

GENERATION AND CHARACTERIZATION OF INDUCED PLURIPOTENT
STEM CELL LINES FROM MULTIPLE SCLEROSIS PATIENTS AND
HEALTHY INDIVIDUALS

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PATIENTS AND HEALTHY INDIVIDUALS**

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ABSTRACT

GENERATION AND CHARACTERIZATION OF INDUCED PLURIPOTENT STEM CELL LINES FROM MULTIPLE SCLEROSIS PATIENTS AND HEALTHY INDIVIDUALS

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Multiple Sclerosis (MS) is an autoimmune disease characterized by inflammation, demyelination, and axonal damage resulting in neurodegeneration in the central nervous system (CNS). Relapsing-remitting MS (RRMS) is the most common form of the disease known to affect more than 2 million individuals globally, and the prevalence of the disease is increasing worldwide. As a chronic condition without a cure, RRMS manifests in a relapsing-remitting form with sporadic attacks suddenly appearing, causing neurological dysfunction to proceed by a recovery phase where all symptoms suddenly disappear. Although the factors causing or contributing to MS initiation/progression are not well understood, previous literature has implicated many potential risk factors for the etiology of the disease, including Epstein-Barr virus, smoking, and Vitamin D deficiency. Another potential risk factor implicated in RRMS is sex. Epidemiological studies strongly suggest that MS is a sexually biased disease that is more common in females. Moreover, the disease progression and severity appear to be sexually dimorphic since the progression and the symptoms are worse in males; however, more inflammatory in females. Importantly, research suggests that biological sex differences beyond gender-related factors may be crucial

in RRMS; however, how biological sex differences affect the disease initiation or progression remain unknown. The hallmark of MS is demyelination, which is the loss of the myelin sheath around axons in the CNS. Oligodendrocytes are the glial cells generating myelin sheath to axons in CNS. It is well established that autoimmune response in MS leads to damage and potentially loss of oligodendrocytes, eventually leading to neurodegeneration. Current therapeutic approaches for MS are only limited to eliminating the symptoms caused by the autoimmune attacks. While such disease-modifying therapies help suppress autoimmune attacks and eventually lower the frequency of attacks, there are no available therapeutics to induce remyelination. Clearly, there is a need for more advanced approaches in MS that would induce remyelination besides immunosuppression. To develop effective therapeutic options that can induce remyelination to complement existing immunosuppressive drugs, a better understanding of cellular processes in remyelination is needed. To facilitate such studies, reliable model systems are necessary for mechanism and drug discovery studies. Human induced pluripotent stem cells (iPSCs) based approaches can provide such systems. Due to their nature and ability to form every possible cell type from the three embryonic germ layers, they can now be easily generated from patients' somatic cells to generate patient-specific cell types. iPSCs can be utilized to obtain cell types that are normally very hard to obtain on a large scale, especially for CNS disease modeling. Indeed, numerous studies have demonstrated that iPSCs can be successfully differentiated to neuronal and glial cells, and importantly such cells exhibit disease-specific phenotypes in culture. MS iPSCs have also been generated by various groups, and cells differentiated from the iPSCs exhibit disease characteristics, suggesting that these cells can be utilized for MS studies. The main goal in this thesis was to generate and fully characterize iPSC lines from male and female patients and matched controls to facilitate future studies. Towards this goal, blood samples were obtained from age, and sex-matched RRMS patients (3 females and 3 males) and 6 healthy individuals (3 females and 3 males) and Peripheral Blood Mononuclear Cells (PBMCs) were isolated. Following, the PBMCs were

reprogrammed using Sendai virus-based reprogramming method, and a total of 12 iPSC lines were established, and their pluripotent characteristics were experimentally characterized. The established iPSC lines will serve as excellent tools for modeling RRMS, enabling us to test multiple approaches to understand the mechanisms governing sex-specific differences and remyelination.

Keywords: Multiple Sclerosis, Disease Modeling, Human Induced Pluripotent Stem Cells, Sendai Virus Based Reprogramming



ÖZ

MULTIPL SKLEROZ HASTALARINDAN VE SAĞLIKLI BİREYLERDEN UYARILMIŞ PLURİPOTENT KÖK HÜCRE HATLARININ ÜRETİMİ VE KARAKTERİZASYONU

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Multipl Skleroz (MS), merkezi sinir sisteminde (CNS) nörodejenerasyon ile sonuçlanan inflamasyon, demiyelinizasyon ve aksonal hasar ile karakterize otoimmün bir hastalıktır. Relapsing-remitting MS (RRMS), 2 milyondan fazla kişiyi etkilediği bilinen, hastalığın en yaygın şeklidir ve hastalığın prevalansı dünya çapında artış göstermektedir. Tedavisi olmayan kronik bir hastalık olan RRMS, aniden ortaya çıkan sporadik ataklarla tekrarlayan-düzelten bir formda kendini göstererek nörolojik işlev bozukluğuna neden olur ve hemen akabinde tüm semptomların aniden kaybolduğu bir iyileşme evresiyle devam eder. MS başlangıcına/ilerlemesine neden olan veya katkıda bulunan faktörler iyi anlaşılmamış olsa da önceki literatür, Epstein-Barr virüsü, sigara ve Vitamin D eksikliği dahil olmak üzere hastalığın etiyojisi için birçok potansiyel risk faktörünü sebep göstermektedir. RRMS'de yer alan bir diğer potansiyel risk faktörü cinsiyettir. Epidemiyolojik çalışmalar, MS'nin kadınlarda daha sık görülen cinsel açıdan yanlı bir hastalık olduğunu kuvvetle önermektedir. Ayrıca hastalığın ilerlemesi ve şiddeti cinsel olarak dimorfik görünmektedir, öyle ki hastalığın ilerlemesi ve semptomları erkeklerde daha kötü seyrederken, kadınlarda daha inflamatuvarıdır. Daha da önemlisi, araştırmalar cinsiyete bağlı faktörlerin ötesinde biyolojik cinsiyet

farklılıklarının RRMS'de çok önemli olabileceğini düşündürmektedir; ancak, biyolojik cinsiyet farklılıklarının hastalığın başlamasını veya ilerlemesini nasıl etkilediği bilinmemektedir. MS'nin ayırt edici özelliği, CNS'deki aksonların etrafındaki miyelin kılıfının kaybı olarak bilinen demiyelinizasyondur. Oligodendrositler, CNS'deki aksonlara miyelin kılıfı oluşturan glial hücrelerdir. MS'deki otoimmün yanıtın, sonunda nörodejenerasyona yol açan oligodendrositlerin hasar görmesine ve potansiyel olarak ölümüne yol açtığı iyi bilinmektedir. MS için mevcut terapötik yaklaşımlar, yalnızca otoimmün atakların neden olduğu semptomları ortadan kaldırmakla sınırlıdır. Bu tür hastalık modifiye edici tedaviler, otoimmün atakları bastırmaya ve nihayetinde atakların sıklığını düşürmeye yardımcı olurken, yeniden miyelinizasyonu ve/veya nörorejenerasyonu indükleyecek mevcut terapötikler bulunamaktadır. Açıkçası, MS'de immünosupresyonun yanı sıra remiyelinizasyon ve nörojenezi indükleyecek daha gelişmiş yaklaşımlara ihtiyaç vardır. Mevcut immünosupresif ilaçları tamamlamak üzere remiyelinizasyonu indükleyebilecek etkili terapötik seçenekler geliştirmek için remiyelinizasyondaki hücresel süreçlerin daha iyi anlaşılmasına ihtiyaç vardır. Bu tür çalışmaları kolaylaştırmak için mekanizma ve ilaç keşif çalışmalarında güvenilir model sistemler gereklidir. İnsan uyarılmış pluripotent kök hücreler (iPSC'ler) tabanlı yaklaşımlar bu tür sistemleri sağlayabilir. Doğaları ve üç embriyonik germ tabakasından olası her hücre tipini oluşturma yetenekleri nedeniyle, artık hastaların somatik hücrelerinden hastaya özel hücre tipleri oluşturmak için kolaylıkla üretilbilirler. Bir kez oluşturulduktan sonra, iPSC'ler, özellikle CNS hastalık modellemesi için, normalde canlı bir organizmadan elde edilmesi çok zor olan birtakım hücreleri elde etmek için kullanılabilir. Gerçekten de çok sayıda çalışma, iPSC'lerin nöronal ve glial hücrelere başarılı bir şekilde farklılaştırılabileceğini ve daha da önemlisi, bu tür hücrelerin kültürde hastalığa özgü fenotipler sergilediğini göstermiştir. MS için de iPSC'ler çeşitli gruplar tarafından oluşturulmuştur ve hastalık özelliklerini sergilemeleri, bu hücrelerin MS çalışmaları için kullanılabileceğini düşündürmektedir. Bu tezdeki ana amaç, gelecekteki çalışmaları kolaylaştırmak için erkek ve kadın hastalardan ve eşleştirilmiş kontrollerden iPSC

hatları oluřturmak ve tam olarak karakterize etmektir. Bu amaca ynelik olarak yař ve cinsiyet uyumlu RRMS hastalarından (3 kadın ve 3 erkek) ve 6 saėlıklı bireyden (3 kadın ve 3 erkek) kan rnekleri alınarak ve Periferik Kan Mononkleer Hcreleri (PBMC'ler) izole edilmiřtir. Ardından, PBMC'ler Sendai virs tabanlı yeniden programlama yntemi kullanılarak yeniden programlanmış ve toplam 12 iPSC hattı oluřturularak ve pluripotent zellikleri deneysel olarak karakterize edilmiřtir. Oluřturulan iPSC hatları, RRMS'yi modellemek iin mkemmел aralar olarak hizmet edecek ve cinsiyete zg farklılıkları ve remiyelinizasyonu yneten mekanizmaları anlamak adına ok sayıda farklı yaklařımı test etmemizi saėlayacaktır.

Anahtar Kelimeler: Multipl Skleroz, Hastalık Modellemesi, İnsan Uyarılmış Pluripotent Kk Hcreler, Sendai Virs Temelli Yeniden Programlama



To the future of regenerative medicine

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LIST OF ABBREVIATIONS

ABBREVIATIONS

MS = Multiple Sclerosis

RRMS = Relapsing-Remitting Multiple Sclerosis

CNS = Central Nervous System

EAE = Experimental Autoimmune Encephalomyelitis

iPSC(s) = Induced Pluripotent Stem Cell(s)

hESC(s) = Human Embryonic Stem Cell(s)

TC = Tissue Culture

PBMC(s) = Peripheral Blood Mononuclear Cell(s)

RBC(s) = Red Blood Cell(s)

MEF(s) = Mouse Embryonic Fibroblasts

pMEF(s) = Primary Mouse Embryonic Fibroblasts

mmc-MEF(s) = Mitomycin C-treated Mouse Embryonic Fibroblasts

DPBS = Dulbecco's Phosphate-Buffered Saline

EES = Erythroid Expansion Supplement

EB(s) = Embryoid Bodie(s)

NPC(s) = Neural Precursor/Progenitor Cell(s)

OPC(s) = Oligodendrocyte Precursor/Progenitor Cell(s)

OL(s) = Oligodendrocyte(s)

bFGF = Basic Fibroblast Growth Factor

FBS = Fetal Bovine Serum

KSR = KnockOut Serum Replacement

HSC(s) = Hematopoietic Stem Cell(s)



CHAPTER 1

INTRODUCTION

1.1 Multiple Sclerosis

Multiple Sclerosis (MS) is an inflammatory neurodegenerative autoimmune disease of the central nervous system (CNS) (Dobson & Giovannoni, 2019). MS is characterized by inflammation, demyelination, and axonal damage that eventually led to neurodegeneration (Filippi et al., 2018). Although the factors leading to the onset and the progression of MS are mostly uncertain, Epstein-Barr Virus, smoking, and Vitamin D deficiencies are among the hypothesized factors contributing to the disease together with the genetic background (Ramagopalan, Dobson, Meier, & Giovannoni, 2010).

MS is a chronic condition that manifests in a relapsing-remitting (RR) form, characterized by sporadic attacks causing neurological dysfunction followed by a recovery phase, and a progressive form that usually evolves from the RR form (Baecher-Allan, Kaskow, & Weiner, 2018). Relapsing-remitting MS (RRMS) is a type of MS that starts with sudden exacerbation of the disease but continues with improvement and disappearance of symptoms with certain periods. RRMS is the most common form of the disease compared to primary progressive and secondary progressive forms of MS, and it is known to affect more than 2 million individuals worldwide (Stenager, 2019). The prevalence of MS is increasing worldwide, such that a 10.4% increase in the disease prevalence was reported between 1990-2016, and the population in Turkey was found to carry a high risk of MS with an increase rate of 14.3% (Wallin et al., 2019).

MS is a sexually dimorphic disease, and it is known that various parameters such as age of onset and frequency of attacks differ in male and female patients. The

worldwide statistics suggest that the rate of MS among female and male patients is 3:1, where women are more vulnerable to the disease (Dunn, Gunde, & Lee, 2015; Gilli, DiSano, & Pachner, 2020; Harbo, Gold, & Tintore, 2013). The impact of biological sex on MS was studied in many aspects of the disease. It was found that though women have increased susceptibility for MS, the disease outcomes are exacerbated in men (Gilli et al., 2020). In addition, it has been reported that the disease is more inflammatory in women, while the progression of MS in men is more neurodegenerative (Gilli et al., 2020).

Demyelination (loss of myelin sheath) in the CNS is the hallmark of MS, which can only be detected via magnetic resonance imaging at the pre-clinical stage that is essential for diagnosis (Baecher-Allan et al., 2018; Dobson & Giovannoni, 2019). Disability in individuals affected by MS progresses and worsens over time due to demyelination that eventually leads to neurodegeneration (Filippi et al., 2018). The key driver of the pathogenesis in MS is the adaptive immune system. As the disease progresses, in each relapse, activated cells of the adaptive immunity start migrating and infiltrating through the blood-brain barrier into the CNS, where they begin to attack oligodendrocytes (OLs) and the myelin sheaths produced from them (Baecher-Allan et al., 2018; Filippi et al., 2018; Goldenberg, 2012). Treatment options for RRMS are limited to the elimination of symptoms which were shown to effectively eliminate relapses acting as immunomodulators, where they prevent infiltration of effector cells (B- and T- lymphocytes) into the CNS and decrease the number and function of activated lymphocytes while increasing the number and function of regulatory lymphocytes (Baecher-Allan et al., 2018). Approved drugs suppress the attacking of cells of the adaptive immunity to OLs and the myelin sheaths, lowering or eliminating the frequency of incoming relapses (Cunniffe et al., 2021).

Governed by the activation and migration of existing OLs and their progenitors (Oligodendrocyte Progenitor Cells, OPCs), remyelination is the process of recovering the lost myelin sheath around axons that is essential for restoring nerve connection and prevents neurodegeneration (Cunniffe & Coles, 2019; Franklin,

Frisen, & Lyons, 2020; Lubetzki, Zalc, Williams, Stadelmann, & Stankoff, 2020). One of the most crucial aspects of remyelination is that it is essential for restoring nerve connection and prevents neurodegeneration, which eventually leads to disabilities under the context of MS (Franklin et al., 2020; Lubetzki et al., 2020). There are 16 different approved disease-modifying therapeutics and drugs for RRMS; however, these are only limited to immunosuppression and do not induce remyelination (Cunniffe & Coles, 2019; Cunniffe et al., 2021; Torkildsen, Myhr, & Bø, 2015). There are defects in the remyelination process of MS, but the molecular mechanisms that cause these defects are still largely unknown (Franklin et al., 2020). Therefore, a better understanding of the molecular mechanisms that play a crucial role in remyelination can pave the way for the development of effective treatment options for MS. Physiologically relevant and reliable model systems are required to facilitate such studies.

1.1.1 Experimental Models of Multiple Sclerosis

The limited access to human brain tissue makes model organisms and experimental systems crucial for understanding the mechanisms and disease pathology in neurological diseases (Marton & Paşca, 2020). Most studies investigating the molecular biology of MS use animal models, and the most common of these models is the Experimental Autoimmune Encephalomyelitis (EAE) model (Sanabria-Castro, Flores-Diaz, & Alape-Giron, 2020). Being the most widely used preclinical *in vivo* research model, EAE is usually induced using pertussis toxin combined with adjuvants and MS-specific target antigens (i.e., MBP, MOG and PLP) in rodents which results in inflammation, demyelination, and neurodegeneration in the CNS, and generates a similar pathology to MS (Sanabria-Castro et al., 2020). There are no animal models of EAE of natural or spontaneous origin. However, it is highly controversial how closely EAE models mimic the human pathology in MS for several reasons (Handel, Lincoln, & Ramagopalan, 2011). First, demyelination in EAE models is not consistent. Second, the lesions in the CNS of EAE models are

limited to the spinal cord in most EAE. Lastly, it was shown that there are significant differences in the dynamics of OLs and OPCs upon demyelination in humans versus EAE models of MS (Yeung et al., 2019).

