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Evaluating cell reprogramming, differentiation and conversion technologies in neuroscience

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Abstract

The scarcity of live human brain cells for experimental access has for a long time limited our ability to study complex human neurological disorders and elucidate basic neuroscientific mechanisms. A decade ago, the development of methods to reprogramme somatic human cells into induced pluripotent stem cells enabled the *in vitro* generation of a wide range of neural cells from virtually any human individual. The growth of methods to generate more robust and defined neural cell types through reprogramming and direct conversion into induced neurons has led to the establishment of various human reprogramming-based neural disease models.

Progress in the study of human CNS development and function has been hindered by the inaccessibility of the relevant tissues and cell types. Likewise, the polygenic and multifactorial nature of many neurological diseases has prevented the generation of model organisms that effectively represent relevant aspects of these diseases^{1,2}. The recent advent of technologies that enable adult human somatic cells to be reprogrammed into induced pluripotent stem cells (iPSCs) for the generation of neural cells^{3–5} as well as direct conversion into neural cells^{6–8} have therefore provided a unique opportunity to investigate important aspects of CNS function, development and disease at a cellular level (FIG. 1).

Cell-reprogramming technologies provide potentially unrestricted access to CNS cells in which an individual's unique genetic landscape is represented. These *in vitro*-generated neural cells provide a valuable and unique resource to investigate otherwise inaccessible mechanisms of human neurodevelopment (FIG. 1). Indeed, reprogrammed cells have been used to study the transcriptional, epigenetic and functional signatures of neural cells from different species, revealing several human-specific cellular adaptations^{9,10}. Cell reprogramming can also enable the investigation of patient-specific models of genetic and sporadic diseases and allow researchers to monitor the progression of neuropsychiatric and neurodegenerative diseases in these models^{11,12}. It is hoped that further refinements to the technology to allow the development of specific subtypes of neural cells, in combination with approaches for single cell molecular characterization, will provide a platform for the

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identification and validation of drug targets and also for scalable drug screening and new diagnostics.

To successfully apply reprogramming technologies to disease modelling, the study of human development and other aims, it is necessary to produce defined cultures of specific subtypes of human neurons and glial cells *in vitro*. Differentiation of human pluripotent stem cells (hPSCs), which comprise iPSCs and human embryonic stem cells (hESCs), involves the use of timed combinations of mitogens and morphogens that gradually specify temporal and positional identity by mimicking developmental cues^{13,14}. By contrast, direct conversion utilizes the overexpression of cell type-specific transcription factors to jumpstart lineage changes and direct cellular identity towards the desired cell type, thereby bypassing most developmental stages¹⁵ (FIG. 1). In this Review, we describe recent advances in hPSC neural differentiation and direct conversion, discuss the differences between the two technologies and consider their relative advantages and disadvantages, which depend on the desired application of the generated cells.

Directed hPSC differentiation

During neuronal development, molecular programs progress in a concerted manner to generate distinct neuronal types in specific regions of the nervous system. Neuronal subtypes are defined by several characteristics, including their localization in the nervous system, connectivity, morphology, marker and neuro transmitter expression profiles and electrical firing profile. Researchers aiming to use reprogrammed cells to investigate stem cell neurobiology and neural disease must, therefore, provide the right cues to generate specific neural cell progenitors, functional neurons or glia.

There are several methods currently available to generate stem and progenitor cells that are committed to neural differentiation from hPSCs. These all result in the production of cells with the capacity for limited self-renewal and multipotency, which we here refer to collectively as neural progenitor cells (NPCs). Once neural commitment and regionalization are completed, further specification of different neuronal subtypes from hPSC-derived NPCs can be achieved through additional maturation time and neurotrophic support.

Cues for neural commitment and early regionalization.

Methods for generating NPCs from hPSCs (see REF. 16 for example) are derived from groundbreaking work on animal models of neurodevelopment, which identified the key events in early mammalian neural commitment and regionalization. These studies have shown that the intense proliferation of early neuroectodermal NPCs generates the first wave of neurons as well as other types of NPCs, including the neural rosette NPCs that populate the early neural tube. Later during development, radial glial cells (another type of NPC) produce most neurons¹⁷ (FIG. 2). Morphogens and mitogens confer different positional identities to responsive NPCs in the neural tube^{18–20}. For example, sonic hedgehog (SHH), is secreted from ventral regions — namely, the notochord and floor plate — of the neural tube, whereas WNT proteins and bone morphogenetic protein (BMP) are secreted from dorsal regions^{21–23}. This results in morphogen gradients that specify the different subtypes of NPCs along the dorsal–ventral axis. Along the anterior–posterior axis, a sequence of

timed developmental events and signaling cues, including those downstream of fibroblast growth factors (FGFs) and retinoic acid (RA), specify neural progenitor fates^{24,25}. Importantly, these patterning factors typically fulfil both morphogenic and mitogenic duties at the same time, although their effects depend on localization, developmental stage, concentration and the target NPC.

In hPSC studies, NPCs with a more posterior identity, such as those that generate neurons with the features of spinal cord motor neurons or midbrain neurons, have been produced by adding SHH, RA and FGF2 or FGF8 to the media during the neural rosette stage *in vitro*^{13,26–29} (FIG. 2). By contrast, NPCs similar to those found in the hippocampal dentate gyrus (DG) have been generated by treating embryoid bodies (aggregates obtained from hPSC cultures) with antagonists of the SHH pathway³⁰ and a cocktail of anti-posteriorizing factors: these include Dickkopf-related protein 1 (DKK1), Noggin and small molecules, such as SB431542, which block WNT, BMP and transforming growth factor- β (TGF β) pathways, respectively. Inhibition of the WNT pathway coupled with inhibition of SMAD signalling promoted forebrain identity in hPSC-derived NPCs^{13,31}, and subsequent activation of SHH signalling converted these NPCs to those with characteristics of medial ganglionic eminence (MGE)-derived interneuron progenitors^{32,33}. NPCs committed to generating both deep- and upper-layer cortical excitatory neurons have been derived from hPSCs by inhibiting the SMAD signalling pathway^{18,27,34–38}. Finally, by coupling traditional neural regionalization methods with immuno-isolation of a CD133-positive population, it was possible to generate a long-term self-renewing population of hindbrain or spinal cord radial glia-like NPCs from hPSCs; these cells maintained the ability to differentiate into neurons, astrocytes and oligodendrocytes³⁹.

Specification of excitatory cortical neurons.

In vivo, excitatory cortical neurons are born in the dorsal forebrain, arising from actively dividing radial glia⁴⁰. Deeper-layer neurons are generated first, followed by upper-layer neurons⁴¹. The FEZF2 (FEZ-family zinc finger 2)–CTIP2 (COUP-TF-interacting protein 2; also known as BCL11B) genetic pathway directs the fate choice of cortical projection neurons in the developing cerebral cortex: FEZF2 is necessary for specification of all populations of subcortical projection neurons, whereas CTIP2 is required for axon fasciculation and guidance in these neurons^{42,43}. An example of a well-defined cortical layer-specific transcription factor is SATB2 (special AT-rich sequence-binding protein 2). SATB2-expressing cells project through the corpus callosum to the contralateral hemisphere⁴².

In hPSC-based studies, an adherent culture system can be used to generate functional cortical excitatory neurons³⁴. Recently, a comprehensive characterization of the gene expression, morphology and electrophysiology of cortical excitatory neurons derived from hPSCs demonstrated that the *in vitro* derivation of cortical neurons follows a layer-specific sequential order and that the derived pyramidal cells can integrate into the cortical circuitry of neonatal mice³⁵. In other studies, live antibody staining for the forebrain NPC surface marker forebrain surface embryonic antigen 1 (FORSE1), followed by flow cytometry was used to enrich hPSC-derived NPC cultures with progenitors that preferentially give rise to

neurons with an upper cortical layer identity^{18,28}. Functional glutamatergic neurons of the hippocampus were produced using an hPSC-based protocol³⁰ in which timed exposure of early NPCs to WNT signalling generated hippocampal dentate gyrus-like cells that expressed the marker transcription factor prospero homeobox protein 1 (PROX1). PROX1 labelling in live cells using an engineered *PROX1* promoter-driven enhanced green fluorescent protein (EGFP) reporter construct, enabled these cells to be further enriched by flow cytometry.

