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Generation of RRMS and PPMS specific iPSCs as a platform for modeling Multiple Sclerosis

Naresh Mutukula [a,1], Zhiqiu Man [a,1], Yuta Takahashi [a,1], Francisca Iniesta Martinez [b,c,1], Mariana Morales [a], Ester Carreon-Guarnizo [b,c], Rocio Hernandez Clares [b,c], David Garcia-Bernal [d], Llanos Martinez Martinez [c], Jeronimo Lajara [c], Estrella Nuñez Delicado [c], Jose E. Meca Lallana [b,c,*], Juan Carlos Izpisua Belmonte [a,*]

[a] Gene Expression Laboratory, Salk Institute for Biological Studies, La Jolla, CA, USA
[b] Clinical Neuroimmunology Unit and Multiple Sclerosis CSUR, Department of Neurology. "Virgen de la Arrixaca" Clinical University Hospital, IMIB-Arrixaca, Murcia, Spain
[c] Universidad Católica San Antonio de Murcia (UCAM), Murcia, Spain
[d] Hematopoietic Transplant and Cellular Therapy Unit, Instituto Murciano de Investigación Biosanitaria Virgen de la Arrixaca, Medicine Department, University of Murcia, Murcia, Spain

A B S T R A C T

The advent of cellular reprogramming technology converting somatic cells into induced pluripotent stem cells (iPSCs) has revolutionized our understandings of neurodegenerative diseases that are otherwise hard to access and model. Multiple Sclerosis (MS) is a chronic demyelinating, inflammatory disease of central nervous system eventually causing neuronal death and accompanied disabilities. Here, we report the generation of several relapsing-remitting MS (RRMS) and primary progressive MS (PPMS) iPSC lines from MS patients along with their age matched healthy controls from peripheral blood mononuclear cells (PBMC). These patient specific iPSC lines displayed characteristic embryonic stem cell (ESC) morphology and exhibited pluripotency marker expression. Moreover, these MS iPSC lines were successfully differentiated into neural progenitor cells (NPC) after subjecting to neural induction. Furthermore, we identified the elevated expression of cellular senescence hallmarks in RRMS and PPMS neural progenitors unveiling a novel drug target avenue of MS pathophysiology. Thus, our study altogether offers both RRMS and PPMS iPSC cellular models as a good tool for better understanding of MS pathologies and drug testing.

1. Introduction

Multiple Sclerosis (MS) is a chronic neuroinflammatory disease of central nervous system (CNS) in which the immune system infiltrates into and attacks the myelin sheath that wrapped around the axons, causing progressive demyelination of axons ultimately resulting in axon atrophy and death of the neurons { (Lassmann et al., 2012); (Compston and Coles, 2008) ; (Franklin, 2012) }. Though neurodegeneration has been established as a result of immune mediated inflammation, recent evidences suggest that these two processes might occur in parallel and independent of each other { (Minagar et al., 2004); (Friese et al., 2014) ; (Stys et al., 2012) }. In spite of decades of research and resources invested yet the cause of MS is still elusive, implicating the complex nature of this disease. Accumulating evidences suggest that MS is a multifactorial disease where the genetic predisposition is triggered by immunological and environmental factors, altogether contributing to the disease onset, progression and outcome { (Reich et al., 2018); (Goldenberg, 2012) ; (Didonna and Oksenberg, 2015) ; (Sawcer et al., 2014) }. The etiology of MS disease is unknown but largely involves both a complex genetic trait associated with more than 100 loci and several environmental risk factors { (Sospedra and Martin, 2016)}. Particularly, two polymorphisms of the tumor necrosis factor (TNF) receptor superfamily 1A (*TNFRSF1A)* gene, rs1800693 and rs4149584 were found to be associated with an increased MS risk { (Comabella et al., 2013)}. MS is a heterogeneous disease consisting of two main subtypes: relapsing-remitting MS (RRMS) — a most common MS subtype, begin with temporary relapses followed by remission phase and with subsequent years, evolves into a phase with worsened symptoms lacking remissions. Although less common, another subsequent portion of MS patient suffer a severe form of MS subtype known as primary progressive MS (PPMS)

* Corresponding authors at: Clinical Neuroimmunology Unit and Multiple Sclerosis CSUR, Department of Neurology. "Virgen de la Arrixaca" Clinical University Hospital, IMIB-Arrixaca, Murcia, Spain (J.E. Meca Lallana).
E-mail addresses: jmeca@ucam.edu (J.E. Meca Lallana), belmonte@salk.edu (J.C. Izpisua Belmonte).
[1] Authors contributed equally.

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with worsened symptoms from the beginning without any in between remissions { (Reich et al., 2018); (Lassmann, 2019) ; (Gholamzad et al., 2019) }. However, despite this classification into subtypes, there is a wide range of disease severity among the patients within these two subtypes. The reasons for these varied outcomes are not well understood. There are no reliable markers known to predict the disease subtype, severity and response to treatments. Hence developing appropriate disease subtype specific model systems that sheds light on subtype specific novel markers evaluation is required and as well posing questions about specific mechanisms contributing to each disease subtype is necessary.

