Tampereen yliopisto

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MODELLING MULTIPLE SCLEROSIS WITH HIPSC-DERIVED ASTROCYTES

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ABSTRACT

Oona Kukkonen: Modelling multiple sclerosis with hiPSC-derived astrocytes
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Multiple sclerosis (MS) is an inflammatory autoimmune disease affecting the central nervous system (CNS) by demyelinating axons and causing neurodegeneration. MS onsets between the ages of 20 and 40 being the leading cause of neurological disability among young adults. It has been indicated that certain genetic and environmental factors together increase the risk of developing MS. There is no complete cure for the disease, but several treatments are available that mitigate the symptoms and slow the disease progression.

Astrocytes are star-shaped glial cells that make up the majority of cells in the CNS. They have several important homeostatic functions in the development and the maintenance of CNS. They support functions of neurons and take part in brain metabolism. In the inflammatory environment, astrocytes can respond and transform on the inflammatory phenotype. Depending on the condition astrocytes can turn into detrimental or neuroprotective phenotype. Studies have highlighted the important role of astrocytes in MS pathology, but it is still poorly characterized. Animal models have been used to study astrocytic functions, but they don't tell the whole truth due to the complexity of the human body, which underlines the need for human-based models. Human induced pluripotent stem cells (hiPSCs) have shown to be a great option for modelling different diseases. The aim of this bachelor's thesis is to describe the role of astrocytes in MS and use of hiPSC-derived astrocytes from patients with MS.

Currently, four articles have reported the generation and use of hiPSC-derived astrocytes obtained from patients with different types of MS. Using varied reprogramming and differentiation protocols they have produced hiPSC lines and mature astrocytes which have taken from 3 to 4 months. Astrocytes have been stimulated with different MS-associated cytokines and the modifications of astrocyte phenotypes have been detected with commonly used methods and then compared to healthy controls. Articles focused on different topics and aspects and ended up with contradictory outcomes.

Studies have revealed some changes in the phenotypes of MS astrocytes. Cytokine stimulation has induced specific transcriptome profiles. Astrocytes have showed neuroprotective properties but only with astrocytes derived from certain MS subtypes. One study has reported that MS astrocytes have altered metabolic profiles with mitochondrial dysfunction, higher superoxide production and changes in the inflammatory response. In some studies, the differences between control and MS astrocytes haven't been significant, which questions the need of MS-specific lines. However, more knowledge about astrocytes' role in MS should be gained in the future due to their numerous functions in the CNS. hiPSCs seem to be a powerful tool to study disease mechanisms which is why they should be utilized to understand MS pathology better.

Keywords: multiple sclerosis, astrocyte, hiPSCs, disease model

TIIVISTELMÄ

Oona Kukkonen: Indusoiduista pluripotenteista kantasoluista erilaistetut astrosyytit multippeliskleroosin mallinnuksessa
Kandidaatintutkielma
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Multippeliskleroosi eli MS-tauti on tulehduksellinen keskushermostoa rappeuttava autoimmuunisairaus, jossa elimistö hyökkää omia kudoksia vastaan aiheuttaen hermosolujen demyelinaatiota sekä tulehdusmuutoksia. Sairastumisikä on 20–40 vuotta ja tauti on yleisin nuorten aikuisten toiminta- ja liikuntakykyä rajoittava sairaus. MS-taudin tarkkaa syntymekanismia ei tiedetä, mutta sen on todettu puhkeavan yhdessä sekä perintö- että ympäristötekijöiden seurauksena. Tautiin ei ole parantavaa hoitoa, mutta sen oireita ja etenemistä voidaan hillitä useilla lääkkeillä.

Astrosyytit ovat tähden muotoisia tukisoluja keskushermostossa. Ne ovat runsain solutyyppi keskushermoston alueella, jossa niillä on monia tehtäviä kehityksessä ja homeostaattisessa ylläpidossa. Ne tukevat hermosolujen toimintaa sekä osallistuvat aivojen aineenvaihduntaan. Astrosyytit voivat pahentaa tulehdusta, mutta tilanteesta riippuen ne kykenevät myös muun muassa korjaamaan vaurioitunutta kudosta. Astrosyyteillä on osoitettu olevan tärkeä rooli MS-taudin patologiassa, mutta tästä on kuitenkin toistaiseksi rajoitetusti tietoa. Astrosyyttien toimintaa on tutkittu pääasiassa eläinmalleilla, mutta ne eivät kuitenkaan täysin vastaa ihmiskehon monimutkaista toimintaa, jolloin tulokset eivät ole täysin verrattavia. Tästä syystä ihmisperäisiä malleja tarvitaan, jotta MS-tautia voidaan tutkia tarkemmin. Indusoidut pluripotentit kantasolut (human induced pluripotent stem cells, hiPSCs) ovat osoittautuneet hyväksi tavaksi mallintaa erilaisia tauteja. Tämän kandidaatintutkielman tavoitteena on kuvailla astrosyyttien roolia MS-taudissa sekä tutkia MS-potilailta saatujen hiPSC-johdettujen astrosyyttien toimintaa.

Tähän päivään mennessä on julkaistu neljä artikkelia, joissa kerrotaan hiPSC-johdettujen astrosyyttien valmistamisesta sekä niiden käytöstä MS-taudin tutkimuksessa. Alkuperäiset solut on otettu potilaista vaihtelevilla tautityypeillä. Erilaisilla uudelleenohjelmointi- ja erilaistamismenetelmillä on tuotettu hiPS-soluja sekä kypsiä astrosyyttejä prosessissa, joka kestää kolmesta neljään kuukautta. Stimuloimalla astrosyyttejä MS-taudille tyypillisillä sytokiineilla, astrosyyttien fenotyyppimuutokset on voitu huomata käyttämällä yleisesti tunnettuja menetelmiä. Näitä astrosyyttejä on sitten verrattu terveisiin verrokkeihin. Tutkimukset ovat keskittyneet hieman erilaisiin aiheisiin ja tulokset ovat olleet osittain ristiriitaisia.