Specification of dopaminergic neurons.

Dopamine neurons that reside in the substantia nigra pars compacta (SNc) of the midbrain have been extensively studied because their progressive loss is directly involved in Parkinson disease pathology^{44,45}. Human neurons that express tyrosine hydroxylase (an indicator of dopaminergic identity) have been generated by passaging hESC-derived NPCs in the presence of FGF2 or FGF8, to allow posteriorization, and SHH, which acts as a ventralizing agent⁴⁶⁻⁴⁸. The generation of dopaminergic neurons with actual ventral midbrain identity from hPSCs requires floor plate patterning and activation of WNT signalling to activate the transcription factors forkhead box protein A2 (FOXA2; also known as HNF3 β) and LIM homeobox transcription factor 1 α (LMX1 α). This protocol produces tyrosine hydroxylase-positive cells that express pituitary homeobox 3 (PITX3) and NR4A2 (also known as NURR1), which are markers of the SNc²⁰. Such dopaminergic populations can be enriched by using specific genetic markers such as NR4A2 or PITX3 promoter-driven fluorescent proteins⁴⁹. Protocols that promote enrichment of dopaminergic neurons have not only the potential to impact Parkinson disease research, but they potentially allow for a better understanding of the dopaminergic reward circuit that is affected in mood disorders such as depression.

Specification of motor neurons.

Motor neurons are of great interest because there are many neurodegenerative diseases that exclusively affect this population. Numerous genes required for the specification of corticospinal motor neurons (CSMNs) and other cortical projection neurons have been identified, and the molecular mechanisms controlling CSMN development have been characterized⁵⁰⁻⁵². However, the refinement of methods to generate specific CSMN populations from hPSCs is still in progress. For example, multistep differentiation protocols using chemically defined media have allowed researchers to monitor the generation of human hPSC-derived subtypes of cortical neurons such as CSMNs^{34,38}. The use of FEZF2 reporters to enrich CSMNs from differentiated hPSCs has been described⁵³; however, FEZF2 expression *in vivo* is not restricted to CSMNs, as all cortical projection neurons express FEZF2 at different levels^{48,49}. This highlights the importance of using multiple markers to accurately define cell identity (BOX 1). Optimizing the generation of CSMN-specific cells will be a crucial next step in developing specific models of human CSMNs from iPSCs.

Protocols for generating α -motor neurons (AMNs) from hPSCs have been developed and expanded for several years and are often used for disease modelling^{54,55}. Such protocols usually use a combination of posteriorizing factors (such as SHH and RA) and selection

markers such as Hb9 promoter-driven reporters to distinguish spinal AMNs^{53,56–58}. AMN protocols have been widely used to model neurodegenerative disorders, and drug-screening platforms have also been developed using AMN survival as readout^{59,60}.

Specification of interneurons.

In vivo, interneurons are born in the ventricular and subventricular zones of the MGE and caudal ganglionic eminence (CGE)⁶¹. Using a combination of small-molecule approaches to induce ventral telencephalic fate, coupled with inhibition of WNT and SMAD signalling, two groups were able to induce MGE-like progenitors from hPSCs^{32,33}. Cortical interneurons take a remarkably long time to reach functional maturation in the embryonic brain and in culture³³. Further optimization of *in vitro* maturation protocols will therefore be important when designing experiments to dissect the different subtypes of inhibitory neurons that have been directly implicated in neuropsychiatric disorders such as autism and schizophrenia^{62,63}.

Generating glial cells from hPSCs.

Glial cells provide metabolic and trophic support to neurons and have important roles in many aspects of neurodevelopment and in neurodegenerative diseases^{64–67}. Glial differentiation from hPSCs requires neural regionalization, coupled with an extended time in culture and the addition of morphogens involved in glial fate.

Astrocyte progenitor enrichment can be achieved through initial treatment of hPSCs with FGFs and RA (for posterior patterning) followed by the addition of ciliary neurotrophic factor (CNTF) and further functional maturation that can last up to 6 months^{68,69}. Generation of hPSC lines expressing reporter genes under the control of astrocyte-specific promoters allows for enrichment of desired populations and could potentially reduce the time required for differentiation in culture⁷⁰.

In vivo, oligodendrocytes develop from radial glia, then form long branches and undergo sub-branch ramification and extension of myelin membranes^{71–73}. The markers A2B5 and platelet-derived growth factor receptor- α (PDGFR α) identify oligodendrocyte progenitors, and O4 sulfatide-specific antibody labels ramified, but immature, oligodendrocytes⁷⁴. These cells acquire galacto-cerebrosides (GalC) and later express the oligodendrocyte marker O1 as they finally reach the postmitotic mature oligodendrocyte stage in which myelin components such as proteolipid protein (PLP) and myelin basic protein (MBP) are synthesized. Existing protocols for generating myelinating oligodendrocytes *in vitro* from hPSCs are extremely inefficient. Current protocols use a combination of factors to drive initial neuralization, followed by the addition of oligoglia-inducing factors (such as, PDGF α , neurotrophin 3 (NT3) and insulin-like growth factors (IGFs)) to generate oligodendrocyte progenitor cells (OPCs)^{39,75,76}. Sufficient myelination is typically only achieved following *in vivo* transplantation⁷⁷. Thus, improvement of *in vitro* myelination protocols will have a marked impact on the field of disease modelling.

Microglia are CNS-resident macrophages that perform important tasks during inflammatory processes in ageing and disease^{78–81}. The signalling steps involved in microglial

development are not well understood, and further characterization of this process will therefore be essential to enable production of patient-specific microglia cells from hPSCs⁸².

Direct conversion to neural cells

The demand for live, functional human neural cells has triggered the development of alternative technologies to generate specific human cell types⁸³. The first demonstrations that transgenic expression of certain transcription factors can shift the identity of a cell directly into another identity took place in the 1980s^{84,85}, and transcription factors that mediate conversion of glial cells into neurons were also explored^{86,87}. More recently, the first conversion of mouse fibroblasts into induced neurons (iNs) sparked a new wave of enthusiasm about direct conversion (FIG. 1). A combination of only three transcription factors — BRN2 (also known as POU3F2), achaete-scute homologue 1 (ASCL1) and myelin transcription factor 1-like protein (MYT1L), collectively known as BAM — was sufficient to convert embryonic or adult mouse fibroblasts into iNs that were capable of synapse formation and action potential firing⁶ (FIG. 1). Only 1 year later, several laboratories reported the generation of iNs from human fibroblasts^{7,88,89}. Since then, work in this field has shed light on the mechanisms involved in direct conversion and the most efficient set of factors and media compounds for conversion. It has become possible to generate specific neural and neuronal subpopulations and to compare properties of the converted cells to their *in vivo* counterparts or to neurons generated by established methods, such as iPSC-based differentiation protocols^{90,91}.

Transcription factors driving direct conversion.

Any change in cell identity requires a transition between epigenetic states, mediated by transcription factors and epigenetic modulators. During *in vivo* differentiation, the combination of a genetically programmed cascade of transcription factor-mediated protein expression patterns with the modulation of exogenous signalling factors controls stepwise changes in cell fate (FIG. 3a). Thus, a pluripotent blastocyst cell becomes a somatic cell by activating a previously repressed epigenetic state. During reprogramming, so-called pioneer transcription factors access nucleosomes directly, bind to closed chromatin structures and coordinate the binding of secondary transcription factors to initiate a new cell fate⁹². In iPSC reprogramming, the ‘Yamanaka-factors’ OCT4 (also known as POU5F1), SOX2 and Krueppel-like factor 4 (KLF4) act as pioneer transcription factors, whereas MYC only binds to already opened chromatin and acts as a secondary enhancer^{3,93,94}. During BAM-mediated conversion of mouse fibroblasts to iNs, ASCL1 or ZFP238 can act as a pioneer transcription factor that later recruits BRN2 to the sites of ASCL1 binding, whereas MYT1L is only required at later stages for maturation⁹⁵ (FIG. 3b). Thus, iN conversion follows a hierarchical mechanism in which a sequential series of epigenetic events occurs⁹⁶. Notably, several laboratories have shown that transcription factor expression can be temporal and is not continuously required for iN conversion, thus overcoming the potential risk that permanent overexpression of neurodevelopmental transcription factors might interfere with mature neuronal phenotypes, functionality or other relevant phenotypes of the generated iNs^{97–100} (FIG. 3b,c).

