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Direct somatic lineage conversion

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The predominant view of embryonic development and cell differentiation has been that rigid and even irreversible epigenetic marks are laid down along the path of cell specialization ensuring the proper silencing of unrelated lineage programmes. This model made the prediction that specialized cell types are stable and cannot be redirected into other lineages. Accordingly, early attempts to change the identity of somatic cells had little success and was limited to conversions between closely related cell types. Nuclear transplantation experiments demonstrated, however, that specialized cells even from adult mammals can be reprogrammed into a totipotent state. The discovery that a small combination of transcription factors can reprogramme cells to pluripotency without the need of oocytes further supported the view that these epigenetic barriers can be overcome much easier than assumed, but the extent of this flexibility was still unclear. When we showed that a differentiated mesodermal cell can be directly converted to a differentiated ectodermal cell without a pluripotent intermediate, it was suggested that in principle any cell type could be converted into any other cell type. Indeed, the work of several groups in recent years has provided many more examples of direct somatic lineage conversions. Today, the question is not anymore whether a specific cell type can be generated by direct reprogramming but how it can be induced.

1. Cell fate conversion between related cell lineages

Early studies in *Drosophila* revealed the existence of 'master' transcriptional regulators that can initiate large genetic programmes such as the transformation of antennas into legs or the ectopic induction of a well-structured, complex eye [1,2]. Working with mouse embryonic fibroblasts (MEFs) that were known to give rise to muscle cells upon treatment with the DNA demethylating agent 5-azacytidine, Weintraub and co-workers [3] isolated a single cDNA encoding the bHLH transcription factor MyoD that was alone sufficient to induce myogenic cells from fibroblasts. MyoD was found to also convert other mesodermal cell types into muscle, but cells of ectodermal origin were largely resistant to this reprogramming [4,5]. The discovery of MyoD sparked renewed interest in the efforts to find equivalent master regulators for other lineages. A resulting impressive finding was that B-lymphocytes could be converted into functional macrophages using just the single transcription factor C/EBPα [6,7]. Subsequently, more such lineage-conversion examples were described within the endodermal, mesodermal and ectodermal lineage compartments [8–11]. However, all of these examples were limited to cell lineage conversions between closely related lineages that share an immediate common precursor cell and it was debated whether direct lineage conversion may be limited to such closely related cell types.

2. Nuclear transfer and the discovery of induced pluripotent stem cells

In the 1950s, nuclear transplantation of amphibian somatic cells into oocytes led to the successful generation of live animals. This remarkable finding showed that all the information necessary for proper embryonic development is indeed stored in already specialized, somatic cells and these programmes can

be reactivated or 'reprogrammed' [12]. However, ensuing attempts to replicate this finding in mammalian cells remained unsuccessful which led people to conclude that somatic cells of higher organisms cannot be reprogrammed, akin to the higher restriction in regenerative potential of higher organisms. However, four decades later, Wilmut and co-workers [13] successfully cloned Dolly the sheep. This was a transformative discovery for the field of reprogramming because it unequivocally demonstrated that, even in mammals, somatic cells can be reprogrammed towards totipotency. Once it was clear that reprogramming of mammalian cells is generally possible, the search for reprogramming factors and their mechanism began. After another 10 years of intensive research by multiple groups, it was Yamanaka and co-workers [14–18] who identified a combination of four defined factors that was sufficient to reprogramme mouse and human fibroblasts into induced pluripotent stem (iPS) cells which were later shown to be molecularly and functionally indistinguishable from blastocyst-derived embryonic stem (ES) cells. The previous establishment of mouse and human ES cells from the inner cell mass of blastocysts was another important basis for the iPS cell discovery, as these unique cells could be maintained virtually indefinitely in culture in an undifferentiated, pluripotent state [19–21]. Unexpectedly, it turned out that iPS cell reprogramming was comparatively simple. Viral infection with just four factors (Oct3/4, Sox2, Klf4 and c-Myc) was sufficient to induce iPS cells in various somatic cells such as fibroblasts, keratinocytes, hepatocytes and mononuclear blood cells [22–25].

In addition, to provide a first approach to generate human cell types for disease modelling and transplantation-based therapies, the iPS cell discovery also suggested that lineage conversions even between distantly related cell types may be possible. More specifically, it raised the possibility that a specific combination of transcription factors unique to the target cell type would be sufficient to reprogramme other cell types into this lineage, challenging the notion at the time that somatic lineage conversions is restricted to closely related cell types. Extrapolating from lessons from iPS cell reprogramming, however, a single factor appeared to be insufficient and it may indeed require a combination of multiple important pluripotency factors. We explored this idea and specifically attempted to generate postmitotic neurons from fibroblasts.