Current therapies aim to suppress immune attack are only effective in reducing relapses and delaying the disease progression { (Compston and Coles, 2008); (Comi, 2013) ; (Killestein and Polman, 2011) ; (Torkildsen et al., 2016) }. However, there is no effective treatment that could halt and/or reverse the demyelination and neuronal damage. The potential to repair myelin loss and remyelinate the bare axons that could restore the neurological conditions has currently become the focus of new age MS therapeutics { (Huang et al., 2011); Baecher-Allan et al. (Baecher-Allan et al., 2018); Wooliscroft et al., 2019}. The success of potential remyleination therapies relies on the better understanding the biology of relevant neural cell types especially myelin generating cell types such as oligodendrocytes (OL) and their progenitor—oligodendrocyte progenitors (OPC) in a healthy and diseased milieu. Therefore, stem cell technologies that has the potential to generate any cell type of the body provides an exciting platform for the MS researchers to study relevant affected cell types and potentially sheds further insights on the MS pathogenesis and drug discovery.

Major findings of MS disease pathogenesis have come from the studies of *in vivo* MS animal models such as rodent experimental autoimmune encephalomyelitis (EAE), cuprizone induced MS mouse models and from patients' post-mortem brain tissue samples {Franklin et al., 2002; Wekerle, 2008; Lutz et al., 2014; Mix et al., 2010}. However, they contain many limitations in modeling human MS disease pathology and inappropriate for drug testing. Therefore, patient derived cellular models are relevant for our basic understanding of MS disease. The path breaking discovery of induced pluripotent stem cells (iPSCs) by Shinya Yamanaka in 2006 has revolutionized stem cell and regenerative medicine fields {Takahashi and Yamanaka, 2006; Takahashi et al., 2007}. The ability to derive iPSCs from any somatic cell by the forced expression of Yamanaka factors (OCT4, SOX2, KLF4 and c-MYC) and their subsequent differentiation into any cell type provides a remarkable opportunity to model diseases, drug screenings and even cell-based replacement therapies {Nishikawa et al., 2008; Ebert et al., 2012; Shi et al., 2017; Li and Izpisua Belmonte, 2016; Vadodaria et al., 2020}. This iPSC technology enables to generate relevant diseased cells with MS genetic background from MS patients in large scale and thereby allows performing in depth analyses of development and progression of MS. In addition, these patient specific MS cell lines would act as valuable tools for testing new drug treatments. Henceforth, iPSCs technologies hold a great promise and hope to recapitulate MS disease pathology and identify novel treatments for MS.

Herein, we report as a proof of concept, the successful generation of both MS subtypes—RRMS and PPMS iPSCs from patient peripheral blood mononuclear cells (PBMCs) followed by their differentiation into neural stem cells i.e., building units of central nervous system. We propose that the iPSC lines generated in this study can serve as valuable and powerful references to model MS subtype specific pathogenic mechanisms, potentially leading to novel therapeutic targets.

2. Results

2.1. Derivation of RRMS and PPMS iPSC lines from patient peripheral blood mononuclear cells

Peripheral blood mononuclear cell (PBMC) samples were obtained from 4 RRMS, 4 PPMS patients and 4 healthy control individuals. These PBMC were reprogrammed to respective MS subtype RRMS, PPMS and control iPSC lines using integration free episomal vector reprogramming system (Supplementary Fig. 1A). Episomal plasmid vectors (pCXLE) consisting of transcription factors (*OCT-3/4, SOX2, L-MYC, LIN28* and *KLF4*) were introduced in to PBMC through nucleofection. At around 12–15 days, colonies exhibiting morphology similar to embryonic stem cells (ESC) were observed. After 25 days, these individual colonies were picked and dissociated mechanically for further passaging. Furthermore, these colonies were maintained in both MEF-feeder and matrigel coated feeder-free conditions (Supplementary Fig. 1B).

2.2. Characterization of MS and control iPSC lines

To confirm the generated MS and control iPSC lines exhibit pluripotent stem cell characteristics, we looked at the morphological features and pluripotency marker expressions of the generated Control (CTRL1-4), RRMS (RRMS1-4) and PPMS (PPMS1-4) iPSC lines. All the generated iPSC lines displayed distinct ESC morphological features such as compact colony formation with defined edges and cells with prominent nuclei (with high nuclear-to-cytoplasmic ratio). We next performed the immunofluorescence analysis on undifferentiated iPSC lines with antibodies against OCT4, SOX2 as well as NANOG and confirmed the expression of these pluripotency markers in each of the four derived Control (Fig. 1A), PPMS (Fig. 1B) and RRMS (Fig. 1C) iPSC lines. We next extracted mRNA from these undifferentiated iPSC lines and their parent PBMCs, and then carried out qPCR analysis for pluripotency markers such as OCT4, SOX2 and NANOG (Fig. 1D). This qPCR analysis confirmed the expression of pluripotency markers in Control (Ctrl2 and Ctrl3), RRMS (RRMS3 and RRMS4) and PPMS (PPMS3 and PPMS4) compared to their parent blood cells. Taken together, in line with previous studies {Takahashi et al., 2007; Martí et al., 2013}, all the generated iPSCs in this study displayed ESC like morphology and pluripotency markers expression.

