Stem Cell Therapy for Neurological Disorders: From Bench to Bedside

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Introduction

According to the National Institute of Neurological Disorders and Stroke (NIH), there are found to be 488 neurological disorders present in the world at the time of writing, ranging from acid lipase disease to Zellweger syndrome [1]. No book chapter is equipped to tackle all of these in detail; however, it will focus upon the five major neurological diseases: Alzheimer's disease, Parkinson's disease, Huntington's disease, Multiple Sclerosis and Amyotrophic Lateral Sclerosis. The chapter will begin by summarising the epidemiology, pathology and disease susceptibilities for each of these five major neurological diseases. The chapter will then look in detail at generating patient-specific cell lines for personalised medicine, genome editing and the differentiation protocols necessary for cell replacement therapy related to the aforementioned neurodegenerative diseases. This chapter will then look at the alternative cell sources that have been used as existing and current cell therapeutic strategies before summarising with the advantages and constraints of stem cells in research and clinical translation. Finally, the chapter will conclude on the current research findings with a particular focus on patient-derived research in Parkinson's disease and how different therapeutic strategies can be targeted at different neurological diseases focusing on – Parkinson's disease and multiple sclerosis – before summarising on the challenges for stem cell therapy in neurological disorders: from bench to the bedside.

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Neurological Disorders

Alzheimer's Disease

Epidemiology and Pathology

Alzheimer's disease (AD) is the most common age-related neurodegenerative disease in the world currently estimated to affect 30 million people, a figure expected to quadruple in 40 years [2]. In 2010 alone AD was estimated to have cost the world $604 billion [3]. This staggering figure will only increase.

Pathologically AD is characterised by three cardinal changes in the brain: the presence of amyloid β (Aβ) plaques, intra-neuronal hyper-phosphorylated microtubule-associated protein tau and the loss of specific neurons and synapses, principally pyramidal neurons that are located in the cerebral cortex and cholinergic neurons of the basal forebrain [4]. However, as the disease progresses serotoninergic and noradrenergic neurons are also affected, with post-mortem tissue showing further loss of GABA and somatostatin cell types. Loss of these

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neuronal subtypes leads multiple atrophy of these affected brain regions, often beginning with the hippocampus and including the entorhinal cortex, frontal, parietal and temporal cortices [5]. For further reading on the progressive AD stages, see Braak and Braak. AD is symptomatically characterised by a progressive loss in learning, memory and cognitive decline.

Disease Susceptibility

AD is largely thought to be sporadic with genetic mutations thought to account for 0.5 % of all AD patients worldwide [1]. Mutations in three autosomal dominant Mendelian risk genes – *APP*, *PSEN1* and *PSEN2* – are highly penetrant and lead to an early onset of this disease [1–4]. Mutations in *APP* result in increased levels of Aβ and also change the ratio of cleaved Aβ peptides from 40 amino acids ($A\beta_{40}$) to increasing levels of the more toxic $A\beta_{42}$ peptides [5]. The $A\beta_{42}$ peptide is more hydrophobic and amyloidogenic than the $A\beta_{40}$ form and leads to increasing amyloid fibrillogenesis [1]. Mutations in *PSEN1* and *PSEN2* have been found to impair the activity of γ-secretase that is involved in the cleavage of *APP*, which also results in an increased $A\beta_{42}$ to $A\beta_{40}$ ratio [6–9]. Mutations in *APP*, *PSEN1* and *PSEN2* account for only 13 % of all early-onset AD patients [5]. *APOE* is a moderately penetrant gene with semi-dominant inheritance [10]. Inheriting the ε4 allele, homozygous *APOE* ε4ε4 and heterozygous *APOE* ε3ε4, increases the risk of AD 15x and 3x, respectively, compared to the most common *APOE* ε3ε3 form of the gene [11].

Parkinson's Disease

Epidemiology and Pathology

Parkinson's disease (PD) is a multifactorial neurodegenerative disease characterised by the loss of A9 dopaminergic neurons in the substantia nigra pars compacta (SNc) of the midbrain. The presence of intra-cytoplasmic inclusions (Lewy bodies) is another characteristic feature in those midbrain dopaminergic neurons (mDA) in the SNc that remain [12]. PD is the second most common neurodegenerative disease worldwide; the results from a 2010 census in the USA found PD to affect 630,000 people in the USA costing $14.4 billion in 2010 alone; as the prevalence to PD is projected to double by 2040 due to an increased elderly population, this cost will only increase [13].

The dopaminergic (DA) neurons project from the SNc to the dorsolateral striatum, caudate and putamen forming the nigrostriatal pathway that releases the neurotransmitter dopamine; it is thus the reduction in the dopamine following the progressive loss of DA neurons that allows the disease to manifest and become symptomatic. The four cardinal motor symptoms that signify the manifestation of the disease remains the primary tool of clinical diagnosis: bradykinesia, resting tremor, rigidity and postural instability. However, there are many other non-motor symptoms that are associated with the disease and can often precede the initial diagnosis, such as depression and gastrointestinal difficulties. As no PD patient presents with a homogenous aetiology, PD remains a difficult disease to diagnose without any of the motor symptoms. At the stage when the PD has manifested into the characteristic motor symptoms that precede diagnosis, up to 70 % of the mDA neurons of the SNc have already degenerated [14].

Disease Susceptibility

Approximately 90 % of all PD is idiopathic and thus of an unknown aetiology [15]. However, there are genetic susceptibility loci and risk genes attributed to PD, known as the PARK genes, of which there are 18 identified to date [16]. Two of these key PARK genes are PARK1/4 (*SNCA*) and PARK8 (*LRRK2). SNCA* encodes α-synuclein, a monomeric protein that in its mutated form undergoes a conformational change from an α-helical structure into β-sheets, which aggregate and oligomerise to form toxic protofibrils that then fibrilise [12, 17]. Fibrils of α-synuclein have been identified as the main components of LBs and LNs [13, 14]. Duplications and triplications of *SNCA* is directly correlated to the earlier onset and disease severity of familial PD [18–20], with single nucleotide polymorphisms associated with an increased risk in sporadic PD [21, 22].

The most common (>10 %) autosomal dominant locus of PD is *LRRK2*. *LRRK2* is also the locus most commonly associated with idiopathic PD where spontaneous mutations in this gene accounts for 3.6 % of all idiopathic PD; the *LRRK2* (G2019S) mutation is responsible for 1–2 % of all these cases [23]. Mutations in *LRRK2* have been implicated with impairments of lysosomal packaging and chaperone-mediated autophagy [24], reducing the clearance of α-synuclein and ubiquitin, leading to presynaptic accumulation and subsequent neuronal toxicity [25]. Thus dysfunction in both *SNCA* and *LRRK2* represents two of the main sources of PD; it is unknown, however, how the dysfunction of these genes is triggered.

Recent evidence, however, has shown that tau, a protein heavily associated with Alzheimer's disease (AD), enhances the aggregation and toxicity of α-synuclein [26]. This research article follows a publication [27] that suggests that tau and Aβ interact synergistically with α-synuclein in vivo to promote aggregation and accumulation of each other leading to cognitive dysfunction.

Huntington's Disease

Huntington's disease (HD) is a progressive, fatal, monogenic neurodegenerative disorder. HD is caused by an expansion of a triplet region of polyglutamine (CAG) repeats in the huntingtin (*HTT*) protein; this trinucleotide repeat then results in the addition of a long stretch of glutamines (polyQ) near the N-terminus of the protein [28]. Initially, HD causes loss of medium spiny neurons (MSN) in the neostriatum before progressing to loss of entire cortical structures [29, 30]. Another pathological feature of HD is astrogliosis [31]. HD is autosomal dominant and displays age-dependent penetrance with the increased length of CAG repeats inversely proportional to age of onset with CAG repeat lengths greater than 36 repeats considered a pathological threshold [32, 33]. The prevalence of Huntington's disease is 4–10 per 100,000 in the Western world; the mean age of onset is 40 years, with death occurring 15–20 years from time of onset. HD patients have progressive motor dysfunction, cognitive decline and psychological problems such as suicidal ideation [34].

Multiple Sclerosis

Epidemiology and Pathology

Multiple sclerosis (MS) is a chronic neurodegenerative demyelinating disease. MS affects 2.5 million people worldwide with 80 % of patients developing a progressive disability and costs the EU economy €9 billion per year [35]. There are two disease-specific symptoms of MS: Lhermitte's symptom, an electrical sensation that runs down the spine on neck flexion, and Uhthoff's phenomenon, a worsening of symptoms due to higher than usual temperatures [36]. MS is not localised to any specific region of the brain are affects the cerebellum and cerebellar pathways, optic nerve, brainstem and also the spinal cord. MS is a demyelinating neuronal disease caused by the loss of the oligodendrocytes that normally create and maintain the myelin sheath. MS is in part a disease of the innate immune system, as following inflammation the blood–brain barrier becomes dysregulated, transendothelial leukocytes cross the blood–brain barrier and become autoreactive: attacking oligodendrocytes; this leads to the eventual demyelination of neurons and MS pathology [37].