Interestingly, there seems to be species-specific differences in the optimal set of transcription factors required for direct conversion. ASCL1-based strategies have been shown to be less efficient in the human system than in rodents: four factors (BAM plus neurodifferentiation D1 (NEUROD1)) are required to convert human fibroblasts into functional iNs⁷. By contrast, neurogenin 2 (NGN2), a well-studied pro-neuronal transcription factor¹⁰¹, efficiently converts human fibroblasts into iNs⁹⁸. In the search for the smallest and most efficient set of factors sufficient for reprogramming⁹⁰, it is not surprising that a combination of the two pioneer transcription factors, ASCL1 and NGN2, yields high conversion efficiencies, even in fibroblasts derived from adult and ageing humans^{102,103}. To purify iNs from the fibroblasts that remain after conversion, flow cytometry sorting following live antibody staining for polysialylated neuronal cell adhesion molecule (PSA-NCAM) has been shown to be practical because fibroblasts, unlike NPCs, show very little background staining of neuronal markers¹⁰².

Although it has not yet been decisively demonstrated whether fibroblast-based iNs pass through an intermediate progenitor-like epigenetic state (as studies based on glia as starting cells suggest^{104,105}), it is well established that iN conversion requires no proliferation^{98,106}. Cell division is deemed essential during *in vivo* and hPSC differentiation because it stimulates the chromatin remodelling required to provide transcription factor access to previously closed regions⁹². However, the iN pioneer transcription factors seem to overcome the need for cell division-based chromatin remodelling. However, it has been shown that replication-induced cellular senescence as well as p53 overexpression inhibit direct conversion, whereas depletion of the p53 stabilizers p16 and p19 enhances conversion¹⁰⁷. Thus, a non-senescent quality of fibroblasts is crucial for iN conversion.

Transcription factor-free approaches.

Alternative strategies for direct conversion, including targeted gene repression, have been explored (FIG. 3b–d). The pro-neuronal microRNAs (miRNAs) miR-124 and miR-9* can convert human fibroblasts into immature iNs through activation of NEUROD2, although ASCL1 and MYT1L are required for functional maturation of these cells¹⁰⁸. In further work, miR-124, in combination with BRN2 and MYT1L, was shown to convert adult human fibroblasts into functional iNs without the need for transcription factor pioneers¹⁰⁹. Although miRNAs are likely to act by mildly repressing a very large number of target genes, it is important to determine whether short interfering RNA (siRNA)-mediated knockdown of only one target could mediate or initiate direct conversion. Indeed, siRNA-mediated polypyrimidine tract-binding protein 1 (PTBP1) knockdown is sufficient for the direct conversion of mouse fibroblasts into iNs¹¹⁰. PTBP1 suppression leads to the de-repression of miRNAs that target components of the RE1-silencing transcription factor (REST) complex, thus allowing for the expression of epigenetically silenced pro-neural genes^{111,112}. Likewise, direct siRNA-mediated REST knockdown can convert embryonic mouse fibroblasts into iN-like cells¹¹⁰. Another strategy based on the principle of epigenetic de-repression involves the use of small molecular signalling pathway inhibitors (FIG. 3d). Inhibition of TGF β or SMAD signalling using Noggin or inhibition of ALK — in combination with glycogen synthase kinase 3 β (GSK3 β) inhibition, forskolin-mediated activation of adenylyl cyclase and direct application of cell-permeable cyclic AMP —

dramatically enhances iN conversion efficiencies^{98,102}. Interestingly, two recent studies identified cocktails of small molecules that can convert fibroblasts into functional neurons without the need for exogenous genetic factors^{113,114}. Both studies used slightly varying cocktails of inhibitors of GSK3 β and ALK as well as cAMP enhancers and achieved iN conversion of mouse fibroblasts by adding only two additional compound classes: the pro-neurogenic small molecule isoxazole 9 (ISX9)¹¹⁵ and I-BET151, a putative inhibitor of fibroblast-specific gene expression¹¹³. For the conversion of human fibroblasts, addition of the histone deacetylase inhibitor valproic acid, the JUN amino-terminal kinase (JNK) inhibitor SP600125, the protein kinase C (PKC) inhibitor GO6983 and the widely used RHO-associated protein kinase (ROCK) inhibitor Y-27632 was required to generate human iNs with expression profiles similar to ASCL1- or NGN2-based iNs¹¹⁴ (FIG. 3d). Although both procedures have yet to be reproduced in other laboratories, they set a milestone for transgene-free direct lineage reprogramming efforts.

Direct conversion into specific neuronal subtypes.

A major aim is the generation of human donor-specific, region-specific and neurotransmitter subtype-specific iNs. The majority of iNs produced by most human iN protocols have glutamatergic properties (although some human protocols generate a minor fraction of GABAergic cells)^{7,102,108,109}. As outlined above, during human hPSC differentiation, specialized NPCs are responsive to exogenous morphogens that activate lineage-specific transcription factors^{28,116}. As there is no morphogen-responsive NPC intermediate in direct conversion, the co-overexpression of general pro-neuronal transcription factors with lineage-specific transcription factors has been explored (FIG. 3c). Combinations of transcription factors known to be key during midbrain development (FOXA2)¹¹⁷ and SNc development (LMX1 α , NR4A2 and PITX3)¹¹⁸ have been shown to induce a dopaminergic phenotype in human iNs^{89,119,120}. Similarly, the combination of LMX1 β and FEV (known as PET1 in mice) either with FOXA2, or with NKX2.2 and GATA2 (which are instructive factors during the development of midbrain raphe populations¹²¹), produced iNs with a serotonergic neurotransmitter identity¹²². Further studies showed that cholinergic iNs⁹⁸ and spinal motor neuron-like iNs can be generated⁸⁸. Another study showed that a combination of CTIP2 and DLX1 and/or DLX2, which orchestrate striatal development *in vivo*, could direct iN conversion towards a GABAergic fate that seems to closely resemble striatal medium spiny neurons¹²³. In addition to different CNS phenotypes, peripheral neuronal cell types, such as different classes of sensory neurons, could be directly generated from human fibroblasts using NGN1 and/or NGN2 and BRN3A (also known as POU4F1)¹²⁴. The many successful approaches to directly convert fibroblasts into different defined subtypes of neurons indicate a surprisingly high plasticity of the iN process that seems to be amenable to directional control through developmental transcription factors.

Direct conversion into glial cells and NPCs.

Direct conversion of fibroblasts into other neural cell types has also been explored (FIG. 1). Nuclear factor 1A (NFIA), NFIB and SOX9 can convert mouse fibroblasts into induced astrocyte-like cells¹²⁵. To generate oligodendroglial cells, expandable induced OPCs (iOPCs) have been generated through overexpression of SOX10 and oligodendrocyte transcription factor 2 (OLIG2), in combination with either zinc-finger protein 536 (ZFP536)

or NKX6.2 (REFS 126,127). Given the strong interest in functional human glial cells for disease modelling and regenerative approaches, it will be important to translate these findings to the human system.