2.3. Generation of neural progenitors from MS and control iPSCs

We next looked at the differentiation potential of the derived MS and control iPSC lines by subjecting them to neural induction according to previously published protocol {Chambers et al., 2009}. Briefly, these iPSCs were exposed to small molecule inhibitors such as SB-431542 and LDN-193189 that inhibit TGF-beta and BMP pathways respectively. After the completion of neural induction, the generated neural progenitor cells (NPCs) were then maintained in a proliferative neural medium consisting of FGF2 and EGF2. We then tested these NPCs for the presence of neural stem cell markers such as PAX6, NESTIN, HES5 and SOX1 (Fig. 2). Quantitative PCR analysis confirmed that all the generated iPSC derived NPC lines including control (Ctrl2 and Ctrl3), RRMS (RRMS3 and RRMS4) and PPMS (PPMS3 and PPMS4) exhibited higher expression of neural stem cell marker expression compared to their respective undifferentiated iPSC counterparts. This result indicates the successful differentiation of both healthy and patient derived iPSC lines to neural lineages. Furthermore, we have observed the decreased PAX6 expression in RRMS and PPMS neural progenitors compared to control neural progenitors (Fig. 2B, and 2C). However, as the analysis is restricted to only two iPSC lines each, we therefore suggest that there could be possible differences in diseased neural stemness but required inclusion of more no. of iPSC lines and high-through put analyses to pin down the disease phenotypes. Altogether, these results display successful differentiation of MS iPSC lines to neural lineage and suggest the possibility of compromised neural progenitor fate specification in the case of MS diseased cells and warrants further in-depth analysis.

