Review
Future Neurology

Overview of recent advances in the generation of human neurons and neural stem cells through reprogramming and differentiation techniques

Melanie Swan
MS Futures Group, PO Box 61258, Palo Alto, CA 94306, USA ■ Tel.: +1 650 681 9482 ■ Fax: +1 504 910 3803 ■ m@melanieswan.com

Stem cell research could have a significant near-term public health impact with applications in cell-replacement therapies (treatments are in development for over 50 diseases), disease modeling and drug discovery. Recent advances have been achieved in techniques for reprogramming somatic cells directly to neurons and neural stem cells, and in differentiating pluripotent cells to neurons and neural stem cells. Neurons and neural stem cells are of particular translational interest due to the lack of effective clinical therapies for neurodegenerative disease. This analysis reviews recent reprogramming and differentiation research advances that relate to human neuron and neural stem cell generation. While these emerging techniques are promising, some of the processes are relatively new, and the fidelity and functionality of generated neurons and neural stem cells in clinical application is yet to be confirmed.

Background

Research regarding stem cells (undifferentiated cells of a multicellular organism that are capable of giving rise, indefinitely, to more cells of the same type, and from which certain other kinds of cell arise by differentiation) and their use in medicine is a field that has been steadily advancing. However, it is still in the early phases of development and widespread clinical application is not immediately imminent. There are issues at a number of levels including whether terminally differentiated cells, which are derived through some of the newer reprogramming and differentiation techniques, are really the same as their naturally occurring counterparts, and furthermore, that no matter how derived, delivering and directing the behavior of stem cells *in vivo* is tricky and nascent. If these kinds of issues can be resolved, stem cell research and cellular therapy development could have a significant near-term impact on worldwide public health, and many individuals living today could experience stem cell-related therapies.

The main applications of stem cell research are cell-replacement therapies, disease modeling, drug discovery and drug toxicity assessment. One indication of the growth in stem cell research is the wide range of diseases (over 50) being targeted [1]. Dozens of stem cell treatments are in different stages of development ranging from clinical availability to early stage testing. Some of the diseases targeted include leukemia, HIV/AIDS, sickle cell disease, glioblastoma, Parkinson's disease, Alzheimer's disease, spinal cord injury, macular degeneration, heart disease and ischemic stroke. Another sign of the maturation of stem cell research is the contemporary focus on large-scale commercialization and the translational mechanics of implementing stem cell therapies into clinical practice. Recent research advances have been achieved in improved techniques for obtaining both pluripotent cells (stem cells that have the potential to differentiate into any of the three germ layers: endoderm, mesoderm or ectoderm, giving rise to any fetal or adult cell type) and differentiated cells, for example the emerging ability to directly reprogram somatic cells to final differentiation (e.g., cardiomyocytes and neurons) omitting pluripotency as an intermediary step.

The importance of neurons & neural stem cells

Neurodegenerative disease is a particularly relevant area for stem cell research, investigating conditions such as Parkinson's disease, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis. There are few effective clinical therapies, the cause and progression of these diseases is not well understood,

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and investigating the conditions *in vivo* is prohibitively invasive. For example, the current treatments for Parkinson's disease: levodopa, dopamine agonists, monoamine oxidase inhibitors-B inhibitors and deep-brain stimulation may be symptomatically helpful to some extent but are not curative. Likewise in amyotrophic lateral sclerosis, the best drug, riluzole, is only estimated to extend life for 3–4 months, and does not change the progression of the disease; death generally occurs 3–5 years after diagnosis as motor neuron capability is steadily lost. Stem cell research is already being used as a technique for testing hypotheses on live cells as scientists create *in vitro* laboratories for modeling both healthy and diseased cells [2]. Stem cell research is also critical in creating cellular therapeutics that may help with neurodegenerative disease. Cellular therapies are sought for neuroreplacement in several diseases, for example to replace nigral dopaminergic neurons lost in Parkinson's disease.

Principal aim of analysis

The principal aim of this analysis is to provide a general narrative and overview of research published in the last few years relating to human neuron and neural stem cell (NSC) generation. A characterization of the nature of current activity as a whole and a specific description of emerging techniques employed and results achieved is discussed so that the progress, status and prospects of human NSC generation may be seen more clearly. The paper is structured to discuss two recent and promising approaches to generating human neurons and NSCs: reprogramming somatic cells and differentiating pluripotent cells.