Tutkimukset ovat paljastaneet muutoksia MS-potilaista johdettujen astrosyyttien fenotyypeissä. Sytokiinistimulaation on huomattu johtavan MS-astrosyyteissä ominaisiin transkriptomi-profiileihin. Astrosyyteillä on todistettu olevan hermoa suojaavia ominaisuuksia, mutta vain tietyillä MS-tyypeillä. Jotkin tulokset ovat myös osoittaneet MS-astrosyyttien metabolisen profiilin muuttuneen, mikä näkyy muun muassa mitokondrioiden toimintahäiriöinä, lisääntyneenä superoksidin tuottona sekä muutoksina tulehdusvasteessa. Osassa tutkimuksista merkittäviä eroja terveiden astrosyyttien ja MS-astrosyyttien välillä ei huomattu, mikä voi kyseenalaistaa niiden tutkimisen tarpeen. Astrosyyteillä on kuitenkin lukuisia rooleja keskushermostossa, minkä vuoksi tarvitaan enemmän tietoa niiden roolista MS-taudissa. hiPS-solut ovat osoittautuneet tehokkaaksi välineeksi tautimekanismeja tutkittaessa, miksi niitä pitäisi myös hyödyntää enemmän MS-taudin patologian selvittämisessä.

Avainsanat: multippeliskleroosi, astrosyytti, hiPSCs, tautimalli
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CONTENTS

1. INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) characterized with demyelination and neurodegeneration. It is estimated that globally about 2.5 million people have MS disease, and it is the main cause of neurological disability among young adults. The ultimate cause of MS is poorly known but it is approved that both genetic and environmental factors increase the susceptibility to disease. There are available treatments that slow the disease progression of MS but there is currently no complete cure for it. (Thompson et al. 2018)

Stem cell culture models are used to study neurodegenerative diseases, like MS. Rodent models have been widely utilized but they don't fully recapitulate the human conditions and the complexity of the human cells, so they don't always give reliable results. Human induced pluripotent stem cells (hiPSCs) have shown a great potential modelling several diseases and understanding their pathophysiology. Novel protocols have been developed to obtain all major CNS cell types from hiPSC: neurons, oligodendrocytes, astrocytes, and microglia. Still, research has been relatively limited concerning MS and the function of CNS cell types during the disease. Over 20 studies have been published that describe the use of MS hiPSCs. (Fortune et al. 2022)

Astrocytes are a sub-type of glial cells in the CNS. They are the most abundant cell type in the CNS and have an important role in maintaining homeostatic functions in the brain and supporting other cells, like neurons. They have been shown to be involved already in the early stage of MS development. Astrocytes are the main recruiters of lymphocytes and play a pivotal role in the formation of MS lesions. These lesions are caused by the functional changes of astrocytes when they become active. In addition to neurotoxic features, astrocytes can also restrict inflammation and support lesion repair. Gaining knowledge of astrocytes in MS is crucial due to their numerous functions in the CNS. However, astrocytes are not as well studied as other CNS cell types. (das Neves et al. 2021) The aim of this review is to understand how the function of astrocytes is studied with MS hiPSC-derived astrocyte models and what kind of astrocyte stem cell models are already done to study MS.

2. MULTIPLE SCLEROSIS

MS is a chronic inflammatory autoimmune-mediated disease of the CNS that is a leading cause of neurological disability of young adults. It causes focal lesions in the white matter of the CNS leading to demyelination and axonal loss. (Lassmann 2018) MS usually onsets between the ages of 20 and 40 years, having a higher prevalence in women. Clinical symptoms of the disease include vision problems and nystagmus, motor dysfunction, tremor, cognitive dysfunction, fatigue, and loss of coordination. Muscle weakness can be severe when it may lead to partial or complete paralysis. Currently, there are available several MS treatments that can mitigate symptoms and relapses and slow down the progression of the disease. (Thompson et al. 2018)

The etiology of MS is still unknown, but it has been noted that both genetic and environmental factors determine the risk of getting the disease. The most significant risk factors are known to be insufficiency of vitamin D, obesity, and cigarette smoking. Also, specific infections can be associated with the disease: for example, Epstein-Barr virus (EBV) infection has been shown to increase the risk of MS development. MS is not directly inherited but the risk has been shown to greatly increase when related to someone who has MS. Several disease-associated risk alleles have been found. (Thompson et al. 2018)

Even though the ultimate cause of MS remains unclear, there are main steps in MS pathology that are known to occur in the beginning of the disease. According to current knowledge, the disease initiates in the periphery when CD4+ T lymphocytes interact with antigen presenting cells (APCs) which are activated by some pathogen activated molecules. CD4+ cells start to differentiate into different T helper (Th) cell phenotypes, such as Th1, Th2 or Th17 phenotype, induced by multiple cytokines. Th1 and Th17 cells secrete several cytokines that are capable of promoting inflammation. Differentiated CD4+ cells infiltrate into CNS by passing the blood-brain barrier (BBB) endothelium. In the CNS, CD4+ T cells are recruiting other immune cells, such as CD8+ T cells, B cells and monocytes to the CNS as well as activating glial cells of the CNS like microglia and astrocytes. This leads to neurodegeneration and demyelination of axons forming MS plaques to the white and gray matter tissues. (Dendrou, Fugger, and Friese 2015)

3. ASTROCYTES

3.1 Homeostatic functions of astrocytes in CNS

The CNS is a part of our nervous system that includes the brain and the spinal cord. The main tissue component in CNS is nervous tissue that is composed of neurons and neuroglial cells, like astrocytes, oligodendrocytes, and microglia. The morphology of astrocytes is star-shaped with radially arranged foot processes which help them to communicate with surrounding environment. Neuroglia's function is to provide metabolic support and structure for neurons. Among them, astrocytes are cells that have many important homeostatic functions, like stabilization and regulation of the BBB and absorption of excess neurotransmitters. Microglia are immune cells of the CNS. They are phagocytic cells that work as brain macrophages. Oligodendrocytes generate and maintain myelin around the axons ensuring efficient and fast transfer of action potential through the axon. In MS, the function of neuroglia is disrupted which partly cause demyelination and neurodegeneration, but these dysfunctions still aren't fully understood. (Quan, Uyeda, and Muramatsu 2022)