2.4. Elevated cellular senescence in MS iPSC derived NPCs

Many neurodegenerative diseases such as Alzheimer's, Parkinson's

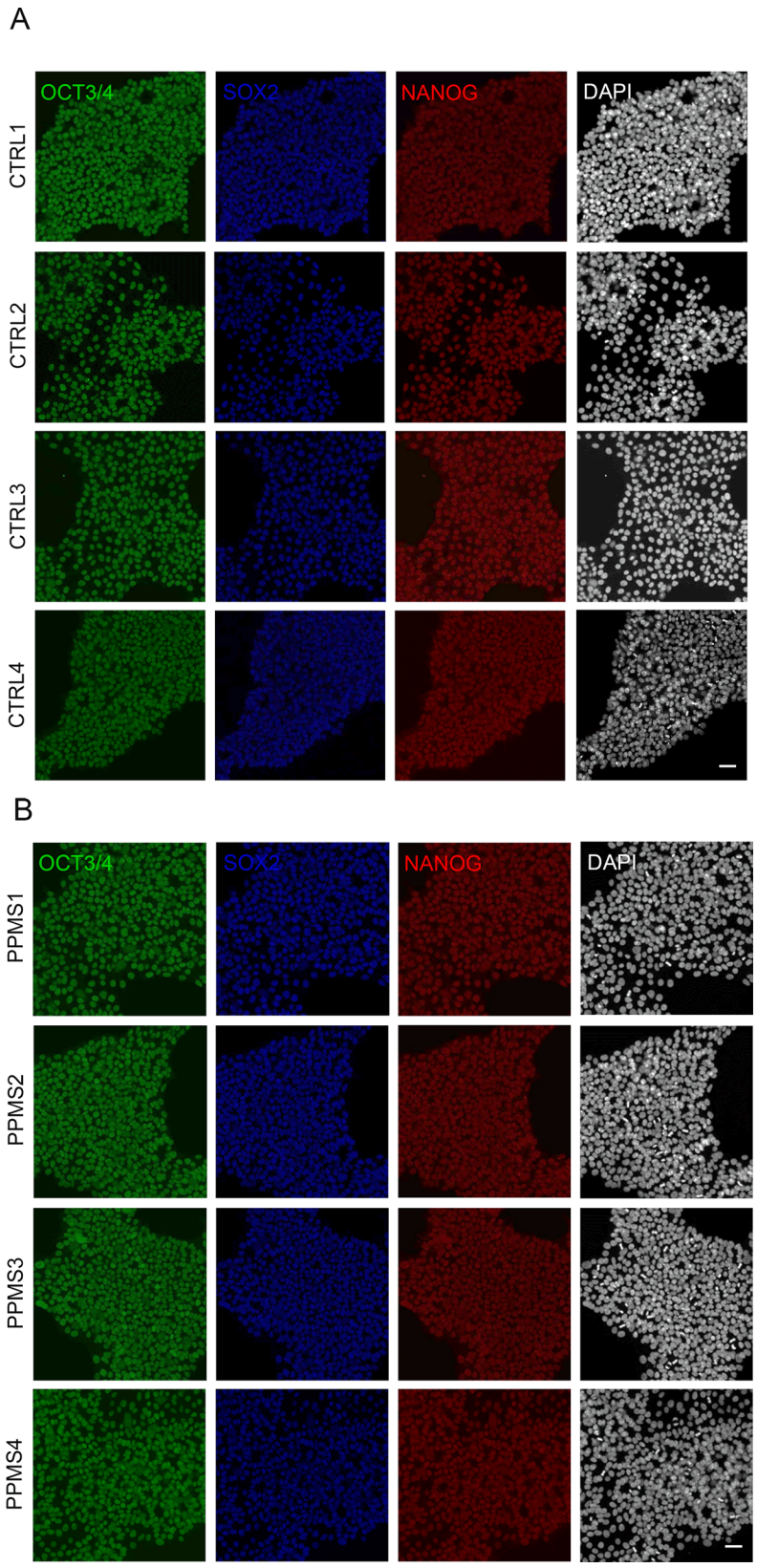


Fig. 1. Characterization of the Control and MS Patient derived iPSC lines. (A)Immunostainings of pluripotency markers OCT3/4, SOX2, NANOG as well as with respective nuclear staining DAPI in undifferentiated Control iPSC lines generated (CTRL1-4). Scale bar: 50 μm. (B) Immunostainings of pluripotency markers OCT3/4, SOX2, NANOG as well as with respective nuclear staining DAPI in PPMS patient derived undifferentiated iPSC lines generated (PPMS1-4). Scale bar: 50 μm. (C) Immunostainings of pluripotency markers OCT3/4, SOX2, NANOG as well as with respective nuclear staining DAPI in RRMS patient derived undifferentiated generated (RRMS1-4). Scale bar: 50 μm. (D) Quantitative PCR (qPCR) analysis of transcript levels of pluripotency markers OCT4 (top), NANOG (middle) and SOX2 (bottom) in Blood cells (PBMC), Control (CTRL2 and CTRL3), RRMS (RRMS3 and RRMS4), and PPMS (PPMS3 and PPMS4) iPSC lines generated. All transcript levels are normalized to the respective GAPDH levels in each sample and expressed as fold change.

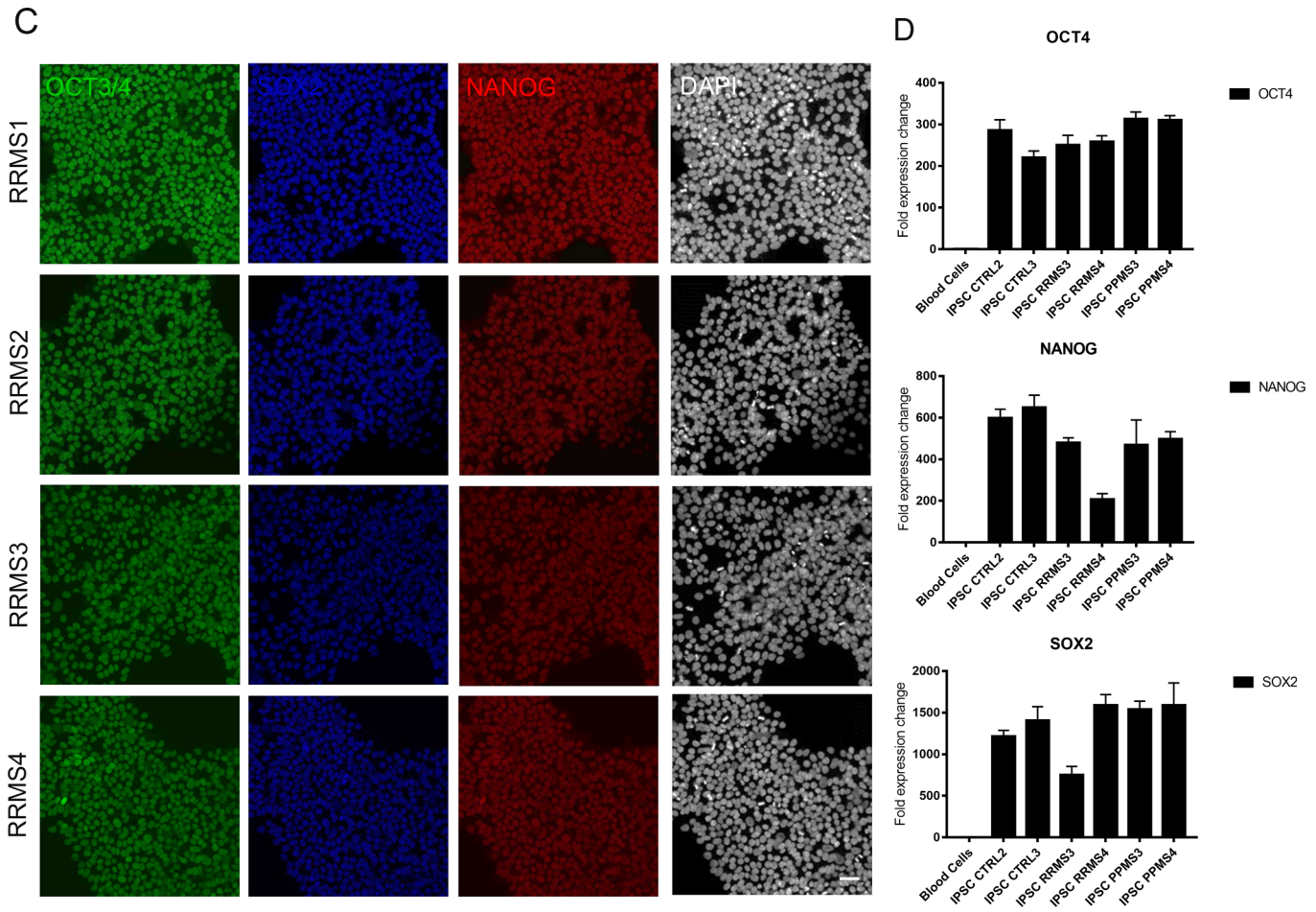
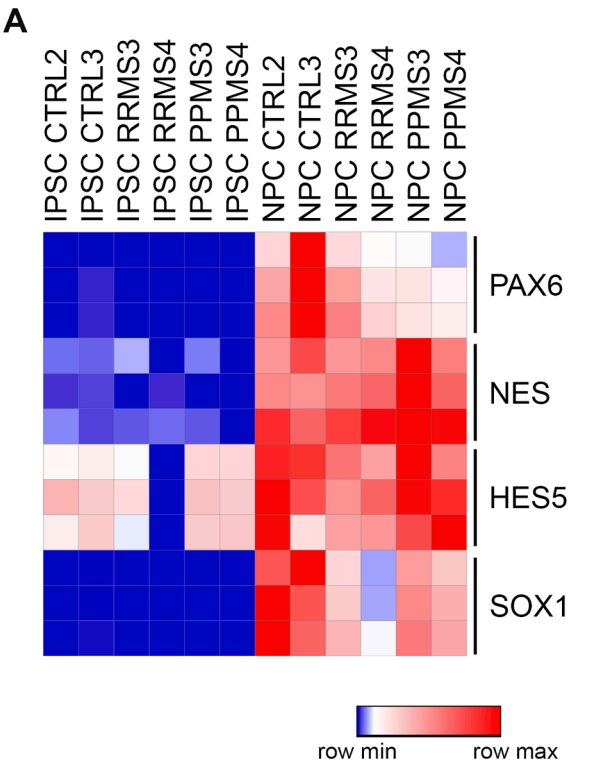


