and glutamatergic neurons for use in disease mechanism studies, and potentially for future cell replacement therapies. However, this field is still in its infancy and there are many issues to be overcome. Most importantly, the molecular and/or epigenetic mechanisms underlying these cell fate changes remain to be elucidated. For example, it is intriguing that significantly different combinations of transgenes and/or miRNAs

synergistically or sometimes antagonistically lead to a distinct neuronal cell fate or fates. It remains to be seen whether these hiNs have acquired a long-term sustainable genetic program that will function both in vitro and in vivo. Furthermore, the identification of additional factors (e.g., general neuronal induction factors, specific lineage factors, and noncoding RNAs) and their optimal stoichiometric and kinetic combinations will likely improve the efficiency of specific neuronal conversion. Notably, in another study, Sheng Ding and his colleagues elegantly showed that transient expression of the four reprogramming factors efficiently reprogrammed mouse fibroblasts into neuronal progenitors that could proliferate and then differentiate into functional neurons, including DA neurons (Kim et al., 2011). While the direct conversion strategy discussed above appears to bypass the pluripotent/multipotent stem cell stages and thus may avoid the potential risk of tumor formation, it can also limit the final number of cells that can be obtained. Thus, the potential to extend the findings of Kim et al. (2011) to the conversion of human fibroblasts into expandable progenitors with minimal or no risk of tumor formation is very attractive, and should be pursued. Also, while the direct conversion studies used human fibroblasts as the starting material, it remains to be seen whether hiNs can be similarly generated from alternate cell types (e.g., lymphocytes). Last but not least, in light of recent findings that significant numbers of gene mutations and copy number variants can be induced during reprogramming (Barrialeaux and Knoepfler, 2011), it will be

critical to develop nonviral, genome non-integrating conversion methods such as direct protein delivery (Kim et al., 2009).

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FoxO: A New Addition to the ESC Cartel

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DOI 10.1016/j.stem.2011.08.003

The forkhead box O (FoxO) family is involved in diverse cellular processes such as tumor suppression, stress response, and metabolism. In a recent *Nature Cell Biology Letter*, Zhang et al. (2011) uncover a novel role for FoxO proteins in regulating the identity of human ESCs.

Embryonic stem cells (ESCs) are distinguished by the features of pluripotency (the ability to give rise to all cell types in and derived from the embryo proper) and self-renewal (the capability to be grown indefinitely in an undifferentiated state in vitro). This unique ESC state is defined by a distinct gene expression profile crafted by the ESC transcription network, wherein the trio of OCT4, SOX2, and NANOG lies at the core (reviewed in Young, 2011). The ESC state is nonetheless sustained by many other transcription factors, establishing an integrated transcription network, which, when perturbed, triggers the loss of ESC identity.

The extended pluripotency network includes numerous families of transcription factors, including the Krüppel-like transcription factors, orphan nuclear receptors, the Myc transcription factor cluster, SMAD proteins, and PR domain-containing transcriptional regulators to highlight

a few (reviewed in Gonzales and Ng, 2011). In a recent issue of *Nature Cell Biology*, Ghaffari and colleagues (Zhang et al., 2011) introduce a new component to this network of pluripotency regulators: the forkhead box O (FoxO) family (Figure 1).

FoxO proteins are transcription factors that act as stress-response tumor suppressors by blocking cell cycle progression and inducing apoptosis (reviewed in van den Berg and Burgering, 2011). FoxO proteins also induce expression of enzymes involved in protection from oxidative stress and are important for metabolic processes in liver and muscle cells.

Despite the wide scope of function of the FoxO family throughout the body, its role in early embryonic development had not yet been studied in detail. In this respect, Ghaffari and colleagues have found that FOXO1 is expressed at abundant levels in both mouse ESCs (mESCs) and human ESCs (hESCs) and is depleted

during differentiation (Zhang et al., 2011). For assessing the functional relevance of FOXO1 in hESCs, a short hairpin RNA (shRNA)-mediated knockdown experiment was performed. They observed the acquisition of an epithelial flattened morphology accompanied by a loss of pluripotency markers and an increase in endomesoderm markers. This phenotype can be rescued by immediate FOXO1 overexpression, demonstrating the specificity of the shRNA knockdown. However, when hESCs were cultured under FOXO1 knockdown conditions for a few passages, the rescue did not work. This finding indicates that FOXO1 depletion can trigger irreversible differentiation within several passages, emphasizing the importance of FOXO1 in ESCs.

Interestingly, differences were observed between mouse and human ESCs in the role of FOXO3A/Foxo3, another FoxO family member tested in