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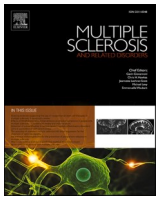

Review article

Using MS induced pluripotent stem cells to investigate MS aetiology

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ABSTRACT

Multiple sclerosis (MS) is a complex disease, and its pathophysiology impacts the function of immune and central nervous system cell types. Despite extensive investigation into the aetiology of MS, the underlying cause/s remain elusive and consequently, faithful *in vitro* or *in vivo* preclinical models of MS do not exist. Advances in human stem cell technologies have enabled the generation of induced pluripotent stem cells (iPSCs) from people with MS. This review summarises the discoveries made using iPSCs derived from people with MS and explores their current and potential application/s in MS research.

1. Overview of multiple sclerosis

Multiple sclerosis (MS) is a complex neuroinflammatory and neurodegenerative disease that affects central nervous system (CNS) function. The initial events that lead to CNS inflammation, demyelination and neurodegeneration are unknown (Fischer et al., 2013), but there are two competing hypotheses (Stys et al., 2012): the 'inside-out' hypothesis proposes that primary damage to the CNS precedes and primes an autoimmune attack from peripheral immune cells; while the 'outside-in' hypothesis suggests that the immune system mounts a response that is misdirected against the CNS, perhaps due to molecular mimicry (Titus et al., 2020). It is accepted that a combination of genetic, environmental and lifestyle factors underpin MS susceptibility (Gresle et al., 2020). The largest genome wide association study (GWAS) of MS, that has been carried out to date, identified 233 independent genetic variants that reached genome-wide significance (International Multiple Sclerosis Genetics Consortium, 2019), underscoring the polygenic complexity of this disease. Alone, each variant represents a minute increase in MS susceptibility, but together they are estimated to account for 39% of the genetic component of MS risk. These genetic risk variants could also interact with environmental and lifestyle risk factors, such as viral infection, particularly Epstein Barr virus (Bjornevik et al., 2022; Rang et al., 2022), smoking (Arneth, 2020) and adolescent obesity (Mokry et al., 2016), however, more research is required to learn how they work together to impact the function/s of specific cell types and increase MS susceptibility.

MS is a human disease and the small effect sizes of the identified MS-associated genetic variants (Burrows et al., 2019) preclude the generation of strictly genetic models of MS. Models have been created however, that replicate distinct aspects of MS pathology and allow researchers to interrogate specific disease processes in isolation. The primary rodent models used are the experimental autoimmune encephalomyelitis (EAE) model, which has been used to gain knowledge about leukocyte infiltration of the CNS and inflammatory demyelination (Constantinescu et al., 2011), and the toxin-induced models of demyelination, particularly cuprizone-feeding and lysolecithin injection, which induce oligodendrocyte death and allow the study of oligodendrogenesis and remyelination (Hall, 1972; Torkildsen et al., 2008). *In vitro* systems have historically involved the culture of primary cells derived from the rodent CNS (Sanabria-Castro et al., 2020), due to the difficulties associated with obtaining human CNS tissue. iPSC technology has enabled the generation of large numbers of human cells for research purposes (Takahashi et al., 2007). Protocols have been developed and refined to differentiate iPSCs into neural progenitor cells (NPCs) (Chambers et al., 2009), oligodendrocytes (Garcia-Leon et al., 2020), neurons (Gunhanlar et al., 2018), microglia (Abud et al., 2017), astrocytes (Tcw et al., 2017) and vascular cell types (Faal et al., 2019). If appropriately utilised, iPSCs could be used to facilitate research into MS pathophysiology and fast-track the identification of novel therapeutics.

Patient-derived iPSCs were first used to model an inherited disease, spinal muscular atrophy, in 2009 (Ebert et al., 2009), but have since been used to interrogate the cellular and molecular mechanisms of complex neurological diseases, including amyotrophic lateral sclerosis (Livesey et al., 2016) and schizophrenia (Brennand et al., 2011). In 2012, the first iPSC line from an individual with MS (MS iPSC line), was generated from a woman with relapsing-remitting MS (RRMS) (Song

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et al., 2012). Over the past decade, 52 MS iPSC lines have been generated and reported in the literature (Table 1 and Fig. 1A) and 23 studies have been published that report the generation or use of MS iPSCs (Fig. 1B). MS iPSCs have been generated from peripheral blood mononuclear cells, fibroblasts, mesenchymal cells, and renal proximal tubule epithelial cells (Fig. 2A), using a variety of reprogramming techniques, most commonly the episomal vector transduction and Sendai virus reprogramming methods (Fig. 2B). In line with MS prevalence and age of onset, the MS iPSC lines have been predominantly generated from women (Fig. 2C), individuals with RRMS (Fig. 2D) and individuals aged 30-50 years (Fig. 2E). As more MS iPSC lines are generated, it becomes increasingly important that we consider how this resource can be used to better understand MS pathophysiology.

2. Material and methods

2.1. Study design

Papers were accessed through Pubmed, Biorxiv and Google Scholar. The search terms used were "Multiple Sclerosis AND iPSCs OR Neurons OR Astrocytes OR Oligodendrocytes OR Microglia OR Endothelial Cells OR Pericytes OR Blood brain barrier". The reference lists of identified MS iPSC papers were also examined for additional MS iPSC papers. 23 MS iPSC papers were identified in total.