Beyond animal models, MS has been widely studied in the past decade using in vitro models. More specifically, five distinct models utilizing different cell types and approaches have been utilized in MS disease modeling. These are primary rodent cells, primary human cells, human immortalized cell lines, and human induced pluripotent stem cell (hiPSC) and human embryonic stem cell (hESC) derived lines of the CNS, including brain organoids (Martinez-Larrosa, Matute-Blanch, Montalban, & Comabella, 2020). Due to the complex nature of the disease in humans, most of the models used in MS studies fail to recapitulate the disease, which may be responsible at least in part for the lack of development of effective therapeutic options. There has been growing interest in human model systems to study MS, and among these, iPSC-based approaches are considered highly attractive model systems to study the disease, which offers unique advantages as discussed below.

1.2 Human Induced Pluripotent Stem Cells

Advancements in stem cell fields have demonstrated that human pluripotent stem cells (i.e., human embryonic stem cells (hESCs) and iPSCs) can provide versatile culture systems for disease modeling (Avior, Sagi, & Benvenisty, 2016). Human ESCs are derived from the inner cell mass of blastocysts (Moon, Park, Kim, Oh, & Kim, 2006), they have the ability to grow indefinitely while maintaining pluripotency (Evans & Kaufman, 1981; Martin, 1981). The first hESC lines were derived in 1998 (Thomson et al., 1998), and it is now well established that hESCs can be differentiated to all cell types in the human body, making it an excellent tool for disease modeling. Later advancements in stem cell biology demonstrated minimal essential transcription factors that are needed for making a cell “pluripotent”, starting with somatic cells. More specifically, both mouse and human

somatic cells were successfully reprogrammed into induced pluripotent stem cells (iPSC) using simultaneous transfer and expression of 4 transcription factors (Yamanaka factors): Oct3/4, Sox2, Klf4, and c-Myc (K. Okita, Ichisaka, & Yamanaka, 2007; Takahashi et al., 2007; Takahashi & Yamanaka, 2006). In the same year, the establishment of iPSCs from somatic cells has also been achieved using another set of 4 transcription factors which essentially yielded similar outcomes after reprogramming: Oct3/4, Sox2, Lin28, and Nanog (Yu et al., 2007). Established iPSCs share two distinct properties of embryonic stem cells in definition: self-renewal and pluripotency. iPSCs can indefinitely give rise to daughter cells upon division while maintaining all the progenitor properties. The capacity of a pluripotent stem cell refers to the pluripotency, where under defined conditions cells can differentiate and give rise to any cell type of the three embryonic germ layers: endoderm, mesoderm, and ectoderm. Human iPSC technology has certain advantages over hESCs as there are ethical issues concerning deriving hESC (Lo & Parham, 2009), and the cells with patients' genetic material may be highly useful for disease mechanism studies.

Biological similarities and differences between hESCs and iPSCs have been the focus of many studies. Despite the similar morphology (round-shaped and single-layered colonies with intact boundaries, high nucleus to cytoplasm ratio, and prominent nucleoli in every cell within the colony) and culture conditions of hESCs and hiPSCs, there are also several differences, including cell surface markers, epigenome, gene expression patterns and age-affected mitochondria and telomeres (Doi et al., 2009; Laurent et al., 2011; Phanstiel et al., 2011). Since such differences are very common among different ESC lines as well as iPSCs, the quality of an established iPSC should be continuously tracked with routine karyotype and gene expression analyses (Buganim, Faddah, & Jaenisch, 2013). Moreover, while working with hESCs and their derivatives may raise ethical concerns, it is much easier to handle iPSCs and their derivatives in research (Condic & Rao, 2010; Lo & Parham, 2009). Thanks to numerous studies contributing to optimal conditions, reagents, and technologies, iPSC generation has been a streamlined process.

Theoretically, cells of any somatic origin can be reprogrammed using various alternative methods, including viral vectors with excisable, integrating, and non-integrating forms. Non-viral systems have also been reported to be used in somatic cell reprogramming (Buganim et al., 2013). Beyond genomic materials for somatic cell reprogramming for iPSCs generation, proteins of recombinant origin and whole-cell extracts from either engineered HEK293-T cells or hESC lines were used (Cho et al., 2010; Kim et al., 2009; Zhou et al., 2009). However, the reprogramming efficiency was found to be extremely low when reprogramming efficiencies of different types of somatic cells were compared with the recombinant protein technology (Stadtfield & Hochedlinger, 2010).

Among many other somatic cell sources, dermal fibroblasts and peripheral blood mononuclear cells (PBMCs) are the most common origin of cells that are suitable for reprogramming at high efficiencies (Febbraro, Chen, & Denham, 2021; Fidan, Ebrahimi, Çağlayan, Özçimen, & Önder, 2015; Hiramoto et al., 2020; Liu et al., 2019; Omer, Hudson, Hudgins, & Boyd, 2021; Ross, Fraser, Bagnall, & Semsarian, 2017; Tang et al., 2020). PBMCs are preferred as it is less invasive to obtain these cells from the donors. Once established, there are several characterizations steps that the established iPSC lines must meet before being utilized in any further research. Fully established human iPSCs express a specific set of antigens including SSEA-3, SSEA-4, TRA-1-60, DNMT3 β , and REX1 (Deyle, 2015). An established pluripotent stem cell should also have endogenously upregulated expression of pluripotency related markers such as *OCT4*, *SOX2*, *NANOG*, and *TRA-1-60* and Alkaline Phosphatase. They must be capable of differentiating into cells of the three embryonic germ layers: endoderm, mesoderm, and ectoderm, which is usually achieved either by embryoid body (EB) formation *in vitro* or by teratoma formation *in vivo*. Established iPSCs should retain a normal karyotype, and the exogenous reprogramming particles and genes should be cleared out from the lines (Quintanilla, 2013).

1.3 Modeling Multiple Sclerosis Using Induced Pluripotent Stem Cells

In general, it is challenging to model disorders affecting neurons or glial cells because of the limitations on accessing the nervous tissues from live mammals. For MS, there has been a significant increase in the generation and characterization of exclusive iPSC lines, which are used to develop model systems for drug screening and advanced molecular characterization. Such cell lines were derived from either healthy or diseased individuals and differentiated mostly into astrocytes (Perriot et al., 2018) and OLs (Lopez-Caraballo, Martorell-Marugan, Carmona-Saez, & Gonzalez-Munoz, 2020; Morales Pantoja et al., 2020; Thiruvalluvan et al., 2016). In a recent study, iPSCs generated from a cohort of RRMS, PPMS, and the control group were differentiated into neural progenitor cells (NPCs), and it was found that NPC marker expression levels were significantly reduced in the diseased individuals, and the NPCs were found to undergo senescence upon differentiation when compared to healthy controls (Mutukula et al., 2021). Remyelination failure is another important hallmark of MS and understanding OPC and OL dynamics during MS pathogenesis is crucial. For this purpose, multiple studies were conducted to effectively generate fully functional (both *in vivo* and *in vitro*) human OLs either from control or diseased samples in the field (Czepiel et al., 2011; Douvaras & Fossati, 2015; Douvaras et al., 2014; Ehrlich et al., 2017; Garcia-Leon et al., 2018; Hu, Du, & Zhang, 2009; Kawabata et al., 2016; Lopez-Caraballo et al., 2020; Morales Pantoja et al., 2020; Mozafari et al., 2020; Wang et al., 2013; Yamashita et al., 2017). Despite such advances, it is still unclear why new OL generation through the maturation of OPCs is impaired in MS. Since the generated iPSCs carry the same genetic background (disease-relevant genes and alleles) when derived from the diseased individuals, it would be a better model for understanding the actual causes of OL and OPC insufficiencies under the context of MS.

1.4 Aim of the Study

There is no cure for MS, and the underlying cause of the disease is not well understood. There are defects in remyelination processes in MS, and therefore better understanding of underlying molecular mechanisms of remyelination in MS is crucial for both basic research and translational studies. Another critical research focus in MS is studying the effects of biological sex differences using the cell types (i.e., oligodendrocytes) affected by the disease. To facilitate such studies, the generation of physiologically relevant, species-specific, and potentially unlimited cell types can provide robust model systems to study MS. The establishment of hiPSC from MS patients, in a sex-matched manner, has numerous advantages, including the fact that these cells will carry patients' genetic backgrounds. Here, we aimed to generate 12 age and sex-matched human iPSC lines from 6 RRMS patients (3 females and 3 males) and 6 healthy individuals (3 females and 3 males) to model MS *in vitro*. Although there are different iPSC lines established and published from various donor sources, including those with RRMS and healthy controls, they are insufficient for mechanistic studies of oligodendrocyte pathophysiology involving samples that are not adequately matched with their age and biological sexes. Additionally, MS is a disease that shows a significant difference in the prevalence and disease phenotype at different locations worldwide, suggesting the importance of generating iPSCs from different geographical locations. To the best of our knowledge, this work established the first iPSC lines derived from Turkish MS patients. These 12 different sex and age-matched iPSC lines can be utilized to generate various cell types, including oligodendrocytes, to study molecular mechanisms important for remyelination and sex-specific differences. Having unlimited number of CNS cells that can be derived from iPSCs, high-throughput drug screening could also be possible (Csöbönyeiová, Polák, & Danišovič, 2016; Elitt, Barbar, & Tesar, 2018; Farkhondeh et al., 2019).

CHAPTER 2

MATERIALS AND METHODS

2.1 Selection of Patients and Healthy Individuals

Ethical approval was obtained from the Ethics Board of the University of Health Sciences, Turkey (Approval No: 19/83), and all the blood samples were collected with individuals' informed consent. Blood samples were obtained from 3 female and 3 male patients who were clinically diagnosed with RRMS after thorough neurological examinations (i.e., magnetic resonance imaging) according to 2017 McDonald Criteria (Begentas et al., 2021). All the patients enrolled in this study were recently diagnosed with RRMS, and the blood samples were collected before they started taking any disease-modifying medication. Both the patients and control groups were selected from the age range of 25-40. As the control group, 3 female and 3 male individuals, whose ages were also matched with the previously enrolled patients', were selected among healthy individuals who were not related to RRMS patients, did not have any known neurological disorders other than headache, and did not have a history of any autoimmune-related disorders (**Table 1**). All participants enrolled in this study were free of blood coagulation problems, which can severely decrease the PBMC yield during isolation.

Table 1 Information of individuals enrolled in this study either as RRMS patient or as healthy control.

SAMPLE CODE	SEX	AGE	DIAGNOSIS
MSp#3	Female	36	RRMS
MSp#4	Female	31	RRMS

Table 1 (cont'd)

MSp#7	Female	35	RRMS
MSp#10	Male	25	RRMS
MSp#11	Male	32	RRMS
MSp#15	Male	38	RRMS
C#3	Female	40	HEALTHY
C#6	Female	30	HEALTHY
C#7	Female	40	HEALTHY
C#1	Male	32	HEALTHY
C#8	Male	25	HEALTHY
C#12	Male	37	HEALTHY

2.2 Isolation of PBMCs from Freshly Drawn Whole Blood

Blood samples were collected in 3-ml tubes containing anticoagulants (i.e., sodium citrate, EDTA, or sodium heparin) and immediately transferred to our laboratory. The blood tubes and Histopaque-1077 (Sigma, 10771) reagent were set aside for approximately 30 minutes for getting both reagents to room temperature before proceeding. Histopaque-1077 and the blood sample were added to a 15-ml centrifuge tube at a ratio of 1:1. First, Histopaque-1077 was added to the tube. After inverting the blood tubes several times, the blood was aspirated with a serological

pipette and carefully and slowly layered onto the reagent without disturbing the layer. After tightly wrapping with parafilm, the samples were centrifuged at 400g for 30 minutes at room temperature with acceleration and brake values set to their lowest. After centrifugation, the upper layer (**Figure 1**) containing plasma was aspirated within 0.5 cm of the opaque interface (buffy coat) containing PBMCs and discarded. The buffy coat containing PBMCs was carefully collected without disturbing the layers and transferred to a 15-ml centrifuge tube with a serological pipette. PBMCs were washed with 10 ml DPBS (Biological Industries, 02-023-1A) 3 times using centrifugation at 250g for 10 minutes at room temperature, with acceleration and brake settings maximized. After the final washing, the supernatant was discarded, and the pellet was resuspended in 10 ml DPBS, and cell counting was performed. After calculating the total number of PBMCs isolated from the whole blood, cells were centrifuged at 250g for 5 minutes at room temperature and resuspended in a freshly prepared, prechilled freezing medium (**Appendices B**) to obtain 1×10^7 cells/ml of freezing media per cryovial. Each vial was immediately put in a freezing container (Mr. Frosty) and frozen at -80°C overnight. The next day, frozen PBMCs were transferred to the vapor phase of the liquid nitrogen tank until expansion and reprogramming.

2.3 Cell Culture Experiments

2.3.1 Preparation of Mitotically Inactivated Mouse Embryonic Fibroblasts

Primary mouse embryonic fibroblasts (pMEFs), isolated from D13.5 mouse embryos as described elsewhere (Tessarollo, 2001), were readily available in our laboratory as described elsewhere. For mitotic inactivation of pMEFs as feeders in iPSC culture, pMEFs at or below passage 10 was used. Although MEFs are highly popular cells utilized in pluripotent stem cell culture, other cell types, such as SNLP cells, can also be utilized as feeder cells. We have expanded and cultured SNLP cells

and explored their utility in pluripotent stem cell culture, which can provide a versatile feeder cell system. However, in this study, we chose to utilize MEFs for our hiPSC culture throughout the study as these cells are highly established in the literature for hiPSC culture and relatively cost-effective in our hands.

2.3.1.1 Expansion of Primary Mouse Embryonic Fibroblasts

One vial of pMEFs at passages 2-6 was thawed, resuspended in MEF medium (**Appendices B**), counted using a hemacytometer. Cells were centrifuged at 200g for 10 minutes, resuspended in MEF medium and seeded on a 15-cm tissue culture (TC)-treated plate at a density around 5×10^6 cells per plate with a final volume of 25 ml and cultured in a 37°C incubator with a humidified atmosphere of 5% CO₂. On the next day, full medium change was performed. The cells were cultured for approximately 2 more days before reaching up to 90% confluency without media change. The plate was examined under a light microscope daily, and when they reached 90% confluency, cells were passaged at a ratio of 1:5. For passaging, medium was aspirated, and the plate was washed once with DPBS, and aspirated. 5 ml of Trypsin-EDTA, 0.05% (25300054, Gibco) was added, and the plate was incubated at 37°C for 5 minutes or until all the cells are detached. After incubation, 5 ml of prewarmed MEF medium was added for trypsin inactivation, and the plate was thoroughly washed to collect all detached cells and transferred in a 15-ml centrifuge tube and resuspended with a serological pipette. 20 ml of MEF medium was added in 5 new 15-cm plates. The 10 ml cell suspension was then split into 5 new 15-cm plates where each plate received 2 ml from the suspension. Plates were incubated in a 37°C incubator until they reach 90% confluency without changing the medium. When these plates reached 90% confluency, all plates were trypsinized, and the cell suspension was split evenly onto 20 new 15-cm plates at a passaging ratio of 1:4. When those 20 plates of pMEFs reached 90% confluency, 15 of the plates were used in mitotic inactivation, and the remaining 5 of these plates served as source plates to grow more pMEFs for the next round of mitomycin C treatment and

passed into 20 new plates until they reach passage 10. When there were 20 confluent plates of pMEFs at passage 10, mitotic inactivation was performed on all plates, and further pMEF expansion was terminated. The expansion and mitotic inactivation cycle were reinitiated with a fresh vial of frozen pMEF stocks from lower passages.

2.3.1.2 Mitomycin C Treatment for Mitotic Inactivation of Primary Mouse Embryonic Fibroblasts

For mitotic inactivation of expanded pMEFs, a frozen Mitomycin C aliquot was thawed at room temperature, diluted to 0.5 mg/ml with DPBS and sterilized through a 0.22 μ m filter. An inactivation medium containing 0.01 mg/ml Mitomycin C in MEF medium was prepared and set aside. The medium was aspirated from the expanded pMEFs, and approximately 15 ml of inactivation medium was carefully added to each plate. Plates were incubated for 3 hours in a 37°C incubator for mitotic inactivation. After incubation, inactivation media was aspirated, and plates were washed 3 times with DPBS (containing Ca^{++} and Mg^{++}). Plates were trypsinized, and Mitomycin C-treated MEFs (mmc-MEFs) were collected from the plates with MEF medium in a 50-ml centrifuge tube and centrifuged at 200g for 10 minutes at room temperature, and the supernatant was aspirated. Cells were briefly resuspended in 10 ml MEF medium, and the cells were counted to freeze 7×10^6 cells/cryovial. After centrifuging at 200g for 5 minutes at room temperature, cells were resuspended in prechilled freezing medium (**Appendices B**) and split into cryovials. Vials were transferred to -80°C in a freezing container (Mr. Frosty) overnight. Frozen stocks were kept at -80°C for short-term storage up to 6 months or transferred to a liquid nitrogen tank's vapor phase for long-term storage.