Fig. 1. (*continued*).

including Multiple Sclerosis are age associated adult onset, chronic and progressive neuropathies {Reeve et al., 2014; Hou et al., 2019}. Aging is an important risk factor for the progression of these chronic neurodegenerative disorders. Cellular senescence is an important hallmark of aging process and is increasingly reported for its association with several human neurodegenerative diseases {Song et al., 2020; Tacutu et al., 2011; Childs et al., 2015}. Therefore, we next asked whether patient cells with MS genetic background could show any precocious cellular senescence especially in the neural progenitors which are the building blocks of whole central nervous system. Hence, we performed senescence associated β-galactosidase (SA-β-gal) staining on control (Ctrl2 and Ctrl3), RRMS (RRMS3 and RRMS4) and PPMS (PPMS3 and PPMS4) neural progenitor cells (Fig. 3A). Indeed, we observed an elevated number of senescent cells in PPMS and RRMS neural progenitor culture compared to control neural progenitor cells (Fig. 3B). This result indicates that cellular senescence could play a potential role in MS pathogenesis. Interestingly, neural progenitors of PPMS (PPMS2 and PPMS3) subtype which is the severe form of MS have shown fourfold higher the number of positive senescent cells compared to RRMS neural progenitors (Fig. 3B). This result suggests a positive correlation between the level of cellular senescence and the severity of MS disease. We next carried out qPCR analysis of known markers of cellular senescence such as p16, IL6, ATF3 and GADD25B and confirmed their elevated expression in PPMS and RRMS neural progenitors (Fig. 3C). The levels of cellular senescence are pronounced in PPMS subtype but less differences were observed in RRMS compared to Control NPCs. However, we suggest that these results could be limited and an additional data including extra MS iPSC lines and further in-depth high through put RNA-Sequencing and immunostaining studies is required to confirm the role of senescence in MS disease. Altogether, these results suggest mechanisms such as elevated cellular senescence might contribute to the MS disease pathology and further research in this line depict more insights about MS pathomechanism and might result in novel MS drug targets.