2.2. Inclusion criteria

The inclusion criterion was that each paper must have generated or used at least one iPSC line from an individual diagnosed with MS. All papers that met the criterion are listed in Supplementary Table 1.

2.3. Data acquisition

Following identification of MS iPSC papers, details of the MS iPSC lines were extracted from the methods and supplementary information. The following information was recorded: the number of MS iPSC lines generated/used, the starting cell type, type of iPSC reprogramming, sex and age of the individual(s) and the type of MS. If information was unavailable, the term "unspecified" was used.

3. Are brain cells intrinsically different between people with and without MS?

The roles of MS susceptibility genes identified through GWAS have largely implicated cells of the immune system in disease development (International Multiple Sclerosis Genetics Consortium, 2019). However recent mapping of established MS susceptibility genes onto multiple single cell RNA sequencing datasets from MS brain tissue, has revealed enrichment of MS-susceptibility genes in astrocytes, vascular cells and excitatory neurons (Absinta et al., 2021). Additionally, family-based studies and a novel machine learning approach implicate variants in CNS related genes in affecting MS susceptibility and progression, respectively (Fazia et al., 2021; Jokubaitis et al., 2022; Mascia et al., 2022). This supports the idea that cells of the CNS, not only immune cells, are involved in MS development. To date, MS iPSCs have been used to generate neural stem / progenitor cells (NPCs) (Nicaise et al., 2017), neurons (Song et al., 2012), oligodendrocytes (Douvaras et al., 2014), astrocytes (Perriot et al., 2018; Ponath et al., 2018) microglia (Douvaras et al., 2017) and brain microvascular endothelial cells (BMECs) (Supplementary Table 1). These early studies indiate that MS iPSCs have the capacity to differentiate into each CNS cell type, that MS iPSC-derived cells differ from those generated from healthy control iPSCs, and highlight the potential use of MS iPSCs for identifying cell types and molecular pathways integral to MS initiation.

3.1. MS iPSC-derived NPCs show signs of cellular senescence

NPCs are multipotent cells capable of producing neurons and glia in the developing and adult CNS (Martinez-Cerdeno and Noctor, 2018). In MS, cells expressing NPC markers, Nestin and Musashi-1, have been identified within lesions (Snethen et al., 2008). In animal models of demyelination NPCs can perform both cell replacement and neuroprotective functions (Einstein et al., 2006; Xing et al., 2014). Following cuprizone-induced demyelination, NPCs in the adult mouse subventricular zone generate new oligodendrocyte progenitor cells (OPCs) for the corpus callosum (Xing et al., 2014). Furthermore, these

Table 1
Papers that report the generation of new MS iPSC lines.

Type of MS	# of Lines Generated	Sex	Age	Starting Cell Type	Method of Reprogramming	Reference
PPMS(4)/ RRMS(4)	8	-	-	PBMCs	Episomal vectors	Mutukula et al. (2021)
PPMS(2)/ RRMS(4)	6	F	26, 42, 45, 47, 54, 56	Fibroblasts	Tempo Bioscience proprietary reprogramming protocol	Ponath et al. (2018)
PPMS	4	F/ M	50F, 56M, 61M, 62F	Fibroblasts	StemGent mRNA/miRNA kit	Douvaras et al. (2014)
PPMS	3	F/ M	45F, 61M, 62F	PBMCs	Sendai virus	Nicaise et al. (2017)
PPMS	1	M	65	PBMCs	Sendai virus	Mehta et al. (2021)
RRMS	4	F/ M	15M, 17F, 21F, 31F	PBMCs	Episomal vectors	Perriot et al. (2018)
RRMS	3	F/ M	35F, 56F, 61M	PBMCs	Episomal vectors	Ghirotto et al. (2022)
RRMS	3	F/ M	32F, 34F, 71M	Fibroblasts	Sendai virus	Starost et al. (2020)
RRMS	1	M	25	PBMCs	Sendai virus	Begentas et al. (2021)
RRMS	1	M	32	Renal proximal tubule epithelial cells	Episomal vectors	Massa et al. (2016)
RRMS	1	F	35	Fibroblasts	Supernatant from transfected HEK293 cells	Song et al. (2012)
SPMS	4	F	37, 39, 42, 43	Menstrual blood stromal cells	Supernatant from transfected HEK293 cells	Lopez-Caraballo et al. (2020)
-	6	F/ M	33F, 41M, 42F, 44M, 49F, 49M	Fibroblasts	Retrovirus	Miquel-Serra et al. (2017)
-	6	-	-	PBMCs	Episomal vectors	Morales Pantoja et al. (2020)
-	1	M	73	-	-	Linville et al. (2019)