3. Induced neuronal cells: direct lineage conversion between distantly related somatic cell types

Following the rationale outlined above, we hypothesized that somatic cell fate conversion across different germ layers is possible by overexpressing the right combination of transcription factors. As we desired to induce neuronal cells from fibroblasts, we searched for neuron-specific genes that could accomplish such a conversion. Since most reprogramming studies were based on transcription factors, we decided to only include this class of genes into our candidate list. We selected 19 candidate genes that showed specific expression or were otherwise related to reprogramming processes and introduced them in combination into MEFs [26]. Forced co-expression of these 19 factors induced a neuronal morphology and the expressions of a pan-neural marker albeit only with very low efficiency. Assuming that a smaller combination of factors may increase the conversion efficiency, we sought to identify the critical factors within the pool. We found that one of the 19 factors, the bHLH factor Ascl1 (also known as Mash1), was sufficient to induce cells with immature neuronal morphologies and expression of the neuronal markers Tuj1 and Tau. Adding each of the remaining 18 genes in two-factor combinations was then used successfully to identify those critical factors that further improve the induction of fully reprogrammed neurons. This experiment revealed that five additional genes (Brn2, Brn4, Myt1l, Zic1 and Olig2) increased the number of Tuj1-positive cells and improved the morphological complexity of the neuronal cells. After successive testing of various combinations of these five factors, we determined that the combination of the three factors Ascl1, Brn2 and Myt1l is sufficient and the most optimal way to induce neuronal cells from MEFs. Detailed electrophysiological characterization showed that these cells possessed all principal functional properties of neurons including the ability to fire action potentials as well as to form functional synapses. We therefore termed the cells induced neuronal (iN) cells. These three-factor MEF-iN cells appeared to be exclusively excitatory neurons, as we could detect only excitatory and no inhibitory postsynaptic events even though the cells expressed functional GABA receptors. Moreover, the cells expressed markers of excitatory neurons such as vesicular glutamate transporters but no inhibitory markers such as glutamic acid decarboxylase [26]. Remarkably, the reprogrammed cells became postmitotic within 24 h after induction of the reprogramming factors. With about 20% in MEFs, the reprogramming process was surprisingly efficient. In addition, the reprogramming dynamics were very rapid as neuronal genes were induced as early as 24 h after induction of the three reprogramming factors.

This paper demonstrated that somatic cells can be directly reprogrammed into distantly related lineages, as distant as cell types representing different germ layers. It remained open whether more cell types, other than fibroblasts, can be also converted into iN cells, for instance cells representing yet another germ layer. To address this question, we sought to reprogramme genetically marked hepatocytes and observed that the same three factors that work in fibroblasts can also convert primary hepatocytes into functional neurons [27]. Thus, similar to iPS cell reprogramming, iN cells can be generated from different lineages using the same combination of reprogramming factors and both mesodermal-to-ectodermal and endodermal-to-ectodermal conversion can be accomplished. Moreover, the genetic lineage tracing system employed in this study allowed us to carefully investigate the expression of the donor cell transcriptome. Based on bulk and single-cell RNA expression studies, we concluded that the hepatocyte-specific transcriptional programme is robustly silenced in iN cells [27]. A more recent survey of various reprogrammed cell populations with the aspiration to measure the authenticity of target cell types came to the conclusion that the degree of transcriptional similarity to target cell types varies between different reprogrammed cells, which is mostly driven by non-proper silencing of the donor cell programmes highlighting the importance of transcriptional repression in reprogramming [28].

4. Generation of human-induced neuronal cells

Encouraged by the promising advances in neuronal reprogramming of mouse cells, several groups set out to find ways to generate human iN cells. Despite a principal similarity in brain development between mice and humans, inter-species differences in gene regulatory networks affecting neuronal differentiation had been reported suggesting that the cues identified in mouse cells likely require adjustment for human cell reprogramming [29–31]. On the other hand, iPS cells were successfully generated from mouse and human cells using the same four factors [15]. We therefore tried initially to induce human iN cells from fibroblasts using the same three transcription factors that were successful in mouse cells. Although the neuronal marker Tuj1 and simple neuronal morphologies were induced upon transduction with the factors, the cells remained immature and lacked functionally mature properties. Thus, we screened for additional factors that in combination with the three original factors may enhance neuronal reprogramming of human cells. Consequently, we found that NeuroD1 together with the three factors Brn2, Ascl1 and Myt1l (BAMN) was able to generate functional iN cells from fetal and postnatal human fibroblasts [32]. NeuroD1 is a bHLH transcription factor downstream of Ngn1 and Ngn2 and essentially involved in neuronal specification and pancreatic islet cell development [33]. These human iN cells showed electrophysiological features and gene expression patterns similar to primary excitatory neurons just like the mouse iN cells generated before. When co-cultured with primary mouse cortical neurons, human iN cells formed functional synapses and showed both spontaneous and evoked postsynaptic currents. A recent study demonstrated that iN cells can be generated from non-human primate skin fibroblasts using the same combination of transcription factors [34].