Methods

A review of over 25 human neuron and NSC generation studies published mainly in the last 2 years was undertaken through a literature search and analysis. The literature review consisted of first generating a list of potential published studies for inclusion by searching for the keyword terms 'neural stem cell generation, neural stem cell review, neural stem cell overview, neural stem cell, stem cell generation, dopaminergic stem cell' and other related permutations in Medline/PubMed, ISI Web of Science, Microsoft Academic Search and Google Scholar. Additional papers were selected from the bibliographies of the initially retrieved articles, and per identification of leading worldwide laboratories working in the field. Searches of the internet in general (e.g., Google) and social media (e.g., Twitter, blogs [using Google Blog Search and other tools]), Facebook, Google Plus, YouTube and Vimeo) were also conducted with the same keywords related to NSC generation. Searches were initially conducted between December 2011 and January 2012. Papers were selected for review per the inclusion criteria of human NSCs or neurons having been generated, or on the basis of seminal work that could quickly lead to progress in human cells.

Results

Reprogramming human fibroblasts directly to neurons

Reprogramming (converting cells from one type to another) is a technique that has been used for some time to erase the epigenetic marks of adult somatic cells to turn the cells back to an embryonic state. In 2006, scientists from the Yamanaka laboratory reported the ability to reprogram mouse fibroblast cells to a pluripotent state using four transcription factors (Oct4, Sox2, Klf4 and c-Myc) [3]. Since then, the successful reprogramming of somatic cells to induced pluripotent stem cells (iPSCs) has also been achieved with other and fewer transcription factors. In the last few years, an advance has been the conversion of somatic cells not just to pluripotent cells, but directly to other cell lineages, such as cardiomyocytes and neurons, bypassing the intermediate step of pluripotency [4]. Recent activity in reprogramming human fibroblast cells directly to neurons is described in Table 1 and below.

Seven teams converted human fibroblasts directly to neurons by reprogramming embryonic, postnatal and adult skin cells with a variety of canonical and novel transcription factors, and applying growth factors, miRNAs and small molecules. Transcription factors are important as they control the gene expression that maintains or determines a cell's identity. Introducing and forcing the expression of certain transcription factors influences pluripotent cells to become different kinds of downstream cell types. Transcription factors work together in interacting networks, so a number of transcription factors are required, and a precise sequence of their application may be required. Small molecules and miRNAs influence whether transcription factors are expressed or blocked by turning on or off intracellular signaling cascades. In these studies, viral transduction was typically used to generate the induced neurons (iNs). Some of the advantages of direct conversion are that it can be shorter (differentiating cells from

Table 1. Reprogramming human fibroblasts directly to neurons by study, result and technique.

Study (year)	Result	Technique	Ref.
Pang *et al.* (2011)	Human embryonic and postnatal fibroblasts → neurons	Reprogram with four transcription factors: Ascl1, Brn2, Myt1l and NeuroD1	[5]
Qiang *et al.* (2011)	Human adult fibroblasts → neurons	Reprogram with five reprogramming factors: Ascl1, Brn2, Myt1l, Olig2, and Zic1, with support factors BDNF, NT3 and GCM	[6]
Ambasudhan *et al.* (2011)	Human adult fibroblasts → neurons	Reprogram with miRNA (miR-124) and two transcription factors: Brn2, Myt1l	[8]
Yoo *et al.* (2011)	Human fibroblasts → neurons	Reprogram with miRNA (miR-9/9* and miR-124) and three transcription factors: Ascl1, Myt1l and NeuroD2	[10]
Son *et al.* (2011)	Human embryonic fibroblasts → spinal motor neurons	Reprogram with eight reprogramming factors: Ascl1, Brn2, Myt1l, Lhx3, Hb9, Isl1, Ngn2 and NeuroD1	[11]
Pfisterer *et al.* (2011)	Human embryonic and postnatal fibroblasts → dopaminergic neurons	Reprogram with three transcription factors: Ascl1, Brn2 and Myt1l for neurons, plus two genes: *Lmx1a* and *FoxA2* for dopaminergic neurons	[12]
Ladewig *et al.* (2012)	Human postnatal fibroblasts → functional neuron-like cells	Reprogram with two transcription factors: Ascl1 and Ngn2, and small molecule inhibition of SMAD signaling and glycogen synthase kinase-3β	[13]

pluripotency can take 1–2 months), and more efficient as more cells may be converted. On the other hand, some of the disadvantages include challenges in establishing the desired properties of neurons, incorrect karyotyping, reproducibility and large-scale manufacture. However, overall, direct reprogramming could be quite useful in potential translational application as a more expedient technique than alternatives as skin cells could be easily collected from patients, converted into desired lineages and transferred autologously.