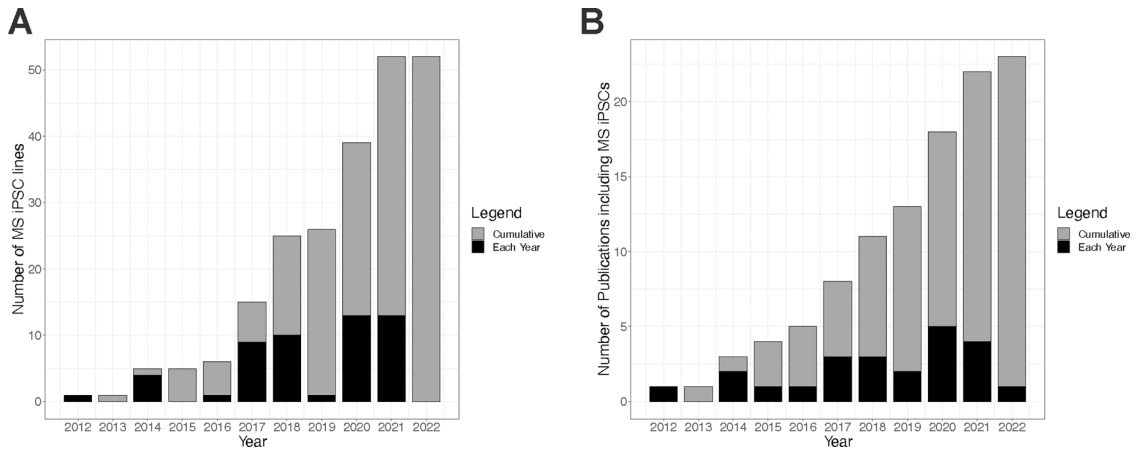

Fig. 1. Summary statistics of MS iPSC usage. A. The number of MS iPSCs lines generated overtime. B. The number of papers published that make use of MS iPSC lines overtime. A, B. Studies were found using PubMed between 3/5/21 – 22/3/22 with the key search terms "multiple sclerosis" and "iPSCs".

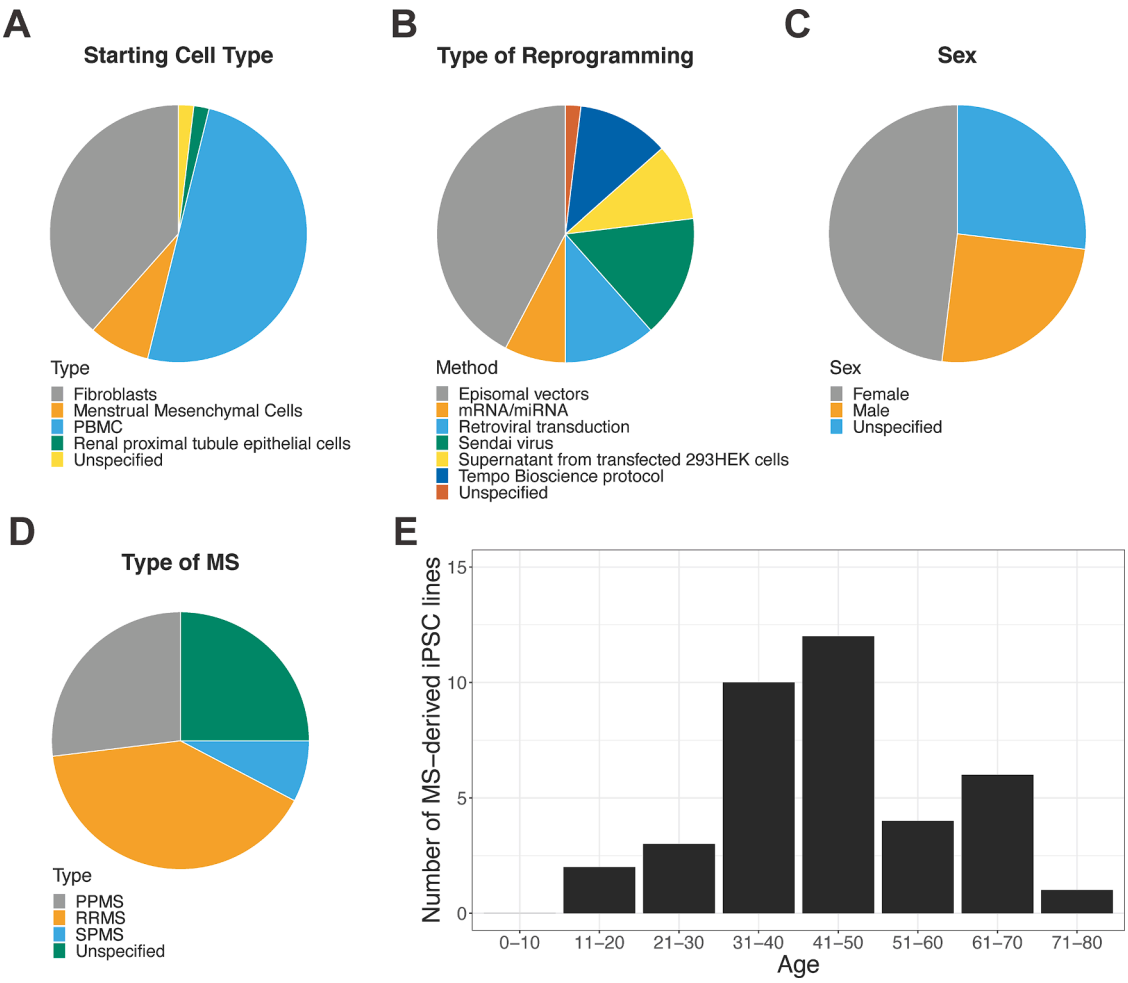

Fig. 2. Details about MS iPSC lines and their generation. A. The proportion of MS iPSCs that were reprogrammed from fibroblasts, menstrual blood mesenchymal cells, peripheral mononuclear blood cells (PBMCs) and renal proximal tubule epithelial cells B. The proportion of MS iPSCs that underwent different reprogramming protocols. **C.** The proportion of MS iPSC lines that have been generated from women and men. **D.** The proportion of MS iPSC lines that have been generated from people with relapsing-remitting MS (RRMS), secondary-progressive MS (SPMS) and primary-progressive MS (PPMS). **E.** The distribution of the age at which cells were collected from the donors. **A-E.** Studies were found using PubMed between 3/5/21 – 22/3/22 with the key search terms "multiple sclerosis" and "iPSCs".