Disease Susceptibility

The interaction of the Epstein–Barr virus (EBV) is heavily implicated in aetiology of MS with almost 100 % of patients analysed in the study by the Ramagopalan group having previous serological markers of a past EBV infection [38]. The environment plays a hugely significant factor in determining disease susceptibility in MS with the biggest risk factor being latitude [39]. There is also an increasing female to male bias that has markedly increased in 50 years [40]. Although the role of genetics are not as pivotal in MS compared to other highly penetrant Mendelian diseases such as HD, with only a 30 % likelihood of MS if both parents are sufferers [41], there is still a genetic link with heterogeneity on the

major histocompatibility antigen (HLA) class II complex gene locus *MHA-DRB1* being associated with increased risk of the disease [42, 43].

Amyotrophic Lateral Sclerosis

Epidemiology and Pathology

Amyotrophic lateral sclerosis (ALS) is the most common adult motor neuron disease, affecting 1:500,000 people worldwide per year [44]. ALS is fatal, is idiopathic and varies from patient to patient with loss of both upper motor neurons (UMN) in the motor cortex and lower motor neurons (LMN) in the brainstem and spinal cord. Loss of neurons leads to muscle atrophy with patients additionally presenting with dysphagia, dysarthria, spasticity and hyperreflexia symptoms and an abnormal reflex commonly called Babinski's sign. The age of onset of ALS is variable but tends to be after 40 years of age; only 4 % of ALS patients survive longer than 10 years [45].

Disease Susceptibility

Approximately 10 % of the cases of ALS are familial [46]. Of those familial cases, mutations in three genes – superoxide dismutase *SOD1* [47, 48], TAR DNA-binding protein-43 (TDP-43) *TARDBP* [49] and fused in sarcoma *FUS* [28, 29] – account for 30 %.

Mutations in TDP-43, encoded by *TARDBP*, enhance protein aggregation, fibril formation and neurotoxicity in ALS [30]. TDP-43 also behaves as a prion with intracellular TDP-43 exhibiting seed-dependent and self-templating aggregation, with propagation of TDP-43 aggregates via the exosome [31]. The *SOD1* mutations in ALS are fascinating as they show that the motor neurons are selectively degenerated via astrocytic- and microglial-mediated toxicity [32, 33]. The original study by the Przedborski group and follow-up papers have shown that when the mutant *SOD1* is carried on motor neurons, fibroblasts, cortical neurons and myocytes, they do not cause toxicity; similarly *SOD1* mutated astrocytes and glial are not toxic to spinal GABAergic, dorsal root ganglion neurons or hESC-derived interneurons, therefore implicating ALS as a non-autonomous neurodegenerative disease [32–37].

Stem Cell Therapy for Neurological Disease – Introduction

An advantageous route in which to research and subsequently treat neurological disorders and neurodegenerative diseases is to recapitulate in vitro the endogenous, patient-derived cell type where the disease is present and prevalent in vivo, thus determining the reason(s) for their specific vulnerability and selective degeneration.

Existing ways to elucidate and research the disease phenotype typically involve post-mortem tissue sections, neuroblastoma cell lines, non-human animal models, including small rodents, yeast, drosophila and zebrafish and non-human primary culture cell lines. All of these various research sources have been useful and valuable in studying the disease further; however, no research strategy is without limitation. The increasing use of stem cell therapy, particularly from patient-derived induced pluripotent stem cells (iPSCs) in countless groups around the world, should lead to further understanding and hopefully better treatment of these complex progressive chronic neurological diseases.

The reprogramming of the patients' somatic cells, typically fibroblasts from a skin biopsy, to a pluripotent, neural precursor or terminally differentiated cell type, or the differentiation of the pluripotent or neural precursors cells into the terminally differentiated cell type.

Once the terminally differentiated cells such as mature, electrophysiologically active neurons are generated, they can be used in drug screening to elucidate the efficacy of novel or pre-existing drugs or neurotrophins that can be subsequently used in patient therapy. These de novo neurons or neural precursor cells have the long-term potential to be utilised as cell replacement therapies. Fundamentally, however, the generation of these patient-derived disease lines has the ability to enhance research and understanding of the disease aetiology, pathogenesis and manifestation; to ascertain the selective vulnerability and thus disease mechanism; and to halt the disease progression and prevent further cell loss. An alternative strategy to modelling the disease by generating patient-derived in vitro de novo

neurons could be using adult stem cells, such as in the bone marrow, to mobilise endogenous protection to treat the selective vulnerabilities of the disease.

Generating iPS Cell Lines to Study Neurological Disease

Induced pluripotent stem cells were first derived in 2006 in a seminal paper in Cell by Takahashi and Yamanaka [50]; this ground-breaking work was based upon Sir John Gurdon's work on frogs in 1962 that challenged the dogma that mature cells are irreversibly committed to their fate [51]. Yamanaka and Takahashi proved this in a mouse model using a retrovirus encoding *Oct4*, *Sox2*, *Klf4* and *c-Myc* to induce pluripotency from dermal fibroblasts. One caveat however with the paper was that the cells failed to produce a viable chimera, a hallmark of pluripotent stem cells [50]. However, 1 year later Yamanaka and Takahashi were the first to generate iPS cells using the same four factors in human cells, this time being able to produce viable chimeras [52]. A month later using lentiviruses encoding the four factors of *Oct4*, *Sox2*, *Nanog* and *Lin28*, James Thomson's group also generated iPS cells including a viable chimera from human fibroblasts [53]. What these two landmark papers proved and repeated was that pluripotency can be induced from terminally differentiated mature somatic cells, developing the possibility of personalised medicine: using the patient-specific cells to treat his individual disease. The pioneering work of iPS generation based on the vectors discovered by Yamanaka and Thomson has been replicated in many laboratories throughout the world using different tissue sources to generate iPS cell lines including the amnion [54], dental pulp [55], adipose tissue [56], blood [57–59] and urine [60].

There are caveats with the original Yamanaka and Thomson studies, however, such as by using integrating viruses to reprogram somatic cells; both the vector backbone and transgenes are integrated into the genome. These integrating vectors have the potential to create mutations upon genome insertion that interfere with the normal function of the cell. The integrating vectors can also result in residual transgene expression that can influence and affect the differentiation propensity of cells to specific lineages [53]. Also two of the Yamanaka factors – *Klf4* and c-*Myc* – are oncogenic and have previously resulted in tumourigenicity due to reactivation of the *c-Myc* oncogene [61]. Consequently, as summarised in Table 1, research was undertaken to either use nonintegrating transduction strategies [62, 63, 65–68], integrating vectors that can be excised out of the genome [69–71], DNA-free delivery of vectors in the form of RNA [64], proteins [72], mRNAs [73], microRNA (miRNA) [74] and a chemical only induction of pluripotency [75]. However, all of these strategies have their own specific constraint of using each method; in particular the reprogramming efficiencies of the non-integrating adenoviral and episomal methods are very low.

The Sendai virus being an RNA virus will never produce DNA that integrates into the host genome during transduction; it can easily be removed by antibody-mediated negative selection and generates iPS cells with a high efficiency [64]; as a result the Sendai virus method of iPS reprogramming is commonly used. Unfortunately the transgenes from the Sendai virus can only be removed by diluting, i.e. multiple passages. In the original research paper by Fusaki et al., the transgenes however were still there after 20 passages [64]. A caveat in using Sendai viruses to reprogram to pluripotency is the time the iPS cell line will need to spend in in vitro culture to facilitate the removal of these transgenes. Consequently reprogramming to pluripotency using this technique becomes a long process. The consequence of an extended time in in vitro culture is that there is greater chance of losing genomic stability and acquiring karyotypic abnormalities such as amplifications or trisomy on chromosome 8,12, 17q, 20q and X found in both iPSCs and hESCs [71, 76–78]. Genes found on these chromosomes such as the pluripotency gene *Nanog* and anti-apoptosis gene *Survivin* is encoded in 12 and 17q, respectively, where increasing dosage of these genes can confer a selective advantage [76].

Another strategy used to generate iPS cell for clinical therapy was the excision of the already integrated transgenes. The two systems available

Table 1 Strategies for generating iPS cells

Method	Species	Vectors	Days to generate iPS	Reprogramming efficiency (%)	References
Integrating vectors					
Retrovirus	Human	OSKcM	30	2×10^{-4}	[52]
Lentivirus	Human	OSNL	20	2.2×10^{-4}	[53]
Nonintegrating vectors					
Episomal	Human	OSNLKcM40	30–35	$3–6 \times 10^{-6}$	[62]
Adenovirus	Human	OSKcM	25–30	2×10^{-6}	[63]
Sendai	Human	OSKcM	7+ p5–p20[a]	$10^{-3} – 10^{-2}$	[64]
Minicircle	Human	OSNL	28	5×10^{-5}	[65]
Episomal plasmids	Human	OSKLlM	26–32	$1 \times 10^{-5} – 3 \times 10^{-4}$	[66]
Liposomal magnetofection	Mouse	OSKcM	7	4×10^{-2}	[67]
Doxycycline inducible	Human	OSKcM	7	92 %	[68]
Excised integrated vectors					
PiggyBac	Human	OSKcM	20–30	3×10^{-4}	[69, 70]
Cre/loxP (lentiviral)	Human	OSKcM	15–20	0.5 %	[71]
Viral-free delivery					
Proteins	Human	OSKcM	56	1×10^{-5}[b]	[72]
mRNA	Human	OSKcML	17–24	2.9 % (4.4 % – Hypoxia)	[73]
miRNA	Human	mir-200c, 302 s, 369 s	20	5×10^{-5}[c]	[74]
Small molecule-only induction					
Chemicals	Mouse	VC6TFZ	36–48	0.2 %	[75]

Abbreviations: *O* Oct4, *S* Sox2, *K* Klf4, *cM* c-Myc, *N* Nanog, *L* Lin28, *40* SV40LT, *lM* L-Myc, *VC6TFZ* small molecules taken from [75]

[a]The numbers of passages (p) required before transgenes are silenced

[b]Transduction repeated 6 times

[c]Transduction repeated every 48 h 4 times

that use this are Cre/loxP recombination and *piggyBac* transposons. In the Cre/loxP system even though the viral cassette containing the Yamanaka factors were removed following reprogramming using transfection of the Cre recombinase, the initial vector sequences still remained integrated into the genome; thus, the Cre/loxP system still has a risk of insertional mutagenesis [71]. In the mouse, *piggyBac* has been shown to be a viable strategy for iPS generation without leaving a genomic footprint [69, 70]. Unfortunately, there is a lack of information to date regarding the removal of the *piggyBac* insertions in the human suggesting more work needs to be done before it can be used routinely as the strategy for personalised medicine.