Besides neurogenic transcription factors, the gene class of microRNAs increasingly received recognition and their role in regulating cell fate decisions has been suggested in numerous reports [35,36]. With regard to neuronal reprogramming, two independent studies succeeded in converting human fibroblasts into neurons by replacing transcription factors of the BAMN combination with microRNAs. An elegant study by Yoo et al. [37] showed that the combination of AMN together with miR-9 and miR-124 was effective in producing functional excitatory neurons with synaptic competence while forced expression of unspecific microRNAs did not produce neuronal cells. Remarkably, just the two microRNAs alone produced cells expressing a neuronal marker. It was further shown that human iN cells induced the neuron-specific (n)BAF chromatin-remodelling complex, which is likely mediated by the microRNAs miR-9* and miR-124 by repressing the subunit BAF53a, which in turn facilitates its replacement by BAF53b. Another study used the two transcription factors BM together with the most abundant microRNA in the human brain, miR-124, to successfully reprogramme postnatal and adult human fibroblasts into iN cells with some functional properties [38]. The cells produced using these three protocols appeared all of excitatory neurotransmitter phenotype. Around the same time, two additional groups reported the generation of mouse and human dopaminergic iN cells (see below). After just another year, a slightly different and more efficient approach for direct reprogramming of human fibroblasts to functional neuron-like cells applied forced expression of Ascl1 and Ngn2 in combination with small molecules. Upon optimized dual inhibition of the SMAD pathway as well as glycogen synthase kinase-3β signalling, the authors demonstrated neuronal conversion resulting in up to 75% of the cell population expressing immature neuronal markers like Tuj1 after three weeks [39]. Similarly, the two small molecules forskolin and dorsomorphin (a bone morphogenetic protein (BMP) pathway inhibitor) supported the Ngn2-mediated generation of human iN cells [40].

In general, these studies showed a substantial overlap of the essential reprogramming factors and mechanism for successful cell-fate conversion between human and mouse cells. However, iN cell reprogramming appears to be slower and less efficient in human cells. Neuronal reprogramming of human fibroblasts requires a maturation time of at least five weeks in order to form functional synapses while postsynaptic currents can be recorded in murine iN cells after only two weeks. Moreover, the reprogramming efficiency of human iN cells using proneural transcription factors was 10 times lower than mouse neuronal induction (2–4% versus 10–20%). This remarkable resistance of human somatic cells against iN cell reprogramming appears to be a more general phenomenon as it is also observed to be reduced in iPS cell, induced hepatocyte and induced cardiomyocyte reprogramming. Similar to our observations with iN cell reprogramming, the direct conversion of mouse cardiac fibroblasts was efficiently achieved with a combination of three transcription factors (Gata4, Mef2c and Tbx5), whereas reprogramming of human cardiac fibroblast required the co-expression of two additional factors (Mesp1 and Myocd) [41,42]. By contrast, direct reprogramming of mouse and human fibroblasts into hepatocytes was successfully demonstrated with mostly overlapping combinations of three transcription factors. For mouse cells, the factor combination Hnf4α, Foxa1, Foxa2/Foxa3 [43] or Gata4, Hnf1α, Foxa3 [44] showed similar results and the combination FOXA3, HNF1A, HNF4A was effective in human cells although the maturation stage of induced hepatic cells may not be equivalent [45]. Of note, another study by Du et al. claimed that a combination of HNF1A, HNF4A and HNF6 together with the factors ATF5, PROX1 and CEBPA was required to generate more mature hepatocytes [46]. These findings confirm modest inter-species differences in regulation of transcriptional programmes for lineage differentiation and further suggest that the complexity of an organism is partially reflected in lineage identity specification on the cellular level.

5. Generation of induced neuronal cells with specific neuronal subtype properties

The mammalian central and peripheral nervous system consists of thousands of different types of neurons with distinct properties regarding morphology, receptor and ion channel composition, firing behaviour and neurotransmitter usage. The major classification is based on the usage of neurotransmitters such as glutamate, GABA, serotonin, dopamine, other catecholamines like adrenaline and epinephrine, and acetylcholine. One important question that arose was whether direct reprogramming can accomplish the generation of iN cells with specific neurotransmitter phenotypes and specific regional identity. In the following, we will discuss the current progress towards this goal (table 1).