3. Discussion

Inaccessibility to relevant diseased human MS cell types and lacking culture systems that generate them in large scale for intensive high throughput investigations had impeded advances in MS disease biology and drug testing. The generation of clinically relevant human cellular models with patients MS genetic background would thus offer an exciting and promising opportunity to model MS pathophysiology and find novel drug targets. Here in this study, we have successfully converted patient PBMCs of both the clinical forms of MS i.e., RRMS and PPMS into their respective iPSCs using integration free episomal reprogramming vectors. All the generated iPSCs in this study including Control, RRMS and PPMS have shown ESC like morphology and pluripotency markers consistent with the previous studies {Takahashi et al., 2007; Martí et al., 2013}. Similar to previous reports, MS iPSCs were successfully differentiated to neural progenitor cells {Song et al., 2012; Massa et al., 2016; Nicaise et al., 2017} albeit we found reduced expression of PAX6—pan-neural stem cell marker in RRMS and PPMS neural progenitors implicating compromised stem cell fate specification. Furthermore, we found increased cellular senescence in MS neural progenitors especially pronounced elevation in the PPMS subtype. Either normal aging process or pathological cellular stress or inflammation results in cellular senescence. Sequentially cellular senescence



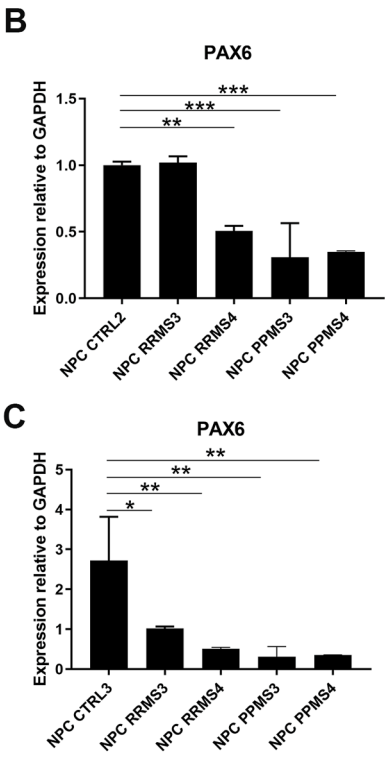


Fig. 2. Expression of neural stem cell markers in control and MS patient iPSC derived NPC. (A) Quantitative PCR (qPCR) analysis of transcript levels of neural stem cell markers PAX6, NESTIN, HES5 and SOX1 in Control (CTRL2 and CTRL3), RRMS (RRMS3 and RRMS4), and PPMS (PPMS3 and PPMS4) iPSC lines and respective NPC cultures generated. All transcript levels are normalized to the respective GAPDH levels in each sample and fold change is converted to Z-Scores and expressed in the form of a heat map. Color-coded scale represents relative expression levels of each gene in triplicates (row) across different samples. (B) Quantitative PCR (qPCR) analysis of PAX6 transcript levels obtained for neural progenitor cells of Control (CTRL2), RRMS (RRMS3 and RRMS4), and PPMS (PPMS3 and PPMS4) lines. All transcript levels are normalized to the respective GAPDH levels in each sample. Statistics: Bars represent mean ± S.D. Statistical test: one-way ANOVA followed by Tukey's post hoc test: *P < 0.05; **P < 0.01 ***P < 0.001; ****P < 0.0001. (C) Quantitative PCR (qPCR) analysis of PAX6 transcript levels obtained for neural progenitor cells of Control (CTRL3), RRMS (RRMS3 and RRMS4), and PPMS (PPMS3 and PPMS4) lines. All transcript levels are normalized to the respective GAPDH levels in each sample. Statistics: Bars represent mean ± S.D. Statistical test: one-way ANOVA followed by Tukey's post hoc test: *P < 0.05; **P < 0.01 ***P < 0.001; ****P < 0.0001.

further inflames the surrounding tissue environment through the release of inflammatory SASP components {Zhu et al., 2014; Ovadya and Krizhanovsky, 2014}. Our results thus suggest that elevated cellular senescence in MS NPC might aggravate inflammation, increase demyelination as well as reduce OPC differentiation and OL remyelination capacity. However, this study is confined to the analysis of two iPSC lines for healthy and each disease subtype and thus, the possible differences observed in neural stemness and cellular senescence need to be characterized in-depth further with the inclusion of extra iPSC lines and high-throughput analyses. These findings thus further require an in-depth research on how cellular senescence links to inflammation and demyelination in MS and would offer an exciting new avenue to probe in and find novel drug therapies modulating cellular senescence in MS.

4. Materials and methods

4.1. Isolation of PBMC from healthy individuals and MS patients

Density centrifugation (Ficoll-Paque) method is used to isolate PBMCs following previously established protocols {Pham et al., 2004, 2008}. Briefly, 5 ml of blood was obtained from patient and healthy individuals using pre-heparinized syringes and was diluted with an equal volume of phosphate-buffered saline, pH 7.4 (PBS). 10 ml of diluted blood was layered over 5 ml of the Ficoll-Paque PREMIUM (GE Healthcare, cat. no. 17–5442-02) and centrifuged at 400 × g for 30 min at 18 ℃. The resultant PBMC interface layer was carefully removed and washed once with PBS-EDTA by centrifuging at 200 × g for 10 min at 18 ℃. Cell number and viability were determined using hemocytometer and approximately 5–10 million PBMCs were isolated from 5 ml of peripheral blood. The PBMCs were cryopreserved using STEM-CELLBANKER® GMP Grade (Zenoaq) and stored in liquid nitrogen until required for further downstream analyses.