Astrocytes are the most abundant cell type in the CNS. They have many important functions in the development and maintenance of CNS where they maintain homeostatic functions by upkeeping and repairing the BBB and supporting the functions of neurons (Figure 1). BBB is formed of endothelial cells, pericytes and astrocytes. Endothelial cells are attached to each other with tight junctions. Around them are pericytes that are embedded in the vascular basement membrane. Astrocytic endfeet surrounds these cells and regulate tight junctions and produce molecules that regulate the CNS blood flow (Figure 1A)**.** Astrocytes control pH and the composition of the extracellular fluid by expressing water and ion channels, such as AQP4 and Kir4.1, at the BBB. (das Neves et al. 2021)

Astrocytes also support the function of neurons by giving metabolic support. At the synaptic cleft, the processes of astrocytes form tripartite synapse, where they can make a connection with pre- and post-synaptic neurons (Figure 1B). The function of this structure is to participate in many processes, like synaptogenesis, synaptic stability, neurotransmitter uptake, glutamate uptake, and buffering of ions like sodium (Na^+), potassium (K^+) and protons (H^+). For example, astrocytes remove extracellular glutamate from the synaptic cleft and then convert it to glutamine (Figure 1B). Glutamate is the major excitatory neurotransmitter in the CNS. Neurons can take glutamine up and reconvert it to active neurotransmitters. In addition to synapse formation, astrocytes are involved in the elimination of synapses by phagocytosis. They also have an important role in displaying Ca^{2+}

signals. Astrocytes have a glycogen storage, whereof they can degrade glucose to pyruvate, convert it to lactate, and provide it to neurons as an energy supply (Figure 1C). Glucose can also be taken straight from the circulation. All these functions are allowed because of the location of astrocytes between blood vessels and neurons. (das Neves et al. 2021; Kim, Park, and Choi 2019)

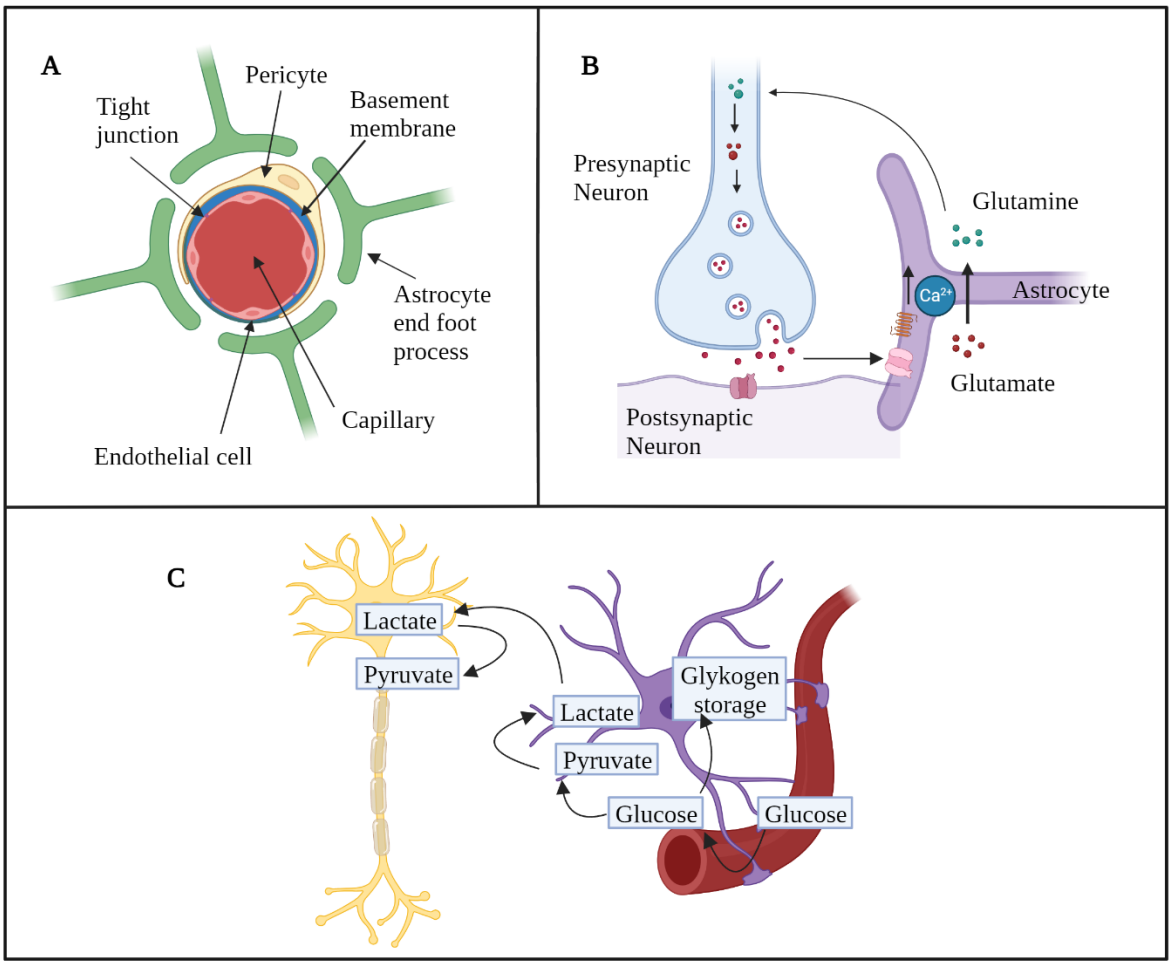

Figure 1. *Physiological functions of astrocytes. A. Astrocytic end-feet support the integrity of BBB by regulating the CNS blood flow and maintaining tight junctions between endothelial cells of the blood vessels. B. At the synaptic cleft astrocytes are in contact with presynaptic and postsynaptic terminals of neurons forming a tripartite synapse. One of the important functions of astrocytes there is to remove excess glutamate and convert it to glutamine which is transferred back to neurons. Neurons reconvert glutamine back to active glutamate. C. Astrocytes can uptake glucose from the circulation and degrade it to pyruvate which is converted to lactate. Lactate is provided to neurons which can use it as an energy supply. Glucose can also be stored in astrocytes as glycogen. Image from (das Neves et al. 2021) modified with Biorender.*