2.3.2 Expansion of PBMCs Prior to Reprogramming

One vial of PBMCs (for each of the 12 samples) was removed from the liquid nitrogen tank, thawed in a 37°C water bath until only a small piece of ice remains and taken into the biosafety cabinet after cleansing with 70% ethanol. Immediately after thawing, 9 ml of PBMC medium (without StemSpan Erythroid Expansion Supplement (EES)) was taken into a serological pipette and the thawed content of cryovial containing PBMCs was slowly aspirated within the same pipette. The cell/medium mixture was dispensed slowly into a 15-ml centrifuge tube, resuspended briefly and cell counting was performed. After calculating the total number of viable cells, the suspension was centrifuged at 250g for 10 minutes at room temperature and the medium was aspirated. Cells were resuspended in PBMC medium (without EES) to obtain 5×10^5 cells/ml in PBMC medium. On 2 wells of a 12-well plate (for each of the 12 samples in total), 1 ml from this suspension was seeded to obtain 5×10^5 cells in each well. The media was supplemented with 1X EES and cells were incubated in a 37°C humidified incubator with 5% CO₂. The next day, the cells were harvested from the wells and seeded on new wells within the same 12-well plate to eliminate any adherent cells. After that, half medium change was performed: 500 µl of the media was removed from each well and replaced with freshly prepared 500 µl PBMC medium supplemented with 1X EES. Half-medium change was performed every day for 10 days following the initiation of PBMC expansion. After 10 days of expansion, PBMCs were collected from each well and counted. After the expansion is completed, PBMCs were subjected to reprogramming.

2.3.3 Generation and Feeder-Dependent Culture of iPSCs from Expanded PBMCs

After the expanded PBMCs were counted, cells were centrifuged at 250g for 10 minutes at room temperature. Meanwhile, 3 vials of CytoTune vectors (KOS, hc-Myc, h-Klf4) were removed from -80°C and thawed by firstly immersing each vial

in 37°C water bath for 10 seconds and then completely thawed at room temperature in approximately 1 minute. After thawing each vial, they were briefly spun in a tabletop centrifuge and immediately immersed on ice. For each vector, the volumes needed to transduce 1×10^5 cells for each of the 12 samples were calculated (**Appendices C, Figure 17**) at a multiplicity of infection (MOI) of 5:5:3 (KOS:hc-Myc:h-Klf4, respectively). The calculated amount of each reprogramming vectors was transferred to a 15-ml centrifuge tube containing 1 ml of prewarmed PBMC medium supplemented with 1X EES and briefly resuspended with a serological pipette. After setting up the viral vectors in media, the transduction must be initiated within 5 minutes. After centrifugation was complete, the pellet containing expanded PBMCs was resuspended in prewarmed PBMC medium supplemented with 1X EES to obtain 1×10^5 cells in 300 μ l of media. Cells were then transferred to round-bottom centrifuge tubes at a volume of 300 μ l for each of the 12 samples to be transduced for reprogramming. Next, the 1 ml mixture of CytoTune vectors were immediately transferred onto the PBMCs in round-bottom centrifuge tubes and briefly resuspended with a serological pipette. The tube was tightly capped and wrapped with parafilm and centrifuged at 1000g for 30 minutes at room temperature. At this point, cells were being transduced by the CytoTune Sendai virus vectors and indicated as Day 0, signifying the day of transduction. After centrifugation, transduced cells were transferred directly to 1 well of a 12-well plate and incubated in a humidified atmosphere of 37°C with 5% CO₂ overnight. The next day (Day 1), cells were collected from the plates, and each well was further rinsed with PBMC medium to recover as many cells as possible. Collected cells were centrifuged at 200g for 10 minutes at room temperature to get rid of the excessive virus. Supernatant was removed, and the pellet was resuspended in 0.5 ml of PBMC medium supplemented with 1X EES and transferred to 1 well of a TC-treated 24-well culture plate. The cells were cultured for 2 days in an incubator at 37°C with 5% CO₂ without changing the medium. On Day 2, TC-treated 6-well plates were coated with 0.1% gelatin (**Appendices A**) by adding 1 ml gelatin in each well and incubating at room temperature for 30 minutes under UV-light in a biosafety cabinet.

Gelatin was aspirated and 1 ml of MEF medium was added to each well. On gelatin coated 6-well plates, mmc-MEFs were seeded at a density of 3×10^5 cells/well and incubated overnight at 37°C with 5% CO₂. On Day 3, MEF medium was replaced with PBMC medium without 1X EES. Transduced PBMCs were collected from 24-well plates in PBMC media, and each well was rinsed with the PBMC medium to collect as many cells as possible. Transduced PBMCs were counted and centrifuged at 200g for 10 minutes at room temperature. Cells were resuspended in PBMC medium and split onto at least 2 wells of mmc-MEF coated plates where each well was seeded with 5×10^4 cells. PBMC medium was no longer supplemented with 1X EES from Day 2 onwards. All the remaining cells were subjected to RNA extraction to be used as a positive control in the verification step of clearance of CytoTune reprogramming vectors. Every other day (Days 4-6), a half-media change was performed by gently removing 1 ml of spent PBMC medium gently and slowly with a P1000 pipette and adding fresh PBMC medium. On Day 7, after removing half of the media, 1 ml of freshly prepared prewarmed hiPSC media (**Appendices B**) supplemented with 4 ng/ml bFGF (**Appendices A**) was added on each well. On Day 8 and onwards, complete media change was performed every day by aspirating the spent medium and replacing it with 2 ml of hiPSC medium supplemented with 4 ng/ml bFGF. From Day 8-20, initial iPSC colonies started to form and grow (**Figure 1B,1C,1D and 1E**). The plates were examined under the microscope each day until the colonies grow large enough (around 1 mm in diameter) for the passaging

2.3.4 Culture, Maintenance and Passaging of iPSCs

2.3.4.1 Clonal Expansion and Manual Passaging of iPSCs

Emerging and established clones of iPSCs were routinely maintained and cultured on 0.1% gelatin and MEF-coated TC-treated culture plates with hiPSCs medium supplemented with fresh 4 ng/ml bFGF, and the medium was replaced daily. The day before passaging, 12-well plates were coated with 0.1% gelatin and seeded

with mmc-MEFs. Next day, MEF medium was replaced in each well, to which a colony will be passaged, with hiPSC medium supplemented with 4 ng/ml bFGF and incubated for at least 3 hours prior to the passaging for conditioning of the media at 37°C with 5% CO₂. When emerging iPSC colonies grow around 700 – 1000 µm in diameter, the morphologically excellent colonies were selected and passaged manually. A single colony that is well isolated from rest of the other colonies was passaged individually into 1 well of a 12-well plate for clonal expansion and each clone was marked with a different number. At least 6 clones were harvested for each of the 12 samples reprogrammed. A light microscope was installed inside the biosafety cabinet, and colonies were marked from under the plates. With a P200 pipette, MEF cells were pulled away from around the colony and with a new pipette tip, the colony was cut into smaller pieces carefully without lifting the pieces away from the bottom of the plate. Pieces of a single colony were carefully collected with a P200 pipette and gently transferred on 1-well of a 12-well plate. Each clone of all samples was manually passaged up to passage 5, or until they are at least 50% confluent for enzymatic passaging.

2.3.4.2 Enzymatic and Mechanical Passaging of iPSCs

2.3.4.2.1 Enzymatic Passaging of iPSCs Using Dispase II

iPSC colonies above passage 5 with more than 50% confluency and minimal differentiation were routinely passaged using Dispase II (**Appendices A**). Before passaging, fresh gelatin and MEF-coated plates were prepared at least one day before passaging. On the day of passaging, MEF medium was replaced with hiPSC medium supplemented with 4 ng/ml bFGF at least 3 hours prior to passaging for conditioning. The differentiated colonies with undefined boundaries were eliminated by aspiration under the biosafety cabinet. Dispase was prepared by thawing an aliquot at room temperature and adjusted it to 1 unit/ml (U/ml) (**Appendices A**) in prewarmed DMEM/F12. The medium was aspirated from each well to be passaged and washed

with 1 ml DMEM/F12 for once. Each well to be passage of a 6-well plate was added 1 ml of 1 U/ml dispase (or just enough to cover the cells if any other plate format was utilized). Plates were incubated in a 37°C incubator for 15 minutes, or until the edge of colonies starts to pull away from the MEF layer (**Figure 1A**). Colonies were examined under the light microscope every 5 minutes. When the colonies start detaching from the MEF layers, dispase was aspirated, and 2 ml of DMEM/F12 was added in each well. Colonies were harvested from the MEF layer by gently blowing the DMEM/F12 on colonies with a serological pipette several times. Once most of the colonies are removed from the surface, they were transferred to a 15-ml centrifuge tube containing 10 ml of prewarmed DMEM/F12 and allowed to sediment by gravity for 2 minutes. Once colonies are pelleted, DMEM/F12 was aspirated, and colonies were washed once with 5 ml of DMEM/F12 and sedimented by gravity. After aspirating DMEM/F12, cells were resuspended in 1.5 ml of hiPSC medium supplemented with 4 ng/ml bFGF and gently pipetted several times with a P1000 pipette to break large colonies into smaller pieces. Colony pieces were then passaged onto fresh MEF-coated dishes at a ratio of 1:3 or 1:5, depending on the initial confluency.

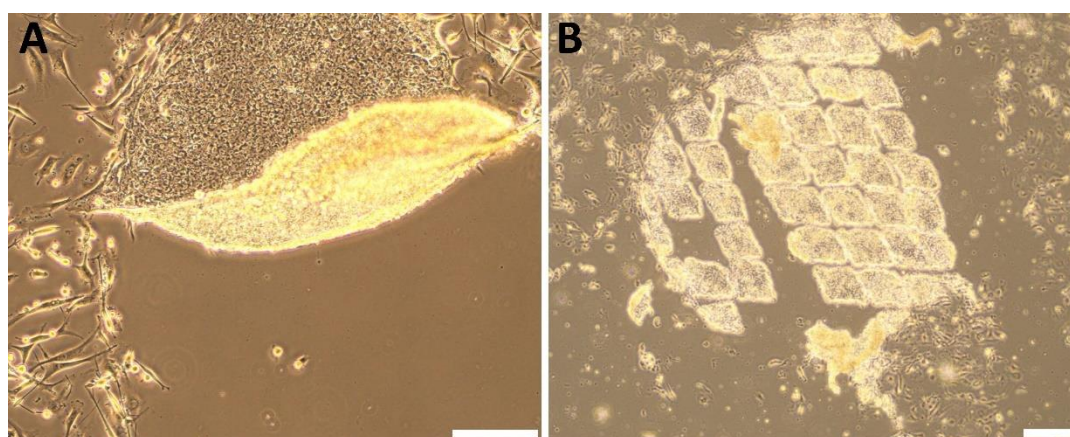


Figure 1 Enzymatic and mechanical passaging of two different clones of iPSCs.

(A) Passaging of iPSCs (C#8) using dispase, after 15 minutes of treatment with 1 U/ml enzyme. (B) Mechanical passaging of iPSC colonies (MSp#4) using StemPro EZPassage Disposable Stem Cell Passing Tool. Scale bars: 200 μ m

2.3.4.2.2 Mechanical Passaging of iPSCs

When colonies in the culture plate become at least 50% confluent with none to minimal differentiation, colonies were either mechanically or enzymatically passaged. For mechanical passaging, differentiated areas were removed under the light microscope, and cells were passaged using StemPro EZPassage Disposable Stem Cell Passaging Tool (Gibco, 23181010). For this, a passaging tool was removed from its cover under the biosafety cabinet and inserted in one side of a 6-well or a 12-well plate. The tool was rolled over in one direction to cover all the area of the well and removed from the well, then the plate was rotated 90 degrees in any direction and the tool was rolled over the plate again. This yielded equally sized, smaller, and rectangular pieces of colonies in each well to be passaged (**Figure 1B**). After the cutting is complete with the tool, the colonies were removed from the surface by gently pipetting medium several times on the colonies until most of the pieces are fully detached. Collected pieces were passaged in fresh MEF-coated plates at ratio of 1:3 or 1:5 depending on the initial confluency.

2.3.4.3 Cryopreservation and Thawing of iPSCs

At least 6 colonies resembling a unique clone of a sample were selected after reprogramming is completed and expanded by manual passaging up to passage 5. Afterward, one clone of each sample was selected for further expansion and characterized. The remaining clones of each sample were passaged once they reach at least 50% confluency at or around passage 5 and frozen.

2.3.4.3.1 Cryopreservation of iPSCs

Before freezing, cryovials were carefully labeled, describing each clone with sample and clone numbers as well as their passage number. For freezing, each clone was subjected to dispase as described earlier. iPSCs that were removed from the

MEF layer were sedimented by gravity and washed once with DMEM/F12. After aspirating DMEM/F12, colonies were resuspended briefly in freshly prepared hiPSC freezing medium supplemented with 10 μ m rock inhibitor (**Appendices A**). The amount of freezing medium was determined according to the density of cells such that 1 well of a 6-well plate was frozen in 1 ml of freezing media per cryovial. Cryovials were immediately transferred to a freezing container and stored overnight at -80°C. The next day, cryovials were transferred to the vapor phase of the liquid nitrogen tank for storage. For the clones selected for further expansion and characterization, cell stocks were taken every 2-3 passages for backing up as many cell stocks for a colony as possible for each sample.

2.3.4.3.2 Thawing of iPSCs

Frozen iPSCs were thawed by first immersing the vials in a 37°C water bath until only a small piece of ice remains. Afterward, the vials were sprayed with 70% ethanol and taken inside the biosafety cabinet. With a serological pipette, 9 ml of prewarmed hiPSC medium supplemented with 4 ng/ml bFGF was aspirated, and then the content of the cryovial was taken slowly into the same pipette. The cell mixture was gently dispensed in a 15-ml centrifuge tube and very slowly resuspended once. Colonies were allowed to sediment by gravity for up to 3 minutes, and the supernatant was aspirated. Cells were resuspended in 1 ml hiPSC medium supplemented with 4 ng/ml bFGF and transferred on fresh 0.1% gelatin and MEF coated dishes. It took nearly 1 week for colonies to be ready for passaging after thawing.

2.4 Characterization of iPSCs

2.4.1 Verification of Clearance of CytoTune Vectors

Clones expanded at or above passage 6 were routinely checked for the clearance of reprogramming vectors by RT-PCR. For total RNA isolation, NucleoSpin RNA Isolation Kit (Macherey-Nagel, 740955.50) was utilized according to the manufacturer's instructions. For this reaction, cells collected at Day 3-post transduction were utilized as a positive control. iPSCs at or above passage 6 were routinely spared aside for RNA isolation (cells were lysed in the lysis buffer from the RNA isolation kit and stored at -80°C) to be used in verification experiments. RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, K1622) and random hexamers as primers were utilized for the cDNA synthesis. In each reaction, 500 ng cDNA was used as template for primers (**Table 1**) targeting *GAPDH* and Sendai virus specific SeV, KOS, c-Myc, and Klf4 genes. 5X FIREPol Master Mix Ready To Load (12.5 mM MgCl₂) (Solis BioDyne, 04-12-00125) premixed solution was used for PCR reactions. Templates, primers, and the premix were mixed, and the PCR reactions were run at 95°C for 3 minutes for initial denaturation, followed by denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 1 minute, and final elongation at 72°C for 2 minutes on T100 Thermal Cycler (BIO-RAD). The PCR products were run on 2% agarose gel together with the DNA ladder (**Appendices D, Figure 18**) and visualized under UV light.

Table 2 Primers used in PCR reactions.