4.2. Derivation of Control, RRMS and PPMS iPSCs

PBMCs were incubated in PBMC complete medium (StemPro-34 SFM, Life technologies, cat. no. 10639–011) supplemented with GlutaMAX (Gibco, 35050–061), 100 ng/ml SCF (R&D, 7466SC010CF), 20 ng/ml TPO (Biolegend, 763702), 10 ng/ml IL-6 (Biolegend, 570802),100 ng/ml Flt3 (Stemcell, 78009.1)) and dynabeads Human T-Activator CD3/CD28 (Thermo Fisher Scientific, DB11161) for 1 h and T cells were isolated using magnetic cell separation.

Pluripotent stem cell lines were generated using episomal vectors following previously published protocol {Okita et al., 2011} with minor modifications. Briefly, T Cells were transfected with non-integrating episomal vectors (pCXLE-hSK, pCXLE-hUL, pCXLE-hOCT3/4-shp53-F, pCXWB-EBNA1) expressing reprogramming transcription factors (Oct3/4, Sox2, c-Myc, Klf4, shRNA against p53, LIN28, and EBNA-1) using P3 Primary Cell 4D-Nucleofector X kit (Lonza, V4XP-3012) according to the manufacturer's instructions. Transfected cells were then seeded onto six-well plates covered with a mouse embryonic fibroblast (MEF) feeder layer. Post transfection cells were cultured in ESC Medium [DMEM-F12 (Gibco, 11320033) supplemented with 20% KnockOut™ Serum Replacement (Thermo Fisher Scientific, 10828010), GlutaMax (1:100), non-essential amino acids (Life Technologies, 11140050, 1:100), beta-mercaptoethanol (Gibco, 21985023, 1:1000) and 20 ng/ml FGF2 (Gibco, PHG0367)]. Between Day 27 to Day 35 post-transfection, iPSC colonies were manually picked based on their ESC like morphology and further cultured and passaged. Human iPSC cultures were then either maintained on MEF layer (feeder-dependent culture) or on tissue culture plates coated with Matrigel in mTESR-1 medium (feeder independent culture).