Overexpression of the four Yamanaka factors in fibroblasts was shown to generate a source of proliferating neutrally committed cells through direct conversion by destabilizing the somatic cells into a pre-iPSC stage^{128,129}. This paradigm has been utilized in both mouse and human fibroblasts. In these studies, transient expression of a subset of the Yamanaka factors and subsequent transfer of the destabilized intermediate cells into a pro-neurogenic medium containing mitogens produced a population of induced NPCs (iNPCs) (FIG. 1). Other protocols further combined Yamanaka factor expression with early neural transcription factors such as FOXG1 or BRN4 (also known as POU3F4), which then helped to push the unstable intermediates into the iNPC lineage^{128,130–134}. To generate cells of the peripheral nervous system, SOX10 expression combined with WNT signalling was shown to convert fibroblasts into induced neural crest cells¹³⁵. In the absence of reprogramming factors, suppression of let-7 and activation of HMGA2 (high mobility group protein AT-hook 2; also known as HMGI-C) were shown to convert human fibroblast and blood cells into iNPCs¹³⁶. It will be important to clarify the regional identity and developmental potential of the iNPCs generated by these protocols.

iPSCs versus iNs

As described above, cellular neuroscientists benefit from the expansion of a large number of novel differentiation and conversion protocols. At the same time, they also face a difficult decision: which protocol to choose? There are more protocols available for hPSC differentiation than for direct conversion; however, the number of published iN protocols is rapidly increasing, and the relatively simple idea of combining pro-neuronal factors with factors to specify the desired lineage in one step opens new possibilities for non-specialized laboratories. If the cell type of interest can be generated by both methods, we can choose either one of them or combine them to mitigate model system bias in our experiments. However, there are technical as well as conceptual differences between iPSC differentiation and direct iN conversion that may influence the choice of system for particular *in vitro* projects (TABLE 1; FIG. 4). Recent views on the merits of each strategy for *in vivo* transplantation and regenerative approaches have been discussed elsewhere^{15,137}.

Donor cells.

Fibroblasts are the cell type of choice for both iPSC and direct conversion strategies for several reasons. Skin biopsy samples are easy to obtain from donors and can be expanded in culture. Indeed, several existing cell repositories have already banked hundreds of fibroblast lines from diseased and healthy donors. Several other somatic cell types have been successfully reprogrammed into iPSCs and used for disease modelling with varying efficiency, including keratinocytes¹³⁸, dental pulp cells^{139,140}, several blood cell types^{141–143} and exfoliated renal epithelial cells present in urine¹⁴⁴ (TABLE 1). Each has advantages and disadvantages; for example, blood is easy to obtain, but the efficiency of reprogramming is rather low. In the direct conversion field, it has been shown that

hepatocytes, B cells, astrocytes, pericytes and adipocytes are amenable to iN conversion^{97,145–148}.

Interestingly, one attractive source for direct conversion is hPSCs — particularly iPSCs. Unlike somatic cell types, iPSCs are completely de-differentiated cells that intrinsically possess the potential for neural differentiation. Several studies have shown that generation of iPSC–iNs is comparatively easy because only one pro-neurogenic factor (either ASCL1 or NGN2) is needed^{149–151} (FIG. 1). Furthermore, a few studies have used forced expression of neuronal subtype-specific transcription factors in iPSCs or early differentiating cells to generate subtype-specific iPSC–iNs. For example, the overexpression of the lineage determinants LHX8 and GBX1 in human iPSCs enriched for basal forebrain cholinergic neurons¹⁵². Dopaminergic¹⁵³, noradrenergic and cholinergic¹⁵⁴ iPSC–iNs have also been reported. It will be interesting to explore combinatorial approaches in which lineage-specific transcription factors and small molecules or morphogens are applied in a timed, concerted manner. However, it is possible that extended maturation times might be required to generate truly mature neurons, and accelerated maturation might lead to cells stuck in a functionally premature state^{33,35,155}.

Duration.

The time and money spent to generate a cell type of interest can be a decisive argument for or against a particular protocol (FIG. 4a; TABLE 1). The differentiation of fibroblasts into neural cells via iPSCs typically requires 4–6 months before the first functional neurons are generated. Of this period, around 2 months are needed for the generation and validation of iPSC clones. Once this is accomplished, however, iPSCs represent a stable hPSC intermediate that researchers can use for each new round of experiments. From hPSCs, functional neurons can be repeatedly generated within 2–6 weeks, although high proportions of truly functional cells may require extended culture periods³³. As an alternative, a class of well-characterized, patternable rosette-type NPCs can be generated from hPSCs within 2–3 weeks and can serve as expandable tri-potential intermediates^{156,157}. Such rosette-type NPCs exhibit stem cell properties such as long-term self-renewal, tri-potential differentiation and responsiveness to regionalization cues, and they can be differentiated into cultures of functional neurons in 1–6 weeks¹¹.

Compared with hPSC differentiation, direct iN conversion is markedly faster. Functional human iNs can be generated from fibroblasts within 1–3 weeks plus maturation time^{7,98}. Although direct conversion is a one-step process, many iN protocols make use of inducible expression vectors that first generate an ‘iN-ready’ transgenic fibroblast as an intermediate. This extends the production time but substantially reduces the effort spent on viral transductions and probably reduces variability between iN batches¹⁰².

Expandability and cell numbers.

Although most neuroscience techniques such as electrophysiology, fluorescence staining, imaging, enzymatic and colorimetric assays, microarrays and most DNA and RNA ‘omics’ technologies do not demand large cell numbers, others, such as mass spectroscopy and other proteomic assays, may require larger amounts of biological material. Whereas iPSCs are a

virtually inexhaustible source of neural cells, direct iN conversion of fibroblasts yields postmitotic cells that cannot be expanded in culture, which is a major disadvantage (FIG. 4a). iN cell numbers largely depend on the expandability of the donor cell culture as well as conversion efficiency. As typical fibroblast cultures reach around 50 population doublings with around 2–3 doublings per week¹⁵⁸, and conversion efficiencies range between 30% and 80%, repeatedly performed iN conversions from ‘iN -ready’ fibroblasts can yield a few hundred thousand to a million iNs per week until the fibroblasts are exhausted and senescence sets in. Although these limitations may be overcome by immortalizing the fibroblasts before iN, projects that require large cell numbers that go beyond the expandability of fibroblasts may favour iPSC-based strategies or alternatives such as iOPC and iNPC technologies (FIG. 1).

Genomic and epigenetic identity.

The possibility of generating donor-specific brain cells from patients is one of the most important contributions of the stem cell field. However, many aspects of an individual’s identity can be recapitulated in culture, and the preservation of these unique features may differ between the different methods (TABLE 1). This is particularly important for attempts to model and understand complex multigenic, sporadic and epigenetic diseases. iPSC reprogramming has made it possible to study familial and sporadic forms of diseases such as schizophrenia¹², bipolar disorder¹⁵⁹, amyotrophic lateral sclerosis¹⁶⁰ or Alzheimer disease¹⁶¹ in the relevant human cell type side by side.

Environmental and epigenetic risk factors have emerged as important determinants of disease. In this context, chromatin remodelling during iPSC reprogramming is known to cause the epigenetic state of the cell to ‘reset’ into an embryonic-like state^{162–166}. Furthermore, competitive clonal growth of iPSC colonies as well as numerous rounds of cell division probably select for, repair and dilute out macromolecular damage^{167,168}, leaving researchers with a completely rejuvenated cell^{169–171}. Because human age is the most significant risk factor for several neurodegenerative diseases, this rejuvenation represents a major challenge for modelling these late-onset diseases using iPSC-based models¹⁷². By contrast, direct iN conversion does not erase putative cellular ageing markers in fibroblasts derived from old mice¹⁷³, and fibroblast-derived iNs obtained from a cohort of ageing human donors show important transcriptomic and functional signatures of ageing¹⁷⁴. This conceptual difference might turn out to be very important for future comparative models of sporadic late-onset diseases. It should be noted that some disease variants are currently regarded as sporadic forms might turn out to be due to (partially) inherited multigenic causes that are likewise accessible through both iPSC and direct conversion strategies.

Recapitulation of neurodevelopmental phenotypes.