Pang *et al.* converted human embryonic fibroblasts directly into neurons [5]. The team used reprogramming, first generating neuronal cells from mice fibroblasts and then from human fetal and perinatal fibroblasts. In the case of the mouse fibroblasts, three transcription factors (Ascl1, Brn2 and Myt1l) were applied to convert them into functional neurons. In the case of the human cells, the same three transcription factors plus the proneural gene *NeuroD1* were added to convert fetal and postnatal human fibroblasts into neuronal cells. The evidence for the reprogramming was the fact that the derived neuronal cells had the expected form, structure and gene expression of neurons, and generated action potentials and synaptic contacts.

Qiang *et al.* and Ambasudhan *et al.* worked with human adult fibroblasts, converting them directly to neurons. The Qiang team used five reprogramming factors: Ascl1, Brn2, Myt1l, Olig2 and Zic1, together with support factors BDNF, NT3 and GCM [6]. The three canonical transcription factors (Ascl1, Brn2 and Myt1l) used to convert mouse fibroblasts directly to neurons in foundational work by Vierbuchen *et al.* [7] were tried but were found to be insufficient, perhaps since adult fibroblasts were being converted. The three support factors employed were related to neuronal survival: BDNF, NT3 and GCM. The substantiation for having created neurons was that the generated cells displayed neuronal morphology, and expressed expected neuronal markers, including Tuj1, MAP2, Tau1, NeuN, NCAM and Neurofilament-160 kd.

The Ambasudhan team reprogrammed with miRNA (miR-124) and two of the canonical forebrain transcription regulators used by Vierbuchen *et al.*, Brn2 and Myt1l, using first human postnatal fibroblasts and then human adult fibroblasts [8]. Confirmation for having obtained neurons was the expected physical morphology, neuronal marker expression (e.g., MAP2 [a neuron-specific cytoskeletal protein]), synapse production and the ability to fire action potentials. The miRNA miR-124 was selected since it is upregulated in neurons and the most abundant miRNA found in mammalian CNSs. Other work from the Studer laboratory also confirmed the potentially influential role of miRNAs, finding that the presence of miR-371-3 may predict neuronal differentiation from human pluripotent stem cells [9]. Yoo *et al.* also employed miRNAs to convert human fibroblasts to neurons, expressing miR-9/9* (involved in gene regulation) and miR-124, along with transcription factors Ascl1, Myt1L and NeuroD2 [10].

Son *et al.* and Pfisterer *et al.* worked with human embryonic and postnatal fibroblasts,

reprogramming them directly to certain types of fully differentiated neurons: spinal motor neurons and dopaminergic neurons. Son *et al.* converted human embryonic fibroblasts into functional spinal motor neurons [11]. The reprogramming was first carried out in mouse fibroblasts, using seven transcription factors (Ascl1, Brn2, Myt1l, Lhx3, Hb9, Isl1 and Ngn2). To determine that spinal motor neurons had been created, the team evaluated the cells for properties of motor neurons, gene expression and electrophysiological profiles. A further confirmation was that the cells behaved like motor neurons upon transplantation in mice and formed the synapses and began projecting axons. To generate human motor neurons, the same seven mouse motor neuron transcription factors were employed plus *NeuroD1*. *NeuroD1* is a proneural gene that enhances the conversion efficiency of human fibroblasts into motor neurons. The team claimed that functional human motor neurons had been created since the cells had the requisite enzymatic, electrical and behavioral characteristics of motor neurons.