TARGET AND PRODUCT SIZE	FORWARD / REVERSE	PRIMER SEQUENCE (5' – 3')
CytoTune Vector: SeV (181 bp)	FORWARD	GGATCACTAGGTGATATCGAGC
	REVERSE	ACCAGACAAGAGTTTAAGAGATATGTATC
CytoTune Vector: hKOS (528 bp)	FORWARD	ATGCACCGCTACGACGTGAGCGC
	REVERSE	ACCTTGACAATCCTGATGTGG
CytoTune Vector: hc-Myc (532 bp)	FORWARD	TAACTGACTAGCAGGCTTGTCG
	REVERSE	TCCACATACAGTCCTGGATGATGATG
CytoTune Vector: hKlf4 (410 bp)	FORWARD	TTCCTGCATGCCAGAGGAGCCC
	REVERSE	AATGTATCGAAGGTGCTCAA
Pluripotency Marker: OCT4 (63 bp)	FORWARD	GGGTTTTTGGGATTAAGTTCTTCA
	REVERSE	GCCCCCACCCTTTGTGTT
Pluripotency Marker: SOX2 (63 bp)	FORWARD	CAAAAATGGCCATGCAGGTT
	REVERSE	AGTTGGGATCGAACAAAAGCTATT

Table 2 (cont'd)

Pluripotency Marker: NANOG (190 bp)	FORWARD	CCTGAAGACGTGTGAAGATGAG
	REVERSE	GCTGATTAGGCTCCAACCATA
House Keeping Gene: GAPDH (197 bp)	FORWARD	GGAGCGAGATCCCTCCAAAAT
	REVERSE	GGCTGTTGTCATACTTCTCATGG
Mycoplasma Detection: MGSO & GPO3 (270 bp)	MGSO	TGCACCATCTGTCACTCTGTTAACCTC
	GPO3	GGGAGCAAACAGGTTAGATACCCT

2.4.2 Analysis of Alkaline Phosphatase Activity

When iPSC colonies were grown around 0.5 – 1 mm in diameter in 12-well plates, media was aspirated, and cells were washed twice with 1 ml DMEM/F12. For staining, 500X Alkaline Phosphatase Live Stain (Invitrogen, A14353) was diluted to 1X in prewarmed DMEM/F12. 1 ml of this mixture was used to stain 1 well of a 12-well plate. The 1X mixture was added to each well to be stained and incubated at 37°C for 30 – 45 minutes. After incubation, cells were washed twice with DMEM/F12, aspirated, and added 1 ml of fresh DMEM/F12 in each well. Plates were visualized with a FloID Cell Imaging Station using the FITC filter. After visualization, DMEM/F12 was aspirated and replaced with fresh prewarmed hiPSC

medium supplemented with 4 ng/ml bFGF, and cells were continued to be used in routine culture.

2.4.3 Analysis of Pluripotency Markers

2.4.3.1 RT-qPCR for Comparative Analysis of Pluripotency Markers

cDNAs obtained during verification of clearance of CytoTune Sendai vectors were also used for RT-qPCR analysis of pluripotency markers for each different sample. Using these cDNAs as template, primers targeting *OCT4*, *SOX2*, *NANOG*, and *GAPDH* (**Table 2**) were utilized. In each reaction, total reaction volume was set to 10 μ l where cDNA was added with 1:2.5 dilution and with 10 μ M primer concentration. qPCR reactions were performed with at least 3 inter-run replicates for each target gene using Qiagen RotorGene. As a positive control, cDNA derived from H9 hESC line was utilized, and as a negative control, cDNA derived from PBMCs that were expanded for 10 days with PBMC medium, that are not used in transduction and reprogramming. SsoAdvanced Universal SYBR Green Supermix (BIO-RAD, 1725271) was used for running RT-qPCR reactions. Reactions were run at initial denaturation at 95°C for 3 minutes, followed by denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds (for all primers) and elongation at 72°C for 30 seconds and a melting step (50°C – 99°C), rising by 1°C each step. The data obtained from the negative control (PBMCs) was used for normalization and analyzed by $2^{-\Delta\Delta C_t}$ method. GAPDH data served as a reference for data interpretation in GraphPad Prism.

2.4.3.2 Immunostaining for Pluripotency Markers

iPSCs were routinely passaged using dispase into 6- or 12- well TC-treated dishes; however, for staining, they were seeded on 0.1% gelatin, and mmc-MEF coated 4-well cell culture chamber slides and cultured for 2 days in 500 μ l hiPSC medium

supplemented with 4 ng/ml bFGF, and the spent media was replaced daily. One well of 60% confluent iPSCs cultured in a 6-well plate was split into 4 wells of a 4-well chamber slide. When the colonies grow around 0.5 mm in diameter, the medium in each well was aspirated and washed gently with DPBS (with Ca^{++} and Mg^{++}) 3 times. DPBS was aspirated, and cells were fixed using 4% formaldehyde (prepared by ~1:10 dilution of 37% formaldehyde with DPBS) for 10 minutes at 37°C. Formaldehyde was washed for 3 times with DPBS and 0.3% Triton X-100 (prepared by diluting Triton X-100 in DPBS (v/v)) was added for permeabilization. Cells were permeabilized by incubating for 15 minutes at room temperature. After permeabilization, cells were washed 3 times with DPBS, and blocking was performed by adding of 3% BSA and incubating at room temperature for 1 hour. Primary antibodies were prepared in prechilled 0.3% BSA solution and 1 antibody (**Table 3**) was added in each well. After blocking, BSA was aspirated, and primary antibodies were directly added on each well and incubated at +4°C overnight. Next day, primary antibodies were collected from each well and washed with 0.3% BSA solution 3 times. Secondary antibody (**Table 3**) was diluted in prechilled 0.3% BSA and added in each well to be stained. The chamber slide with secondary antibody was incubated at room temperature for 2 hours in the dark. After that, secondary antibody was aspirated, and wells were washed 3 times with DPBS. DPBS was aspirated, and the chamber portion was dismantled for recovering the slide with stained cells attached. The slide was dried with a piece of tissue paper from the edges. ProLong Gold Antifade Mountant with DAPI (brought to room temperature) was then applied in each well dropwise, and a clean coverslip was placed carefully at a 45-degree angle to avoid bubble formation. The slides were cured in the dark for 24 hours, and after that, they were sealed from the edges with nail polish. Slides were stored at +4°C.

Table 3 Antibodies used in immunostaining experiments

ANTIBODY	DILUTION	COMPANY	CATALOG NUMBER
Mouse anti- Oct-3/4	1:100	Santa Cruz Biotechnology	sc-5279
Mouse anti- Sox-2	1:100	Santa Cruz Biotechnology	sc-365823
Mouse anti- TRA-1–60	1:100	Santa Cruz Biotechnology	sc-21705
Mouse anti- SSEA-4	1:100	Developmental Studies Hybridoma Bank	MC-813-70 (SSEA-4)
Mouse anti- AFP	1:100	Santa Cruz Biotechnology	sc-8399
Mouse anti- SMA	1:100	Santa Cruz Biotechnology	sc-53142
Mouse anti- Beta-III Tubulin	1:500	R&D Systems	BAM1195
Mouse anti- PAX6	1:100	Developmental Studies Hybridoma Bank	pax6
Goat anti-Mouse IgG (H + L) Highly Cross- Adsorbed Secondary Antibody, Alexa Fluor 488	1:200	Thermo Fisher Scientific	A-11029

2.4.4 Analysis of Differentiation Capacity of iPSCs

iPSCs that are 60% confluent in 3 wells of a 6-well plate were removed from the MEF layer using dispase for embryoid body (EB) formation. After washing with DMEM/F12, colonies were resuspended in hiPSC medium without bFGF. Colonies were briefly broken into smaller pieces with a serological pipette and transferred to a 12-cm petri dish with 15 ml final volume. Cells were cultured for 2 days in a 37°C incubator without changing the medium. On day 3, cells were collected into a 15-ml centrifuge tube and allowed to sediment by gravity for 1 minute. The supernatant was removed, and cells were resuspended in 10 ml hiPSC medium without bFGF. Cells were transferred to a new 12-cm petri dish and cultured at 15 ml final volume for 3 weeks allowing them to spontaneously differentiate, with a half-medium change every other day. On day 21 of culture, EBs were collected into a 15-ml centrifuge tube, and the supernatant was aspirated. EBs were washed once with DPBS and added with 1 ml of prewarmed trypsin supplemented with 10 μ M rock inhibitor. The tube was tightly capped and wrapped with parafilm and incubated in a 37°C water bath for 10-15 minutes until the solution becomes turbid, indicating single cell dissociation. After incubation, 10 ml DMEM with 100 μ l FBS was added to inhibit trypsin activity, and cells were resuspended several times until it is mostly single cells in the suspension. Cells were centrifuged at 200g for 10 minutes at room temperature. The supernatant was aspirated, and cells were resuspended in 2 ml hiPSC medium without bFGF and split into each well of a 0.1% gelatin-coated 4-well chamber slide at a volume of 500 μ l. Half-medium change was performed every other day for 7 days. 7 days later, wells were largely covered by differentiated cells, and immunostaining was performed as described previously using primary antibodies against 3 embryonic germ layers (**Table 3**) using AFP for endoderm, α -SMA for mesoderm, and TUBB3 (β -III Tubulin) or PAX6 for ectoderm.

2.4.5 Karyotype Analysis

Karyotype analysis was performed by INTERGEN Genetics and Rare Diseases Diagnosis Research & Application Center and by MBGENLAB Genetic Diseases Diagnosis Center at their cytogenetics laboratories. Karyotyping was performed for each of the 12 samples that were prepared at or above passages when they were verified to be free of exogenous expression of the reprogramming factors. iPSCs from each sample were treated with dispase, separated entirely from the MEF feeders, and seeded on Matrigel-coated 60-mm TC-treated culture plates. The next day, plates were tightly wrapped with parafilm and transported to the cytogenetics laboratories in thermostable styrofoam boxes for karyotyping. More than 20 metaphase spreads were analyzed with standard G-banding analyses at a 550–600 band resolution.

2.5 Mycoplasma Analysis

For mycoplasma analysis, a PCR reaction was performed based on a previous study (Young, Sung, Stacey, & Masters, 2010) using 1 μ l of 24-hour old media from each sample as a template with primers (MGSO and GPO3, **Table 2**) targeting more than 40 different species of mycoplasma. 5x FIREPol Master Mix was utilized in this reaction with 10 μ M of each primer and the reaction was run at 95°C for 3 minutes, followed by 35 cycles at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute, and final elongation at 72°C for 2 minutes. The PCR products and the DNA ladder (**Appendices D, Figure 18**) were run on 2% agarose gel and visualized under UV light.

CHAPTER 3

RESULTS

3.1 Reprogramming and Morphological Analysis of iPSCs

PBMCs were successfully isolated from freshly drawn whole blood and expanded in PBMC medium supplemented with 1X EES. PBMCs were grown in suspension, and adherent cells were avoided by transferring the cells to new wells. PBMCs were greatly expanded in number in 10 days and morphologically excellent for each of the 12 samples, as shown in **Figure 2B**. After transduction, PBMCs were floating in smaller clusters indicating reprogramming and transduction to be successful. At the first 4 days post-transduction, cells were only weakly adherent to the feeder layer either as single cells or as small clusters; however, successfully reprogrammed cells produced their own colonies by Day 8 post-transduction (**Figure 2D**). By 3 weeks of culture, the first iPSC colonies were ready for passaging for each sample, and most of the emerging colonies were morphologically excellent (**Figure 2E**). As colonies were selected and picked simultaneously for each sample, new colonies were still emerging up to Day 45-post transduction, after which all colonies at passage 0 were scraped from the wells with feeders and frozen as a mixed cell stock for each sample as a backup. For obtaining high-quality stem cells with feeder-dependent culture, the quality of and the density of the feeder cell layer is also crucial as morphologically and physiologically excellent feeder cells in turn increases the quality of the co-cultured stem cells. One representative image depicts this fact as shown in **Figure 3**, panel **A** represents mmc-MEFs that were seeded at an ideal density that are looking morphologically excellent, and on panel **B**, an established iPSC colony which possesses all morphological characteristics of a human pluripotent stem cell with defined borders, prominent nucleoli in all cells within the colony, and with undifferentiated, tightly packed cells.

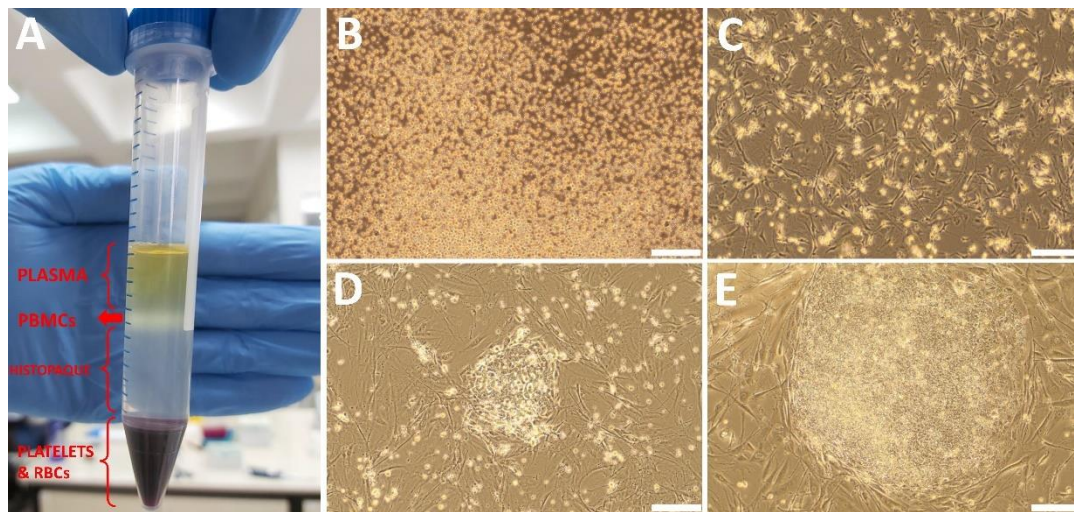


Figure 2 Representation of progression during reprogramming, from PBMC isolation to the emergence of iPSC colony (MSP#4).

(A) Layers produced after initial centrifugation during PBMC isolation; from top to bottom are labeled as blood plasma, buffy coat or opaque interphase containing PBMCs, Histopaque-1077 reagent, platelets, and red blood cells (RBCs). (B) PBMCs expanded for 10 days, on the day of transduction (Day 0). (C) Transduced PBMCs cultured on MEF-coated plate (Day 4 post-transduction). (D) An emerging primary iPSC colony on Day 8 post-transduction. (E) iPSC colony ready for passaging on Day 18 post-transduction. Scale bars: 200 μ m

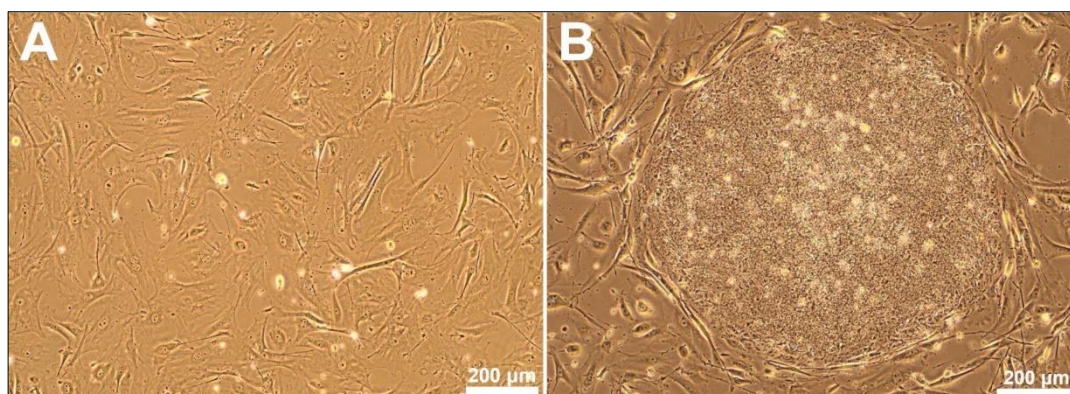


Figure 3 Morphological examination of established iPSCs generated and cultured on mmc-MEFs.

(A) mmc-MEFs seeded at the ideal density for feeder-dependent iPSCs culture. (B) An established iPSCs colony (MSp#3, Passage 15) on mmc-MEF cells on day 5 post passaging.

3.2 Clearance of Exogenous CytoTune Vectors

As the first step of characterizing an iPSC cell line, a single clone from each sample was expanded up to passage 10 and utilized to test the absence of exogenous reprogramming factors that were expressed from the CytoTune vectors. For the first two samples that were reprogrammed (MSp#3 and MSp#4), we started testing for the clearance at and above passage 5 but could not confirm the loss of all CytoTune vectors by no earlier than passage 10. Afterward, for all reprogrammed samples, clearance of CytoTune vectors were tested at or above passage 10. At varying passages, all samples were confirmed to be free of any exogenously expressed transcription factors by performing RT-PCR as depicted in **Figures 4-15A**.