Differentiation of hPSCs including iPSCs into neural tissues follows developmental pathways and thus recapitulates several cellular stages of human neural development¹⁷⁵ (FIG. 4b; TABLE 1). This is particularly interesting for neurodevelopmental diseases such as familial dysautonomia¹⁷⁶, Rett syndrome¹⁷⁷ and ring chromosome-associated disorders¹⁶⁷, and may prove to be relevant to other neurodevelopmental diseases such as microencephaly and lissencephaly. Furthermore, the pathology of neuropsychiatric diseases such as autism

spectrum disorders¹⁷⁸, schizophrenia¹⁷⁹ or bipolar disorder¹⁸⁰ may have an important neurodevelopmental component that can be recapitulated through iPSC differentiation models such as neural organoids¹⁸¹ (BOX 2).

As opposed to the possibility of recapitulating neural development with iPSC-based models, direct conversion skips all developmental precursor cell stages and generates neurons that have never been in an NPC-like cell stage (FIG. 1; TABLE 1). Neuronal phenotypes that are caused by differences in a precursor cell stage are thus circumvented by iN. Thus, if the disease phenotype of interest is thought to originate during the NPC stage of neurodevelopment, it may be better not to use iNs or perhaps to use iNs together with iPSCs (which might provide the perfect control to prove that this theory of origin is valid) (FIG. 4b).

Diversity and mosaicism.

Existing genetic mosaicism is an emerging concern when reprogramming new patient lines. Somatic copy number variation (CNV) in human primary fibroblasts before reprogramming has been demonstrated^{182,183}. It has been estimated that approximately 30% of fibroblast cells have somatic CNVs in their genomes, suggesting widespread somatic mosaicism in the human body (FIG. 4c). It is important to consider this when interpreting results from iPSC line derivatives: owing to their clonal origin, one iPSC clone only represents one donor fibroblast cell. To minimize possible misinterpretations, several iPSC clones from a given donor should be derived and analysed for similar phenotypes. Variation, which is a big issue in human disease modelling, may in turn originate from the donor's genetic mosaicism, which should not be regarded as a mere artefact but rather illustrates the complexity of the human 'cellome' that might determine an individual's health and disease. By contrast, a population of 100 iNs resembles 100 different fibroblasts and is thus likely to capture the donor's mosaicism. Furthermore, single-cell analyses and in-depth genomic DNA sequencing can be used to identify potential mosaic CNVs in fibroblasts, iNs and iPSC clones (FIG. 4c). In the future, iNs could be used to identify and quantify a patient's CNVs, whereas high-throughput genome editing technology in iPSCs could be used to test the impact of the identified variants. However, especially when looking at small genomic alterations in cultured cells, it is important to keep in mind that *in vitro* culture of not only iPSCs and their derivatives but also of fibroblasts and iNs can lead to culture-induced genomic artefacts that may contaminate the results^{184,185}. Controlling the results with cells from the primary biopsy sample or other tissues such as blood samples is thus essential when performing such studies.

Summary

Recent advances in the generation of vital human neural cells *in vitro* have enabled the study of human neurodevelopment, cellular neurophysiology and neural diseases at the cellular level in a donor-specific manner. Since the invention of iPSCs one decade ago, our understanding of the cues required to differentiate pluripotent cells into specific neural precursor cells and functional neural subtypes has grown tremendously. The past 5 years have also seen substantial progress in the development of alternative strategies to generate

human neural cells, such as the direct conversion of fibroblasts into iNs or iNPCs. The expanding pool of various protocols warrants the consideration of practical differences such as time and cost efficiency and the difficulty of the method. Furthermore, a keen awareness is demanded with regard to the respective conceptual advantages and disadvantages associated with each method, such as clonogenicity, preservation of genetic and epigenetic identity or neurodevelopmental aspects. However, attention to these differences will open avenues to tackle complex scientific questions regarding human development or disease by combining different human cell reprogramming technologies. Adequate comparison of *in vitro*-generated neural cells to their putative counterparts *in vivo* still represents an important challenge for the human stem cell field. Furthermore, the vast diversity of human individuals in comparison to inbred mice, as well as the virtually unlimited complexity of neurological diseases, remains a difficult problem. Although not all reported cell reprogramming technologies will prove useful to neuroscientists, a significant number will and, with time, are likely to contribute to new discoveries to unravel important cellular mechanisms of the complex human nervous system in health and disease.

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Box 1 | Defining cell types *in vitro*.

The accurate characterization of specific subsets of *in vitro*-generated neurons is important for the accurate interpretation of data derived from these cells, irrespective of whether they are used for modelling disease states, developmental studies or circuitry. Cell type validation of human neurons *in vitro* represents a challenge in the field given that direct comparison with their endogenous counterparts is often difficult or impossible. Most studies rely on the isolated expression of one or two ‘markers’ as a criterion to suggest cell identity (usually measured using antibodies transposed from mouse studies). However, as various cell types may (transiently) express these markers during development, a solid interpretation of induced pluripotent stem cell-based results based on one or two markers is somewhat limited. In direct conversion paradigms, it is conceivable that some markers are directly downstream of the genes that are overexpressed, which may also cast doubt on the validity of the suggested identity. It is thus of crucial importance for future studies to build neuronal profiles based on multimodal analyses, including broad molecular signatures and deep electrophysiological properties. For example, bulk and single-cell transcriptomic technologies provide an in-depth molecular signature that can be directly compared to the human brain transcriptome database⁹⁹. For functional evaluation, patch clamping remains the gold standard but only permits us to assess a handful of selected neurons within several thousands of cells. The development of standardized electrophysiological maturity markers to classify functionality levels of neurons would help to allow the interpretation of electrophysiological data even between laboratories. Furthermore, because patch clamping is of low-throughput design, alternative techniques such as calcium imaging or multi-electrode arrays provide more efficient ways to examine larger networks of neurons. As more rigorous ways to define cell subtype identity and functional maturity become available, controlling the identity and quality of generated cultures will allow increasingly accurate experiments to address phenotypic differences between cell types and diseases that may be specific for certain brain regions.

Box 2 | Cellular environment and neural organoids.

In vitro-generated neural cells are typically grown in standard base media supplemented with hormones, antioxidants and growth factors that promote neuronal differentiation and survival but that are quite distant from the natural microenvironment in the brain. One promising trend is to optimize the culture conditions for maturing human neurons and other cell types to expose human cells to more physiological environments *in vitro* to better support neuronal functions^{186,187}. Further, *in vitro* neural cells are typically cultured as 2D monolayers that lack the juxtacrine environment of the brain, including dense synaptic and glial contacts. This limitation has to be taken into account when interpreting results from monolayer cultures. The generation of 3D cerebral organoids from human pluripotent stem cells has been recently proposed as a means to overcome these limitations and to also recapitulate aspects of early human brain cortical development that still appear inaccessible through direct induced neuron conversion paradigms³⁶. Although early results seem promising for the study of neural progenitor migration and cortical expansion, current hurdles include a broad variety of floating aggregates that contain notable degree of heterogeneity of cell types as well as a lack of nutritious support for cells located in the centre of these aggregates^{188,189}. Reduction of variation through a higher degree of structural control, together with live imaging of specific migrating progenitors, will be essential to promote a better understanding of the potential applications for the organoid technology and will provide a unique opportunity to study human development that is otherwise inaccessible.

Induced pluripotent stem cells

(iPSCs). Pluripotent stem cells created from differentiated somatic cell types, such as fibroblasts, by reprogramming with a set of transcription factors or other approaches.

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Direct conversion

This term (also known as direct cell fate conversion, lineage conversion or transdifferentiation) describes the forced identity change of a somatic cell — for example, a fibroblast — directly into another related or unrelated somatic cell — such as a neuron — by means of transcription factors or other approaches.

Epigenetic

Describes changes in gene function that occur through changes to the genome that do not involve altering the DNA sequence. Examples of epigenetic events include DNA methylation, histone acetylation or X-chromosome inactivation. Epigenetic changes control biological processes such as differentiation, cell type identity or ageing.

Human pluripotent stem cells

(hPSCs). Stem cells that have the potential to form any cell type of the human body. They include human embryonic stem cells (hESCs) and human induced pluripotent stem cells (iPSCs). All hPSCs have the same ability to differentiate into cells of distinct lineages, such as neurons.

Human embryonic stem cells

(hESCs). Pluripotent stem cells that are derived from the inner cell mass of the developing blastocyst.