NPC-derived OPCs differentiate and make a significant contribution to callosal remyelination following cuprizone withdrawal. When primary mouse or iPSC-derived NPCs are injected into the lateral ventricles or cisterna magna of EAE mice, the NPCs are recruited to lesions where they reduce the pathological and clinical signs of disease (Einstein et al., 2006; Laterza et al., 2013). The NPCs attenuate immune cell infiltration to the CNS rather than contributing to cell replacement (Laterza et al., 2013). While these studies do not point to NPCs playing a role in MS

initiation, they suggest that NPCs could influence the MS disease course by influencing neural repair and neuroinflammation. iPSC-derived NPCs are transcriptionally and functionally similar to primary foetal and adult human NPCs (Hofrichter et al., 2017; Lorenz et al., 2017). MS iPSC-derived NPCs have been generated and studied to determine whether there are intrinsic differences in NPCs from people with MS that could contribute to MS pathology (Mutukula et al., 2021; Nicaise et al., 2017; Nicaise et al., 2019). iPSCs can be differentiated into NPCs by adding SMAD (suppressor of mothers against decapentaplegic) inhibitors to the culture medium to mimic developmental signalling and specify cells towards the neuroectoderm lineage. This takes approximately 14 days (Chambers et al., 2009), is the first step in many neuron, oligodendrocyte and astrocyte differentiation protocols, and results in a population of self-renewing NPCs that can be cryopreserved, thawed, and readily expanded for experimental and differentiation protocols (Cheng et al., 2017).

Early studies of MS iPSC-derived NPCs suggest that they undergo premature cellular senescence and are less capable of providing neuroprotection than NPCs derived from control iPSCs (people without MS). Primary progressive MS (PPMS) or control iPSC-derived NPCs were injected into the tail vein of mice after two weeks of cuprizone feeding. These mice underwent a further two weeks of cuprizone feeding before tissue collection, at which point the corpus callosum of MS-NPC injected mice had less myelination and a larger number of apoptotic cells than control-NPC injected mice (Nicaise et al., 2017). Interestingly, PPMS-iPSC-derived NPCs were also more likely to differentiate into astrocytes and not oligodendrocytes. This suggests there is an intrinsic deficiency in the capacity for PPMS iPSC-derived NPCs to differentiate into cells of the oligodendrocyte lineage to directly promote myelin repair, and that they fail to support myelin repair from other sources. PPMS iPSC-derived NPC paracrine signalling is also dysregulated, as culturing primary rat OPCs with conditioned medium harvested from PPMS iPSC-derived NPCs, increases their susceptibility to glutamate-induced cell death and impairs their differentiation into oligodendrocytes (Nicaise et al., 2017). In MS lesions and normal appearing white matter, approximately half of all $SOX2^{+}$ NPCs express $p16^{Ink4a}$ (Nicaise et al., 2019), a marker of cellular senescence, and PPMS iPSC-derived NPCs also express genes associated with cellular senescence at a higher level than control iPSC-derived NPCs (Mutukula et al., 2021; Nicaise et al., 2019). This appears to be the result of aberrant mTOR signalling, as the gene expression profile of PPMS iPSC-derived NPCs, and their capacity to secrete factors that promote OPC differentiation *in vitro* is restored to that of control iPSC-derived NPCs when they are treated with the mTOR inhibitor, rapamycin (Nicaise et al., 2019). Together these studies examined NPCs from 7 different MS iPSC lines and provide the first evidence that NPCs may undergo accelerated cellular senescence in people with MS, a phenotype that could feasibly contribute to MS development and progression.

3.2. Properties of MS iPSC-derived cells of the oligodendrocyte lineage

Oligodendrocyte death, demyelination, and a failure of remyelination are key features of MS pathology. Oligodendrocytes are the cell type most often produced from iPSCs for MS research (Lopez-Caraballo et al., 2020; Morales Pantoja et al., 2020; Mozafari et al., 2020; Starost et al., 2020). The earliest protocol to successfully differentiate human iPSCs into cells of the oligodendrocyte lineage took 6 months and produced cells that successfully myelinated CNS axons when transplanted into *shiverer* mice (Wang et al., 2013). Alternative protocols for oligodendrocyte differentiation have since been published that are faster, simpler, have an improved differentiation efficiency (Douvaras et al., 2014; Ehrlich et al., 2017; Garcia-Leon et al., 2020; Gorris et al., 2015), and result in iPSC-derived oligodendrocytes with a gene expression profile comparable to that of adult human primary oligodendrocytes (Ehrlich et al., 2017).