4

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Table 1. Summary of studies on direct reprogramming to specific neural subtypes.

species	neural subtype	factors	original cell	efficiency	action potential	synaptic competence	ref
mouse	excitatory neuron	Pax6	postnatal astrocyte	Tuj1 (+): 50-60%	ND	ND	[8]
		Ngn2		Tuj1 (+): 71%	OK	OK	[47]
		Ascl1		Tuj1 (+): 37%	OK	OK	[47]
		Ngn2		MAP2 (+): 91%	OK	OK	[48]
		Ascl1, Brn2, Myt1l	MEF	Tuj1 (+): 20%	OK	OK	[26]
		Ascl1		Tau (+): 10%	OK	OK	[49]
		Ascl1, Brn2, Myt1l	hepatocyte	Tau (+): 5%	OK	OK	[27]
	dopaminergic neuron	BAM, Lmx1a, FoxA2	MEF	TH (+): 10%	OK	ND	[50]
		Ascl1, Lmx1a, Nurr1	MEF	TH (+): 18%	OK	ND	[51]
		Ascl1, Lmx1a, Nurr1, Foxa2, En1, Pitx3,	TTF	TH (+): 9.1%	OK	ND	[52]
	inhibitory neuron	Dlx2	postnatal astrocyte	Tuj1 (+): 93%	OK	OK	[48]
	spinal motor neuron	BAM, Lhx3, hb9, Isl1, Ngn2	MEF, TTF	Hb9 (+): 5-10% (MEF)	OK	OK	[53]
	retinal ganglion like cells	Ascl1, Brn2, Ngn2	MEF, ETF (adult ear fibroblast)	RPF1 (+): 5% (MEF), <1% (ETF)	OK	ND	[54]
	neural precursor cell	Sox2, Brn2, Foxg1	MEF	ND (proliferating)	OK (iNPC derived neuron)	ND	[55]
	oligodendrocyte precursor cell	Sox10, Olig2, Zfp536	MEF	ND (proliferating)	ND	ND	[56]
human	excitatory neuron	Ascl1, Brn2, Myt1l, NeuroD1	embryonic, postnatal fibroblast	TUJ1: 2-4%	OK	OK	[32]
		Ascl1, NeuroD2, Myt1l, miR-9/9*, miR-124	postnatal, adult fibroblast	postnatal: Tuj1 (+) 4-8 %; adult: Tuj1 (+) 1-11%	OK	OK	[37]
		Brn2, Myt1L, miR-124	postnatal, adult fibroblast	postnatal: Tuj1 (+) 4-5 %; adult: Tuj1 (+) 1.5-11.5%	OK	ND	[38]
	immature neuron	Ascl1	fetal fibroblast	ND	OK	ND	[49]
	dopaminergic neuron	BAM, Lmx1a, FoxA2	embryonic, postnatal fibroblast	TH (+): 10%	OK	ND	[50]
		Ascl1, Lmx1a, Nurr1	fetal, adult fibroblast	TH (+): 3%	OK	ND	[51]
	spinal motor neuron	BAM, Lhx3, Hb9, Isl1, Ngn2, NeuroD1	embryonic fibroblast	Hb9 (+): 0.03%	OK	ND	[53]
	inhibitory neuron	Bcl11B, Dlx1, Dlx2, Myt1l, miR-9/9*, miR-124	postnatal, adult fibroblast	MAP2: 90%; TUJ1: 87%	OK	OK	[57]