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Mitogens

Proteins or chemical compounds that induce cell division by triggering mitosis. Proliferation of stem cells during development *in vivo* and *in vitro* depends on mitogen-induced signalling, whereas mitogen-deprivation induces stem cell differentiation.

Morphogens

Proteins or chemical compounds that define the relative position of stem and progenitor cells in the developing organ *in vivo* and thereby lay out the pattern for the spatial organization of cellular subtypes within the same tissue. *In vitro*, morphogens are used to pattern stem and progenitor cells such as neural progenitor cells (NPCs) to promote the generation of a desired cellular subtype that is associated with a certain tissue region *in vivo*.

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Neural progenitor cells

(NPCs). A broad term describing stem and progenitor cells of the nervous system. There are many types of NPCs during neural development and in the adult brain that all share the characteristics of proliferation and multipotency, meaning that they can give rise to enlarged numbers of differentiated neurons and glia.

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Neural rosette

The developmental signature of neural progenitors in cultures of differentiating human pluripotent stem cells; rosettes are radial arrangements of columnar cells that express many of the proteins expressed in neuroepithelial cells in the neural tube.

Radial glial cells

A subtype of neural progenitor cells that span the radial axis of the developing cortex and serve as precursors or guides for newly born postmitotic neurons on their way into the mantle zone.

Embryoid bodies

Aggregates of pluripotent stem cells that are in the process of differentiation. The 3D structure of embryoid bodies is thought to provide a cellular environment that promotes the differentiation of pluripotent cells into desired cell types in several differentiation protocols.

Short interfering RNA

(siRNA). Short double-stranded RNA molecules that silence gene expression in a sequence-specific manner by a process termed RNA interference.

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Genetic mosaicism

The presence of a variety of genetically distinct populations of cells within one individual. Differences in the cellular genotypes may comprise single-nucleotide polymorphisms (SNPs), indels, copy number variations and loss of heterozygosity. Such genetic changes can be caused by viral insertions, endogenous retrotransposition, DNA damage or repair and other mechanisms and may arise during development or later in life.

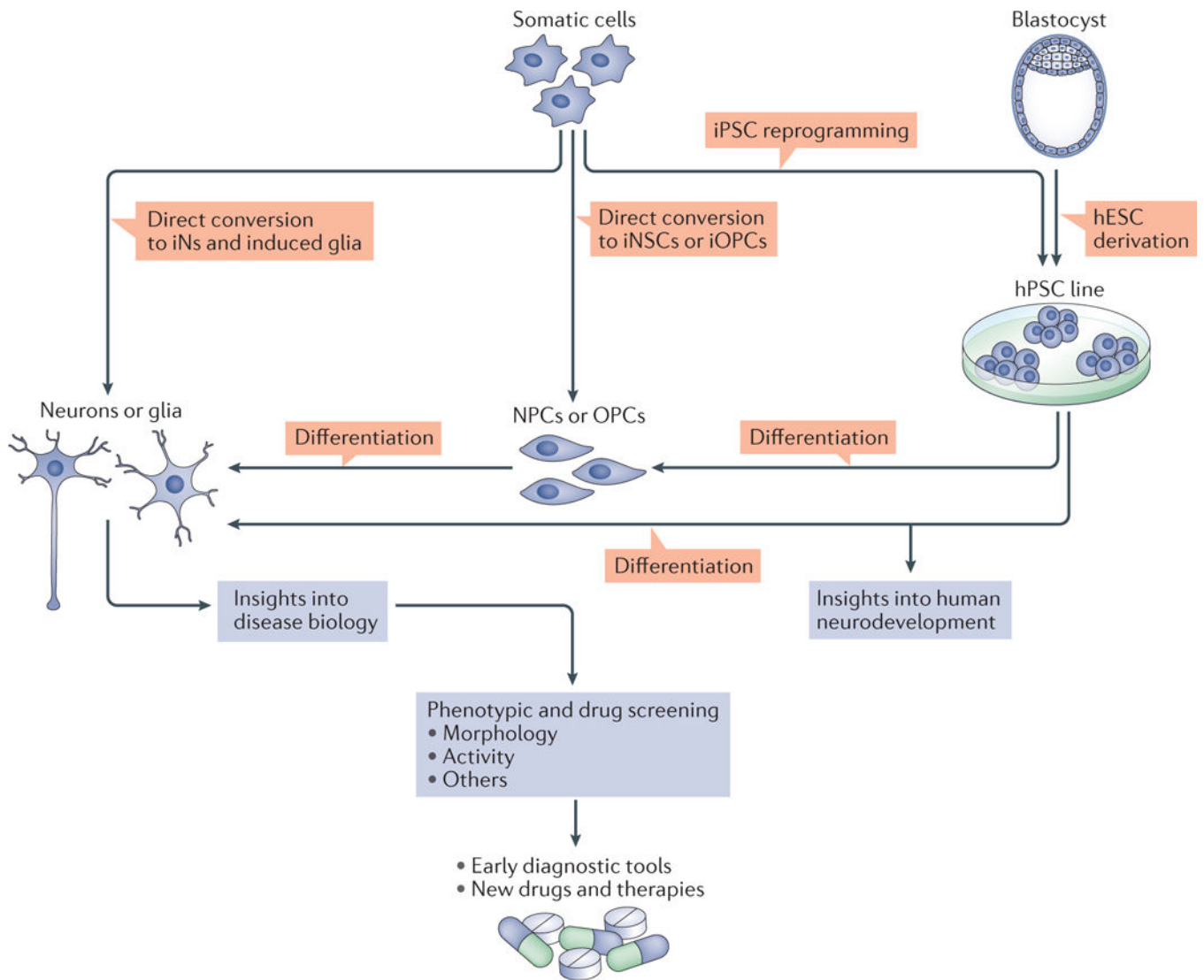


Figure 1 |. Reprogramming or direct conversion to generate neural cells.

Neural cells can be generated from somatic cells through somatic tissue reprogramming, which produces induced pluripotent stem cells (iPSCs), or by direct conversion. Neural progenitor cells (NPCs) and oligodendrocyte progenitor cells (OPCs) can be generated through the differentiation of human pluripotent stem cells (hPSCs), which can comprise human embryonic stem cells (hESCs) or iPSCs, or by direct neural conversion of somatic cells such as fibroblasts. Differentiation-derived NPCs as well as direct conversion-derived induced NPCs (iNPCs) can further be differentiated into neurons and/or glial cells and can allow the study of aspects of human neurodevelopment. When somatic cells or iPSCs are directly converted into induced neurons (which are then known as iNs or iPSC-iNs, respectively), the NPC stage is bypassed. Cultures of neurons and glia can be used for studying disease-related biology and to develop phenotypic assays and screening to evaluate patient- or disease-specific phenotypes. For example, cellular morphology, activity patterns and connectivity can be assessed. Once a distinct disease-related phenotype is identified that can be reliably monitored, drug-screening platforms can be developed to test compounds

that improve cellular phenotype. New diagnostic tools and therapeutic compounds could emerge from the screenings.