Pfisterer *et al.* converted human embryonic and postnatal fibroblasts directly to dopaminergic neurons, finding that the canonical Vierbuchen transcription factors (Ascl1, Brn2 and Myt1L were sufficient to reprogram somatic cells to neurons [12]. Two additional genes were then expressed to obtain dopaminergic neurons: Lmx1a and FoxA2 (factors related to dopaminergic neuron development). The evidence for having obtained neurons was morphology, neuronal marker expression profiles and electrophysiological activity. Specifically, the markers expressed were βIII-tubulin (a microtubule found nearly exclusively in neurons), MAP2 (although less so in converted postnatal cells than in embryonic cells), and synaptophysin (a marker present in synapses).

Ladewig *et al.* converted human postnatal fibroblasts directly to functional neuron-like cells [13]. The fibroblasts were reprogrammed by using two transcription factors (Ascl1 and Ngn2), and small molecules to inhibit SMAD signaling and glycogen synthase kinase-3β. The small molecules were selected since they have been used to facilitate neural differentiation from pluripotent stem cells. The team focused on the yield and purity of the generated neurons to substantiate their work, looking for the expression of expected neural markers, such as βIII-tubulin, GABA (the major inhibitory neurotransmitter in the brain) and VGLUT1 (a vesicular glutamate transporter found in glutamatergic neurons).

Transdifferentiation of mouse fibroblasts to neural progenitor cells/NSCs

As discussed in the 'Reprogramming human fibroblasts directly to neurons' section, the successful reprogramming of somatic cells from one mature lineage to another (e.g., fibroblasts to induced neurons) has been demonstrated. As a result, a contemporary research focus is examining the deployment of the same techniques to convert somatic cells to earlier precursors of terminally differentiated cells, which would then have a wider range of uses. In the neural context, so-called transdifferentiated neural progenitor cells (NPCs) would be a more versatile product of reprogramming. NPC populations could be expanded in experimental situations, and further directed to generate different kinds of neurons and glia as required. Research advances are quite recent, and have occurred mainly in mouse cells so far as described in Table 2 and below.

Four teams have shown results in reprogramming somatic cells to NPCs or NSCs (neural progenitor stem cells or NSCs) as a preliminary step to working with human cells. Kim *et al.* from the Ding laboratory converted mouse fibroblasts to NPCs with four reprogramming factors (Oct4, Sox2, Klf4 and c-Myc) [14]. Lujan *et al.* from the Wernig laboratory converted mouse fibroblasts to neural precursor cells with three reprogramming factors (Brn2, Sox2 and FoxG1) [15]. Han *et al.* from the Schöler laboratory used seven transcription factors (Brn4/Pou3f4, Sox2, Klf4, c-Myc plus E47/Tcf3) to convert mouse fibroblasts to induced NSCs [16]. Thier *et al.* from the Brüstle laboratory reprogrammed mouse fibroblasts to NSCs with four transcription factors (Sox2, Klf4, c-Myc, and Oct4; limiting Oct4 to the initial phase of reprogramming) [17]. To evaluate the success of these protocols, the different teams examined morphology, expression and other characteristics of the generated cells, and claimed that the cells could be further differentiated into neurons, astrocytes and oligodendrocytes. While the different combinations of transcription factors used is the most obvious difference in results, the protocols employed had other culturing and procedural differences since the NSCs obtained apparently show different properties in regard to proliferation capacity and multipotentiality in further differentiation.

Differentiation of human pluripotent stem cells to neural cells

Aside from reprogramming and transdifferentiation, the longer-existing approach to generating neurons and NSCs has been differentiating them

Table 2. Transdifferentiation of mouse fibroblasts to neural progenitor/neural stem cells by study, result and technique.

Study (year)	Result	Technique	Ref.
Kim *et al.* (2011)	Mouse fibroblasts → NPCs	Reprogram with four transcription factors: Oct4, Sox2, Klf4 and c-Myc	[14]
Lujan *et al.* (2012)	Mouse fibroblasts → NPCs	Reprogram with three transcription factors: Brn2, Sox2 and FoxG1	[15]
Han *et al.* (2012)	Mouse fibroblasts → NSCs	Reprogram with seven transcription factors: Brn4/Pou3f4, Sox2, Klf4, c-Myc plus E47/Tcf3	[16]
Thier *et al.* (2012)	Mouse fibroblasts → NSCs	Reprogram with four transcription factors: Sox2, Klf4, c-Myc and Oct4; limiting Oct4 to the initial phase of reprogramming	[17]

NPC: Neural progenitor cell; NSC: Neural stem cell.

from pluripotent cells, initially from embryonic stem cells (ESCs), and now also from iPSCs. Recent advances in differentiating human pluripotent stem cells to neural cells are discussed in Table 3 and below.