(a) Generation of induced dopaminergic neurons

The quest to generate iN cells with dopaminergic properties was one of the first goals given the clinical importance of the midbrain-type dopamine neurons for Parkinson's disease (PD). Successful induction of dopaminergic neurons (iDANs) from human somatic cells was first shown by two European groups in 2011. Pfisterer et al. [50] reported that forced expression of the BAM factors together with transcription factors for midbrain patterning and specification of dopaminergic neurons, Lmx1a and Foxa2, can covert human embryonic fibroblasts into neuronal cells expressing general dopaminergic neuronal markers. These markers included enzymes required for dopamine biosynthesis like tyrosine hydroxylase and aromatic L-amino acid decarboxylase as well as the orphan receptor Nurr1 which is an important transcriptional regulator for midbrain dopaminergic neurons. Another group led by Caiazzo et al. [51] demonstrated that a three-factor combination of Ascl1, Nurr1 and Lmx1a successfully generated iDAN cells with key dopaminergic features from embryonic and adult human fibroblasts. Also, these cells expressed the marker panel of dopamine neurons and in addition appeared to possess functional properties like the stimulation-dependent release of dopamine as determined by amperometry and high performance liquid chromatography analysis. Both studies, though, lacked evidence of proper midbrain regionalization. This is of critical importance because previous studies suggested that only properly specified dopamine neurons will innervate striatal medium spiny neurons and thus lead to functional recovery after transplantation into Parkinsonian animals, and therefore Kim et al. [52] sought to identify factors that were able to induce more midbrain-like features in iDANs. To this end, the authors used tail tip fibroblasts of Pitx3-EGFP knock-in mice, as Pitx3 is a highly specific reporter for authentic midbrain dopaminergic neurons [58]. After screening a set of 11 candidate factors, the combination of just Ascl1 and Pitx3 was found to be sufficient to produce Pitx3-EGFP positive cells. However, expression analysis of genes involved in the biosynthesis, uptake and storage of dopamine revealed partial reprogramming of these cells and a low similarity to primary midbrain dopaminergic neurons. Upon including additional reprogramming factors (Lmx1a, Nurr1, Foxa2 and En1) together with Ascl1 and Pitx3, i.e. a total of six factors, in combination with the neurothrophic factors Sonic hedgehog (Shh) and fibroblast growth factor 8 (FGF8), the efficiency of Pitx3-EGFP cells drastically increased (9.1% at 18 days after induction) and expression of more mature dopaminergic neuron markers was enhanced. Importantly, these six-factor iDAN cells were demonstrated to release dopamine and improve behavioural phenotypes in a 6-hydroxydopamine mouse model of PD. However, the functional quality of these iDAN cells in vivo was significantly lower than transplantation of primary dopamine neurons as reflected by the higher number of cells required to alleviate disease burden. These data indicate that fully functional integration of reprogrammed neuronal subtypes in vivo requires a high level of specification exceeding the combined instructive accuracy of as many as six cell-type specifying factors.

(b) Generation of induced motor neurons

Spinal motor neurons are of similarly high interest for disease study and regenerative medicine as dopamine neurons. Amyotrophic lateral sclerosis (ALS) is a severe, progressive neurodegenerative condition that affects these cells. Son et al. [53] showed that the addition of four motor neuron-specific factors (Lhx3, Hb9, Isl1 and Ngn2) to the BAM combination efficiently induced functional motor neurons from mouse embryonic and postnatal fibroblasts. The generated induced motor neurons (iMNs) resembled primary motor neurons in comprehensive transcriptional profiling, exhibited protein marker expression specific for motor neurons, including Hb9 and the enzyme choline acetyltransferase (ChAT), and showed specific electrophysiological properties such as responsiveness to both GABA and glutamate. Intriguingly, iMN were capable of forming functional neuromuscular synapses when co-cultured with muscle cell line-derived myotubes, which could be blocked by an acetylcholine receptor antagonist. Noteworthy, when iMN cells were induced from MEFs carrying a mutant superoxide dismutase (Sod1) gene, implicated in the development of ALS, the viability of mutant iNMs in wild-type-glia co-cultures was reduced compared to control iNMs. These data suggest that iMN cells provide a powerful tool to study the pathophysiology of ALS. Similar to our findings, the addition of NEUROD1 to the factor combination found for mouse cells also facilitated the generation of human iMNs from ES cell-derived fibroblast-like cells.

(c) Induction of inhibitory, striatal medium spiny neurons

Inhibitory neurons are critical regulatory elements in neural circuits. A brain structure predominantly consisting of inhibitory neurons is the striatum. Striatal neurons are also known to be most susceptible to neurodegneration in Huntington's disease and therefore of high clinical interest. Recently, an elegant study succeeded in generating iN cells with many hallmarks of inhibitory striatal neurons [57]. The efficiency from human postnatal and adult fibroblasts was remarkably high when the transcription factors CTIP2, DLX1, DLX2 and MYT1 L and microRNAs miR-9/9* and miR-124 were combined. The transcription factors chosen are highly expressed during development of the striatum, an apparently important criterion for identification of successful reprogramming factors as documented reprogramming factors tend to be highly and specifically expressed in the target cell type. The reprogrammed cells expressed general markers for GABAergic neurons such as GABA, GAD67, and were negative for glutamatergic markers such as VGLUT1. More careful single-cell gene expression analysis confirmed the presence of GABAergic markers and showed the absence of markers for dopaminergic, cholinergic, glutamatergic and serotonergic neurons. Moreover, a substantial overlap of expression profiles between inhibitory, striatal medium spiny neurons (iMSNs) and microdissected human striatal tissue including the specific marker DARPP-32, as well as the lack of doublecortin expression, which is enriched in migratory neurons, suggest that these cells indeed resemble mature interneurons of the striatum. Upon transplantation into mouse brains, these cells showed long-term survival of over six months, functional integration into local circuits and displayed typical electrophysiological properties of MSNs including a typical action potential firing pattern. Transplanted human iMSNs extended projections from their injection site in dorsal striatum to the substantia nigra, the anatomical targets

5

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of MSNs. Notably, addition of the microRNAs and co-expression of the antiapoptotic gene BCL2L1 dramatically increased conversion efficiency from 0.3 to 90% MAP2-positive cells from postnatal and to 82% MAP2-positive cells from adult fibroblasts. These data therefore show that combining different components that potently direct a cell identity and eliminate unrelated cell biological complications like apoptosis can efficiently generate highly specific neuronal subtypes competent of accurate integration *in vivo* and encourage further development of potential regenerative medicine applications as well as similar approaches for generation of other neuronal subtypes.