Initial investigations sought to determine whether the impaired oligodendrocyte differentiation experienced by people with MS, resulted from an intrinsic deficiency in cells of the oligodendrocyte lineage or was due to the inflammatory lesion environment. The proliferation rate of OPCs, capacity for oligodendrocyte differentiation, and myelination was found to be equivalent for control- and MS iPSC-derived oligodendrocytes *in vitro* (Starost et al., 2020). Furthermore, control- and MS iPSC-derived $O4^{+}$ OPCs / immature oligodendrocytes were able to proliferate, differentiate and remyelinate when injected into the forebrain of *shiverer* mice (Mozafari et al., 2020). To determine whether the inflammatory environment was responsible for poor oligodendrocyte differentiation in people with MS, the cytokines IFNγ and TNFα were added to control iPSC-derived oligodendrocyte cultures and were found to impair oligodendrocyte differentiation (Starost et al., 2020). A separate study compared the effect of a chronic, low-dose of IFNγ on control and MS iPSC-derived OPC differentiation and found that oligodendrocyte differentiation was equally inhibited in both groups. These experiments indicate that MS iPSC-derived OPCs show a normal susceptibility to inflammatory differentiation block (Morales Pantoja et al., 2020). While these studies strongly argue against MS-relevant intrinsic differences in OPC and oligodendrocyte behavior, a proteome comparison revealed that MS iPSC-derived OPCs, from people with secondary progressive MS (SPMS), expressed proteins associated with cellular movement and cell-to-cell signalling at a lower level than control iPSC-derived OPCs. Furthermore, SPMS iPSC-derived OPC migration was impaired, and conditioned media from these cells was sufficient to slow control iPSC-derived OPC migration by ~ 50% (Lopez-Caraballo et al., 2020), suggesting that the absence of a normally secreted factor could reduce OPC migration in people with MS. Further work will be required to determine whether intrinsic differences exist in cells of the oligodendrocyte lineage in people with MS and their contribution to the disease pathology.

3.3. Is neuron function inherently impaired in people with MS?

Neuron loss underpins lasting cognitive and motor impairment in people with MS (Dutta and Trapp, 2011) and can be extensive, with some lesions exceeding 60% neuron loss (Mews et al., 1998). Particular neuronal subtypes, such as parvalbumin interneurons, are more vulnerable to MS-induced degeneration (Zoupi et al., 2021). This may result from the loss of oligodendrocyte-derived metabolic support (Lee, Y. et al., 2012; Philips et al., 2021) or the release of pro-inflammatory proteins, such as TNFα, from infiltrating peripheral immune cells or activated glia (Kuhlmann, 2002). iPSCs can be differentiated to produce functional neuronal subtypes (Mertens et al., 2016), making it possible to characterise the intrinsic membrane properties and response of neurons to inflammatory mediators. While each differentiation protocol and neuron subtype will be different, foetal and adult primary cortical neurons display significant overlap with iPSC-derived neurons when examined by single cell RNA sequencing (Handel et al., 2016).

Despite the significant role that neurons play in MS pathology, only two studies have investigated the properties of MS iPSC-derived neurons (Massa et al., 2016; Song et al., 2012). The first study found that MS iPSC-derived neurons had a hyperpolarised resting membrane potential relative to control iPSC-derived neurons and failed to generate spontaneous action potentials (Song et al., 2012), while the later study found that MS iPSC-derived neurons had a typical morphology and normal electrophysiological properties (Massa et al., 2016). It is important to note however, that these studies examined one MS iPSC line each and used differentiation protocols that did not specify a neuronal subtype. To truly determine whether MS iPSC derived neurons possess intrinsic deficiencies that contribute to MS pathology, more cell lines need to be subjected to differentiation methods that produce specific neuronal subtypes. Intrinsic changes in neuron function have been identified using iPSCs generated from people with other neurological conditions, such as Parkinson's disease (Carola et al., 2021), autism spectrum disorder (Lim et al., 2021) and tuberous sclerosis complex (Catlett et al.,

2021). Characterising the earliest drivers of neuron pathology will be critical for developing neuroprotective therapies for MS and this could be achieved by studying MS iPSC-derived neurons.

3.4. MS iPSC-derived astrocytes are functional but have an altered metabolic profile

In the healthy CNS, astrocytes perform a diverse range of functions including the regulation of blood flow, facilitation of synaptic communication, and formation of the glia limitans (Sofroniew and Vinters, 2010). In response to inflammatory demyelination, astrocytes become reactive (Escartin et al., 2021) and while reactive astrocytes can perform pro- and anti-inflammatory functions, the MS lesion environment typically promotes a pro-inflammatory phenotype (Liddelow et al., 2017). Reactive astrocytes engulf myelin debris (Ponath et al., 2017) and contribute to glial scaring, but it is unclear whether these processes are beneficial or detrimental (Anderson et al., 2016; Ponath et al., 2017; Silver and Miller, 2004). Experimental evidence from the EAE mouse model of inflammatory demyelination support reactive astrocytes contributing to CNS damage as they release an array of chemokines and upregulate adhesion molecules (Yi et al., 2019). Complete astrocyte ablation however, results in more severe EAE (Toft-Hansen et al., 2011), suggesting that astrocytes are also capable of reducing the extent of immune infiltration into the CNS. While astrocytes can modulate EAE, this does not shed light on the importance of astrocytes in MS initiation or susceptibility. iPSCs generated from people with vanishing white matter and Alexander disease have been differentiated to generate astrocytes, which were found to have abnormal gene expression profiles and cellular phenotypes including increased astrocytic proliferation and a reduced ability to promote OPC differentiation (Leferink et al., 2019; Zhou et al., 2019).