Three other alternative strategies that avoid the introduction of genetically modifying DNA into the genome include the use of proteins, mRNAs and microRNAs (miRNA). The protein-based method takes the longest out of the all methods summarised in Table 1; it is also the most labour intensive requiring 6 repeated transductions [72]. The use of mRNAs seems to be an attractive way to generate iPS cells for translational research: a high efficiency of up to 4.4 % under hypoxic conditions plus no molecular footprint such as integrating vectors or transposons to remove [73]. The miRNA method is another attractive nonviral solution that can be applied in translation therapy. miRNAs leave no molecular footprint, are reasonably efficient and are quick: can reprogram in under 3 weeks [74]. MicroRNA therapy is already deemed to be safe and is undergoing stage 2b clinical trials in the treatment of Hepatitis C under the brand name Miravirsen [79, 80].

Recently the group led by Hongkui Deng has been able to generate iPS cells from murine cells using only small molecules. Provided this can be replicated in human cells, this is another strategy to generate iPS cells with no footprint [75].

In October 2013, another landmark paper in the field of cellular reprogramming has been published in Nature by the Israeli group led by Jacob Hanna showing the generation of iPS cells at an efficiency of close to 100 % in both human and murine cells in 7 days due to knock-down of the nucleosome repressor complex Mbd3 [68]. The importance of this research is the advancement in the understanding at a molecular level with a huge gain at a practical level. By generating iPS cells at a 92 % efficiency in approximately a week, there is a huge cost benefit by reducing the laboratory hours needed to make iPS cells; also with an efficiency of 92 %, it is the first strategy that could lend itself to automation and scaleup and bring patient-specific medicine a step closer.

Since 2007 when Yamanaka and Thomson showed that somatic human skin could be reprogrammed to pluripotency, iPS cells became instantly more advantageous than hES cells for one significant clinical reason. The genome of the in vitro derived cells matches the in vivo cells; consequently for any future cell replacement therapy, there would be a significant reduction in risk of immune rejection compared to the allogeneic hES cells, which will express the human leukocyte antigen minor histocompatibility complex (HLA/mHC) and low levels of the class I major histocompatibility antigen (HLA/MHC class I) [81]. There are logistical benefits from using iPS cells in that they are easier to derive than hES and are not subject to the ethical concerns or strict financial constraints of federal funding in the USA.

However, although Yamanaka and later Thomson pioneered iPS cell research, enabling the possibility of personalised medicine and a patient-specific clinical resource of iPS lines, there are still many constraints. Many of the strategies developed and in use require integrating vectors and transgenes, with evidence of insertional mutagenesis and transgene reactivation [82, 83]. There is also an increase in copy-number variants and increase in point mutations in protein coding genes as opposed to hES cells [84, 85]. Finally the last constraint that needs rectifying prior to cell therapy is epigenetic memory [86, 87], which not only suggests that these cells are not truly pluripotent but also implies that there is lack of current understanding on epigenetic memory which needs to be fully understood before it is assumed a fact as it is a variable and may influence the results of future studies.

Genome Editing

Yamanaka and Thomson have brought cell-replacement therapy a step closer by allowing it to be patient-specific, provided however that the cells do not need genetic modification. In the cases of a genetic mutation, the cells once derived will also retain that mutation and the disease phenotype, for example, a triplication in the *SNCA* gene is present not only in the dopaminergic neurons but also in the fibroblasts and the iPS cell line [88, 89]. A way to avoid this is by genome editing homologous recombination; the first study on this was by the Jaenisch research group where zinc finger nuclease (ZFN) mediated genome editing was used to edit the genome to correct the A53T point mutation and create an isogenic iPS cell line [90].

There are many methods currently published that have the ability to perform genome editing homologous recombination (Table 2): ZFN [97], bacterial artificial chromosomes (BACs) [98], transcription activator-like effector nucleases (TALENs) [99] and clustered, regularly interspaced, short palindromic repeat (CRISPR) [100]. Helper-dependent adenoviral vectors (HDAdV) have also been used in genome editing being able to edit both the transcriptionally active and inactive loci [92, 94, 95]. HDAdV also have a benefit in that they accommodate up to 35 kb of DNA, as opposed to ~4.7 kb that traditional adeno-associated virus (AAV) system. There are a few concerns however with HDAdV: a possibility of in vivo toxicity from the adenoviral capsid proteins, random integration sites and a genomic footprint [101].

Table 2 Technologies for genomic editing (corrective and inductive) of disease mutations in neurological disease models

Disease	Gene	Defection	Corrective method	References
Parkinson's disease	SNCA (α-synuclein)	A53T point mutation	ZFN	[90]
	LRRK2	G2019S	ZFN[a]	[91]
	LRRK2	G2019S	HDAdV	[92]
Huntington's disease	HTT (huntingtin)	CAG repeats	BAC	[93]
HGPS	LMNA (lamin A)	C1824T point mutation	HDAdV	[94]
AWS	LMNA	A1733T	HDAdV	[95]
Gyrate atrophy	OAT	Base-pair mutation	BAC	[96]

Abbreviations: *HGPS* Hutchinson–Gilford progeria syndrome, *AWS* atypical Werner syndrome
[a]Induction of disease mutation as well as deletion

The biggest concern about genome editing is the potential for off-target double-stranded DNA breaks being introduced into the genome, thus creating non-specific genome variants that are not truly isogenic. There is rationale for this concern as a ZFN study by Hockemeyer there was 1 off-target event per 184 clones analysed [97]. However, considering that the human diploid genome is six billion base pairs, that statistical probability of generating an off-target event is far too high. ZFN are also difficult for non-specialists to design and are associated with high rates of failure [102].

TALENs are cheaper and easier to use than ZFN; however, they are larger molecules, so it can be difficult for them to be efficiently delivered [99]. Although, TALEN technology has been used to correct for mutations in the β-globin gene in the blood disorder disease β-thalassemia [103].

CRISPRs have an advantage over ZRN and TALEN in that using a single vector to guide RNAs in series which can be then processed into individual RNAs allows for simultaneous, multiplexed targeting of multiple sites of the genome in the same cell [100]. The big concern with CRISPR is an increased inherent risk of off-targeting due to the guide RNA being shorter [104]. However, a recent publication by the Zhang group have detailed using 2 guide RNA's in a 'double-nickase' strategy has been able to facilitate a double-strand break and reduce off-target mutagenesis up to 1,000 fold [105].

The use of genome editing in science is a recently emerging field, and it is particularly valuable when the iPS cells derived from the patient also contain the genetic disease. Therefore, by editing out the disease and similarly editing in the disease [91, 106], researchers will understand a lot more about each disease phenotype and how it manifests. Also by editing in a disease to a non-disease control line, it effectively creates a positive disease model that could be of particular use in compound screening (Fig. 1) by being able to distinguish phenotype of the mutation from the genetic background. Additionally depending on the research considered this could minimise the use of animals in research. To be able to correct neurodegenerative diseases using genome editing is a very exciting area of research and could even be used in future clinical therapy in correcting the genetic mutation prior to potential transplantation using the patients' isogenic cells.

Direct Conversion

Pioneered by Marius Wernig and colleagues in their seminal paper published in Nature in 2010, they showed that non-neural adult somatic fibroblasts can be directly reprogrammed to terminally differentiated functional and electrically active neurons without going through a pluripotent intermediary state [108]. These induced neurons (iN) were reprogrammed in 13 days from MEFs using three factors – *Brn2*, *Ascl1* and *Myt1l* (BAM factors) – with an efficiency of 19.5 %, with the iN exhibiting positive immunocytochemical staining for the neuronal markers of Tuj1, NeuN and MAP2.