(d) Generation of induced peripheral sensory neurons

Peripheral neuropathies are frequent clinical complications as primary diseases or secondarily as response to another condition (such as diabetes or adverse drug effects) with little therapeutic options. Therefore, access to authentic human peripheral sensory neurons represents an important scientific goal with potentially large clinical implications. Two groups recently reported exciting findings that suggest that mouse and human fibroblasts can be converted to iN cells with even such a high degree of subspecialization as the three main classes of peripheral sensory neurons, including nociceptive neurons that would be highly relevant for pain research [59,60]. The Baldwin group found that just two transcription factors, either Ngn1 or Ngn2, in combination with Brn3a, a critical lineage determination factor for peripheral neurons, was sufficient to convert both mouse primary and human iPS cell-derived fibroblasts to iN cells with peripheral identity representing the three major classes of sensory neurons: nociceptive, mechanosensitive and proprioceptive, which express the key specific markers TrkA, B and C, respectively [59]. The neurons exhibited a typical pseudounipolar morphology, expressed several additional markers of sensory neurons and responded to appropriate stimuli like capsaicin, menthol and mustard oil.

Another study by the Woolf and Eggan groups focused directly on generation of nociceptive neurons using reporter mice for expression of the capsaicin receptor TrpV1, a gene specific to pain sensing cells [60]. Starting with 10 candidate transcription factors that were systematicallly tested in groups and in combination with the three BAM factors, the authors arrived at a combination of five factors (Ascl1, Myt1 L, Isl2, Ngn1 and Klf7) capable of converting 14% of mouse fibroblasts into neuronal cells that were mainly positive for the peripheral nervous system intermediate filament peripherin and showed gene activation of sensory neuron-specific receptor TrkA and the sodium channel $Na_v1.7$ [60]. Surprisingly, the well-studied transcription factor Brn3a that was identified by the Baldwin group to possess high reprogramming activity did not further improve the reprogramming in the context of these five factors. Moreover, pharmacological stimulation of TrpA1, TrpM8 and TrpV1, and P2X3, as part of the highly specific composition of ionotropic receptors and ion channels of nociceptors, revealed a heterogeneity that is similar to what is found *in vivo*. Already evaluating these cells for applied research, the team found that prostaglandin E2 and oxaliplatin were able to sensitize the generated nociceptive iN cells to capsaicin response suggesting that these cells are able to mimic inflammatory pain and pain associated with chemotherapy-induced

neuropathies, respectively. Finally, the authors demonstrated that similar cells can be obtained from human skin fibroblasts from individuals up to 10 years of age using the same five factors, but efficiencies were decreased several fold compared to mouse fibroblasts. Nevertheless, the authors have already demonstrated the usefulness of the human nociceptive iN cells for detecting morphological phenotypes in cells derived from familial dysautonomia patients such as decreased neurite outgrowth.

6. Direct conversion of human pluripotent stem cells into induced neuronal cells

Since the first successful approaches for neuronal induction of human ES cells via embryoid body and neural rosette formation in defined media [61,62], numerous strategies have been developed to generate functional neurons through stepwise directed differentiation of pluripotent cells. Mostly through the application of small molecules such as dual SMAD inhibitors (blocking both BMP and TGFβ pathways) and selective patterning and growth factor signalling cues, differentiation protocols were refined to produce several different neuronal subtypes including excitatory cortical neurons, forebrain inhibitory interneurons and midbrain dopaminergic neurons [63–65]. A complication of these differentiation methods is the multi-stage and therefore experimentally involved differentiation procedure and the protracted functional maturation of the differentiating neurons, which often require over eight weeks to exhibit the first signs of synaptic activity [64,66]. Given our observation that just a few transcription factors have the remarkable ability to induce the neuronal lineage from even distantly related cell types, we sought to revisit the effects of proneural transcription factors in human pluripotent stem cells. The approach to apply transcription factors to facilitate differentiation steps has been used before, in particular in attempts to accomplish subtype specification, but the specific induction of postmitotic neurons has not been tested and optimized in a systematic manner for human pluripotent stem cells [67–70]. We initially tested the BAM factors in human ES cells and noted a dramatic expedition of adoption of neuronal features including repetitive action potential firing as early as 6 days after infection [32]. We then screened additional neurogenic factors for similar effects and found that forced expression of NeuroD1 or Ngn2 was sufficient to efficiently convert human ES and iPS cells into functional neurons [67]. The vast majority of infected cells robustly developed a neuronal morphology and showed pan-neuronal marker expression as early as one week after infection. When co-cultured on primary mouse glia spontaneous and evoked excitatory postsynaptic currents (sEPSC and eEPSC) were recorded in over 90% of the derived hES-iN cells indicating functional maturity and pronounced competence of synapse formation. Thus, compared to conventional differentiation strategies, the functional maturation is accomplished much faster in ES−iN and iPS−iN cells. Notably, forced expression of transcription factors is not the only way to accelerate neuronal induction from pluripotent stem cells. A recent study demonstrated that specific combinations of several small molecule inhibitors were able to dramatically shorten the timing of neuronal induction towards a