Three studies have characterised MS iPSC-derived astrocytes (Ghirotto et al., 2022; Perriot et al., 2018; Ponath et al., 2018). The generation of mature astrocytes from iPSCs is a lengthy process and requires at least 60 days. As this is an active area of research and protocol optimisation is ongoing, no single method has been broadly adopted. However, there is evidence for overlap in the gene expression profiles of foetal primary astrocytes and iPSC-derived astrocytes (Perriot et al., 2018). Current protocols typically generate NPCs and expose them to growth factors, such as epidermal growth factor and ciliary neurotrophic factors, to specify an astrocytic fate (Perriot et al., 2018). Control and MS iPSCs show similar astrocytic differentiation efficiencies, assessed by quantifying expression of the astrocytic markers *S100B* and *GFAP* (Ghirotto et al., 2022; Perriot et al., 2018). However, qPCR array data suggest that control and MS iPSC-derived astrocytes are intrinsically different (Ghirotto et al., 2022), and the majority of the differentially expressed genes were associated with signalling pathways that regulate cell death, mitochondrial dysfunction, and neurodegeneration. The frequency of mitochondrial fission is also elevated in MS iPSC-derived astrocytes; they produce elevated levels of superoxide and proinflammatory chemokines following TNF-α exposure, and a metabolomic analysis indicates that amino acid synthesis and sphingolipid metabolism is perturbed (Ghirotto et al., 2022). The cellular changes are consistent with those that occur during MS pathogenesis (Hassanpour et al., 2020 (Negrotto and Correale, 2017)(Patergnani et al., 2018), and suggest that, in people with MS, astrocytes may be predisposed to react to inflammatory stimuli, perhaps contributing to CNS damage and preventing repair.

MS iPSC-derived astrocytes have also been used to investigate the biological effect of a known MS risk variant, rs7665090^{G} (International Multiple Sclerosis Genetics Consortium, 2013). rs7665090^{G} is located within an intergenic region between *NFKB1* and *MANBA* and increases NFκB signalling in PBMCs (Housley et al., 2015). When MS iPSC-derived astrocytes carrying the risk variant were exposed to TNFα, IFNγ and IL-1β, they showed a more dramatic induction of NFκB expression and activation of the NFκB target genes, *IL-15, ICAM1, CXCL10, C3* and *CCL5*, than MS and control iPSC-derived astrocytes with the protective allele (Ponath et al., 2018). Elevated NFκB expression was not a general feature of MS iPSC-derived astrocytes, but a functional consequence of the MS-associated risk variant, demonstrating that MS iPSCs can be used in a targeted way to understand how genetic variants contribute to MS risk.

3.5. The generation MS iPSC-derived microglia

Microglia are the resident macrophages of the CNS and exhibit dynamic phenotypic plasticity in response to the local environment (Dubbelaar et al., 2018). Importantly, microglia adopt unique disease-associated transcriptional states in MS (Masuda et al., 2019) and a recent large-scale MS GWAS, reported that the expression of MS risk genes was enriched in microglia (International Multiple Sclerosis Genetics Consortium, 2019). Microglia can be generated from iPSCs (Muffat et al., 2016), however, unlike other CNS cells types, microglia are mesoderm-derived and so iPSCs are first differentiated into hematopoietic progenitor cells. This is followed by microglial specification by exposure to TGFβ and interleukin-34 (Abud et al., 2017). iPSC-derived microglia recapitulate the key transcriptomic states characteristic of microglia purified from human brains (Popova et al., 2021), making them valuable for research purposes, as microglial transcriptomes vary significantly across species (Geirsdottir et al., 2019). Patient-specific iPSC-derived microglia have been studied in the context of other neurodegenerative diseases, with Huntington's disease iPSC-derived microglia being hyper-reactive to proinflammatory cytokines (O'Regan et al., 2021). MS iPSC-derived microglia have been generated (Douvaras et al., 2017), however, they have not yet been functionally characterised with respect to their role in MS pathophysiology.

3.6. Does vascular dysfunction contribute to MS initiation or susceptibility?

The blood brain barrier (BBB) is a specialised structure that is composed of endothelial cells, pericytes and astrocytes, which act together to restrict immune cell or serum protein entry and transport nutrients into the CNS (Daneman and Prat, 2015). In MS, the BBB is compromised, allowing peripheral immune cells and damaging serum proteins, such as fibrinogen, into the CNS (Frischer et al., 2009; Marik et al., 2007; Vos et al., 2005). The mechanism of BBB compromise is not understood, however studies using magnetic resonance imaging indicate that BBB dysfunction is an early event that precedes lesion formation (Tortorella et al., 1999; Vos et al., 2005). As differentiation protocols exist that allow iPSCs to be used to generate each of the key BBB-associated cell types (Faal et al., 2019; Lu, T. M. et al., 2021; Stebbins et al., 2019) it may be possible to model this early pathology using MS iPSC-derived neurovascular cell types.