The structure of astrocytes is star-shaped with radially arranged foot processes which help them to communicate with surrounding vasculature. These processes are marked by a protein named glial fibrillary acidic protein (GFAP) which is a typical marker when identifying astrocytes with immunohistochemical methods. Other astrocyte-specific markers which can be used to distinguish

astrocytes from other glial cells are vimentin, S100 calcium-binding protein B (S100-β), glutamate/aspartate transporter (GLAST) and glutamine synthetase (GS). (Sofroniew and Vinters 2010) There are different forms of astrocytes of which the basic types are the protoplasmic and fibrous astrocytes. Protoplasmic astrocytes exhibit highly branched processes and are located in the grey matter. They have shown to be important in synapse maintenance and modulation, and regulation of blood flow with their endfeet. Fibrous astrocytes have straight and long processes and are mainly found in the white matter. These cells have higher expression of GFAP than protoplasmic astrocytes in which GFAP may be only present in the endfeet on the blood vessels. The function of fibrous astrocytes is unclear, but it is known that they are also connected with blood vessels via their processes. (Tabata 2015)

3.2 The role of astrocytes in MS

Despite all the important homeostatic roles of astrocytes, they can also be noxious due to their contribution to the pathogenesis of many neurodegenerative diseases. Astrocytes can respond to inflammation by being activated and turning into reactive astrocyte phenotype when they can reduce their homeostatic functions and become detrimental to neurons and oligodendrocytes. On the other hand, in certain pathological conditions astrocytes can turn on the neuroprotective phenotype. Studies have shown that there are two types of reactive astrocyte phenotypes termed A1 and A2, of which A1 being linked with the harmful phenotype and A2 beneficial phenotype (Figure 2). The phenotype depends on the neuroinflammatory stimulus. For example, LPS activates microglia that secrete inflammatory cytokines yielding A1 reactive astrocytes. Instead, type A2 astrocytes have been associated with neuroprotective properties. (Baldwin and Eroglu 2017)

Astrocytes have a strong contribution to the MS pathology in connection with the alterations in their structure and function. They are already involved in the early prelesioned stage of the disease that can be recognized by BBB breakdown and focal mild perivascular lymphocyte infiltration. Reactive astrocytes have also been found in the brain parenchyma of animal models before immune cell infiltration proposing their role as active contributors to early lesion development. (das Neves et al. 2021)

Astrocytes are very sensitive to the changes in their environment which can easily cause modifications in gene expression, morphology, and functionalities. They are completely involved in immune responses that happen in the CNS and because of their many tasks, dysfunctions of astrocytes are some ways a part of multiple neurological disorders. In response to inflammation,

infection or trauma in the brain, astrocytes become reactive, have changes in their morphology and upregulate the expression of many cell-type specific markers. Production of cytokines and chemokines activate the peripheral immune cells which start to migrate to the CNS. (das Neves et al. 2021) One of the overexpressed proteins is GFAP which is required for the glial scar formation. Astrocytes that are found from glial scar inhibit axonal regeneration, neurite outgrowth and oligodendrocyte precursor cell (OPC) differentiation. On the other hand, glial scar protects the surrounding healthy tissue from immune cells and limits chemokine production. (Correale and Farez 2015; das Neves et al. 2021)

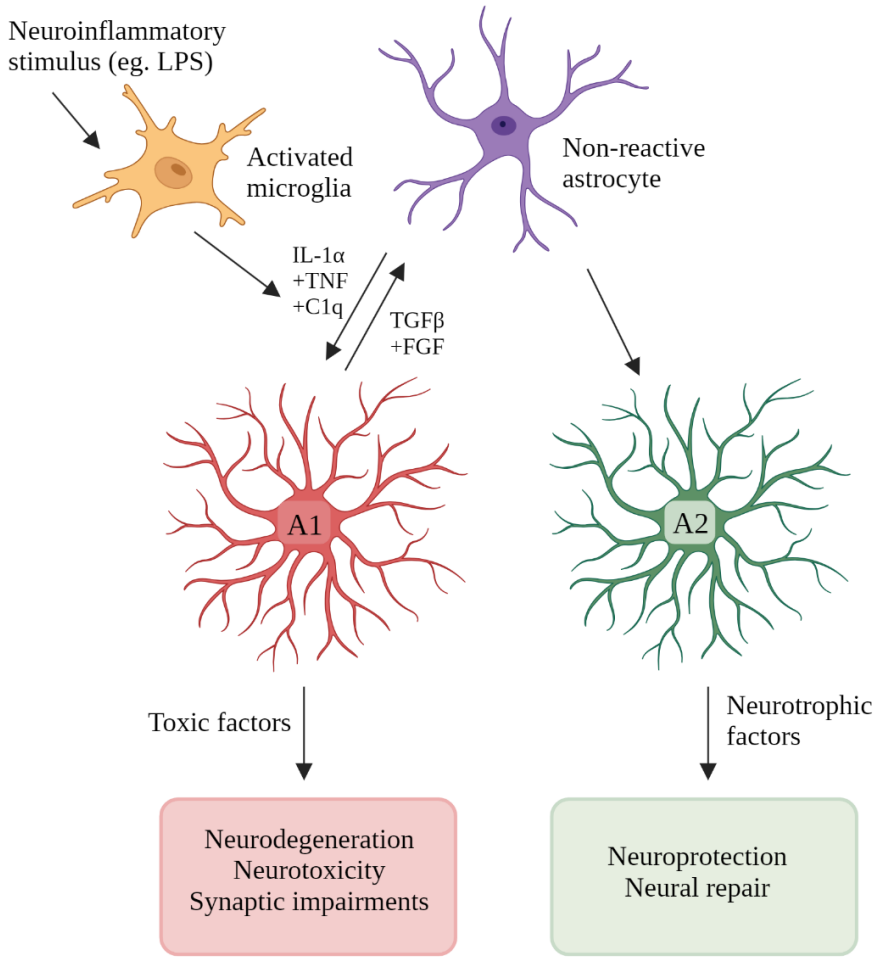

Figure 2. *Reactive astrocytes are heterogenic. Astrocytes can become neurotoxic (type A1) or neuroprotective (type A2) depending on the stimulus. For example, LPS-triggered neuroinflammation activates microglia to secrete inflammatory cytokines IL-1α, TNF and C1q yielding type A1 astrocytes which leads to neurodegeneration, neurotoxicity, and synaptic impairments. On the other hand, addition of TGFβ and FGF can convert astrocytes back to non-reactive type. Astrocytes can also be induced into type A2 astrocytes. These astrocytes release neurotrophic factors making them neuroprotective. Lipopolysaccharide (LPS), interleukin 1-alpha (IL-1α), tumor necrosis factor (TNF), complement component 1q (C1q), transforming growth factor beta (TGF-β), fibroblast growth factor (FGF). Image from (Baldwin and Eroglu 2017) modified with Biorender.*