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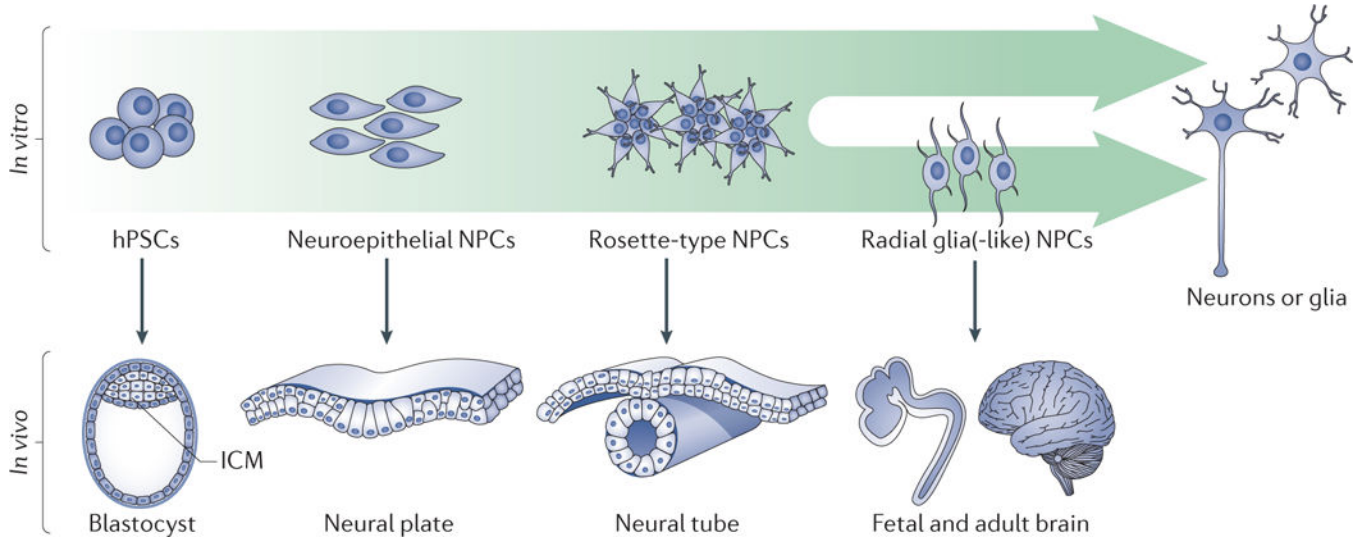


Figure 2 | Stages of neural differentiation *in vitro* and *in vivo*. When human pluripotent stem cells hPSCs (comprising human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs)) differentiate into neurons *in vitro* (upper row), they transit through defined stages during which they resemble distinct neural progenitor cell (NPC) populations present during *in vivo* neurogenesis (lower row). hPSCs resemble the inner cell mass (ICM) of the blastocyst^{3,190}. hPSCs differentiate into neuroepithelial stem cells *in vitro*, corresponding to the neuroepithelial NPCs that form the neural plate *in vivo*¹³. During *in vivo* neurulation, the neural tube closes, patterning along the developmental axes takes place and the first waves of neurons are generated. *In vitro*, the rosette-type NPCs that can also be derived from hPSCs resemble this developmental stage^{28,116,156}. During fetal and adult neurogenesis, radial glia give rise to postmitotic neurons. These correspond to the radial glia-like NPCs that are generated from the rosette-type NPCs *in vitro*^{28,68}.

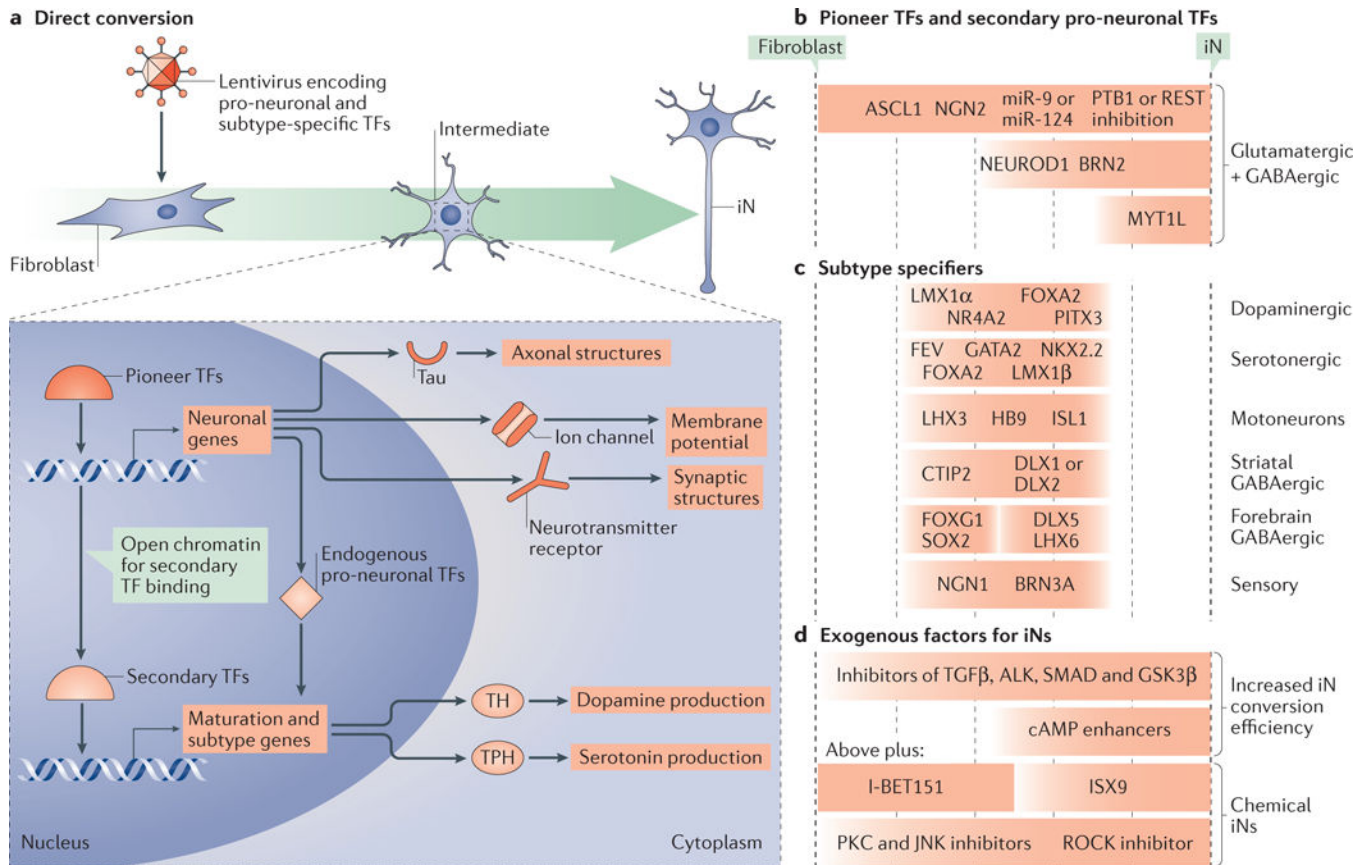


Figure 3 |. Methods for direct iN conversion.

a | During direct conversion into induced neurons (iNs), fibroblasts progressively convert into neurons through an as yet poorly defined transient intermediate state. This process involves dramatic morphological changes but no cell division. General pro-neuronal transcription factors (TFs) such as achaete-scute homologue 1 (ASCL1) and neurogenin 2 (NGN2) act as pioneer transcription factors that trigger the expression of structural neuronal proteins such tau (which help to drive the establishment of neuronal compartments such as the axon or dendrites)¹⁹¹, neurotransmitter receptors (which are required for postsynaptic structures)^{192,193} and ion channels (which build up a neuronal membrane potential)^{194,195}. Pioneer transcription factors also open chromatin structures to allow binding of secondary transcription factors (both transgenic and endogenous) that facilitate the expression of more mature or subtype-specific proteins, such as the enzymes tyrosine hydroxylase (TH) or tryptophan hydroxylase (TPH), which are needed for dopamine or serotonin production, respectively. **b** | The relative timeframe for direct iN conversion using different approaches. Orange bars indicate the stage at which the respective factors or compounds are believed to be effective. General pro-neuronal pioneer transcription factors such as ASCL1 and NGN2 work to reprogramme the cells at the fibroblast stage and tend to result in the production of glutamatergic neurons (the majority) and GABAergic neurons (a minor fraction). Secondary transcription factors such as neurogenic differentiation factor 1 (NEUROD1), BRN2 and myelin transcription factor 1-like protein (MYT1L) are not sufficient to initiate iN conversion but support the process at later stages^{7,102,108}. **c** | To facilitate neurotransmitter-

specific iN conversion, cocktails of specific transcription factors can be added to shape a specific neuronal identity. These lineage-specifying transcription factors are typically well known for their essential roles during the development of the targeted neuronal subtype *in vivo*^{88,89,99,119,122–124,196}. **d** | Manipulation of signal transduction pathways through growth factors and small molecules that inhibit the transforming growth factor- β (TGF β)–ALK–SMAD pathway and glycogen synthase kinase 3 β (GSK3 β), as well as the promotion of cyclic AMP signalling, increases iN conversion efficiencies. Addition of other molecules (such as I-BET151, isoxazole 9 (ISX9) or protein kinase C (PKC), JUN amino-terminal kinase (JNK) or RHO-associated protein kinase (ROCK) inhibitors) to that mix facilitates direct conversion from fibroblasts without the need for transgenes^{98,102,113,114}. CTIP2, COUP-TF-interacting protein 2; FOX, forkhead box protein; LMX1, LIM homeobox transcription factor 1; PITX3, pituitary homeobox 3; PTB1, polypyrimidine tract-binding protein 1; REST, RE1-silencing transcription factor.