Seven teams differentiated human pluripotent stem cells to neural cells as described in a series of methods and discovery papers, working with both ESCs and iPSCs to generate various downstream progenitors, neural cells, neurons and terminally differentiated neurons. Ma *et al.* from the Zhang laboratory differentiated human pluripotent stem cells (ESCs and iPSCs) initially to neuroepithelia, and then to midbrain neural progenitors and functional dopaminergic neurons [18]. A chemical culturing process was used to obtain neuroepithelia, and the application of growth factors was used to obtain neural progenitors and dopaminergic neurons. Specifically, the growth factors employed were FGF8 and SHH (a regulator of organogenesis). The generated cells were evaluated for expected marker expression, electrophysiological properties, and success in rodent implantation. The Zhang laboratory has also established a protocol for the differentiation of astroglia from human pluripotent stem cells by removing mitogens and adding the growth factor CNTF [19].

Chambers *et al.* from the Studer laboratory also differentiated human pluripotent stem cells (ESCs and iPSCs) to neurons [20]. In this case, the team first generated neuroectoderm through culturing processes and the addition of growth factor BDNF and ascorbic acid, and then differentiated the neuroectoderm to postmitotic neurons by adding additional growth factors (SHH and retinoic acid for motor neurons; SHH, FGF8, GDNF, TGF-β3 and cAMP for dopaminergic neurons). Falk *et al.* from the Brüstle laboratory developed a stable protocol for differentiating neurons and glia from multiple human pluripotent (ESC and iPSC) lines in a process that included withdrawing growth factors [21].

Swistowski *et al.* differentiated human iPSCs into dopaminergic neurons, in particular, the midbrain substantia nigra A9 dopaminergic neurons that become impaired in Parkinson's disease [22,23]. First, researchers obtained NSCs by manipulating induced pluripotent cells from different kinds of somatic cells in a multistep xeno-free culturing process (i.e., all components in the cell culture medium are derived from the same organism). The evidence for creating NSCs was that the cells expressed expected markers found in NSCs (e.g., nestin, Sox1 and musashi), and did not express the markers present in further differentiated cells, that is, βIII tubulin in neurons and GFAP and O4 in glia. Then, to obtain dopaminergic neurons, growth factors (SHH and FGF8, then GDNF and BDNF) were introduced to the NSCs, again in a xeno-free culturing process. The evidence for having obtained dopaminergic neurons was that the cells exhibited gene expression profiles similar to their counterparts derived from embryonic stem cells, had an upregulated presence of certain expected markers as compared with NSCs (e.g., En1, Otx2, Lamx1b, Msx1, Nurr1 and Lmx1b) and demonstrated improvement after transplantation in rats. The proof given for obtaining the specifically sought A9 dopaminergic neurons was that an identifiable subset of the dopaminergic neurons generated expressed an expected nigral marker (Girk2).

Denham and Dottari described three different methods for converting human iPSCs to neural cells, and presented a detailed protocol they suggested could be followed by other researchers [24]. The three techniques used a growth factor (noggin), a culturing process (with the stromal [e.g., connective tissue] cell line PA6), and another culturing process (with a laminin

Table 3. Differentiation of human pluripotent stem cells to neural cells by study, result and technique.