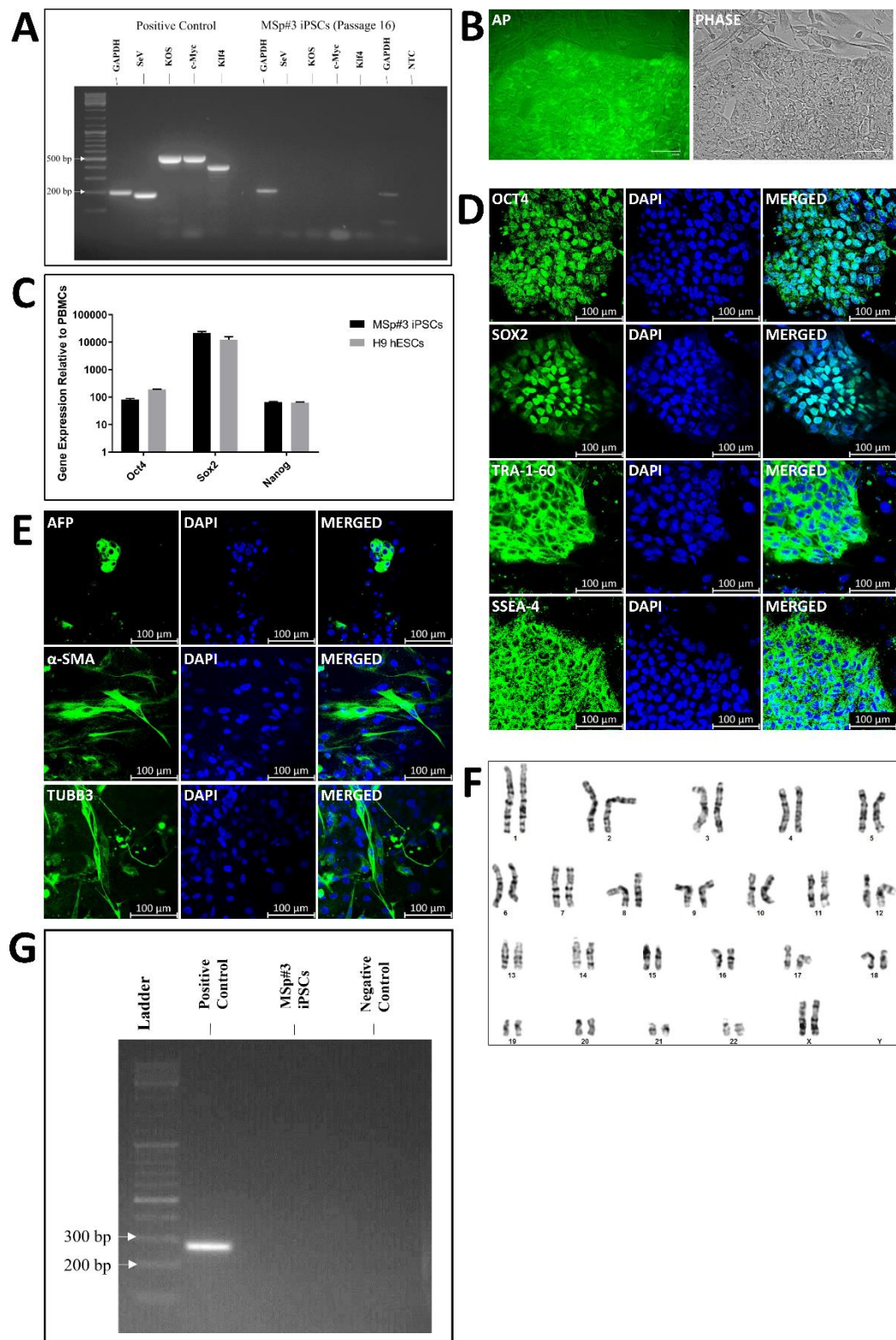


Figure 4 Establishment and characterization of MSp#3 iPSC line, derived from a female RRMS patient.

(Figure 4 cont'd)

(A) Clearance of CytoTune vectors depicted by agarose gel electrophoresis. (B) Established iPSCs stained positive for alkaline phosphatase. On the left, colonies were stained positive for alkaline phosphatase fluorescing under FITC channel, on the right, colony morphology of the alkaline phosphatase positive colony. Images were acquired via Fluid Cell Imaging System or Leica SP8 Confocal Microscope (C) Comparison of endogenous pluripotency marker expression, n=2. (D) Immunocytochemistry for the pluripotency markers, from top to bottom are OCT4, SOX2, TRA-1-60 and SSEA-4, nuclei were stained with DAPI. Images were acquired using Leica SP8 Confocal Microscope. (E) Immunocytochemistry for the markers of the three germ layers. From top to bottom are AFP for endoderm, SMA for mesoderm and TUBB3 or PAX6 for ectoderm. Images were acquired using Leica SP8 Confocal Microscope. (F) Results from the karyotype analysis. (G) Gel image from the mycoplasma analysis experiment.

The legend above corresponds to **Figures 4 – 15**, as similar analyses were conducted for all iPSC lines.

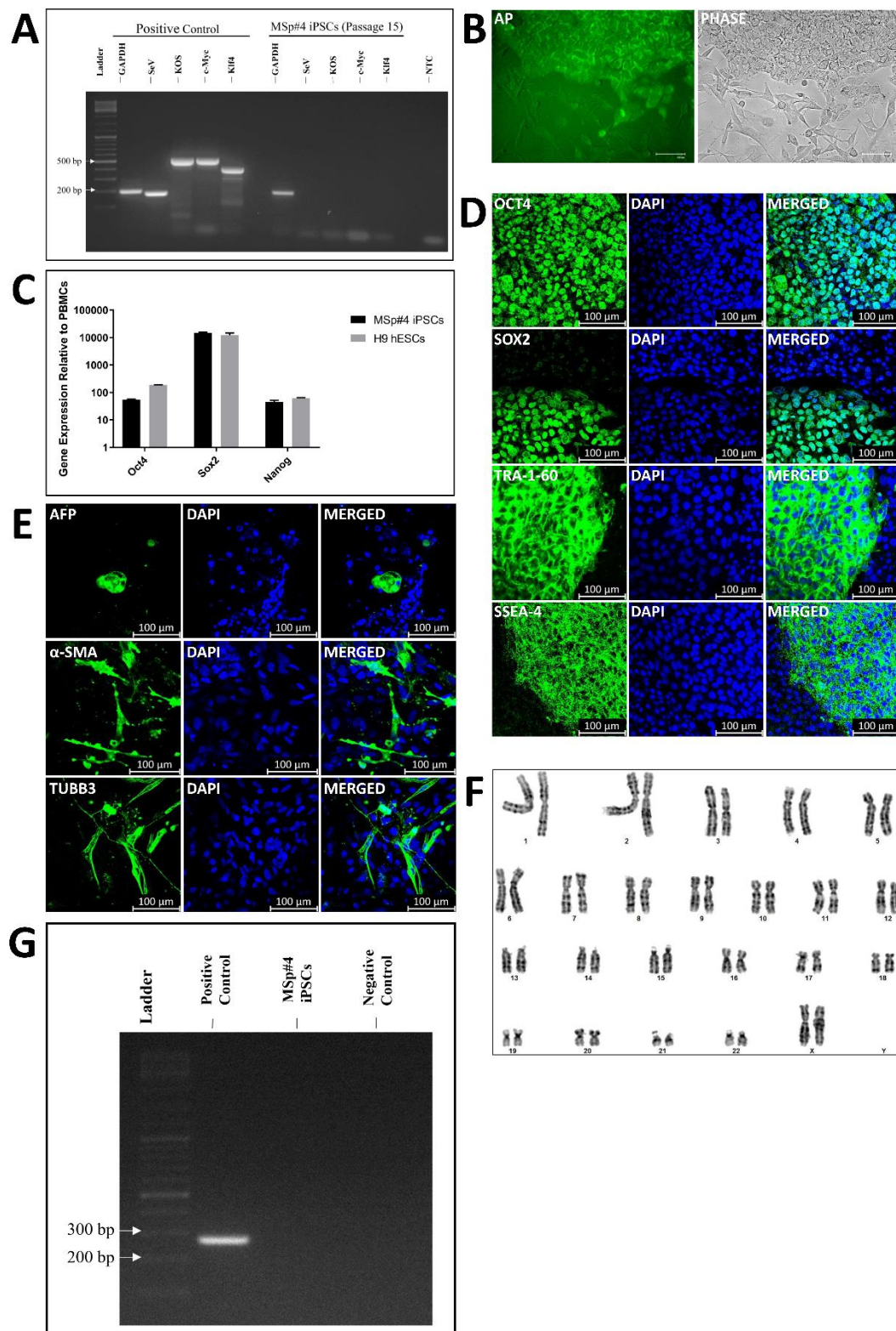


Figure 5 Establishment and characterization of MSp#4 iPSC line, derived from a female RRMS patient.

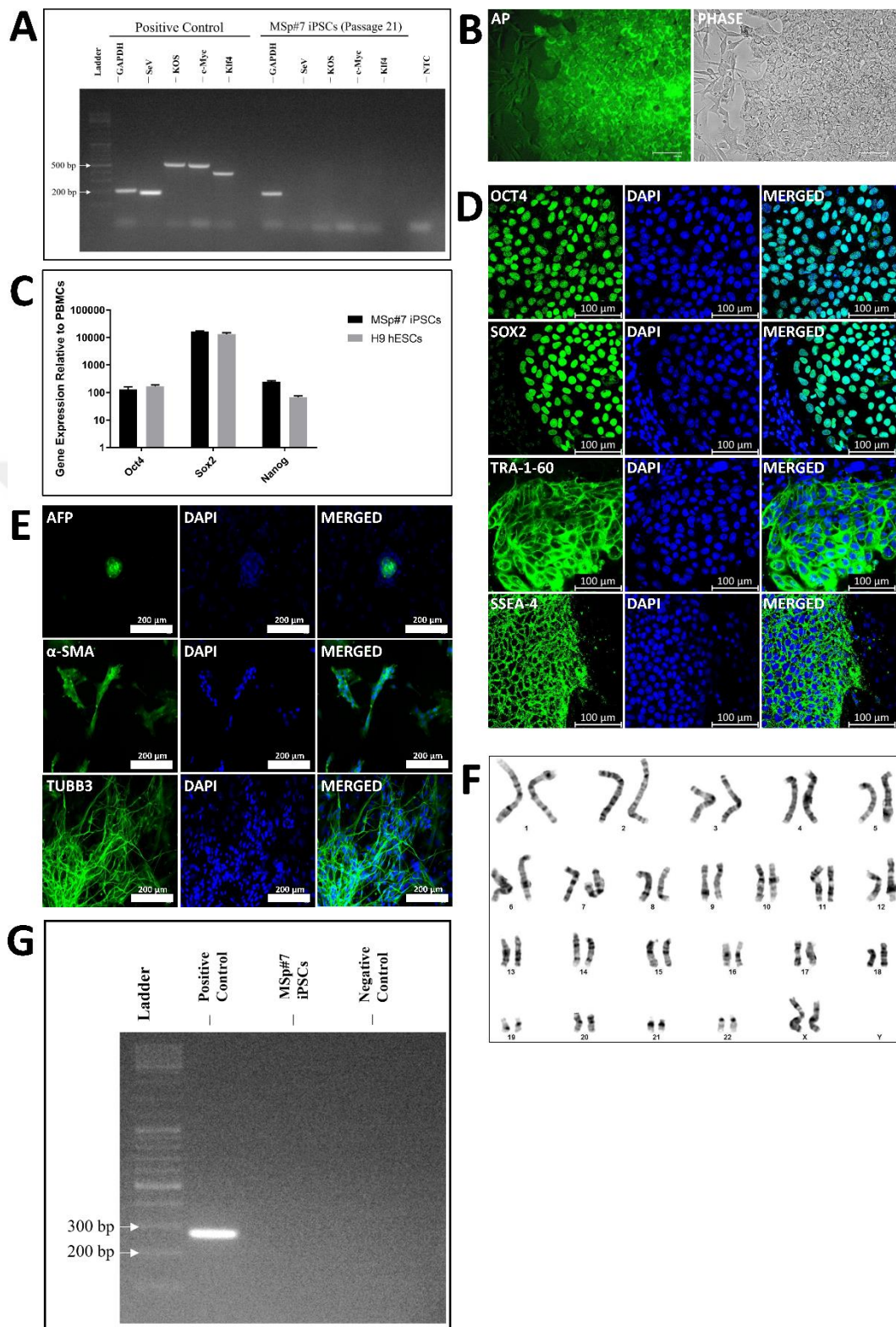


Figure 6 Establishment and characterization of MSP#7 iPSC line, derived from a female RRMS patient.

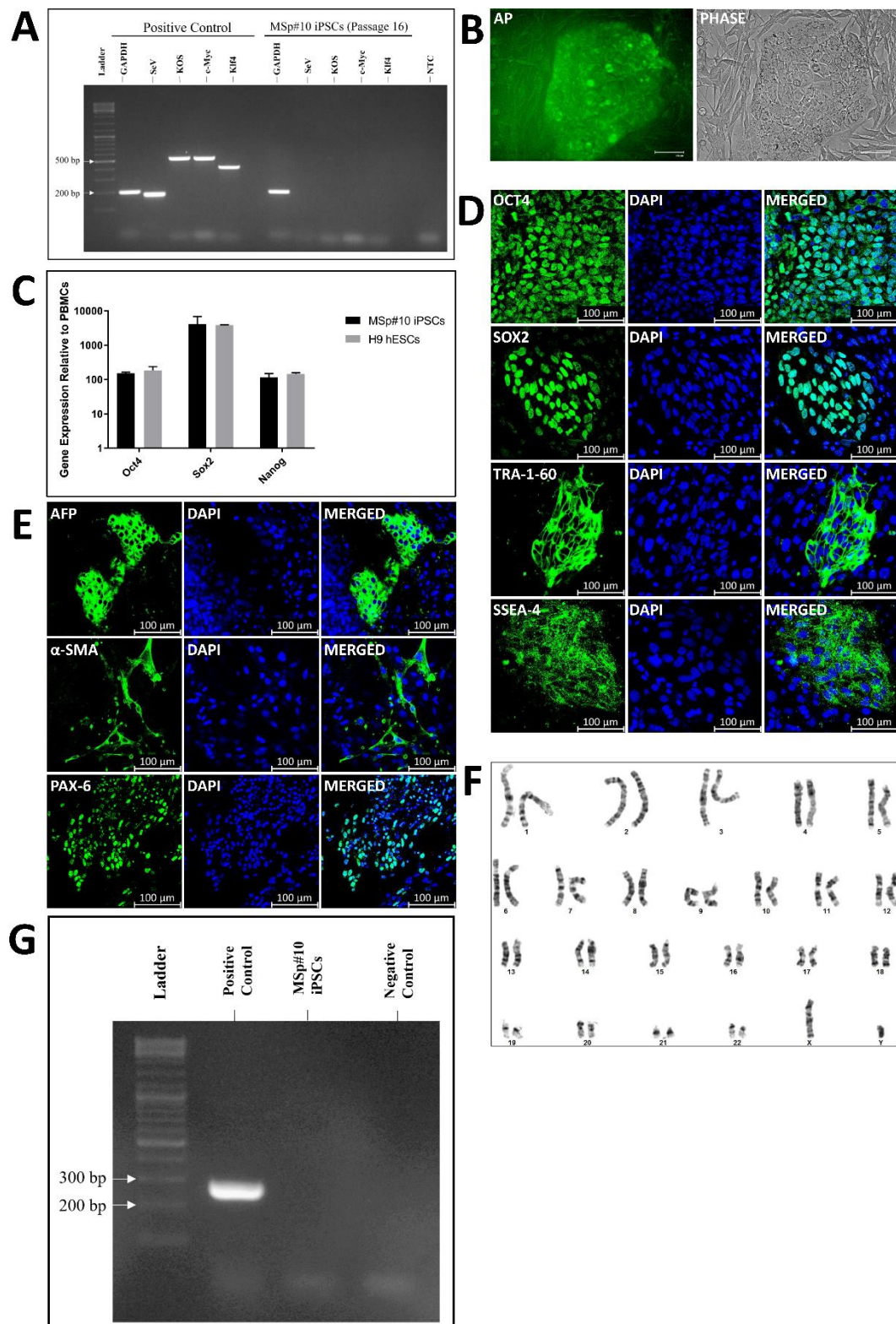


Figure 7 Establishment and characterization of MSp#10 (METUi001-A) iPSC line, derived from a male RRMS patient (Begentas et al., 2021).