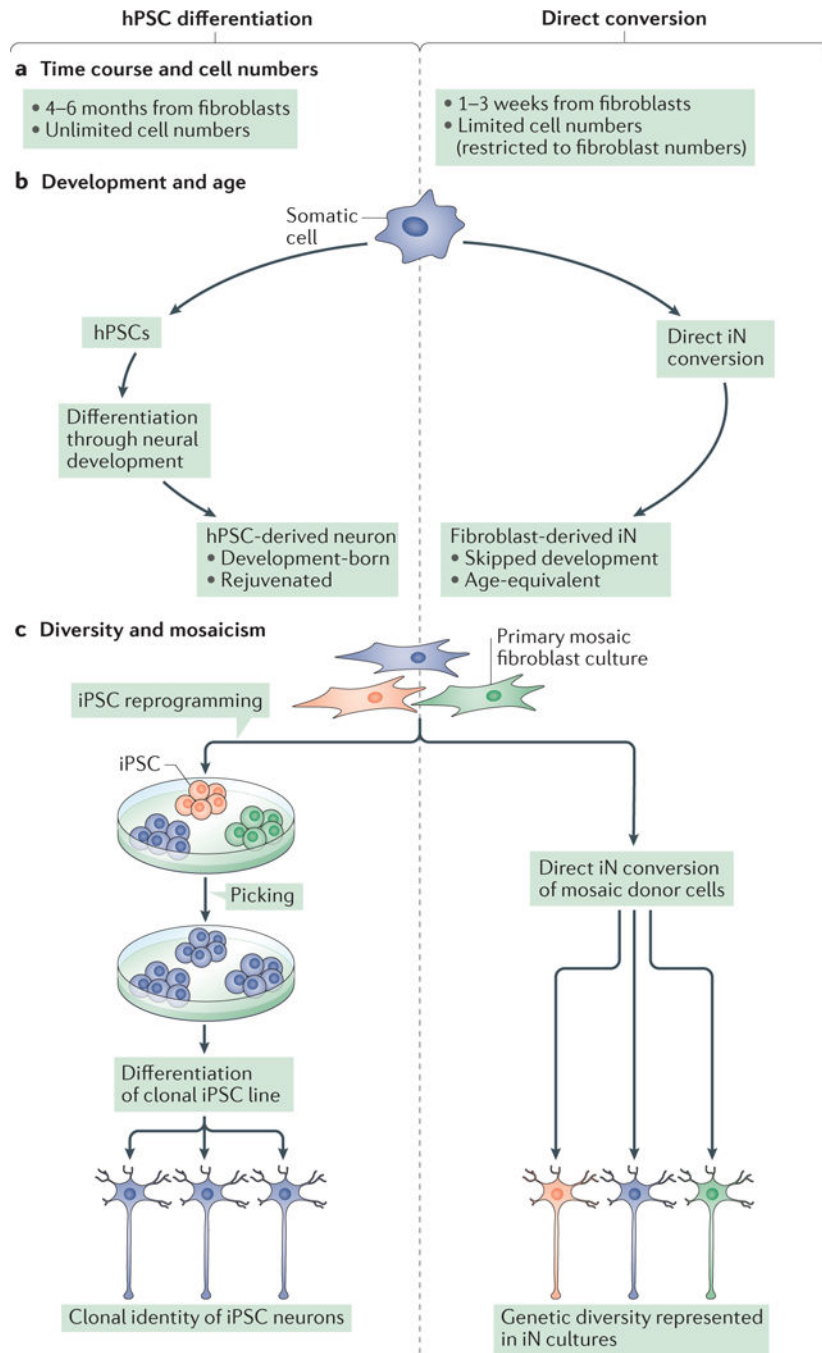


Figure 4 | Comparing iPSC differentiation and direct iN conversion.

a | Time course and efficiency. Direct induced neuron (iN) conversion is a rapid process for the generation of neurons from donor cells. However, human embryonic stem cell (hESC) and induced pluripotent stem cell (iPSC)-based strategies can yield infinite numbers of neurons, whereas iN conversion is limited to the expandability of fibroblasts. **b** | Development and age. In contrast to iNs, human pluripotent stem cell (hPSC) differentiation follows distinct steps of human neural development and transits through cell types corresponding to the various stages of neurulation and neurogenesis. However, hPSC-

derived cells transit through the embryo-like hPSC stage and, as a result, hPSC-derived neurons are regarded as rejuvenated neurons. Direct conversion skips these steps and directly transforms a fibroblast into a neuron, thus maintaining the signatures of their donors' ages. **c | Diversity and mosaicism.** Human somatic cells within an individual do not have identical genomes, and this somatic mosaicism might be an important determinant for biological function of tissues and organs¹⁸³. During iN conversion, a genetically mosaic culture of fibroblasts is converted into a mosaic culture of neurons. By contrast, iPSC lines are clonal cell lines, leading to a culture of neurons that all arise from the same single fibroblast cell.

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Table 1 | Comparing iPSC reprogramming and differentiation with direct conversion technologies

Property (tested to date)	iPSCs and NPCs	iNs	iGlia or iNPCs	Refs
Cell sources	Fibroblasts, keratinocytes, dental pulp cells, blood cells and renal epithelial cells	Fibroblasts, hepatocytes, adipocytes, pericytes, astrocytes and iPSCs	Fibroblasts and blood cells	3,7,97,136,138, 140–148,151
Vectors	Retro-, lenti-, adeno-, Epstein-Barr and Sendai viruses, non-integrating lentiviruses, plasmids, protein transduction, RNA transfection, BACs and piggyBACs	Lenti-, adeno- and Sendai viruses, non-integrating lentiviruses, plasmids and chemicals	Lentiviruses	101,114, 197–201
Time to generate neurons	4–6 months from fibroblasts, 3–6 weeks from iPSCs or 1–3 weeks from NPCs	1–3 weeks, plus maturation	1–4 weeks, plus terminal differentiation and maturation	7,13,102, 119,156
Capacity for expansion	Infinite	Only at fibroblast stage as the derived neurons are postmitotic	High but limited	3,7,98,184, 185,202
Potential numbers of cells	Infinite	Limited by the expandability of fibroblasts and conversion efficiency	High but limited	3,90,102,156
Capacity to generate different neural subtypes?	Yes; many	Yes, but only by using lineage-specific transcription factors	Yes	20,32,39, 57, 68,88,99,119, 122–124
Genomic identity	Donor-specific	Donor-specific	Donor-specific	3,7,56,114
Diversity and mosaicism	iPSC line is a single cell-derived clone	Reflects the cellular diversity of the donor tissue	Clonal and diverse populations possible	182,203,204
Epigenetic status	Identity and age erased	Cell type identity erased; details of epigenome unknown	Unknown	95,162,163,173
Capacity to model neurodevelopment?	Yes	No; neurodevelopmental stages skipped	Partially	176,177,205
Capacity to model ageing?	No; cells are rejuvenated	Yes; ageing signatures preserved	Unknown	168–171,173, 174,204

BAC, bacterial artificial chromosome; iGlia, induced glial cells; iPSC, induced pluripotent stem cell; iN, induced neuron; iNPCs, induced neural progenitor cell; NPC, neural progenitor cell.