BMECs express highly specialised tight junction proteins that regulate the transport of molecules and cells across the BBB (Mittapalli et al., 2010). Recent studies of MS iPSC-derived BMECs (Linville et al., 2019; Nishihara et al., 2022) indicate that the function of these cells is impaired. A monolayer of BMECs, derived from a single MS-iPSC line, was shown to have reduced trans-endothelial electrical resistance (TEER) compared to BMECs derived from a non-MS iPSC line (Linville et al., 2019). This phenotype has since been confirmed in BMECs derived from 4 MS iPSC lines, and is associated with a larger cell size, increased Intercellular Adhesion Molecule 1 expression, a greater permeability to sodium fluorescein and impaired P-glycoprotein efflux pump activity (Nishihara et al., 2022). Adhesion assays using allogenic T helper 1 cells and autologous PBMCs revealed that these cells exhibited stronger interactions with MS iPSC-derived BMECs, indicating a greater capacity for peripheral immune cell invasion into the CNS. Activation of Wnt/β-catenin signalling during endothelial cell differentiation restored the impaired barrier properties of MS iPSC-derived BMECs (Nishihara

et al., 2022). These studies suggest that that MS development could be associated with early and intrinsic changes to vascular cell types and that increased Wnt/β-catenin signalling may be able to reverse the intrinsic deficits. Investigations into other vascular cells types such as pericytes, and their interactions with endothelial cells will be crucial to gain a better understanding of potential vascular deficits in people with MS. Pericytes wrap around endothelial cells and regulate blood flow and BBB permeability (Daneman et al., 2010; Heymans et al., 2020). Numerous differentiation protocols have been developed to produce iPSC-derived neural pericytes, however, these protocols have not yet to be used to investigate MS iPSC-derived pericytes (Faal et al., 2019; Stebbins et al., 2019).

4. Benefits and limitations of utilising iPSCs for MS research

The genetic component of MS risk is incredibly complex and significant efforts to understand the polygenic nature of MS have been conducted through large consortium based case-control GWAS (International Multiple Sclerosis Genetics Consortium, 2019). This approach has successfully identified hundreds of common genetic variants that associate with MS development, each individually of small effect size, but has led to few confirmed MS risk genes. Other study designs, including the employment of exome or genome sequencing strategies to identify rare genetic variation in families with multiple related people with MS, have yielded individual candidate genes and variants, but there has been little functional follow-up to explain and validate their role in MS development (Gharagozloo et al., 2019; Salehi et al., 2021; Wang et al., 2016). The lack of confirmed MS risk genes has prohibited the creation of faithful preclinical genetic models of MS, similar to those developed for other diseases, such as amyotrophic lateral sclerosis, where the development of the *SOD1*^{G93A} mouse, based on familial genetic discoveries, has significantly contributed to the field (Gurney et al., 1994). Indeed, it is not possible to model a polygenic disease like MS in the same way that we model a monogenic disease, for which the introduction of a single mutation or the deletion of a gene can recapitulate the disease phenotype *in vitro* and *in vivo* (Rosen et al., 2018). The complex and largely unknown polygenic nature of MS means that artificial replication of the genetic underpinnings, through tools such as CRISPR, is challenging (Jinek et al., 2012; Mackay-Sim, 2012). The study of primary samples from individuals with MS has been the only way for researchers to truly study MS and access to primary CNS tissue samples is limited to post-mortem and biopsied tissue. The static nature of brain tissue samples and the disease stage at which they are typically acquired, limits the ability of researchers to establish a clear sequence of pathological events, particularly those relevant to early disease stages. MS iPSCs may be an alternative source of disease relevant cell types that possess the full genetic component of MS risk from an individual with MS. iPSCs are ideally suited to studying the genetics of MS, however, the imprint of lifestyle and environmental factors, in the form of epigenetic modifications are lost during reprogramming (Kim et al., 2010). Epigenetic changes likely contribute to MS development and progression (Küçükali et al., 2015), but only direct reprogramming, which converts a mature cell type directly into another mature cell type, can preserve epigenetic signatures, and offer an alternative *in vitro* approach for studying the epigenetics of MS (Chanoumidou et al., 2021; Yun et al., 2022).

Combined advances in iPSC and CRISPR technologies provide a powerful platform on which the genetics of complex diseases can be unravelled (Jinek et al., 2012; Pintacuda et al., 2021). Following genetic analyses, disease associated variants can be investigated in depth using disease relevant iPSC lines carrying candidate risk variants. iPSCs can be differentiated into disease relevant cell types to determine the effect of the variant. However, it is important to acknowledge that there can be significant variability in the behavior of different iPSC lines, and additional variability can be introduced by the differentiation protocols. For this reason, rigorous quality control standards must be maintained, high sample numbers are needed, and each experiment must be carefully designed to support the identification of disease-relevant phenotypes (Volpato and Webber, 2020). If a phenotype is detected, isogenic lines can be generated by removing the candidate variant from the MS iPCSc or introducing the variant into healthy iPSCs. As MS is a polygenic disease, the biological effect of any individual MS-associated variant is likely to be small and result in a subtle phenotype, however, the modification of a single variant on the disease-relevant genomic background, has the potential to disrupt a phenotype reliant on multiple variants working in concert (Coccia and Ahfeldt, 2021). This approach would provide robust validation of the biological effects of candidate variants on specific cell types.