4. STEM CELL-BASED DISEASE MODELLING

Most of the MS studies have been done with rodent models so they make the most of our understanding about the mechanisms of the disease. The most commonly used model to study MS is an experimental autoimmune encephalomyelitis (EAE), where T cell-mediated myelin destruction is induced by immunization of mice with myelin proteins. EAE has helped to understand CNS inflammation, immune-mediated tissue injury and immune surveillance. Although these animal models have been very useful, they don't fully match the human condition so novel models are needed to examine the pathogenesis of MS further. (Martínez-Larrosa et al. 2020) Stem cell-based models are a new way to study MS pathology.

Stem cells are undifferentiated cells in the human body, and they have an ability to differentiate into different types of cells. They are also able to self-renewal. During differentiation cells' potency reduces gradually which means that by every step they have less possible cell types to differentiate. At the pluripotent state cells have an ability to differentiate into all kinds of tissues of the human body, except for embryonic tissue. There are two types of pluripotent stem cells, embryonic stem cells (ESCs) and human induced pluripotent stem cells (hiPSCs). ESCs are derived from the inner cell mass of the human blastocyst-stage of embryos, and they are completely pluripotent. ESCs are used in research because of their high potency to differentiate into many tissues but they have many ethical problems because obtaining them requires the destruction of embryos. (Zakrzewski et al. 2019)

hiPSCs are pluripotent stem cells that are obtained from mature cells in the human body. hiPSC lines are typically produced from fibroblasts or blood cells. These cells are reprogrammed into hiPSCs with reprogramming factors using plasmids or viruses as a vector. The original set of those factors (Yamanaka factors) are the genes Oct4, Sox2, cMyc, and Klf4. Characterization of generated hiPSCs can be done with karyotype analysis to ensure a normal karyotype, and with immunofluorescence staining or flow cytometry to verify pluripotency and differentiation capacity. (González, Boué, and Belmonte 2011) Then, those formed hiPSC lines can differentiate into all cell types of the human body. The cells that are derived from hiPSCs are almost identical to the cell donor which makes them a great option for the research of personalized drug screening and disease modelling. hiPSCs are very similar to ESCs but they don't have ethical issues which makes them better for the research. (Zakrzewski et al. 2019)

There are at least 23 studies that have used hiPSCs derived from MS patients. hiPSCs have been generated from fibroblasts, peripheral blood mononuclear cells (PBMCs), mesenchymal cells, and

renal proximal tubule epithelial cells using different reprogramming methods. At least 52 lines have been made, mostly from women and individuals with relapsing remitting MS (RRMS) disease phenotype. MS-derived hiPSCs have been successfully differentiated into all germ layer derivatives, including astrocytes, oligodendrocytes, functional neurons, and microglia. (Fortune et al. 2022)

5. HIPSC-DERIVED ASTROCYTES

5.1 Reprogramming of hiPSCs and differentiation to astrocytes

When differentiating hiPSCs into astrocytes, the aim is to mimic the environment like the cells have *in vivo* during human fetal CNS development. In gestational week 3 of the development occurs gastrulation, which leads to the formation of three germ layers: endoderm, mesoderm, and ectoderm. From the ectoderm, neural tube is formed during neurulation where the neural progenitor cells (NPCs) arise. These cells can give rise to many of glial and neuronal cell types. (Purves et al. 2001) NPCs (also termed radial glia) differentiate first into neurons, and then glial cells like astrocytes and oligodendrocytes. Neurogenesis onsets around 6-8 gestational weeks, while gliogenesis is taking place around the gestational weeks 16-18, where radial glia undergo gliogenic switch from neurogenesis to gliogenesis which allows them to differentiate into astrocytes. This switch includes activations of Notch1 and Jak/STAT signaling pathways that promote the expression of astrocyte specific genes, such as GFAP. CNTF, LIF and cardiotrophin 1 are cytokines that are involved in the beginning of astrogenesis. Also, the transcription factors, such as SOX9, NF1A and ATF3 are expressed. (Lanjewar and Sloan 2021)

There are many differentiation protocols that are used to differentiate astrocytes from hiPSCs. The first astrocyte differentiation protocol from hiPSCs was published in 2011 utilizing embryonic body (EB) method (Krencik and Zhang 2011) and thereafter several different differentiation protocols have been published that utilized either small molecule- or genetic factor-based protocols. These protocols are continuously being improved to become more efficient, and they differ in multiple aspects, such as media composition, the cell seeding density, the substrate, the timing and concentration of growth factors, and the physical dimension of the culture system (monolayer or EBs). The differentiation process usually takes from 3 to 4 months (Figure 3). The first step of the differentiation is the generation of NPCs with the process called neural induction. Currently, a well-

established serum- and feeder- free protocol for neural induction is dual SMAD inhibition that rapidly convert hiPSCs to NPCs by inhibiting two pathways. This method uses two inhibitors: Noggin (BMP pathway inhibition) and SB431542 (TGF-β pathway inhibition). By inhibiting these pathways, differentiation into endoderm and mesoderm derivatives are repressed, and the surviving cells are differentiating into ectoderm derivatives, including neural progenitors. At the end of this phase NPCs are organized into radially organized cellular structures called neural rosettes that are selected and cultured for many passages. This stage is called NES expansion (Figure 3). Then rosettes are directed toward astroglial progenitors with molecules like FGF, EGF and LIF during NES expansion. To induce mature astrocytes, CNTF has been shown to have a potential role in differentiation process. (Chandrasekaran et al. 2016) Some protocols also use fetal bovine serum (FBS) during the differentiation, but it is known to cause phenotype alterations albeit it would ease the process (Perriot et al. 2021). In addition to these techniques, astrocytes can also be obtained through genetic modification by transferring astrocytes' transcription factors into hiPSCs. (Canals et al. 2018)