Study (year)	Result	Technique	Ref.
Ma *et al.* (2011)	Human pluripotent ESCs and iPSCs → dopaminergic neurons	Generate neuroepithelia in a chemically defined medium; differentiate to midbrain progenitors and dopaminergic neurons with growth factors (FGF8 and SHH)	[18]
Chambers *et al.* (2011)	Human pluripotent (ESCs/iPSCs) → neurons	Differentiate to neuroectoderm through culturing processes and addition of growth factors BDNF and ascorbic acid; differentiate to postmitotic neurons by adding additional growth factors (SHH and retinoic acid for motor neurons; SHH, FGF8, GDNF, TGF-β3 and cAMP for dopaminergic neurons)	[20]
Falk *et al.* (2012)	Human pluripotent (ESCs/iPSCs) → neurons	Generate neurons and glia with growth factor withdrawal	[21]
Swistowski *et al.* (2010)	Human iPSCs → dopaminergic neurons, particularly A9	Generate NSCs: multistep xeno-free culturing process Generate dopaminergic neurons: culture NSCs with growth factors (Shh and FGF8; then GDNF and BDNF)	[22]
Denham *et al.* (2011)	Human pluripotent (ESCs/iPSCs) → neural induction	Treat with growth factor (noggin) and use culturing processes with stromal cell lines, laminin substrate and neural induction media	[24]
Karumbayaramb *et al.* (2012)	Adult human fibroblasts → iPSCs → neurons	Generate iPSCs: multiphase xeno-free culturing and reprogramming process Generate neurons: multiphase culturing process with addition of growth factors (retinoic acid and B27)	[25]

ESC: Embryonic stem cell; iPSC: Induced pluripotent stem cell; NSC: Neural stem cell.

substrate and neural induction media). The proof given for generating neural cells was that cells produced from all three methods expressed expected NSC markers (Pax6, Sox1 and Sox2). The NSCs were further differentiated into neurospheres and then to neurons, glia and neural crest cells with a culturing process. The proof claimed for the final differentiation was the presence of expected markers, such as S100β, βIII tubulin and p75.

Karumbayaramb *et al.* converted human adult skin cells to pluripotency and then to neurons [25]. The team's focus was on creating a process for industrial-volume throughput with commercially available materials as opposed to new basic research findings. In the first step, that is, generating pluripotent stem cells, fibroblasts were obtained from skin biopsies and then cultured and reprogrammed. In the second step, which involved generating NPCs, neurons and glia, the pluripotent stem cells underwent a phased culturing process with commercially available media and growth factors, such as retinoic acid and B27 (a supplement-related hippocampal neuron growth). Evidence for obtaining pluripotent cells was that the cells were morphologically as expected, and exhibited the correct gene expression profile for standard markers (Oct4, Nanog and Sox2), and human pluripotent stem cell surface markers (TRA1-81 and SSEA3). The proof given for generating neural cells was the same: the cells were morphologically similar to their naturally occurring counterparts and expressed expected markers (Sox2, Msi2 and nestin). Some of the novel aspects that could lead to fast commercialization were using nonanimal-derived reagents, technology-aided reprogramming and good manufacturing practices (GMPs).

There are two other research efforts related to human mesenchymal stem cells (MSCs) and tissue. Ma *et al.* generated NSC-like cells from human MSCs taken from bone marrow [26]. The technique used included culturing the MSCs in a medium of human NSCs. Support for generating NSC-like cells was the further differentiation capacity of the cells, purportedly into neurons, astrocytes and oligodendrocytes given the presence of certain expected markers, and into neurons and glia through live transplantation into mice. In addition, the generated cells exhibited the appropriate electrical properties for creating action potentials. Lu *et al.* produced NSCs from Down's syndrome human fetal cortical tissue [27]. The technique used was obtaining human NSCs and growing them in culture with growth factors and proteins. It was suggested that neural cells had been generated since the cells could be further differentiated into neurons, astrocytes and oligodendrocytes.

Conclusion

Discussion & critical evaluation

While the fast pace and degree of useful scientific results is noticeable in using reprogramming and differentiation to generate human neurons and NSCs, there are simultaneously concerns

that need to be addressed in the future. These concerns can be organized into four areas:

- The early stage and risks of techniques for generating human neurons and NSCs;
- The potential need for further characterization of underlying phenomena;
- The need for establishing industry-wide standards;
- The steps needed to progress to translational therapies.