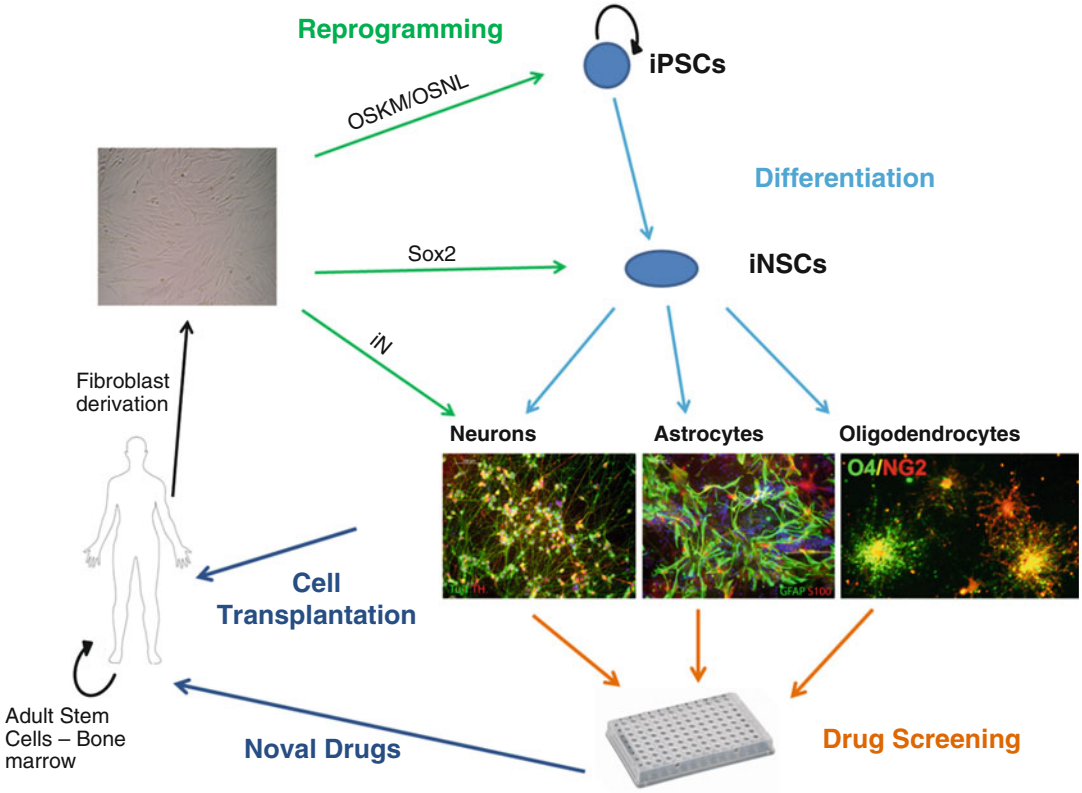

Fig. 1 A schematic representation of the processes that can be undertaken in personalised medicine. Following a patient biopsy, fibroblasts can be derived and expanded into a cell line. The fibroblasts can then be reprogrammed into induced pluripotent stem cells (iPSCs) [52, 53] or an induced neural-progenitor stem cell (iNSCs) state [107] before being terminally differentiated towards a cell type, such as neurons, astrocytes or oligodendrocytes. Alternatively these cells can be directly reprogrammed from the patient somatic cell to the terminally differentiated cell type [108]. These cells can then be used in disease modelling, drug screening and the development on novel drugs or in cell transplantation. Images of fibroblasts and $Tuj1^{+}/TH^{+}$ dopaminergic neurons are provided by the author. The image of the $GFAP^{+}/S100^{+}$ astrocytes is kindly donated by Dr Federica Rinaldi (University of Oxford). The image of the $O4^{+}/NG2^{+}$ oligodendrocytes is used with permission from Professor Nada Zecevic (University of Connecticut) and has previously been published [109]

53 % of the mouse embryonic fibroblasts (MEF)-derived iN expressed Tbr1, a marker of excitatory cortical neurons with both excitatory glutamatergic and inhibitory GABAergic neurons generated. There has been an increase in the number of groups that have used the BAM factors amongst other factors, to directly convert human fibroblasts into terminally differentiated neuronal cell phenotypes which is summarised in Table 3. This includes cell populations specific for neuronal disease such as dopaminergic neurons for PD [114–118], retinal-like ganglion neurons for glaucoma [119] and spinal motor neurons for amyloid lateral sclerosis (ALS) and spinal muscular atrophy (SMA) [120]. All iN for PD exhibited functional dopaminergic neuronal properties: positive expression of midbrain markers, functionally active electrophysiological properties and dopamine release and uptake. In the study led by Abeliovich's group in 2011, the fibroblasts used for the direct conversion were patient-derived from a familial Alzheimer's disease (FAD) patients containing mutations in presenilin-1 and presenilin-2. These FAD-iN were found to show phenotype of AD showing altered processing and localization of amyloid precursor protein (APP) and increased production of Aβ [113].

Table 3 Summary of iN methods following direct conversion

iN-derived subtype	Reprogramming factors	Reprogramming efficiency	Neuronal efficacy	Duration (days)	References
Glutamatergic/ GABAergic neurons	BAM	19.5 % [a]		13	[108]
	BAMN[1]	2–4 %	60 % Tuj1[b]	18	[110]
	BM, miR-124	4–11 %	55 % MAP2[b]	18	[111]
	AM, miR-124, miR9/9	5 %	50 % MAP2[b]	30	[112]
	BAMOZ	7.1–8.9 %	28.4–36.1 % MAP2[b]	21	[113]
Dopaminergic neurons	ANL	3–6 % TH[b] 5–10 % Tuj1[b]		18–24	[114]
	BAMLF	16 %	<95 % MAP2[b]	24	[115]
	AN(Lb)	18.2 %[b]	35.1 % Tuj1[b]	14	[116]
	ANLFEP	9.19 %[a]	2 % Pitx3[b]	18	[117]
	ANPSNg	1–2 %	40 % DDC[b]	20	[118]
Retinal-like ganglion neurons	ANgB[3]	3.5 % Tuj1[b]		14	[119]
Spinal motor neurons	BAMNgHIL[3]	5–10 %[a]		10	[120]

Abbreviations: *B* Brn2, *A* Ascl1, *M* Myt1l, *N*[1] NeuroD1, *O* Oligo2, *Z* Zic1, *L* Lmx1a, *N* Nurr1, *F* FoxA2, *(Lb)* Lmx1b, *E* En1, *P* Pitx3, *S* Sox2, *Ng* Ngn2, *B*[3] Brn3b, *H* Hbx9, *I* Isl1, *L*[3] Lhx3

[a]Relates to reprogramming efficiency in MEFs

[b]Reprogramming efficiency from human astrocytes

Table 4 Summary of various reprogramming methods for iNSC generation

Initial cells	Transcription factors	References
Mouse fibroblasts	(*Oct4, Sox2, Klf4, c-Myc*) initially	[121]
	Oct4 (initially) *Sox2, Klf4, c-Myc*	[122]
	Brn2, Sox2, Klf4, c-Myc	[123]
	Brn2, Sox2, FoxG1	[124]
Mouse and human fibroblasts	*Sox2*	[107]
Murine sertoli cells	*Sox2, Klf4, c-Myc, Brn2, Asc1l, Ngn2, Pax6, Hes1, Id1*	[125]

The field of iN via direct conversion is very exciting and although it does hint at the possibility of far quicker method to reprogram neuronal cells from somatic cells without a pluripotent intermediate, it is still very inefficient. Fibroblasts are not an expandable immortal cell type, with a decreasing capacity to generate iN with every additional passage being exhausted by passage 8 [115]. Therefore, it is questionable if the quantities of cells generated from iN would be sufficient for cell replacement therapy strategies. It is for this reason of scalability and proliferative potential that generating multipotent neuronal progenitors instead of post-mitotic neurons would be a more sustainable long-term strategy in modelling PD.

The multipotent progenitors have been termed induced neural stem cells (iNSCs) and have been generated from murine and human fibroblasts and more recently Sertoli cells (Table 4). After initial research in mouse fibroblasts, there was no consensus on what transcription factors were necessary to generate iNSCs with the Yamanaka pluripotency factors initially used [121, 122]. Han and colleagues then swapped *Oct4* for another POU homeodomain transcription factor in

Brn4 that is normally expressed in the neural tube [126]. Han and colleagues generated stable iNSCs for over 130 passages that could form astrocytes, neurons and oligodendrocytes, although the efficiency for oligodendrocyte generation was extremely poor at approximately 10 % compared to ~100 % efficiency of both neuronal and astrocytic differentiation [123]. The Wernig group found that just *Sox2* and *FoxG1* were necessary to generate iNSCs; however, those iNSCs were bi-potent with the potential to form neurons and astrocytes only, oligodendrocytes were not generated unless the transcription factor *Brn2* was included in the transduction [124]. The first study and only current study published in humans is from the Huang group where transduction with *Sox2* alone could generate stable iNSCs with the potential to differentiate into neurons of multiple subtypes, astrocytes and oligodendrocytes after 2–4 weeks of culture in permissive differentiation conditions. The human iNSC were also able to survive and integrate in mouse brains without any tumourigenicity [107]. The only other published study of iNSC generation is from the Qi Zhou Chinese group using mesoderm-derived Sertoli cells. This study however used 9 transcription factors and needed 1 month of propagation to generate the sufficient amount of cells necessary for analysis [125].