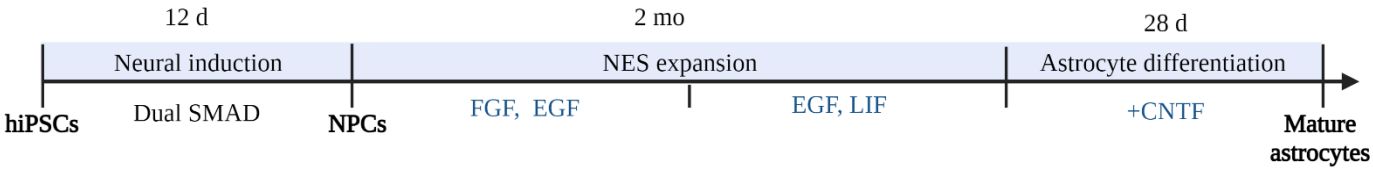

Figure 3. *Timeline representing the differentiation of hiPSCs into mature astrocytes.*

Functions of astrocytes have been studied either under control conditions or under inflammation. Inflammation is induced, for example, by various cytokines. Inflammatory conditions are mimicking those conditions in vivo in the MS brain. There are several methods that are used to ensure the maturity and functionality of differentiated astrocytes. Immunocytochemistry (ICC) is a technique to find a specific protein or antigen in cells by using a primary antibody that can bind to it. In astrocytes this protein marker to characterize the maturation is often GFAP that is found in the astrocytes' foot processes. The sample is visualized under fluorescence microscope with secondary antibody that has a fluorophore to show the localization of a protein. (Burry 2011) Glutamate uptake assays are made to check astrocytes' capability to take up glutamate from the synaptic cleft. If this capability is impaired, it can be seen as increased levels of glutamate that can

cause excitotoxicity. MS patients have been reported to have higher glutamate levels compared to healthy individuals. (das Neves et al. 2021) Ca^{2+} imaging is a main tool to study astrocyte physiology due to the pivotal role of Ca^{2+} signaling in astrocyte functioning. This technique can be used to find dysfunctions of Ca^{2+} signaling in MS astrocytes and neurons. Ca^{2+} imaging requires calcium indicator dyes which bind to calcium-ions. In ultraviolet light, this binding of the dye and calcium emits a light at a specific wavelength that can be detected, and calcium concentration determined. (de Melo Reis, Freitas, and de Mello 2020) One approach to study the function of astrocytes is metabolomics which is an analytical technique for measuring a wide range of metabolites under different biological contexts, such as diseases like MS. This approach combines high-throughput analytical chemistry and data analysis. When transcription products in a certain condition are known, it can be seen which genes are expressed and how has the metabolic function of astrocytes changed. (Castellanos et al. 2022)

5.2 MS-hiPSC-derived astrocytes

There are four studies that have produced hiPSC-derived astrocytes using MS patients as cell donors (Table 1). All of them studied changes in functioning of astrocytes during the development of MS. Total 40 cell lines from MS patients have been produced from different disease subtypes. (Perriot et al. 2018; Ponath et al. 2018; Ghirotto et al. 2022; Kerkering et al. 2023) Most of the produced lines were derived from patients with RRMS subtype, but also 5 lines were produced from progressive MS subtypes, including secondary progressive MS (SPMS) and primary progressive MS (PPMS) patients. hiPSCs were produced using different reprogramming and characterization methods. Reprogramming methods included virus or episomal vectors. Characterization included detection of pluripotency stage, normal karyotype, and differentiation capacity into three germ layers were ensured. Then those cells were differentiated into astrocytes and their functionality was analysed and compared to healthy controls.

Perriot et al. obtained peripheral blood mononuclear cell (PBMC)-derived erythroblasts from patients who had RRMS (n=4). Those cells were reprogrammed into hiPSCs (13 lines) using episomal plasmids as vectors that were encoding for OCT4, SOX2, shRNA-p53, KLF4, L-Myc, and Lin28. Neural induction was done with dual SMAD inhibition on poly-L-ornithine (PLO)/laminin-coated plates. During NES expansion the cells were exposed to FGF2, EGF and LIF. The maturation into astrocytes was done on Matrigel-coated flasks in the presence of CNTF. Mature astrocytes expressed specific astrocyte markers like GLAST, S100β and GFAP and the purity of

the culture was 94%. Astrocytes' capacity of taking up glutamate and displaying calcium transients were tested.

Ponath et al. obtained fibroblasts from MS patients with SPMS (n=2) and RRMS (n=4). Reprogramming into hiPSCs was done with Tempo Bioscience's reprogramming protocol (18 lines) and pluripotency was confirmed with biomarkers including Oct4, Tra-1-80, Nanog and SSEA4. Differentiation to mature astrocytes was done with Tempo Bioscience's serum-, feeder-, integration-, and genetic elements-free, non-viral technology. Astrocytes were plated on PLO/laminin and differentiation was done with medium containing bone morphogenetic protein (BMP-4). The maturation was proved by the expression of markers like GLAST and GFAP, and purity of the culture was 95%. Glutamate and glucose uptake analyses were done to ensure astrocytes' functionality.

Kerkering et al. obtained PBMCs from patients with progressive MS (PMS) which included patients with PPMS (n=2) and SPMS (n=1), and benign MS which included patients with RRMS (n=3). Reprogramming was done by using previously published protocol (Hennig et al. 2019) (9 lines) with Sendai virus as a vector expressing the genes Oct3/4, SOX2, cMyc and KLF4. Phenotype, karyotype, line identity, sterility and the absence of reprogramming vector were analysed. Neural induction was performed with dual SMAD inhibition. Astrocyte differentiation and maturation was done with medium containing DMEM including 1% fetal calf serum (FCS) and CNTF to name a few. Maturity was confirmed with markers S100-β, GFAP and AQP4 using immunofluorence staining and quantitative polymerase chain reaction (q-PCR). Purity of the culture was not mentioned. Functionality of the astrocytes was ensured with Ca^{2+} imaging.