First, regarding the early stage and risks of the techniques for generating human neurons and NSCs, a central claim is that these cells may not be the appropriately exact equivalents of their naturally occurring counterparts [28]. It is unknown whether generated cells may be functionally equivalent to their counterparts, and what the full impact of their behavior may be over time. Additionally, there is concern about the risk of teratoma (in the case of pluripotent cells) and cancer or overproliferation (in the case of NSCs), which needs to be better characterized, understood and prevented prior to translational application. Particularly regarding reprogramming, its foundational mechanisms are not yet understood [29], which contributes to some of the challenges that arise, such as cases of genomic instability [30], DNA rearrangement [31] and harmful epigenetic carryovers [101]. However, technological advances are helping to investigate these claims; in one case a DNA-error-mapping technique was used to tally the number of DNA mutations found in an induced cellular population, and found that few mutations had occurred [102]. Furthermore, even after considering the different issues with derivation source, neurons and NSCs are difficult to deliver, manipulate and measure in live implantation situations.

One approach to resolving these issues would be to have rigorous standards for measuring different cell parameters and how they are maintained over time. The most straightforward areas for standards would include cell morphology, marker expression levels, genetic and epigenetic profiles, and electrophysiological properties. Standards-based measurement techniques could be used in both naturally occurring and generated cells. In addition to characterizing generated cells within standard parameters, what is further needed is to determine the true function of generated cells. Some of these efforts are underway but could be applied more comprehensively. Determining cell function could be accomplished by identifying the region and subtype specificity at the single-cell level and comparing gene expression profiles to counterpart cells. In the case of neurons, function could be assessed by the ability to form action potentials and synaptic competence by electrophysiology. Furthermore, generated cells could be transplanted in order to demonstrate these properties *in vivo*. In the case of NSCs this is even more challenging since astrocytes and oligodendrocytes differentiated from the generated NSCs also need to be tested for functionality (i.e., myelination and support of synapse formation).

A second point is that further characterization of the underlying phenomena of neuron and NSC generation may be needed. Research is continuing to elucidate these areas. For example, regarding the downstream differentiation of NSCs into neurons and glia, researchers evaluated the electrical properties of human and mouse NSCs, and found that as opposed to what was previously thought, membrane capacitance, rather than membrane conductance, was a critical data point in predicting neuronal generation from NSCs [32]. This technique could be useful in distinguishing neurogenic and gliagenic cells in derived stem cell populations where there is currently a dearth of mechanisms for identifying and separating cells. In other research, mathematical modeling was employed to offer a more complete understanding of the functioning of the Delta1/Notch1 pathway, which is involved in cell differentiation [33]. The pathway operates through a series of signaling steps beginning with cell-surface markers Delta1/Notch1, and finishing with the upregulation of *Hes1* and the downregulation of *Mash1*, a gene that promotes neuronal differentiation. Mathematical modeling provided more detail regarding the final Hes1 and Mash1 steps, and was tested through computer-based simulation. The conclusion was that the speed of Hes1 and Mash1 degradation and the presence of another molecule (Mash1-E47) had the most influence on neural differentiation. In another project, molecular signaling pathway studies regarding dopaminergic differentiation (Wnt/β-catenin, Notch and SHH) were investigated [34]. It was concluded that small molecules could be used as an effective means of generating dopaminergic neurons, a finding which is also being seen in other research as discussed earlier [8,10].

A third point is the need to establish standards that can be used in implementing protocols and scaling-up human neuron and NSC production. One challenge in applying the material discussed in several of the research papers presented in

this analysis is its heterogeneity. In general, each study's problem space, techniques and outcomes were all very specific and difficult to compare. Researchers could situate their work more clearly within the overall field, and discuss their own replication of other team's techniques. The research stands more as isolated attempts rather than as a cohesive whole. Given the heterogeneity, it is difficult to compare exactly which methods and techniques may be best, for example, the benefits of certain transcription factors, growth factors and culturing techniques over others. From a proof standards perspective, the general claim for obtaining neurons or NSCs was morphology and gene expression profiles, but more specific industry-wide standards could be developed, perhaps including measurement techniques, expected expression levels and dynamic aspects, such as how cells perform over time. Subsequent neuron and NSC generation research could promulgate a wider comparison of approaches, and ask multiple teams to each test a variety of defined techniques towards the same outcome, which could then be measured objectively.