Differentiation Protocols to Model Selected Neurogenerative Diseases

Generation of iPS cells is the strategy typically used by most research groups as a source of pluripotency prior to the terminal differentiation of the required cell type necessary to the model of the disease. Table 5 shows the most efficient protocol currently published for the generation of the neuronal subtypes lost in the 5 aforementioned most common neurodegenerative diseases: basal-forebrain cholinergic neurons for AD, striatal medium-sized spiny neurons for HD, midbrain dopaminergic neurons for PD, motor neurons for ALS and oligodendrocytes for MS.

The Crompton et al. protocol for AD is long at 90 days, but it has an excellent efficiency. 83 % of the cells generated are Tuj1+ neurons, with the remaining being GFAP+ astrocytes. Of those 83 % of neurons, between 91 and 92.4 % of the hES and iPS cell lines taken through

Table 5 Summary of protocols efficiencies used to generate terminally differentiated cell types for disease modelling

		Efficiency			
Disease	Desired subtype	Neurons	Regional identity	Duration	References
AD	Basal-forebrain-derived cholinergic neurons	83 % $Tuj1^+$	97 % $Nestin^+$ (d30)	90 days	[127]
		17 % $GFAP^+$	**92.3 % $Nkx2.1^+$/$nestin^+$**		
		91–92.4 % $ChAT^+$/$Tuj1^+$			
HD	Striatal medium-sized spiny neurons	51 % $MAP2^+$, 80 % $Tuj1^+$,	**70.6 % $CTIP2^+$/$calbindin^+$**	90 days	[128, 129]
		78 % $GABA^+$/$MAP2^+$			
		60.3 % $CTIP2^+$/$MAP2^+$			
		53 % $Calbindin^+$/$MAP2^+$			
		20 % $DARPP32^+$/$CTIP2^+$			
PD	Midbrain dopaminergic neurons	75 % TH^+	80 % $FoxA2^+$	50 days	[130]
		20 % TH^+/$FoxA2^+$	60 % $Lmx1a^+$/$FoxA2^+$		
			80 % $Otx2^+$/$FoxA2^+$		
			(counts at d25)		
ALS	Motor neurons	**53 % $HB9^+$/$ISL1^+$**	55–70 % FOXP1	31 days	[131]
			63 % $FOXP1^+$/$LHX3^-$		
MS	Oligodendrocytes	**60–80 % $O4^+$**	40–60 % NG2	77 days	[132]
		70 % $A2B5^+$			

Abbreviations: *AD* Alzheimer's disease, *HD* Huntington's disease, *PD* Parkinson's disease, *ALS* amyotrophic lateral sclerosis, *MS* multiple sclerosis

3 replications of this protocol are positive for ChAT: cholinergic acetyl transferase, a marker of cholinergic neurons [127]. The regional identities of the neurons are correct for an AD model being 92.3 % Nkx2.1, a marker of the basal forebrain.

In the HD model study from the Italian group of Elena Cattaneo, striatal medium-sized spiny neurons (MSN) were generated in a very well-described research article that deconstructed each neuronal subtype generated from this differentiation protocol, not all of which are shown in Table 5. A commonly used marker for MSN is DARPP32; however, DARPP32$^+$ neurons are also found outside the striatum. By co-staining with CTIP2, a post-mitotic striatal marker, the regional identity of the MSN can be confirmed. 70.6 % of the calbindin + neurons co-expressed CTIP2, thus confirming the general acquisition of an MSN fate and not of an interneuron identity [128]. The Kriks et al. protocol for PD showed the generation of 75 % of dopaminergic neurons; 80 % of cells differentiated through the protocol were positive for the midbrain marker FoxA2. Although of those 75 % dopaminergic neurons, only 20 % were midbrain dopaminergic neurons [130], suggesting that the regional identity of the majority of those neurons was not specific to the midbrain.

A follow-up dopaminergic differentiation study by Kirkeby et al. trialled the GSK3β inhibitor, CHIR99021, that was used in the Kriks protocol; what was found was at high levels of drug, >1 μM, the floor plate begun to caudalise away from the midbrain fate towards the hindbrain showed by an upregulation in *LEF1*, a gene found at the midbrain–hindbrain border, and also hindbrain genes *HoxA2*, *HoxA4*, *IRX3* and *GBX2* [133, 134]. At 4 μM there was over a 100-fold upregulation in *GBX2*, a key gene expressed in the anterior hindbrain that shares a border in development with the MHB. In the study published in Nature by Millet, *GBX2* was shown to not only repress *Otx2*, a key midbrain gene regulator, but also shift and reposition the MHB, creating a smaller midbrain and larger hindbrain region [135]. Furthermore, shifting of the MHB has a direct consequence on increasing the number of serotoninergic neurons to the detriment of the midbrain dopaminergic neurons [136]. As the Kriks et al. protocol used a very high CHIR concentration of 3 μM, this may explain that of the 75 % of dopaminergic neurons produced by the protocol, 45 % of these neuron did not have a positive identity for the ventral midbrain marker *FoxA2* [130]. Unfortunately, with *Lmx1a* and *Lmx1b* now being shown to have overlap in the formation of the anterior hindbrain roof plate [137], it is quite possible that these remaining 45 % TH positive cells are of an anterior hindbrain identity and not the midbrain, although this is yet to be proven.

However, even though the Kriks protocol may have generated a population of dopaminergic neurons of a midbrain/anterior forebrain identity, it still possess 20 % FoxA2$^+$/TH$^+$ neurons which is currently the best in the literature. Also the issue of non-midbrain contamination is not insurmountable as this can be selected out by methods such as FACS sorting. In addition to this, the neurons from the Kriks et al. protocol have been grafted in mice, rat and monkey models of PD, in all cases showing evidence of survival, integration and behavioural recovery [130].

The diseases of ALS and MS are both nonautonomous diseases; consequently the generation of MN or oligodendrocytes may not necessarily be the most effective long-term strategy for cell replacement therapy. However, it is still advantageous for researchers to obtain regionally specific terminally differentiated cell types to ascertain the specific susceptibility of MNs and oligodendrocytes to astrocytic/microglial disease transmission in ALS and lymphocytes in MS and then try to prevent this susceptibility.

For the study of ALS, the Wichterle group generates limb-innervating lateral motor column motor neurons. The MN quantification shows by immunocytochemistry the generation of 30 % HB9$^+$-specific motor neurons; of those neurons 53 % are HB9$^+$/ISL1$^+$, with ISL1$^+$ specifying spinal motor neurons [131], although ISL1$^+$ neurons are also specific for the cranial ganglia [138]. A FOXP1$^+$ neuronal identity is specific for neurons that innervate with limb muscles [139, 140]. LHX3$^+$ expression determines if a medial motor column that innervates axial muscles

with LHX3$^-$ specifying a lateral motor column with limb-innervating muscles [139]. Of the MN identified there was a 70 % FOXP1$^+$/LHX3$^-$ identity [131]. Thus, the successful generation of limb-innervating lateral motor column motor neurons for ALS research.

Differentiating to oligodendrocytes for the study of MS the protocol by Sundberg et al. generates 60–80 % oligodendrocytes, specified by the commonly used oligodendrocyte specific marker O4 [132]. This paper also confirmed this identity and the prior establishment of oligodendrocyte precursors cells (OPCs) through this 77-day protocol using the markers NG2 and A2B5 to confirm identity. NG2$^+$ positive cells account for the identity of OPCs with NG2 being responsible for directional migration of the OPC through cell polarity [141]. A2B5$^+$ is a marker of the oligodendrocyte–astrocyte shared lineage progenitor cell, shown to be expressed on both OPC and astrocytes; an induction of BMP2 and other BMPs are required for the astroglial switch [142].

In all of these protocols that are used for the treatment of the five major neurodegenerative diseases, the initial recapitulation of development as shown by Crompton et al., is crucial for more efficient differentiation of the terminal cell type of choice. It is of note that the time, necessary generate specific terminally differentiated cells through this protocol is very long. However, if one considers that the gestational period of a human is 266 days, and a mouse is 20 days, a 90-day terminal differentiation protocols that recapitulate the developing embryo would seem accurate. Another important aspect of the differentiation protocols would be to establish maturity of the cells with functional synapses and electrical activity. Consequently it is essential that for disease modelling an accurate recapitulation of the desired cell type must be achieved.

Existing and Future Therapies Using Adult Stem Cells

Since the onset of hES [143] and iPS [52, 53] cells, the field and profile of stem cells has risen exponentially with the potential clinical therapy for every disease ever closer. Unfortunately the progress has been slow, with diseases having increasing layers of complexity and sophistication necessary to first understand before being able to treat; this is in addition to the ethical, logistical and scientific challenges that these hES and iPS cells bring.

Adult stem cells (ASCs) has been used as bone marrow transplants since 1963 [144], with the discovery of what is now known as haematopoietic stem cells (HSCs). Since then there has been the discovery of many more ASC populations: muscle-derived stem cells (MDSCs) [145, 146], mesenchymal stem cells (MSCs) [147], cord blood-derived multipotent stem cells (CB-SCs) [148, 149], neural stem cells (NSCs) [150], adipose stem cells (AdSCs) [151, 152] and most recently amniotic stem cells (AmSCs) [153].