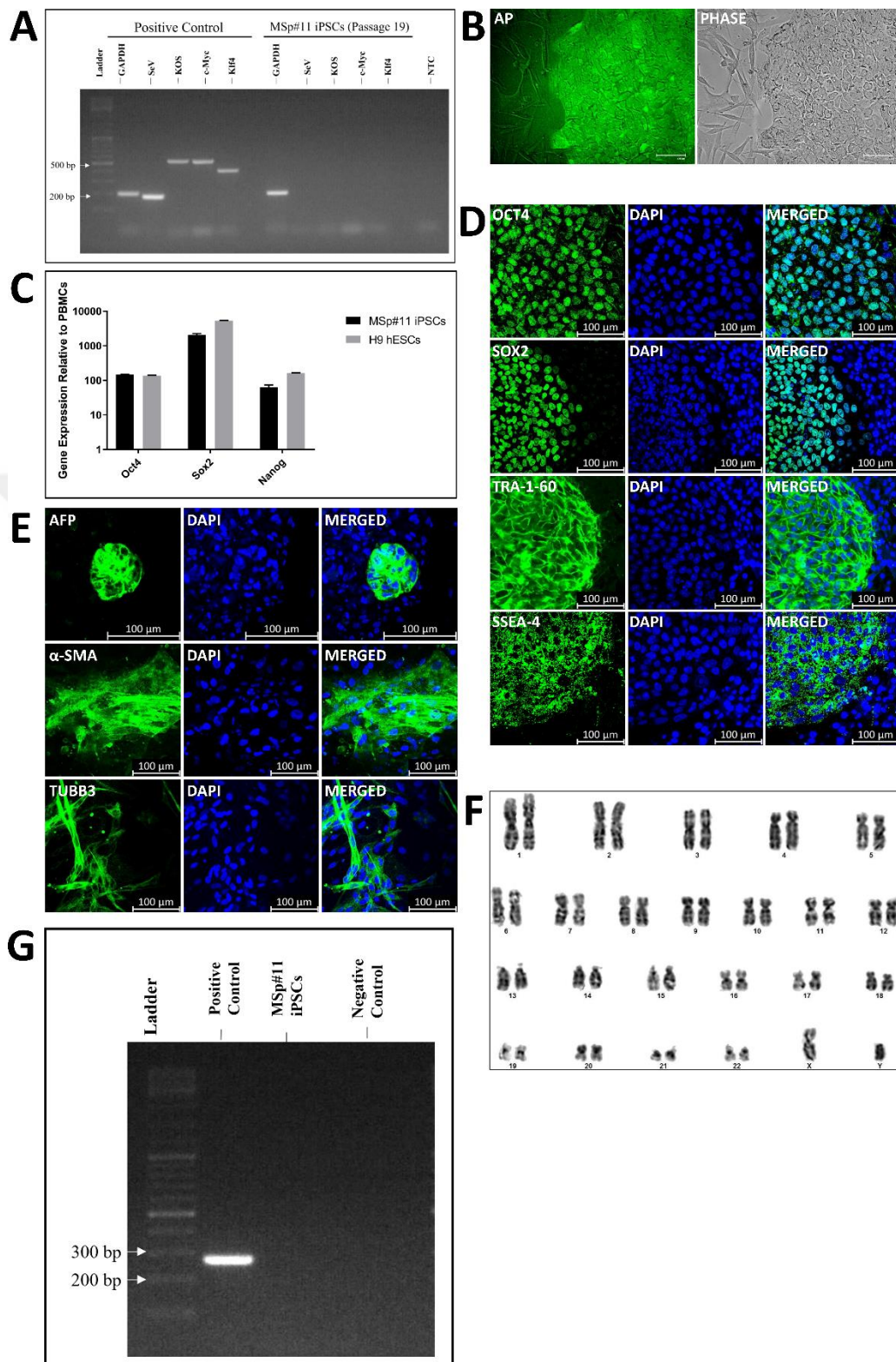


Figure 8 Establishment and characterization of MSP#11 iPSC line, derived from a male RRMS patient.

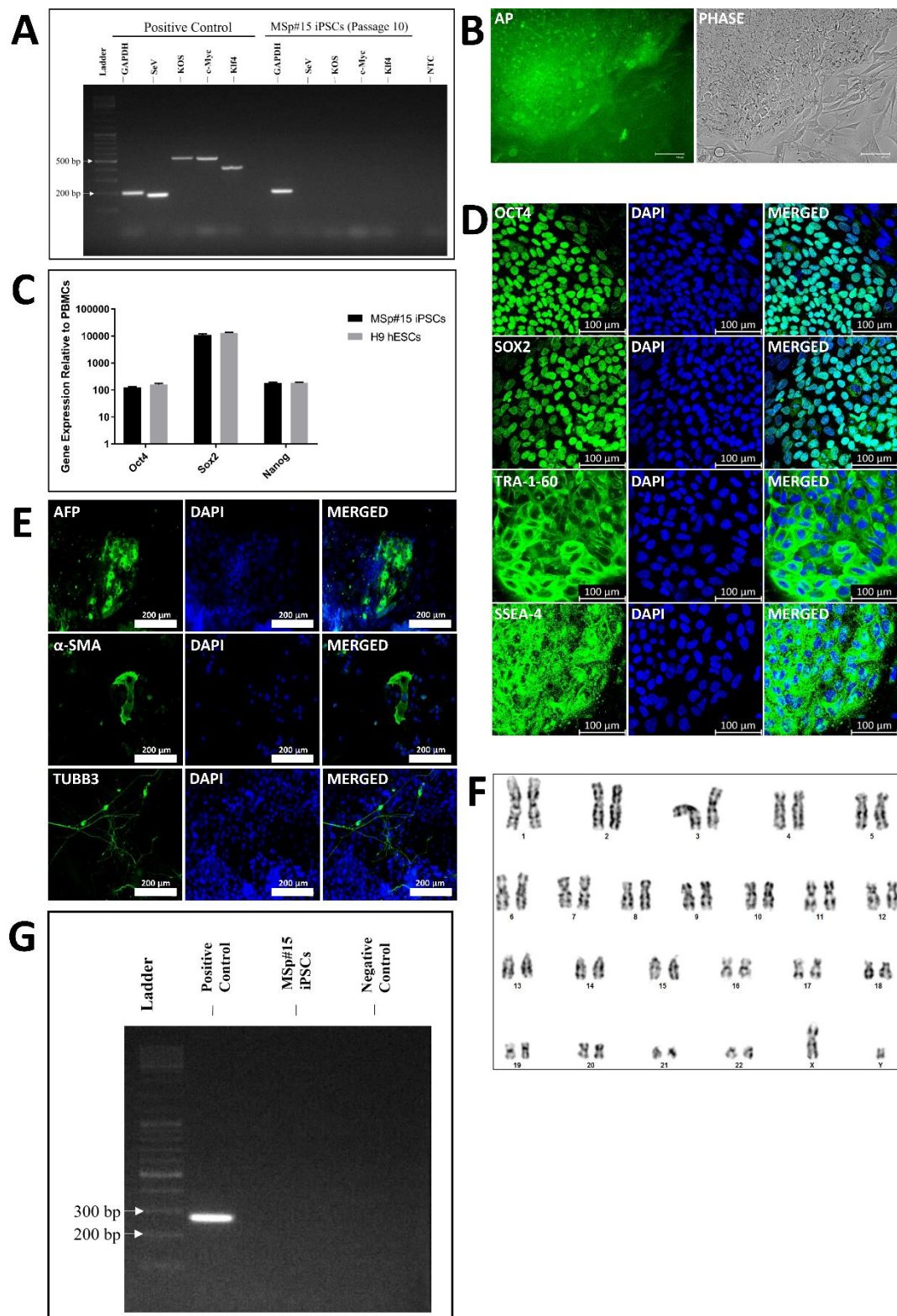


Figure 9 Establishment and characterization of MSp#15 iPSC line, derived from a male RRMS patient.

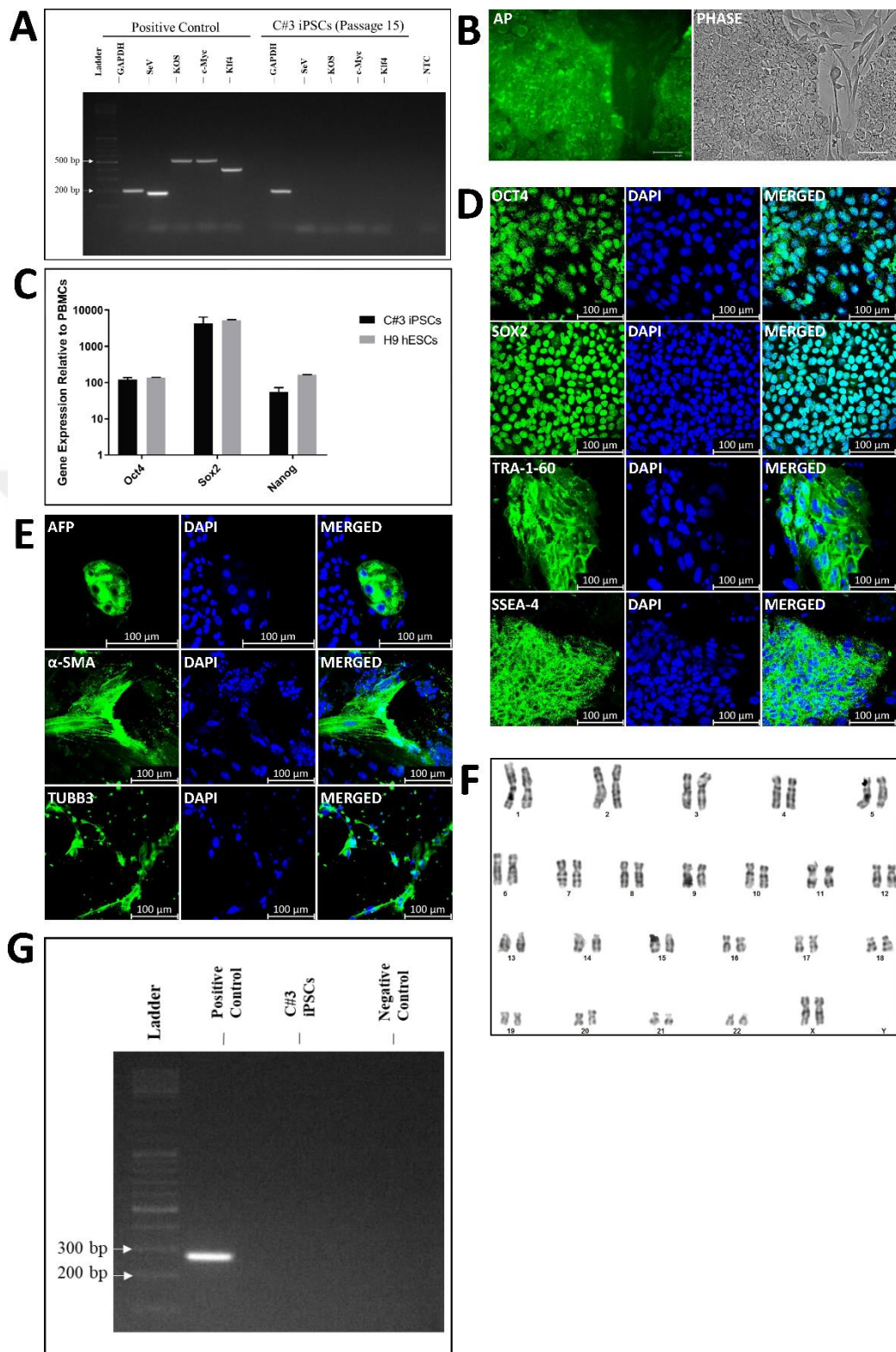


Figure 10 Establishment and characterization of C#3 iPSC line, derived from a healthy female individual.

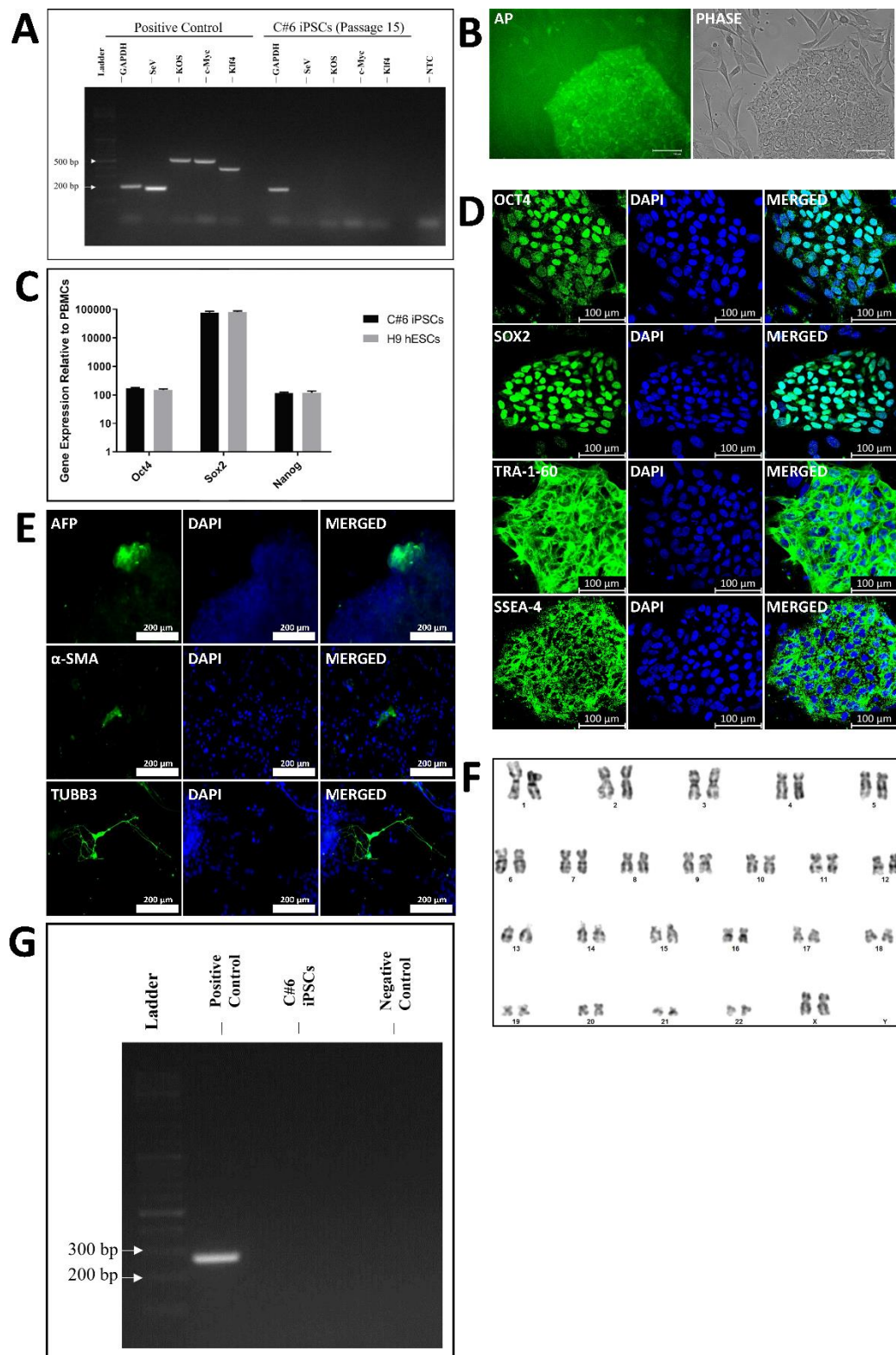


Figure 11 Establishment and characterization of C#6 iPSC line, derived from a healthy female individual.

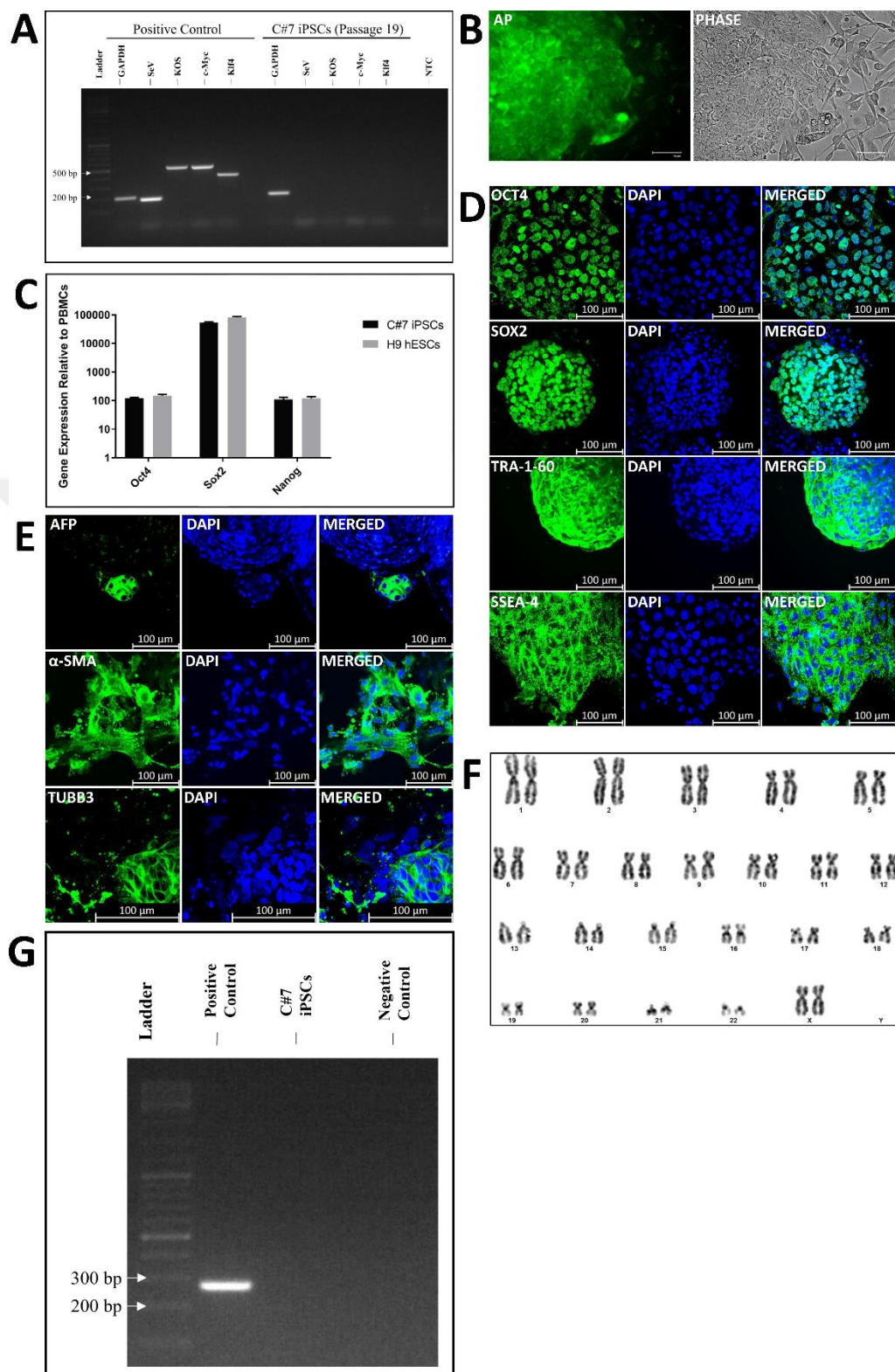


Figure 12 Establishment and characterization of C#7 iPSC line, derived from a healthy female individual.