A fourth point is the required steps for translating this research to therapeutic remedies. One question that emerges from analyzing this research is why the translational steps using these basic research findings are not more in evidence if it is apparently so easy to generate neurons and NSCs. This could have to do with the fact that while the pace of advance is fast, findings have not yet been validated and placed into a translational structure, and also that there are many steps in the process of translating basic research findings to clinical application. On the clinical side, there are 846 NSC clinical trials listed in the NIH clinical trials website as of May 2012 [103]. Perhaps some of these will be successful in ways that earlier clinical trials were not. In earlier stem cell clinical trials involving Parkinson's disease patients, for example, the methods used for NSC delivery were fairly primitive, with the problem that aged and often diseased cells (e.g., in patients with dyskinesia) did not take up the introduced stem cells, and immune suppression and side effects were an issue [104]. At least one dimension that needs to be defined is safety assessment criteria since cellular therapies are different from conventional agents in many ways [35].

Limitations

Some of the limitations of this analysis are that it may not be particularly comparative due to only a few papers being available for analysis, the field of human neuron and NSC generation being relatively new, and that only a few approaches have been employed thus far. It may be too early in the field's development to strive for a comprehensive overview of activity, techniques and results. Furthermore, research may have been unintentionally omitted in the process of identifying papers for inclusion in the review.

Future perspective

Taking a speculative viewpoint regarding how the field of human neuron and NSC generation may evolve in the next 5–10 years, the most obvious potential benefit is in extending basic research findings, such as those described here, to translational and clinical application. Research discoveries in reprogramming and differentiation need to be validated by understanding the behavior of generated cells through *in vivo* integration and human clinical use. Clinical protocols will need to be demonstrated with human adult somatic cells as the standard input rather than embryonic and postnatal fibroblasts. Once therapies are proven scientifically, a focus on the large-scale commercialization and distribution of cellular treatments will be important. Autologous (e.g., derived from the same individual's body, thereby avoiding immune system rejection) stem cell therapies will likely continue to expand in the next several years. The translational application of NSC therapies that is already underway in clinical trials for stroke, brain disorders and spinal injury [36] could be developed for many other neuropathologies in the next 5–10 years. Neurocellular therapies could also be employed for preclinical neuroreplacement and wellness maintenance therapies, and in the longer-term, for neural enhancement. The development of widespread clinical therapies will take time, but in the contemplated era of personalized medicine, there could be enormous benefit in being able to deliver autologous cellular replacement therapies as one core element of the next-generation health maintenance and preventive medicine toolkit. Ultimately, neurodegenerative disease could become a treatable and eradicated condition.

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Executive summary

Background

- Stem cell therapies could have a significant near-term impact on worldwide public health.
- Stem cell research applications include cell-replacement therapies, disease modeling, drug discovery and drug toxicity assessment.
- Neurons and neural stem cells are important due to the destructive impact of neurodegenerative disease and the lack of effective clinical therapies.

Methods

- A review of recent research advances in generating human neurons and neuronal stem cells through the reprogramming of somatic cells and the differentiation of pluripotent cells was conducted.

Results

- There are a variety of protocols for reprogramming human fibroblasts directly to neurons and transdifferentiating mouse fibroblasts to neural progenitor cells and neural stem cells.
- Human embryonic, postnatal and adult skin cells are reprogrammed directly to neurons with canonical and novel transcription factors, and growth factors, miRNA and small molecules.
- The same reprogramming techniques are being used to convert mouse somatic cells to earlier precursors, such as neural stem cells, which would have more versatility.
- A variety of techniques are being used to differentiate human pluripotent stem cells to different kinds of neural cells.
- Human pluripotent stem cells (both embryonic stem cells and induced pluripotent stem cells) are being differentiated to neural cells, mainly with growth factor additions, culturing techniques and small molecules.

Conclusion

- Techniques for generating neurons and neural stem cells may have potential risks, such as questionable functional equivalency to natural counterparts, genomic instability, DNA rearrangement and epigenetic factors.
- Underlying biological phenomena may need further characterization.
- Industry-wide standards are needed for measuring efficacy and safety.
- Steps should be outlined for progress to translational therapies.
- Limitations include difficulty of comparative analysis and possible mischaracterization of overall activity in the field due to the low number of papers available on the topic.

Future perspective

- There is a possibility of extending basic research findings in reprogramming and differentiation to translational and clinical application.
- Potential medium-term widescale commercialization and availability of cellular therapies will be important.
- There is a vision of eventual treatability and eradication of neurodegenerative disease.

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