ASCs in the form of CB-SCs are currently undergoing clinical trials for type I diabetes based on studies in mice reversing the disease [154, 155]. It remains to be seen if CB-SCs to treat disease will be an effective and feasible long-term strategy for the treatment of neurological disease. However, whilst the other methods of cell therapy detailed in this chapter are being advanced and refined for future therapeutic use, adult stem cell therapies appear attractive to patients and clinicians alike. Possibly the greatest benefit of ASCs is their availability, with bone marrow, blood and adipose tissue being readily available sources.

ASCs also have an advantage over iPSCs as they do not have to be reprogrammed. However in the brain, the multipotent adult progenitor cell populations are found in the subgranular zone in the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles [38, 39]; these are not readily accessible areas. Additionally, a constraint of using ASCs compared to hESC or iPSC research is that ASCs are multipotent; however, this dogma is recently being challenged, particularly in AdSCs [40–43].

However, ASCs in the form of MSCs and bone marrow-derived neural crest stem cells (NCSCs) were used in a recent publication that looked at the efficacy of these cell populations when

injected into the striatum of MPTP lesioned mice. At 7 days post ASC intervention, only 3 % of starting 5×10^4 MSCs survived; by 14 days there were no surviving MSCs. The survival rate of the NCSCs was 10 % after 7 days; this lowered to 1 % after 28 days with the same starting cell population. The results of the study showed that those cells that did survive were not able to integrate and migrate through the brain tissue and therefore were unable to modify their initial phenotype and no recovery of any type was observed [156]. The most recent study from the Wislet-Gendebien group has confirmed an earlier implantation study that showed partial and transient survival, poor integration and no neurogenesis or recovery [157]. This study also showed activation of the innate immune system with positive markers for microglial and astrocytes [158]. The most concerning article however is that in vitro expanded neural crest stem cells led to in vivo tumourigenesis in an animal model [159].

ASCs however have also shown to be safe and efficacious; in a study using CB-SCs, the integration and behavioural recovery was shown in rotenone-induced rat models of PD [159]. Furthermore, ASCs have shown efficacy in neuronal protection by secreting protective growth factors such as GDNF and BDNF [160–162]. In October 2013, a publication by a Swedish group has shown that the secretion of BDNF, GDNF, VEGF-A and angiopoietin-1 proteins resulted in axon regeneration, increased vascularity and decreased apoptosis [163]. Consequently it can be seen that CB-SCs show promise as a potential therapy, however more research is necessary to determine its potential use as a bench to bedside therapy.

Foetal Cells – A Case Study from Parkinson's Disease

Foetal cells were first used as a cell therapy in PD. In work pioneered at Lund University which showed in rat models of PD significant functional recovery following foetal nigral transplantations into the host striatum [164–168]. Shortly after, Lindvall and colleagues performed the first transplants of human ventral mesencephalic tissue from tissue collected from elective terminations of pregnancy at 8–10 weeks gestation [169]. In the subsequent paper by the same group six patients had transplanted mesencephalic foetal tissue into the putamen. These foetal cells were able to survive in the brain and produce a significant symptomatic relief, restoring dopamine synthesis and reducing bradykinesia and rigidity [170].

As a consequence of the success results found by Lindvall and colleagues, foetal cell transplantation studies were then replicated with several open-labelled studies taking place across the world [171–176]. Positive results and lack of adverse side effects from these trials led to USA's National Institute of Health (NIH) funding of two double-blind placebo-controlled clinical trials [177, 178]. However, although double-blinded clinical trials were undertaken, there were still concerns that needed to be addressed from the open-labelled studies. In that, although some of the patients showed significant benefit from the foetal transplants, many more did not, with a considerable variation in both intra- and inter-study [179]. Barker and colleagues recently reviewed these open-labelled studies in which variations between these studies were highlighted these included: the age of donor tissue, number of donors, target site for transplantation, immunotherapy and measurable endpoints of each study [179].

Nevertheless, due to positive results in some patients, including a lower Unified Parkinson's disease rating scale (UPDRS), across these open-labelled studies the NIH decided to support two double-blind sham-surgery trials. In 2001, transplantations by Freed and co-workers of mesencephalic foetal tissue from four embryos bilaterally into the putamen were used [177]; what they found was that the younger patients transplanted (<60 years) had a significantly lower UPDRS and an increased ^{18}F-fluorodopa signifying neuronal outgrowth [177]. Unfortunately the older patients (>61 year) had no significant symptomatic improvement over the control group with dyskinesias and dystonias recurring in 15 % of the post-transplanted patients [177].

The second NIH study in 2003 by Olanow and colleagues 1 and 4 foetal donors per transplant

were trialled. Despite an increase in striatal ^{18}F-dopa uptake via PET scans and evidence of graft survival and innervation in autopsy studies, there was ultimately no significance between the foetal transplants and the placebo control, with the transplanted patients from four donors marginally failing to show significance ($P = 0.096$) [178]. The withdrawal of the immunosuppressant cyclosporine and subsequent graft rejection was raised by Olanow and co-workers as a possible reason why the foetal transplants ultimately failed. However, equally if not more concerning was that half of the transplanted patients began to develop graft-induced dyskinesias [178].

The two NIH trials had brought a disappointing conclusion to foetal transplantations as a symptomatic treatment for PD after the initial optimism following the earlier open-labelled studies. Consequently, many questions were raised concerning the future of PD and foetal transplantation such as the PD severity and patient selection, with the potential benefit of the transplant weighed up against the side effects of the post-surgery dyskinesias – the patients with more severe PD (i.e. a higher UPDRS score) possibly being more predisposed to dyskinesias post foetal transplantation. The optimum transplanted area and method of transplant were other questions to be determined with donor quality, quantity, age, storage time and conditions and transplanted region of foetal tissue were other important variables that needed addressing, lest not forgetting that increasing numbers of foetal tissue should be accompanied by an increasing need for immunosuppression.

In 2008 three independent research groups revisited their previous work and released their post-mortem results of PD patients with foetal midbrain transplants. Warren Olanow's research group analysed patient data from an open-labelled study from a PD patient 14 years post transplantation [174]. For 11 years the patient had an improved UPDRS, motor function and less dyskinesias; however, for the last 3 years of her life, her PD symptoms had increased [180]. At the post-mortem the grafted neurons were also found to have positive staining for α-synuclein and ubiquitin, reduced dopamine transporter (DAT) staining and more significantly a Lewy body pathology. Although grafted midbrain did improve function and patient quality of life, it did not arrest the disease and worryingly the disease pathology had spread from the endogenous host cells to the grafted striatum [180].

The earlier Lund trials were revisited by Li and colleagues who found that in patient autopsy's 11 and 16 years post-foetal transplant, the transplanted tissues had survived and provided symptomatic relief. However, what was also observed was α-synuclein and ubiquitin positively stained Lewy bodies and neurites were present in the grafted neurons suggesting host-to-graft disease prion-like propagation [181]. However, as these grafted neurons had provided 16 years of symptomatic relief with an unrelated death, it is debatable if an α-synuclein-driven prion disease propagation significantly affected the patient quality of life. The third set of PD patients post-mortems analysed from Isacson trial was the most successful. After receiving intracerebral transplantation of foetal ventral midbrain grafts, there were no Lewy body of Lewy neurites present [182]. However, serotoninergic neurons were found to be transplanted and thus a mixed non-specific cell population. Using PD patient scans, the group led by Paola Piccini showed that it is the serotoninergic neurons that are responsible for the dyskinesias following neural transplantation [183], confirming prior rat model studies [184].

Recently, a large international consortium headed by Roger Barker from the University of Cambridge has been set up with the aim of determining the efficacy of foetal transplantation in PD as a replacement for dopaminergic neurons [185]. This consortium, TRANSEURO, will attempt to rectify the variability and dyskinesias shown in previous trials with strict controls on patient selection, number, age and storage of donor foetuses, surgical techniques, graft size and placement, the use of immunosuppression and length of time on immunosuppression all being addressed [177, 178, 186]. The results of this trial (NCT 01898390) are due in 2017. The success and long-term viability of the foetal transplantation studies are important not only in PD but in other neurodegenerative diseases

such as HD where foetal transplants have also had mixed success [187–189]. If the foetal transplantations are successful, it could act as a proof of principle and be a precursor for the use of stem cell therapy in neurodegenerative diseases, such as PD. How the TRANSEURO study will address the propagation of the prion-like disease pathology from the host cells to the transplanted tissue however is still to be determined.

Advantages and Disadvantages of Using Stem Cell Therapy in the Treatment of Neurological Disorders

Advantages

There are significant advantages of using stem cell therapy for the treatment of neurological disorders, the ability to generate patient-derived disease lines and then study those degenerated cells of that disease is of unquestionable benefit the researchers aiding the progression and understanding of the disease. The ability to generate in vitro de novo neurons, oligodendrocytes and astrocytes of a specific regional identity enables researchers to look at specific vulnerabilities to specific cell types, also increasing the understanding of the ameliorating ability of the cell types to aid the disease propagation.