peripheral neural identity without the use of transcription factors [71].

7. Direct conversion of somatic cells versus induction of pluripotent cells

In order to evaluate the advantages and limitations of the various approaches to generate human neuronal cells, the starting material, the process itself and the properties of the resulting cells need to be considered in the context of the desired application. Direct reprogramming of somatic cells into a target cell type by forced expression of reprogramming factors without going through an intermediate iPS cell state is a very powerful tool for disease modelling because of the ability to generate specific mature human somatic cells within a short period of time. Our laboratory has demonstrated that fibroblasts could be directly converted into functional mature neuronal cells within two weeks, whereas conventional differentiation of pluripotent cells into somatic lineages (such as neural, hematopoietic, cardiac and hepatic) through exposure to morphogens and small molecules produces cells that are typically immature, resembling fetal stages [72]. Even maturation times of several months are often insufficient to generate mature, adult-like cell types from pluripotent stem cells. For modelling diseases of adult patients, in particular those affecting aged populations such as neurodegenerative diseases, it would be desirable to actually obtain cells of adult maturation levels to assess tissue-specific phenotypes.

A clear advantage of the iPS cell route though, is their unparalleled expandability while maintaining an unaltered pluripotent state. Several somatic cell types can also be expanded in culture (such as fibroblasts, neural stem cells, myoblasts and keratinocytes) but in most cases the cells change epigenetically and typically loose features of stemness and differentiation potential (in the case of somatic stem or progenitor cells). Therefore, in addition to the much higher proliferation capacity, iPS cells can truly self-replicate in culture. The other side of this coin, though, is that iPS cells are clearly not immune to accumulating mutations of various kinds when propagated in culture, including point mutations, copy number variations of small and large genomic fragments, and even gross karyotypic abnormalities [73–79]. In fact, a large survey of hundreds of cell lines found much higher karyotypic stability of directly transdifferentiated cells compared to stem cell-derived cells [79]. The thus far still unappreciated genomic instability of iPS cells in fact puts restrictions on the praised possibility of interrogating the precise genetic background of individual human subjects and patients.

Presumably related to the genomic instability is the widely observed line-to-line variability of iPS cells with respect to growth rate, growth behaviour, transfection/infection rates and differentiation propensities in general and biases towards specific lineages [80]. Importantly, this leads to erroneous conclusions when subjecting those cells to functional assays. This line-dependent variability further causes pronounced experimental noise which complicates and might even preclude meaningful results from high-throughput screens.

Further advantages of direct somatic conversion are timing and homogeneity of target cell types. The generation of a specific cell type from human pluripotent stem cells requires a labour-intensive two-step process of 'reprogramming' of the donor cell and a second often prolonged step of 'differentiation' into the target cell. The single-step 'short cut' approach of direct lineage conversion not only eliminates one step, it also substantially speeds up the generation of mature cell types. Moreover, the advantage of pluripotency to be able to derive many different cell types from one single-cell population is also a disadvantage when it comes to attempts to generate a single defined somatic cell type. Indeed, most iPS cell differentiation protocols typically yield heterogeneous somatic populations such as mixtures of excitatory and inhibitory neurons of various maturation stages in neuronal differentiation protocols. By contrast, iN cells represent a considerably homogeneous population of one predominant neuronal subtype. The homogeneity of the target cell population and a low line-to-line variability is of particular relevance when established diseases models are used for drug discovery where differential responses need to be attributed to specific genetic mutations of the patients.