Chirotto et al. obtained PBMCs from patients with RRMS (n=3). The cells were reprogrammed into hiPSCs (6 lines) using previously published protocol (Araujo et al. 2018) and cells were characterized with markers Oct3/4, SSEA4 and SOX2. Differentiation was also done like previously described. hiPSCs were cultured as EBs and then exposed to neural differentiation factors to form neural rosettes. Rosettes were selected and expanded to get NPCs. Then astrocyte differentiation was done with astrocyte differentiation factors. Also, medium with 3% FBS was used. Mature astrocyte markers used were GFAP, Vimentin, S100-β and AQP4. Purity of the culture was not mentioned. Glutamate uptake assay was done to confirm astrocyte functionality.

Table 1. Reprogramming and astrocyte differentiation methods of the hiPSCs derived from MS patients.

Reference	MS types & number of patients	Starting material	hIPSC reprogramming (manufacturer)	hiPSC characterization with pluripotent markers (method)	Astrocyte characterization	
					Mature astrocyte markers	**Functional analysis**
Perriot et al. 2018	RRMS (n=4) HC (n=3)	Erythroblasts (from PBMCs) (Total 13 lines)	Episomal plasmids (Lonza)	Oct4 shRNA-p53 SOX2 KLF4 L-Myc Lin28	GLAST S100-β GFAP	Glutamate uptake Ca^{2+} imaging
Ponath et al. 2018	RRMS (n=4) SPMS (n=2) HC (n=6)	Fibroblasts (Total 12 lines)	Tempo Bioscience reprogramming protocol	Oct4 Tra-1-80 Nanog SSEA4	GLAST GFAP	Glucose uptake Glutamate uptake
Kerkering et al. 2023	RRMS (n=3) PPMS (n=2) SPMS (n=1) HC (n=3)	PBMCs (Total 9 lines)	Sendai virus (Life Technologies)	Oct3/4 SOX2 cMyc KLF4	S100-β GFAP AQP4	Ca^{2+} imaging Glutamate uptake
Ghirotto et al. 2022	RRMS (n=3) HC (n=3)	PBMCs (Total 6 lines)	Episomal plasmids (Lonza—VPA-1003)	Oct3/4 SSEA4 SOX2	GFAP Vimentin S100-β AQP4	Glutamate uptake

Abbreviations: **MS** multiple sclerosis, **RRMS** relapsing remitting MS, **HC** healthy control, **PPMS** primary progressive MS, **SPMS** secondary progressive MS, **PBMC** peripheral blood mononuclear cell, **Oct4** octamer transcription factor 4, **shRNA-p53** short hairpin RNA-p53, **SOX2** sex determining region Y-box 2 , **KLF4** kruppel-like factor 4, **SSEA4** stage-specific embryonic antigen 4, **GLAST** glutamate aspartate transporter, **S100-β** calcium-binding protein beta, **GFAP** glial fibrillary acidic protein, **AQP4** aquaporin

5.3 MS-specific astrocyte phenotypes

All four articles studied the functions of astrocytes using hiPSC-derived astrocyte models, but they focused on different aspects and topics (Table 2). Astrocytes were activated with cytokines, and the changes in their functioning were examined during MS. While some studies concentrated on the inflammation itself (Perriot et al. 2018; Ponath et al. 2018), other focused on the interaction between astrocytes and neurons (Kerkering et al. 2023), and astrocytes' mitochondrial functions (Ghirotto et al. 2022)

Perriot et al. studied the effect of neuroinflammation on astrocytes. Reactive phenotype was induced with interleukin 6 (IL-6), interleukin 1-beta (IL-1β) and tumor necrosis factor alpha (TNF-α) which are three crucial cytokines in MS. IL-1β and TNF-α were also tested together. The effect of all these cytokines was studied at transcriptomic level. The most significant effect was seen with TNF-α, which upregulated 5001 and downregulated 5488 genes. 7% of the upregulation and 2% of the downregulation were shared with two other cytokines. IL-6 upregulated 209 and downregulated 250 genes of which 24% of the upregulated and 25% of the downregulated genes were shared with two other cytokines. IL-1β had the least notable effect with the upregulation of 481 and downregulation of 264 genes, but 55% of those were shared with TNF-α or IL-6. When stimulated with TNF-α and IL- β together, 1104 additional genes were modulated.

Next, Perriot et al. determined secretion profiles of the MS associated cytokines in reactive astrocytes. hiPSC-derived astrocytes were stimulated with IL-6, IL-1β, TNF-α and IL-1β+TNF-α for 5 days and their secretion was analyzed from the supernatant of astrocyte cultures. IL-1β and TNF-α displayed significant changes in astrocyte phenotypes although IL-1β was seen to be more potent activator secreting large amounts of IL-6 (mean 1301.8 pg/mL) and GM-CSF (mean 214.4 pg/mL) as well as some cytokines that are protective in MS. When stimulating with TNF-α alone, the secretion of GM-CSF (mean=335.6 pg/mL) was larger but also IL-1β (mean=4.3 pg/mL) secretion was significant. IL-6 triggered the secretion of cytokines but in smaller amounts. The largest effect was seen when co-stimulating with TNF-α and IL-1β which enhanced the production of both pro- and anti-inflammatory mediators. Although, there were no significant differences between the MS patients and healthy controls which may prove that the genetic risks that are associated with MS affects to the regulation of immune response, not to the cells in the CNS.

Ponath et al. used astrocyte models to investigate the effect of the known risk variant rs7665090^{G} which is located between MANBA and NFKB1 in the intergenic region. The variant is known to increase nuclear factor kappa B (NF-κB) signalling which is a key regulator of inflammatory immune response and cytokine production. (Fortune et al. 2022) MS hiPSC-derived astrocytes that carried the risk variant were exposed to interferon gamma (IFNγ), IL-1β and TNF-α which showed the increase of NF-κB expression and the activation of the NF-κB target genes, ICAM1, C3, IL-15, CXCL10 and CCL5. These results show that CNS cells must play an important role in the beginning of the MS.