Studies from patient-derived iPS cells have furthermore exemplified some of the limitations of animal models to accurately model disease, with rodent models of PD failing to recapitulate human disease pathology such as the selective loss of midbrain dopaminergic neurons and accumulation of α-synuclein [190]. In addition to this studies have shown that in in vitro neuronal disease models that the disease phenotype is only apparent in the differentiated neurons, not the fibroblasts or the pluripotent iPS cells [89, 191] consequently to study the disease progression and manifestation disease-specific iPS terminally differentiated neurons and also neural progenitors have become an essential tool in research, having the additional potential to minimise the extent of the animal models used in research. Animal models however are still a vital tool for researchers with particular relevance in development and embryology; behavioural studies; ascertaining the effect and side effects of new drugs and treatments on a systemic level; and is also the first step necessary for testing the validity cell replacement therapies prior to human clinical trials and eventually the bedside.

The validity and understanding gauged from these terminally differentiated neurons will become more apparent as the protocols necessary to develop these terminally differentiated diseased cell types are generated with greater specificity and efficacy. The sheer quantity and availability of neurons and neuronal precursor cells that can be differentiated from stem cells lends itself perfectly as a model for toxicity testing via high-content screening and automation in screening new and existing therapies to slow/halt/potentially recover disease manifestation, progression and development of pathology. The ability of an accurate recapitulation of the disease cell type coupled with the exciting potential of genome editing technology such as CRIPSR can be a significant step forward in treating neurodegenerative diseases. In diseases such as HD where a high penetrance monogenic mutation is responsible for aetiology, disease correction by removal of the abnormal CAG repeats, without leaving a genomic footprint can effectively cure the disease in de novo isogenic cells. However to be an effective therapy it can only be used provided 100 % certainty of no off-targeting of the genome, genome editing cannot currently give this guarantee. In AD, PD and ALS, the majority of the cases are idiopathic with no abnormal mutations to correct; thus, it is only by further understanding of the disease aetiology that makes it evidently more treatable.

Challenges

There are many challenges associated with using hES cells for research, such as the ethical issues; legislative funding concerns, particularly in USA; and a risk of immune rejection. Therefore there has been a gravitation towards using

patient-derived iPS cells as the primary tool of research as it enables not only disease modelling but also the onset of personalised medicine. However, there are, if not more challenges associated with iPSCs as there were with hESCs.

The first of these challenges unique to iPSCs is the method of reprogramming; it is tantamount to have a methodology in which miRNA is not randomly integrated into the genome, where reprogramming should be transgene-free leaving behind no genomic footprint. Concerns however still remain regarding the heterogeneity of the iPS cell lines generated compared to the hES cell lines and the variable clones that they produce [89]. There is also the matter that the most common somatic cell type used for reprogramming is skin from patients of all ages. The skin is one of the somatic cells that is more likely to have inherent mutations due to constant exposure to UV damaging sunlight with at least half of the mutations seen from reprogramming already present in the pre-existing fibroblasts [85].

Another more recent concern regarding both iPS-based and direct somatic cell reprogramming of both iN and iNSCs is the phenomenon of epigenetic memory [86, 192], which not only exemplifies the amount of unknown variables in this area of research but also changes any assumption that iPS/somatic cell differentiation is a constant, with epigenetic memory known to vary DNA methylation which in turn can influence differentiation capabilities of directing cell fate in addition to molecular and functional properties of iPS cells [86, 193]. This phenomenon also stresses the importance of having multiple control lines and the use of hESCs as non-iPS control lines. However, hESC lines are also variable, having different characteristics and different lineage differentiation propensities [194]. An additional problem of iNSCs and iN compared to iPSCs is the length of time in culture, lack of cell numbers generated and the variable cell population generated [115, 125].

Consequently, this raises another concern regarding the time and thus number of in vitro passages that cells receive which lead to an increasing genotypic abnormality and karyotypic instability. This raises an important point that needs to be addressed if ever patient-specific personalised medicine is able translate to the bedside, which is cell maintenance in an in vitro environment. The longer cells are cultured in vitro; they are more likely to acquire selective adaptations such as alterations in DNA methylation, X-chromosome instability, imprinting instabilities and partial and full chromosomal aberrations such as trisomy of chromosomes 8, 12 and 17 [195].

Inactivated mouse embryonic fibroblast (iMEF) cells are still used as a supporting matrix for both iPS and hES cell maintenance. However, the complications of using these murine layers is that non-human sialic acid N-glycolylneuraminic acid (Neu5Gc) has been detected on the surface of hESCs maintained on MEF feeder layer which is potentially immunogenic [196, 197]. Subsequently feeder-free iPS/hES cell culture has become more apparent using Matrigel™ and CELLstart™; however, chromosomal abnormalities have still been reported [198, 199] and Matrigel is still derived from mouse sarcoma. An alternative of using murine feeders and non-xeno-free matrices is using autologous feeders [200]. Unfortunately after an increasing number of passages, fibroblasts become less viable and increasingly prone to senescence compared to younger samples [201]; also unlike iPSCs or hESCs, fibroblasts are not immortal and have a limited number of passages. Recently xeno-free clinical grade iPS cell lines have been described, with mandatory procedural guidelines necessary for quality control and good manufacturing practice also published [202, 203].

Results From the Bench

Due to the advent of patient-specific disease cells, there is now a plethora of information being released into the literature from disease modelling. The terminal differentiation of iPS and neural precursors into regionally relevant specific neurons, oligodendrocytes and astrocytes to recapitulate the disease conferring selective vulnerability and disease phenotype in response to toxicity has led to a variety of candidate drugs to be further researched for clinical therapy.

Alzheimer's Disease

In AD, both β- and γ-secretases had led to a reduction of Aβ in patient-derived familial *APP-, PSEN1-* and *PSEN2*-mutated diseased neurons, with β-secretase inhibitors giving a partial reduction in activated GSK3β and phosphorylated tau [204, 205]. Docosahexaenoic acid (DHA) was found to lower the reactive oxygen species (ROS) in addition to a decreased in cell death in an *APP* patient-derived cell line [206]. The use of DHA was previously described in a mouse model to protect against amyloid and dendritic pathology [207].

Parkinson's Disease

In PD there have been three research articles with a familial triplication in *SNCA* (A53T) [88, 89, 106]. In all three articles, phenotype was shown, with a significant increase in the gene and protein expression levels and secretion levels of α-synuclein [88, 89] and a significant increase in stress gene expression levels, including *UCHL1*, one of the PARK genes (PARK5) [88]. The most recent paper looked at nitrosative stress in the *SNCA* A53T line before and after correction by genome editing [106]. Using yeast as an initial platform to model the disease, two markers of ER stress, BIP and PD1, were found to have significant elevation in A53T. ER-associated dysfunction (ERAD) substrates, such as sensitivity to glucocerebrosidase (GBA), another PD-associated risk gene [106, 114], were reversed using a compound called synoviolin, an E3 ubiquitin ligase. Using a small molecule screen based on yeast, a compound called NAB2 was found to increase post-ER forms and ameliorate ER accumulation of ERAD substrates in A53T PD-iPS neurons [106].

In the *LRRK2* mutation in PD, there have been six papers published from patient-derived neurons [91, 92, 191, 208–210]. Overall, it has been shown that there is a link between *LRRK2* dysfunction and α-synuclein accumulation [91, 191, 209]; tau and phosphorylated tau [91]; autophagy impairment [209]; mitochondrial DNA mutations [210]; significantly lower mitochondrial consumption rate than controls [208]; and a decrease in neurite length [91, 191]. After previous suggestions that *LRRK2* (G2019S) is a toxic gain-of-function kinase [211, 212], a small molecular kinase inhibitor significantly lowered numbers caspase-3^+/TH^+ neurons [91] and also rescued defects in nuclear architecture [92].

Huntington's Disease

In the first patient-derived HD-iPS study in both heterozygous and homozygous patients, there was no change in CAG repeat length following reprogramming, which also did not change either after 40 passages of the culture [213]. CAG instability has been previously shown in HD gametes [214], lymphoblasts [215], post-mitotic murine neurons [216] and human striatal neurons [217]. Camnasio et al. also found HD-iPS neurons did not affect neuronal differentiation and had a significant increase in lysosomal activity over control lines [213]. Another HD paper showed that following transplantation of GABAergic neurons of the forebrain LGE identity from a 72 CAG-repeat HD-iPS line into a rat containing a unilateral excitotoxic striatal lesion, a significant behavioural recovery was obtained [218]. However, in this study the in vivo transplanted neurons developed HD pathology 33 weeks after transplantation. The in vivo neurons when exposed to MG132 and thus proteasomal stress also developed HD pathology [218].

Using genome editing via BACS a 72-CAG repeat was replaced with a normal 21-CAG repeat *HTT* gene [93]. The corrected HD line reversed the analysed HD pathology of significantly reduced mRNA levels of BDNF, TGF-β1 and N-cadherin; significantly reduced maximum respiration levels; and significantly increased activated caspase-3. The corrected HD cell line was able to differentiate in vitro into $DARPP32^+$ neurons and integrate and survive in a mouse model. In a study of multiple patient-derived HD-iPS lines, the HD Consortium has also extensively showed that an increased HD

pathology of relative intracellular ATP/ADP ratio and cleaved caspase-3 was associated with the number of CAG repeats [219].