Another point on the list of advantages for pluripotent stem cells is the ability to genetically engineer their genome in an increasingly efficient manner [81]. This is of course based on the great expandability of iPS and ES cells as discussed above. Therefore, disease-associated mutations can be artificially induced in control lines or repaired in disease lines and the exact contribution of the specific genetic lesion can be evaluated in an isogenic control situation which has proved to be a powerful approach [82,83]. Up to now, the majority of studies that model neurological diseases *in vitro* use human neurons generated from pluripotent stem cells. While disease modelling has really just begun to be applied at the larger scale, there are already several excellent proof-of-principle studies that demonstrate feasibility and in some cases new biological insights could be derived. With respect to neurological/neuropsychiatric diseases, iPS cell-derived neural cells have been successfully employed to analyse genetic implications of familial dysautonomia [84], PD [82], schizophrenia [85,86], autism [87,88] and Alzheimer's disease [89,90]. An outstanding question is whether the young neurons produced from iPS cells can reflect aspects of aged neurons, such as the ones in decades-old brains of Alzheimer's disease patients, and whether perhaps fibroblast-iN cells reflect the donor age better than iPS cell-derived neurons [91].

With respect to potential applications in regenerative medicine, direct cell fate conversion of somatic donor cells would have the advantage of minimizing the inadvertent inclusion of undifferentiated cells with high proliferative capacity in the cell population to be transplanted. One of the major concerns regarding pluripotent stem cell-derivatives for transplantation therapies is the potential tumour formation from residual pluripotent stem cells. Even less than 1% of residual undifferentiated pluripotent stem cells in the grafted cell population can lead to teratoma formation calling for efficient purification methods or intricate safety measures to be engineered in pluripotent donor cells [92–94]. Direct conversion of somatic donor cells to any type of mature neuronal cell, on the other hand, avoids the risk of tumour formation because neurons are postmitotic and no oncogenes are being used for reprogramming. Using the BAM factors in reprogramming mouse fibroblasts, we showed that the vast majority of infected cells exited the cell cycle as early as 24 h after transgene activation and that

proliferation was arrested at day 3 [26]. But even direct somatic lineage reprogramming towards proliferative somatic populations like neural stem cells or oligodendrocyte precursor cells represents a much lower risk of cancer formation because of their reduced proliferative capacity compared to iPS cells [55,56,95–99]. Intrinsically linked with this lack of proliferative capacity is the complication of obtaining enough material for transplantation, in particular considering the size of human organs. Therefore, improvement of neuronal reprogramming efficiencies of human somatic cells and protocol adaptions to accessible, replenishing donor tissue (e.g. peripheral blood cells readily available in large quantities) are key issues to work on in the future for successful clinical applications.

The direct induction of neuronal identities in pluripotent cells by forced expression of instructive transcription factors constitutes a promising alternative to both direct cell fate conversion of somatic cells and directed differentiation of pluripotent stem cells for most applications [32,67]. This combined iPS/iN cell approach embraces advantages of both systems and eliminates some of the disadvantages. In particular, the short maturation time, the unparalleled efficiency of induction, and the homogeneity of the neuronal population are key advances in the field. Pluripotent stem cell-derived iN cells are already suitable for robust, functional studies of cellular disease phenotypes as well as high-throughput screens for drug discovery [100].

8. Future challenges

We have discussed several current limitations and challenges that the field faces throughout this review article. Some of the main critical hurdles that will need to be overcome are the ability to induce mature, adult-type somatic cells at high efficiency and of high purity either from pluripotent stem cells or directly from other somatic cells through direct lineage reprogramming. With respect to lineage reprogramming, the efficiencies need to be improved for any larger scale technical or clinical application. Unfortunately, reprogramming efficiencies drop with age of the donor cells and also human cells are harder to reprogramme than mouse cells. Future studies will have to be performed to identify the molecular roadblocks that are apparent in aged and human cells. A rational approach to ultimately increase reprogramming efficiencies is to first spend substantial effort to investigate the molecular underpinnings of the reprogramming mechanisms. With respect to pluripotent stem cells, differentiation protocols will have to be continuously improved and optimized. One possible solution we propose is to not shy away from using DNA or RNA molecules in differentiation approaches which allow the delivery of powerful transcriptional regulators that are capable of regulating important lineage fate decisions with high efficiency and reproducibility as demonstrated by the ES-iN cells obtained with forced Ngn2 expression [67].

In our mind, a major challenge in the future will be genomic stability of iPS cells. This will be critical for both disease modelling (to ensure minimal changes of the genetic background to be interrogated) and regenerative medicine to minimize cancerous transformation or creation of de novo antigens. It is encouraging to know that different culture and dissociation methods have different propensities to develop genomic abnormalities, suggesting that a culture method can be identified that ensures maximal genomic stability.

Finally, disease modelling in two-dimensional culture systems has obvious limitations. The recent development of three-dimensional culture systems that seem to recapitulate self-organizing organoid structures and the prospect to generate human–animal chimeras are exciting new lines of research that will likely enrich our experimental tool sets to study human disease [101–105].

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11

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