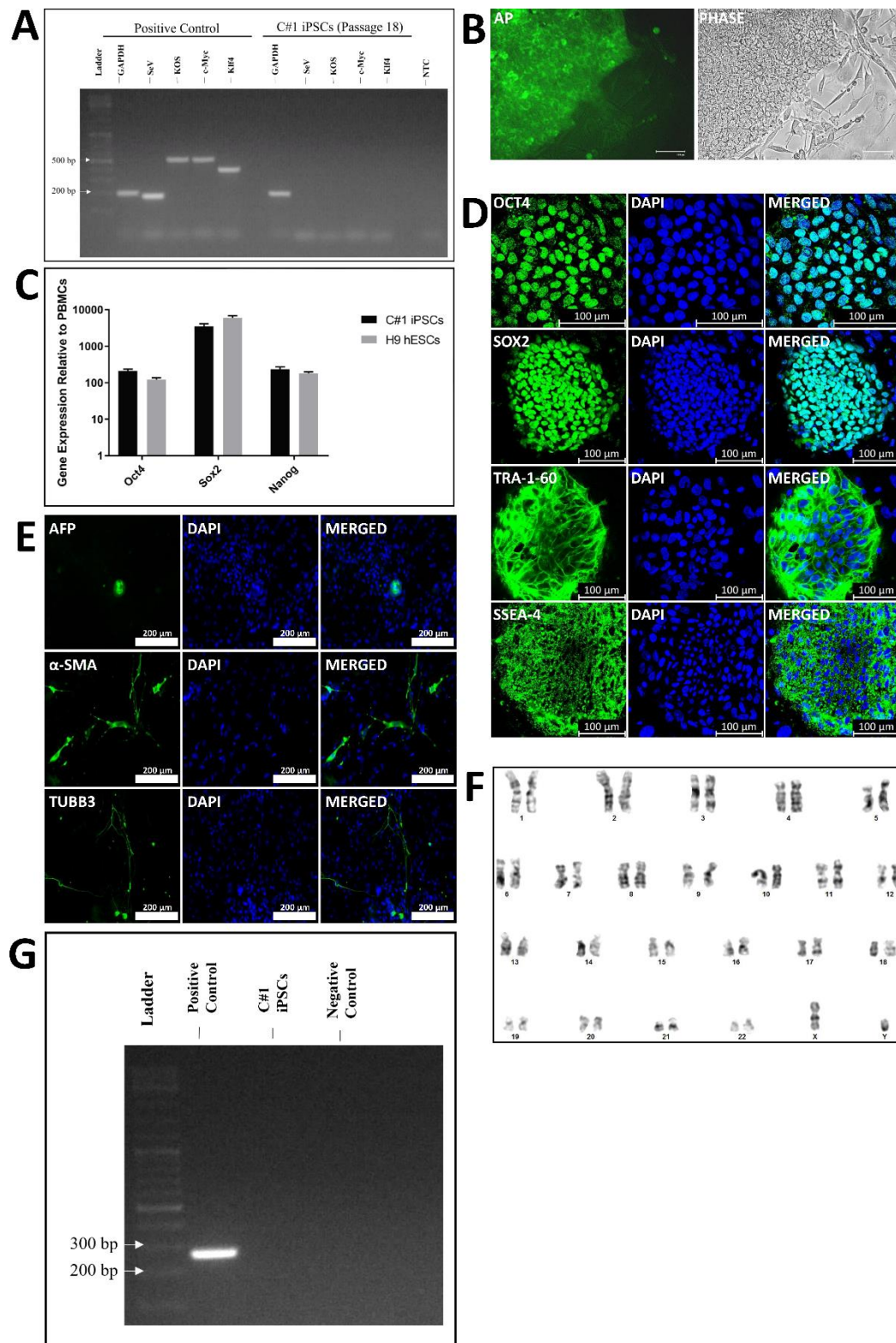


Figure 13 Establishment and characterization of C#1 iPSC line, derived from a healthy male individual.

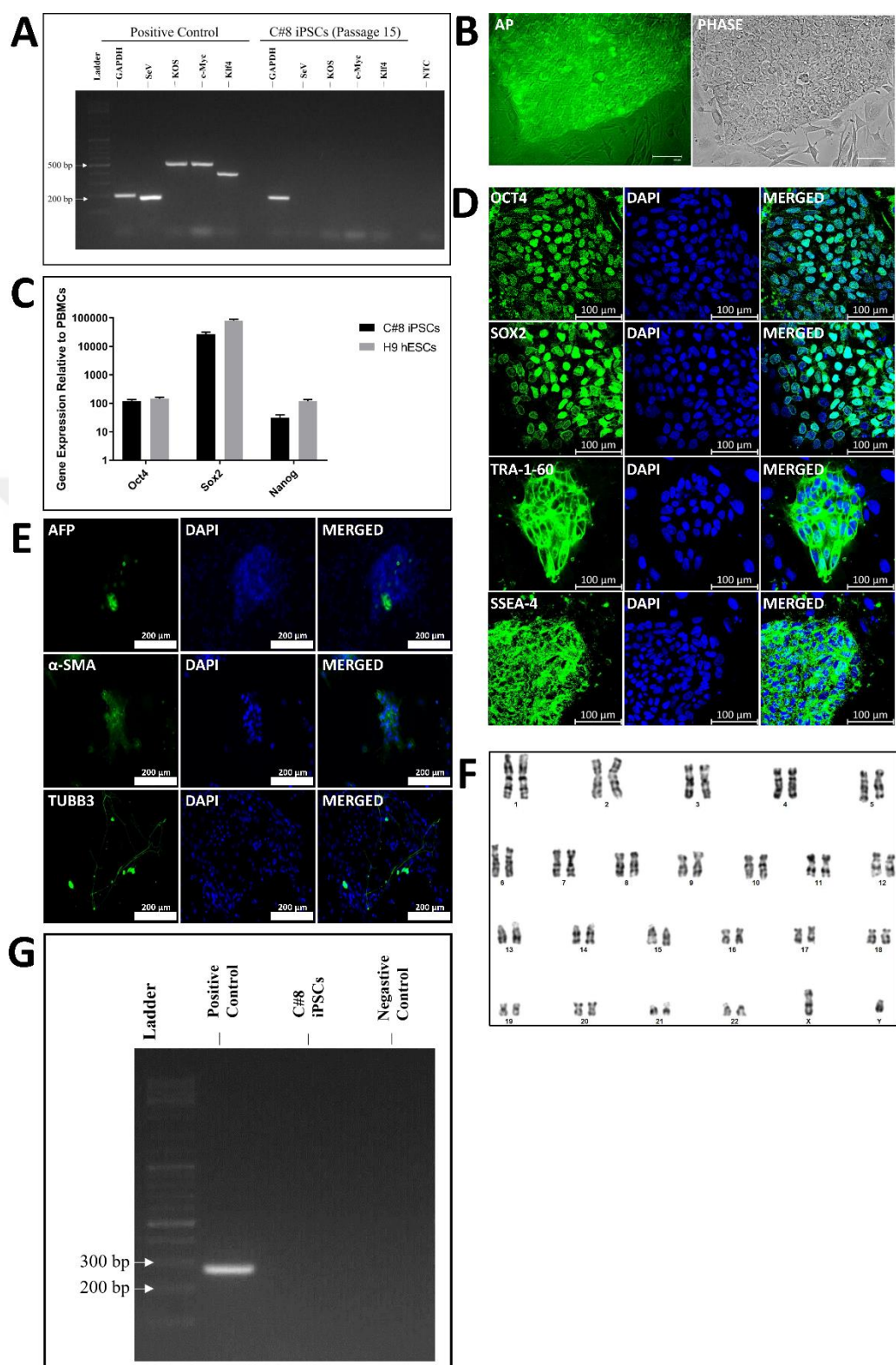


Figure 14 Establishment and characterization of C#8 iPSC line, derived from a healthy male individual.

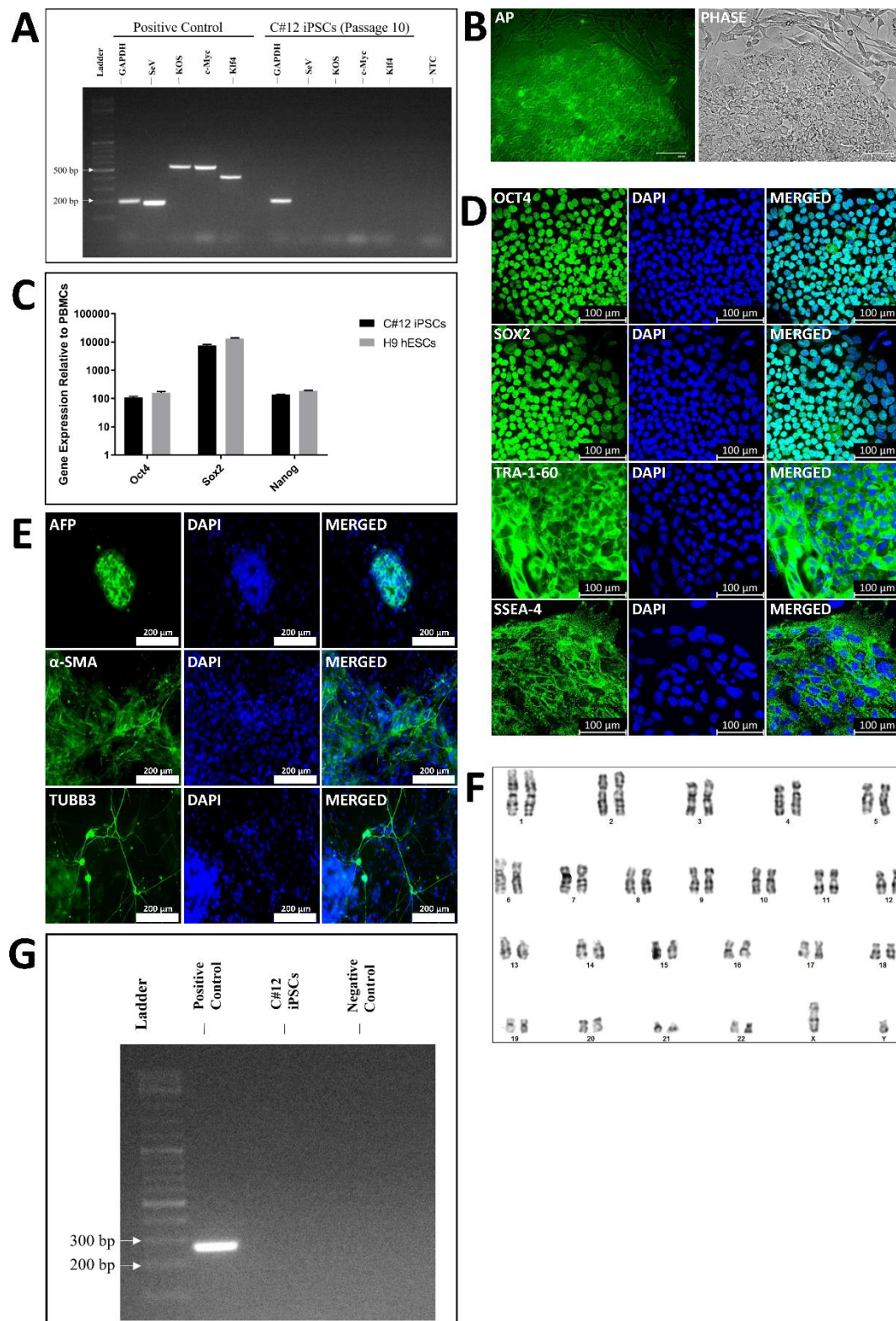


Figure 15 Establishment and characterization of C#12 iPSC line, derived from a healthy male individual.

3.3 Alkaline Phosphatase Activity in Established iPSCs

Alkaline phosphatase is an enzyme known to be elevated in expression and function in pluripotent stem cells (Štefková, Procházková, & Pacherník, 2015). To confirm this phenomenon, established iPSCs for each sample were stained using Alkaline Phosphatase Live Stain Kit without losing the precious iPSC cells, and routine culture was continued following visualization. As shown in **Figures 4-15B**, iPSCs of all samples were stained positive. The surrounding MEF feeders served as a negative control for each sample.

3.4 Expression of Pluripotency Markers in Established iPSCs

3.4.1 Expression of OCT4, SOX2, and NANOG Transcription Factors

For the determination of expression of pluripotency-related genes in each established clone from all the samples, RT-qPCR experiment was conducted. As a positive control cDNA obtained from a previously established hESC line H9 (Thomson et al., 1998), samples were utilized, which were available in our laboratory. However, any established hiPSC or hESC cell line would serve as an excellent positive control for this experiment. As shown in **Figures 4-15C**, all the lines derived from each sample expressed the pluripotency-related genes (*OCT4*, *SOX2*, and *NANOG*) at similar levels compared to hESCs’.

3.4.2 Immunostaining of Oct4, Sox2, TRA-1-60 and SSEA-4

Simultaneously with the RT-qPCR analysis for the pluripotency-related genes, iPSC clones from 12 different samples were used to perform immunocytochemistry to detect pluripotency markers at the protein level. As depicted in **Figures 4-15D**, all 12 samples were positively stained for every cell present within an iPSC colony. As expected, the transcription factors OCT4 and

SOX2 stained all the nuclei within a colony, while TRA-1-60 and SSEA-4 stained the membrane in all established hiPSCs.

3.5 Differentiation of Established iPSCs into the Three Germ Layers

The pluripotency of each of the 12 iPSC lines generated was also tested by their ability to form cells from the three embryonic germ layers. For this, *in vitro* EB formation was performed for induction of spontaneous differentiation. EBs were successfully formed within a 2-weeks period and matured (growing ~500 μ m in diameter (can be affected by the initial size of cell clumps) with an overall dark brown coloration, **Figure 16**) with hiPSC medium without any supplements. After dissociation and further maturation into the three germ layers spontaneously for a period of 1 week, cells were fixed and immunostained for representative markers of the three germ layers. All 12 samples were successfully differentiated and formed representative lineages of the three germ layers as depicted in **Figures 4-15E**, which was confirmed by staining with AFP for endoderm, α -SMA for mesoderm, and TUBB3 or PAX6 for the ectoderm. In concordance with the literature, cells that gave a specific signal to the antibody was imaged using either Leica SP8 confocal microscope or Fluid Cell Imaging System.