Amyotrophic Lateral Sclerosis

In ALS a patient-derived iPS line from TDP-43 mutated neurons displayed phenotype of significantly shorter neurite length and increased insoluble protein of TDP-43 compared to non-disease controls [220]. There was also an upregulation in RNA transcription genes and downregulation in cytoskeletal protein genes, with TDP-43 being involved in RNA metabolism [221, 222]. This study also found that anacardic acid was able to prevent cytotoxicity by arsenite, reversing both neurite length and insoluble protein fraction pathology; it was postulated that this mechanism of action was either via redox reduction or suppression of NF-κB protein complex [220].

Consequently using earlier research done in lower-order mammals and yeast, new pharmacological therapies can quickly be ascertained for their efficacy and therapeutic potential using patient-derived disease-specific cell lines, which have the potential ability to be a causative treatment, reverse pathogenesis, enable better presymptomatic treatment, halt pathogenesis and facilitate better symptomatic treatment and thus improvement in patient quality of life.

Stem Cell Therapy: Readiness to the Bedside I

Parkinson's Disease

PD is a neurological disease where stem cell replacement therapy is a possible strategy for the treatment of the disease in which the precedent has been set transplantation of foetal mesencephalic tissue into the midbrain. Yet PD is not just a motor disorder affecting the dopaminergic neuronal network. PD is a complex and progressive neurodegenerative disease, and PD patients have many non-motor symptoms and often go on to develop dementia. A cell replacement therapy of dopaminergic neurons will not prevent this. Consequently to treat PD, one has to ask what is the aim of the treatment. If the answer is to cure the disease and prevent it, then the field of research is a long way off, as although the disease mechanisms are further being elucidated, such as the increasing number of PARK genes and risk loci from GWAS studies, the accumulation of the α-synuclein and dysfunction of *LRRK2* are still the most common causes. Fundamentally there is still a clear lack of definitive understanding of the PD aetiology and the step-by-step manifestation of the complex and patient-variable disease pathology. Also for much improved outcomes of PD treatment, scientists and clinicians would need to be able to get access to and treat the patient whilst the patient is presymptomatic and has greater than 30 % of the dopaminergic neurons remaining and still functional.

If the purpose of the treatment however is to improve the quality of life, then existing cell therapies have shown to be effective as revealed in the earlier foetal transplant trials [174, 180, 181] with the patient having lower UPDRS scoring indicating improvements in quality of life. The widespread use of foetal transplants in PD however is not a sustainable long-term strategy; therefore, to replace these foetal transplants, stem cell-derived neuronal precursors could be transplanted. The current protocol that produces the greatest amount of TH^+ $FoxA2^+$ neurons is only at approximately 20 % [130], with, the remaining 80 % of cells generated being non-specific. Consequently the current research literature has not advanced sufficiently for iPS-based cell replacement therapy to become a realistic therapeutic strategy yet.

The dangers of transplanting a mixed population of cells have been previously shown in the off-stake dyskinesias in the foetal transplants [177, 223] which has later been confirmed to be a result of contaminating serotoninergic neurons in the transplanted tissue [183]. Although recent evidence has now indicated that it is the loss of cholinergic neurons not dopaminergic neurons that correlates to gait difficulty: one of the cardinal motor symptoms in PD [224]. Therefore, with

further research it is becoming more apparent that transplantation of a pure 100 % dopaminergic neuronal population may not in fact be the best possible treatment. The original therapeutic theory was the cell replacement of the degenerated cells with de novo in vitro generated cells; however, more research and understanding is necessary to determine what all of those cells are. It is currently known that the loss of midbrain A9 dopaminergic neurons of the SNc causes PD, yet PD is implicated in non-dopaminergic multiple pathways: noradrenergic, serotoninergic, glutamatergic and cholinergic within the regions of the cortex, brainstem and basal ganglia [225]. Also with 40 % of all PD patients going on to get dementia [226], the replacement of the dopaminergic neurons would not prevent this.

In addition, cell-replacement therapies do not treat the disease aetiology. Eventually the disease will degenerate the de novo cells as it did the endogenous cells to the prion-like propagation of α-synuclein. There is increasingly evidence that α-synuclein, TDP-43, tau and Aβ are prion diseases [181, 227–233, 240]. This will create a problem for any cell replacement therapies in those diseases in which those toxic proteins take place, PD, ALS and AD. Therefore, for any future cell replacement therapies, the propagation of the disease from host to grafted tissue must be prevented. Recently a group headed by Giovanna Mallucci has published that treatment of a prion-infected mouse model with an inhibitor of the protein kinase PERK (protein kinase RNA-like endoplasmic reticulum kinase) prevented overactivation of the unfolded protein response (UPR) system that otherwise leads to protein synthesis accumulation and prion replication [234]. Provided this result can be replicated in human models, this drug could be used in conjugation with any cell replacement therapy and also given routinely as a therapeutic therapy for these diseases.

Each neurological disorder is different; consequently each requires different treatment. In diseases such as AD and ALS, there is systemic loss of multiple cell types; a cell replacement bench to bedside therapy would not necessarily be the easiest and most effective option with a pharmaceutical intervention being a more realistic method of treatment. Also studies in HD have shown diseases can propagate through astrocytes and glial [235]; in addition to this the disease is not localised to a specific area making transplantation to multiple locations difficult.

There are a lot of pathological similarities in neurodegenerative diseases that warrant further research. For the diseases of protein misfolding, such as PD, AD and ALS, this leads to ER stress; therefore, limiting ER stress by reducing protein synthesis could be a strategy for therapeutic treatment [236]. Another example of this and the closer link between PD and ALS is *FIG4*: a phosphatase that regulates intracellular vesicle trafficking along the endosomal–lysosomal pathway, that mutations lead to ALS and also Charcot–Marie–Tooth neuropathy, has been found in Pick bodies, Picks disease, Lewy bodies in PD and dementia with Lewy bodies (DLB) [237]. This adds further scrutiny to the endosomal–lysosomal pathway, pushing it to a central role in many neurodegenerative diseases. As TDP-43 has been found in ALS, FTN, AD [238, 239] and is can mechanisms known to cause TDP-43 dysfunction implicated in other neurological diseases: Lewy body dementia, Down syndrome, hippocampal sclerosis dementia, familial British dementia and spinal cerebellar ataxia [238]. TDP-43 could be a marker of general neurodegeneration with suppression of aggregated TDP-43 propagation a target for therapy [240].

Stem Cell Therapy: Readiness to the Bedside II

Multiple Sclerosis

A previous strategy on treating MS was the generation of oligodendrocytes as a cell therapy to replace the endogenous myelin-sheath-forming oligodendrocytes. However, it is now established that in MS the OPCs have not been ablated by the activated lymphoblasts of the innate immune system and can still be mobilised to myelin-sheath-forming oligodendrocytes [241]. Due to aging however, the OPCs become less efficient in

differentiating the myelin-sheath-forming oligodendrocytes [242]. Thus, over time the inherent regenerative process that occurs normally and efficiently becomes chronic and inefficient leading to selective vulnerabilities in the motor neuron and subsequent degeneration. It is currently unknown why the motor neuron is selectively degenerated. MS is not a cell autonomous disease; therefore, replacing the degenerated motor neurons in multiple locations would not be a sustainable or realistic source of therapy. To treat MS a synergistic strategy must be employed of suppressing the immune system and repairing the damaged axons by facilitating the endogenous differentiation of the OPCs.

Bone marrow-derived MSCs have shown to be an effective immunomodulator, more so than both adipose-derived and cord blood-derived MSCs, in the suppression of the in vitro proliferation of mitogen or antigen-stimulated T-cell responses [243]. Bone marrow-derived MSCs have previously been shown to promote endogenous repair and functional recovery in animals of the disease [244]. The results from two of the bone marrow-derived phase I clinical therapies indicated that the delivery of the MSCs were safe with no tumourigenicity detected [245, 246]. In one of the studies, there was also evidence of structural, functional and physiological improvement [245]. There are now 13 phase I or phase II clinical trials taking place using bone marrow-, umbilical cord- or adipose-derived MSCs for the treatment of MS [35]. Therefore, using a strategy specific for a disease, it appears that MSCs may be the best effective therapeutic strategy for the treatment of MS.

Conclusion

In conclusion it can therefore be seen that apart from the hopeful and exciting trials in MS, the bench to bedside stem cell therapy for neurological disorders is still a considerable way off. There still remains a great deal of unknowns regarding these neurological diseases. The aetiology, manifestation and subsequent pathology are patient-variable and idiopathic, with common pathways and processes implicated across the different neurodegenerative diseases. The new technologies of genome editing without a molecular footprint represents an exciting chapter in research by being able to correct, control and better model the disease. The progression in differentiation protocols and the advent of direct reprogramming will hopefully speed up the time taken to conduct the research and enable greater study and understanding of the disease. Thus, by being able to understand these neurological disorders, it allows for a better informed rationale when it comes to treatment. Questions still remain about the safety and reproducibility of the patient-derived iPS-cell technology as a route to cell replacement therapies not withstanding the logistical practical and financial implications. However from these patient-derived iPS based cellular models it is inevitable that greater understanding and mechanisms behind the pathology will be found and then hopefully attenuated. Then next question would be how readily and how efficious will these subsequent therapies be in translating from the localised in vitro terminally differentiated cell model to the systemic in vivo environment in which the disease state is in variable stages of progression and is patient-specific. Once the answers to these questions are ascertained the bench to the bedside approach of using stem cell mediated therapy to treat neurological diseases will be realised.

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