Conducting a well-designed iPSC study is expensive, largely due to the cost of culture medium components, culture plasticware and staff costs associated with iPSC maintenance and differentiation. Despite this, they provide a scalable pipeline in which genetic findings can be directly linked to cell specific phenotypes. An example of this is the recent iPSC Neurodegeneration Disease Initiative which is currently working to model over 100 variants associated with Alzheimer's and related diseases, using CRISPR to create highly characterised isogenic cell lines containing or lacking each variant (Ramos et al., 2021). The use of iPSCs for MS genetic studies will require monitoring of the DNA sequence of each iPSC line, as they are prone to accumulate genetic abnormalities over time when undergoing routine culture (Rebuzzini et al., 2015). Genetic aberrations take the form of chromosomal abnormalities, such as a trisomy or loss of a chromosome, and sub-chromosomal abnormalities, such as translocations or regional deletions (Rebuzzini et al., 2015). Steps can be taken to limit the likelihood of accumulated genetic abnormalities, including the adoption of gentle passaging techniques and expanding and cryopreserving low passage cells for later use (Garitaonandia et al., 2015).

iPSCs can be used to investigate biological processes by differentiation into simple monoculture or more complex 3D environments, such as organoids (Wray, 2021), to investigate disease phenotype development and its progression over time. Monocultures provide a unique opportunity to investigate specific cell types in the absence of influencing factors, and co-culture systems, which involve the culture of 2 or more cell types together, facilitate the examination of basic cell-cell interactions or, in some cases, the function of rudimentary structures that resemble those formed *in vivo*, such as the BBB (Lippmann et al., 2020). Organoids are being used to model more complex cellular interactions (Wray, 2021). These self-organising *in vitro* tissue models are made up of multiple cell types, with myelinoids,comprising multiple neuron subtypes, OPCs, oligodendrocytes and astrocytes. Myeloids have been used to recapitulate some disease-relevant phenotypes, with iPSCs derived from a person with Nfasc155 deficiency generating myeloids with impaired paranode localisation (James et al., 2021). Cerebral organoids generated from MS iPSCs are being developed, as evidenced by a conference abstract at the 2021 American Academy of Neurology Annual Meeting, reporting that MS cerebral organoids grow at an accelerated rate and appear to have thicker cortical structures (Chen et al., 2021). The use of organoids is relatively unrefined, due to the infancy of this technology, and have limitations including the lack of vasculature, blood flow and nutrient penetration, and heterogeneity (Wray, 2021). Despite this, they are an attractive tool for MS research, and are likely to be used increasingly in the coming years.

As iPSC technology improves and *bona fide* MS phenotypes are discovered, we anticipate that MS iPSC-derived neural cells will reduce our dependency on *in vivo* animal models for basic research and therapeutic screening. iPSC-derived human *in vitro* models of MS can be used to increase our knowledge of MS disease mechanisms and identify cell types and molecular pathways that can be targeted to prevent disease progression or perhaps even disease initiation (Coccia and Ahfeldt, 2021). This could also allow MS researchers to take advantage of the recent shift in drug discovery away from target-based screening towards phenotypic screening (Lee et al., 2012; Moffat et al., 2017; Shi et al.,

2017). This is partly due to patient iPSC-derived cells being able to recapitulate disease phenotypes, being highly scalable, and avoiding interspecies differences (Mestas and Hughes, 2004). Basic cellular models or more complex organoids have been generated to test drug panels. If an MS relevant phenotype is identified in iPSC-derived cells, this could expedite MS drug development. iPSC- or MS iPSC-derived OPCs may also be an effective method of screening for remyelinating drugs. Silicon micropillars, which are freestanding nanofibers, have been developed that can be used to test the myelinating capacity of cultured oligodendrocytes and enables the testing of compounds that promote remyelination (Mei et al., 2014).

5. Conclusions

Despite the generation of a considerable number of MS iPSC lines (Table 1), the resource remains significantly underutilised by the MS research community. Patient-derived iPSCs are widely used in the study of other neurodegenerative disorders and have been instrumental in the biological characterisation of disease associated variants (Carola et al., 2021; Ortiz-Virumbrales et al., 2017). Studies using MS iPSCs have predominantly examined their differentiation capacity and briefly validated the functional properties of the resulting cell type. Few studies have investigated the effect of MS associated genetic variants on cell biology (Ponath et al., 2018) and none have made use of isogenic controls. Given the recent advances in differentiation protocols that allow the generation of cell types relevant to MS and the feasibility of genetic editing, MS iPSCs present an exciting opportunity to biologically validate MS-associated variants found *in silico* and characterise their cell specific phenotypes.

Author contributions

AF developed the project, carried out the research and wrote the manuscript. JF, NB and KMY developed the project, edited the manuscript, and provided supervision.

Declaration of Competing Interest

The authors declare no competing interests.

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The funding sources had no role in the study design; interpretation of the data; writing the manuscript, or in the publication process.

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Data and materials availability

All data are available in the main text.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.msard.2022.103839.

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