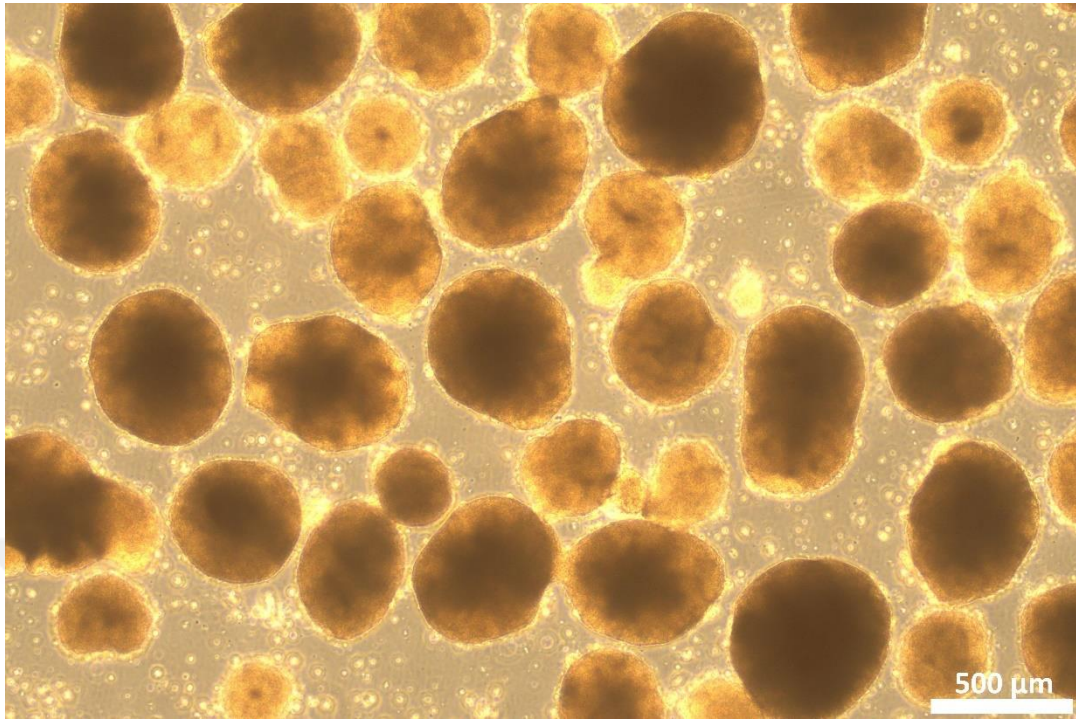


Figure 16 Representative image of embryoid bodies at Day 21 of *in vitro* spontaneous differentiation (MSp#10).

3.6 Karyotyping and Mycoplasma Analysis

Live representative clones from each of the 12 different samples were sent to another laboratory for karyotyping. After verification of clearance of CytoTune reprogramming vectors, iPSCs were grown for 24-hours in Matrigel-coated 60-mm culture plates in MC-hiPSC medium (**Appendices B**). Once ready, the plates were wrapped in parafilm and transported to the laboratory for karyotype analysis. Following colcemid treatment for metaphase arrest, at least 20 metaphase spreads of cells were examined for each of the 12 different samples, and no chromosomal abnormalities were found. All 12 established iPSC lines showed normal diploid male or female karyotype at a resolution of 550-600 bands, as shown in **Figures 4-15F**. All iPSC lines were routinely screened for mycoplasma contamination by conventional PCR. For mycoplasma analysis, a 24-hour old medium from the established iPSCs was collected in tubes and used as a template in PCR experiments

conducted with *Mycoplasma* specific primers. None of the samples were found to be positive for contamination.



CHAPTER 4

DISCUSSION

Human iPSC-based disease models can provide powerful *in vitro* platforms to study MS pathology, demyelination and remyelination mechanisms and evaluate therapeutic interventions (Di Ruscio, Patti, Welner, Tenen, & Amabile, 2016; Martinez-Larrosa et al., 2020). Human iPSCs can be differentiated into cells that are relevant for the disease of interest. Here, we generated hiPSCs from freshly drawn peripheral blood from RRMS patients and healthy controls. Our choice of methodology in reprogramming was based on multiple factors. First, we had to decide on the somatic cell source to be obtained from donors for iPSC generation. Although dermal fibroblasts are among the most common cell sources in the literature for reprogramming, it involves an invasive procedure (skin-punch biopsy) to obtain and derive such cells from the donors (Raab, Klingenstein, Liebau, & Linta, 2014). PBMCs are another conventional alternative somatic cell source that has been shown to be reprogrammed into iPSCs and were utilized in modeling a variety of diseases (Barbeau et al., 2020; Bing et al., 2020; Chu et al., 2020; Deslauriers, Kotini, & Papapetrou, 2021; Gong, Jiao, Zhang, & Yang, 2020; Lim et al., 2020; Yamasaki et al., 2020). For the generation of iPSCs from individuals, PBMCs were selected as the somatic cell source for this study mostly because of the method of obtaining as well as the similar reprogramming efficiencies when compared to other somatic cell sources (Hokayem, Cukier, & Dykxhoorn, 2016; Raab et al., 2014). Moreover, it was shown that the blood sample could be stored up to 48 hours at room temperature for reprogramming purposes (Agu et al., 2015), while storing for 4 hours at room temperature would not affect the number of viable PBMCs nor the characteristics of the cells in the blood sample (Vogelaar, Posthuma, Boomsma, & Kluft, 2002). For an iPSC to be utilized in disease modeling, it is essential that it retains the donor genome. For this reason, reprogramming factor delivery

methodologies that require either permanent or transient integration to the host genome were avoided in this study. Sendai-virus particles packed with RNAs that encode Yamanaka factors (Klf4, Oct3/4, Sox2, and c-Myc) (Fusaki, Ban, Nishiyama, Saeki, & Hasegawa, 2009), episomal vectors that express Yamanaka factors in addition to a few different transcription factors and miRNAs (Keisuke Okita et al., 2011), and mRNA/miRNA reprogramming (Warren et al., 2010) are the 3 major methods that are commonly used in integration-free induction of pluripotency in different types of somatic cells. When the three methods were compared in terms of versatility, efficiency, and post-induction problems such as aneuploidy, they yielded similar results overall (Schlaeger et al., 2015). However, Sendai-virus based reprogramming requires the least amount of work from the beginning of transduction until picking the established colonies when compared to other methods, and clearance of the exogenous reprogramming vectors were relatively faster. Next, it was essential to decide on the growth conditions for the generation and expansion of human iPSCs. Monoculture of human ES or iPSCs *in vitro* requires supplementation of additional growth factors, differentiation suppressors, and an extracellular matrix that supports adhesion (Nakagawa et al., 2014). In defined culture systems utilized for feeder-free culturing, materials that iPSCs demand for retaining pluripotency are highly costly and increases the workload during maintenance. Feeder cells, which can either be derived from humans or mouse fibroblasts, can provide the matrix needed for iPSC attachment in addition to all cytokines and growth factors that iPSCs require for retaining pluripotency. Thus, mmc-MEF feeders were utilized for the generation and maintenance of all iPSC samples. pMEFs were routinely expanded and mitotically inactivated for this project throughout the generation and characterization procedures. In this study, CytoTune-iPS 2.0 Reprogramming Kit from Invitrogen was utilized, and reprogramming was conducted mostly according to the manufacturer's recommendations for feeder-dependent reprogramming of human cells. After obtaining blood samples, PBMCs were isolated within 3 hours using density gradient centrifugation utilizing Histopaque-1077, which helps remove cells and particles from the blood without a nucleus. Based on a previous finding

utilizing fibroblasts for reprogramming with Sendai-virus based method (Beers et al., 2015), we have reduced the number of expanded PBMCs needed for reprogramming to as low as 100,000 cells per sample, enabling us to reprogram at least 6 different samples with the same amount required for reprogramming 1 sample based on the manufacturer's protocol (CytoTune). The number of colonies we obtained after transduction from Day 8 to Day 45 post-transduction ranged from 30 to 180 for each sample (for every 100,000 expanded PBMCs/sample). At the first step of characterization, all samples were verified for the clearance of all exogenous CytoTune Sendai reprogramming vectors between passages 10-21. Next, the presence and activity of alkaline phosphatase, whose expression was known to be elevated in PSCs (ESCs and iPSCs) (Štefková et al., 2015), was tested for all 12 iPSC lines, and all were positive for AP activity based on fluorescence assay that was conducted based on the manufacturer's instructions. Afterward, the levels of endogenous pluripotency markers were analyzed in comparison with cDNA obtained from hESC line H9, which was readily available in our laboratory. All 12 samples yielded similar results in terms of pluripotency gene expression in comparison with the hESC positive control. Next, the presence and the cellular location of 4 pluripotency-related proteins were examined using immunocytochemistry and colonies from all 12 samples stained positive for Oct4, Sox2, TRA-1-60, and SSEA-4. The pluripotency of generated iPSCs was also validated by their capacity to form representative cells from the three embryonic germ layers. The most common method in the literature is to induce teratoma formation where a nude mouse was utilized and injected with generated iPSCs which eventually form a teratoma within 6-8 weeks (Nelakanti, Kooreman, & Wu, 2015). Afterward, the mice are sacrificed, and the teratoma is subjected to immunohistochemistry to analyze the three embryonic germ layers. A more humane and much faster (3-4 weeks) alternative is to perform *in vitro* spontaneous differentiation with EB formation. In this study, all 12 samples were successfully formed EBs and spontaneously differentiated into all three germ layers where they stained positive for AFP, representing the endoderm; SMA, representing the mesoderm; for TUBB3 or PAX6, representing the ectoderm.

There is an approximately 10% chance for a generated iPSC line to have chromosomal abnormality (assuming the somatic source has a normal karyotype), where the major cause is mycoplasma contamination most of the time (Liang & Zhang, 2013). Generating multiple clones for each sample is important because if chromosomal abnormalities were detected within one clone of a sample, there is a great chance that the other clones might be free of such aberrations and serve as a backup assuming the donor's genome was intact. Among all 12 samples that were sent into a cytogenetics laboratory for karyotyping, all of them yielded normal diploid female or male karyotypes based on the donors' biological sex. Having established multiple clones from all 12 samples, mycoplasma analysis was conducted before generating frozen cell stocks of established clones of iPSCs, and all samples were tested negative. Finally, established clones from every sample were frozen and stored in liquid nitrogen tanks at various passages after complete characterization and ready for future studies.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

For modeling MS using iPSCs, many different cell lines were generated (Douvaras et al., 2014; Martinez-Larrosa et al., 2020; Miquel-Serra et al., 2017; Mutukula et al., 2021). In this study, 6 iPSC lines were established from patients diagnosed with RRMS, the most common type of MS worldwide, along with 6 control iPSCs. The patients and healthy individuals were matched based on their sex and age (ranged from 25 to 40). Given that there is a well-established sexual dimorphism in MS, having matched controls are highly useful for further studies. Additionally, this work utilized blood samples obtained from recently diagnosed patients at ages (25-40), right before they started to use disease-modifying medications to eliminate any risks of potential unknown effects of the drugs on the PBMCs. These established and characterized human iPSCs will serve as a great tool for modeling the disease in vitro, which can include studies focusing on remyelination process utilizing iPSC-derived OLs. This is important as the literature suggests a significant difference between rodent OL in the context of remyelination in MS compared to that of humans. For instance, in mice, it was shown that OPCs can get activated upon demyelination and are able to differentiate into mature and functional OLs, which can remyelinate naked axons *in vivo* (Zawadzka et al., 2010), while there is no contribution from the existing OLs for remyelination (Crawford et al., 2016). However, human studies showed that there is a limited number of oligodendrocyte progenitor cells within the CNS (despite being the most abundant progenitors making up approximately 5% of an adult CNS), and they are usually irresponsive for demyelination and does not significantly contribute to the remyelination phenomenon during MS (Dulamea, 2017). Instead, resident, or pre-existing OLs would contribute more to the remyelination process. Studies examining the source of remyelination at demyelinated zones of the CNS (lesions) showed that

newly generated OLs were almost undetectable in the zones of remyelination in MS patients, while the transient remyelination was mostly governed by the pre-existing OLs (Cayre, Falque, Mercier, Magalon, & Durbec, 2021; Yeung et al., 2019). There is still more to understand about what renders OPCs defective in remyelination, what activates them, and the sex-specific differences in OLs when MS initiates and progresses specifically in humans. With the cell lines established in this study, we will be able to differentiate them into neural progenitors and then into OPCs, which will eventually be directed into mature and functional OLs, to utilize in mechanistic studies. In conclusion, the 12 iPSC samples would serve an invaluable tool with unlimited potential for generating 2-D and 3-D culture systems to better understand MS, a sexually biased and incurable neurological disorder.

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APPENDICES

A. Materials and Supplements for Cell Culture

Mitomycin C, 20 mg/ml (Cayman, 11435): Dissolved in dimethyl sulfoxide, DMSO (Serva, 39757.01) at a concentration of 20 mg/ml. Aliquots were stored at -20°C.

Gelatin, 0.1% (Serva, 22151.02): Prepared by dissolving 0.1% (w/v) gelatin in ddH₂O. The mixture was microwaved until it fully dissolves, filtered through a 0.22 µm vacuum filter and autoclaved. Stored at room temperature.

Y-27632 dihydrochloride monohydrate, Rock Inhibitor, 1 mM (Santa Cruz Biotechnology, sc-216067): Prepared by dissolving rock inhibitor in ddH₂O at a final concentration of 1 mM, filter sterilized through a 0.22 µm vacuum filter and stored at -20°C.

Recombinant human FGF-basic, 154aa, Animal-Free, bFGF, 4 µg/ml (Biolegend, 710308): Dissolved in sterile DPBS containing 1% KSR at a concentration of 4 µg/ml and sterilized through a 0.22 µm filter. Stored at -80°C.

Dispase II, 10 mg/ml (Gibco, 17105041): Dissolved in DPBS to obtain 10 mg/ml dispase stock solution, sterilized through a 0.22 µm filter, and stored at -20°C.

B. Media Compositions for Cell Culture

PBMC Medium: For culturing and expanding erythroid progenitors from PBMCs, StemSpan SFEM II (Stem Cell Technologies, 09655) supplemented with 1X StemSpan Erythroid Expansion Supplement, EES (100X, Stem Cell Technologies, 02692) was used.

MEF Medium: Composed of 88% DMEM (Gibco, 11965092), 10% Fetal Bovine Serum, FBS (Gibco, 10270106), 1% Penicillin-Streptomycin (Gibco, 15140122), and 1% Glutamax (Gibco, 35050061).

Human Induced Pluripotent Stem Cell Medium (hiPSC Medium): Prepared by mixing 77% Advanced DMEM/F12 (Gibco, 12634010), 20% KnockOut Serum Replacement, KSR (Gibco, 10828028), 1% non-essential amino acids, MEM – NEAA (Biological Industries, 01-340-1B), 1% Glutamax (Gibco, 35050061), 1% Penicillin-Streptomycin (Gibco, 15140122), and 55 μ M 2-mercaptoethanol (VWR, M131). hiPSC medium was supplemented with 4 ng/ml bFGF freshly before use in hiPSC culture.

MEF-conditioned Human Induced Pluripotent Stem Cell Medium (MC-hiPSC Medium): Prepared by incubating hiPSC medium without bFGF in a mmc-MEF coated 15-cm plate with 24 hours at 37°C incubator. After conditioning of the media, it was sterile filtered using a 0.22 μ m vacuum filter and stored at -20°C in aliquots of 15 ml. MC-hiPSC Medium was used in the feeder-free expansion of iPSCs in Matrigel-coated culture plates for karyotype analysis by freshly supplementing with 10 ng/ml bFGF before use.

Freezing Medium (for MEF and PBMCs): Prepared by mixing 90% FBS with 10% DMSO. DMSO was added immediately prior to use.

iPSCs Freezing Medium: Prepared by mixing 50% hiPSCs medium with 40% KSR and supplemented with 10 μ M rock inhibitor (Y-27632). 10% DMSO was added immediately prior to use.

C. Batch Specific Viral Titers from the CytoTune Reprogramming Kit

CytoTune™ -iPS 2.0 Sendai Reprogramming Kit

Product No. A16517
Lot No. L2160049
Expiration Date 20Feb2022

Component	Titer* (CIU/mL)	Transduction Volume**
CytoTune™ 2.0 hKOS	1.1×10^8	27 uL
CytoTune™ 2.0 hc-Myc	1.0×10^8	30 uL
CytoTune™ 2.0 hKlf4	9.3×10^7	19 uL

* **Titer:** Specification is 8.0×10^7 - 15.0×10^7 Cell Infectious Units/mL. Titer indicates the functional titer to LLCMK2 cells.

Each vial contains 100 uL virus. **Transduction Volume: Indicates suggested volume of virus to be used for 2 wells of a 6 well plate (3×10^5 cells/well) at MOI= 5:5:3 (KOS:c-Myc:Klf4) for BJ cells. See Product Manual for additional information.

Figure 17 A portion from the certificate of analysis from the manufacturer of CytoTune-iPS 2.0 Sendai Reprogramming Kit (Invitrogen), depicting the viral titers of each CytoTune reprogramming vector at the defined multiplicity of infection (MOI is 5:5:3 for the vectors in the given order).

D. DNA Ladder

GeneRuler 1 kb Plus DNA Ladder

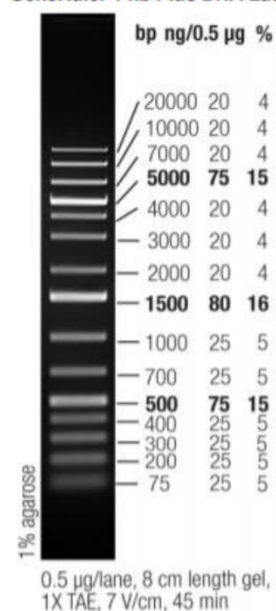


Figure 18 DNA ladder (SM1331, Thermo Fisher Scientific) that was used in agarose gel electrophoresis.