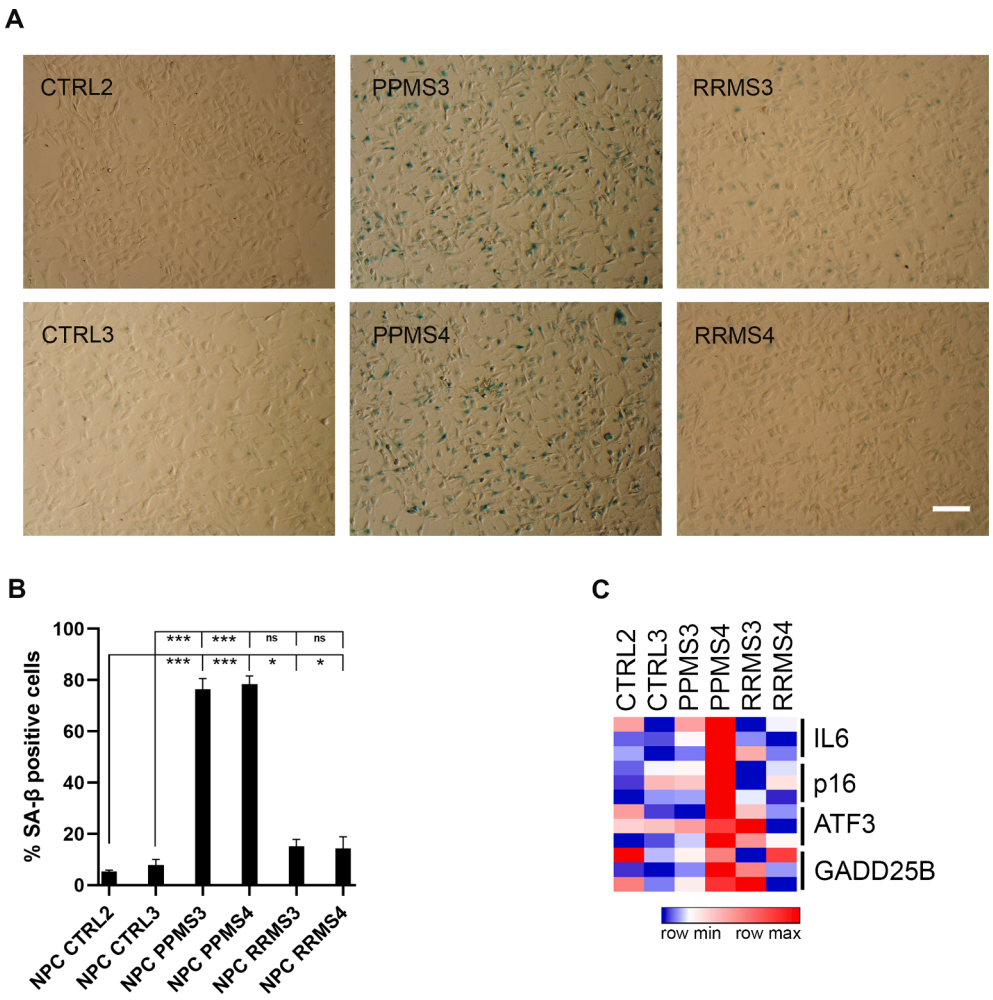


Fig. 3. Elevated cellular senescence in iPSC-derived Neural Progenitor Cells (NPCs) derived from patients with PPMS. (A) Phase contrast images showing senescence associated beta-galactosidase (SA–β-gal) staining in Control, PPMS and RRMS NPC cultures revealed elevated LacZ activity (shown in blue) in PPMS NPC cultures. Scale bar: 50 μm. (B) Quantification of SA–β-gal staining in Control, PPMS and RRMS NPCs shows an increase in LacZ in PPMS cultures. Staining was performed and quantified from three independent differentiations. Bars represent mean ± S.D. Statistical test: one-way ANOVA followed by Tukey's post hoc test: ns—non-significant; *P < 0.05; **P < 0.01 ***P < 0.001; ****P < 0.0001. (C) Quantitative PCR (qPCR) analyses of transcript levels of cellular senescence markers IL6, p16, ATF3 and GADD25B in Control (CTRL2 and CTRL3), RRMS (RRMS3 and RRMS4), and PPMS (PPMS3 and PPMS4) NPC cultures. All transcript levels are normalized to the respective GAPDH levels in each sample and fold change is converted to Z-Scores and expressed in the form of a heat map. Color-coded scale represents relative expression levels of each gene in triplicates (row) across different samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.3. iPSC culture

Human iPSCs were cultured either on MEFs in CDF12 media containing DMEM/F12 (Life Technologies, 11330–032), 20% KnockOut serum replacement (Life Technologies, 10828), 2 mM Glutamax (Life Technologies, 35050–061), 0.1 mM NEAA (Life Technologies, 11140–050), 0.1 mM b-mercaptoethanol (Gibco, 21985) and 4 ng/ml FGF2 (Peprotech), or on plates pre-coated with Matrigel (BD Biosciences, 354248) using mTeSR1 (Stem Cell Technologies, 85850) media.

4.4. Neural differentiation

Human iPSCs were dissociated into single cells by accutase (Innovative Cell Technologies, AM105), and seeded at 20 K cells/cm^2 density on matrigel-coated plates and cultured in mTeSR1 medium containing 10 μM of Rock inhibitor (Y-27632, Tocris) overnight. On day 0, medium was switched to N2B27 medium (DMEM/F12 supplemented with N2 (Invitrogen,17502–048, 1:100), B27 without vitamin A (Invitrogen, 12587–010, 1:50), Glutamax (1:100), NEAA (1:100), beta-mercaptoethanol (1:1000), and 25 μg/ml insulin (Sigma, I9278-5ML)), supplemented with the small molecules SB431542 10 μM (Tocris,1614) and LDN193189 1 μM (MiltenyiBiotec,130–106-540). Medium was changed daily until day 8, and SB431542 and LDN193189 were then withdrawn. On day 14, cells were dissociated and further maintained at high density on matrigel in NPC medium (DMEM/F12, 1x N2, 1x B27 without vitamin A and 20 ng/ml FGF2) and split every week with accutase.

4.5. Senescence-associated β-galactosidase ((SA)–β-gal) assay

Human NPCs were seeded on matrigel coated six well plate at equal densities (2 × 10^6 cells per well), and β-gal staining was performed according to the manufacturer's instructions (Cell Signaling Technology, 9860). Images were captured at 10X magnification using identical phase contrast settings. Staining was performed and quantified in triplicate in each NPC line.

4.6. Immunofluorescence and image acquisition

Cells were fixed for 20 min in 4% formaldehyde in PBS at room temperature (RT). Subsequently, samples were treated with 0.4% Triton X-100 in PBS at RT for 10 min. Cells were blocked with 10% FBS in PBS for 1 h, and then incubated at 4° C overnight with primary antibody. Cells were washed in PBS and incubated at RT for 1 h with the corresponding secondary antibody. DNA was stained with DAPI (Invitrogen). Images were captured with a Zeiss LSM 780 confocal microscope and Olympus IX51 with Olympus S97809 Digital camera. The primary antibodies used were anti-OCT-3/4 (Santa Cruz Biotechnology, sc-5279), and anti-SOX2 (Santa Cruz Biotechnology, sc-17320) and anti-NANOG (Invitrogen, 14–5761-80).

4.7. Quantitative PCR (qPCR)

TRIZOL (Invitrogen) was used to extract total RNA. cDNA was synthesized using iScript ReverseTranscription Supermix for RT-qPCR (Bio-Rad). Quantitative RT-PCR was performed with SsoAdvanced SYBR

Green Supermix (Bio-Rad) using CFX384 Real-Time PCR Detection Systems (Bio-Rad). Primer sequences are shown in Supplemental Table1.

CRediT authorship contribution statement

ZM, YT, FIM, JML and JCIB designed the study. NM, ZM, YT, FIM, and MM, performed the experiments. FIM, ECG, RHC, DGB, LMM, JL, and EN coordinated with patients and arranged for patient samples. NM, YT, JML and JCIB wrote the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2021.102319.

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