Kerkering et al. compared benign MS (BMS) astrocytes and protective MS (PMS) astrocytes by exposing them to MS associated cytokines. Analyzation was done with the marker called non-phosphorylated neurofilament (SMI32) which is a typical marker for axonal damage. When treating with TNF-α+IL-17, neurite damage increased in both clinical groups. When BMS astrocytes were cultured with healthy control neurons, less axonal damage was displayed when comparing with PMS

astrocytes. In other words, BMS astrocytes were neuroprotective, but PMS and HC astrocytes were not. TNF-α+IL-17 treatment led to increased expression of neurotrophic factors via JAK/STAT pathway which have shown to be involved in the neuroprotective role of BMS astrocytes. Also, factors LIF, BDNF and TGF-β were found significantly higher concentrations in BMS supernatants. These factors have an important role in the maintenance of neurons.

Ghirotto et al. focused on mitochondrial function and alterations in the metabolism of MS astrocytes. MS hiPSC-derived astrocytes were activated with TNF-α for 48 hours and after that a PCR array was done to analyse if there would be changes in the genes that are related to mitochondrial function. Results indicated that control and MS hiPSC-derived astrocytes were distinctly different. The genes that are part of signalling pathways regulating neurodegeneration and mitochondrial dysfunction were altered in MS astrocytes. These alterations were seen to cause increased mitochondrial fission, higher superoxide levels and decreased mitochondrial DNA ratio in MS astrocytes. Those astrocytes also secreted an increased amount of pro-inflammatory chemokines which caused changes in inflammatory response. Metabolomics analysis showed a clear difference between the metabolic profiles of control and MS astrocytes.

Table 2. Activation of astrocytes and its impact on astrocyte phenotypes.

Reference	MS types & number of patients	Inducing reactive phenotype	Main findings
Perriot et al. 2018	RRMS (n=4) HC (n=3)	IL-6, IL-1β, TNF-α IL-1β +TNF-α	• RNAseq and secretome analysis showed that MS-associated cytokines induced specific transcriptome and secretome profiles • No significant differences between MS patients and controls
Ponath et al. 2018	RRMS (n=4) SPMS (n=2) HC (n=6)	TNF-α, IL-1β, IFN-γ	• MS lines carried either MS risk variant rs7665090^{G} or protective variant rs7665090^{A} associated to NFκB signalling • Risk variant was associated to upregulation of a subset of NF-kB target genes, like C3, IL-15, CCL5, CXCL10 • No differences were seen between MS patients and controls
Kerkering et al. 2023	RRMS (n=3) PPMS (n=2) SPMS (n=1) HC (n=3)	TNF-α+ IL-17A	• Cytokines effects on the astrocytes not shown, study focused on showing reactive astrocyte effect on neurons • Astrocytes activation with on TNF-α/IL-17A induced less neurite damage in RRMS derived astrocytes compared to progressive MS- and control-derived astrocytes
Ghirotto et al. 2022	RRMS (n=3) HC (n=3)	TNF-α	• In basal stage, MS-astrocytes showed less mitochondria content compared to control-astrocytes • Higher superoxide production and inflammatory cytokines and chemokines in MS vs. controls after TNF-α treatment • Decreased uptake and enhanced release of glutamate • Increased mitochondrial dysfunction • Increased sphingolipid metabolism and decreased amino acid catabolism

Abbreviations: **MS** multiple sclerosis, **RRMS** relapsing remitting MS, **HC** healthy control, **SPMS** secondary progressive MS, **PPMS** primary progressive MS, **IL** interleukin, **TNF** tumor necrosis factor, **IFN** interferon, **NFκB** nuclear factor-κB, **C3** complement factor 3, **CCL5** chemokine ligand 5, **CXCL10** C-X-C motif chemokine ligand

6. CONCLUSIONS

Astrocytes have been shown to have a strong contribution in MS pathology which has raised an interest to study their functioning further. Animal models are not sufficient for a complete understanding of the disease due to the complexity of a human body, and it isn't easy to translate animal study findings into humans. Rodent astrocytes have even shown opposite results compared to human astrocytes. That's why human-based models are needed. This thesis was done to understand astrocytes' role in MS with hiPSC-derived astrocytes obtained from MS patients.

To this day, four articles have analysed hiPSC-derived astrocytes that are obtained from MS patients. They investigated astrocytes' role in the development of MS and the effect of the disease on astrocytes. However, these articles ended up into very contradictory results. Perriot et al. suggested that MS alterations would be associated with the modifications of the immune response when Ponath et al. proposed that cells in the CNS have a very important role in the development of MS. In other words, Perriot et al. thought that MS is initiated outside of the CNS when Ponath et al. suggested that MS is originated from the changes of the cells in the CNS. There are some clinical parameters that may have had an impact on the results. Patients were having different types of MS and were not in the same stage of the disease which may have distorted the results. Perriot et al. obtained the samples from patients that were having their first relapse and hadn't got any treatments, which may be the reason for the lack of differences between control and MS astrocytes. Instead, Ponath et al. got their samples from the patients that were already receiving disease modifying treatments (DMT). Ghirotto et al. had patients who hadn't got any treatments but had a history of several relapses and Kerkering et al. had patients that hadn't been under DMT for the past 6 months.

Defining all the MS-associated risks is difficult due to the complexity of MS pathology. Mechanisms can't be fully known because of the combined effect of many genes that can be part of developing MS. Also, environmental factors have a crucial role in the initiation of MS which makes it much more complicated to study *in vitro*. Environmental factors were not taken into account in these studies which may have had an impact on the results. The research of MS hiPSC-derived astrocytes is in the very beginning of its way, and not much is known about the function of astrocytes in MS. Two of the four studies didn't find a significant differences between healthy and MS astrocytes. In the future it may be profitable to consider is it even worth to make MS hiPSC-derived astrocytes if there are no remarkable differences.

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