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Literature Review

Methodology of neuronal pluripotent stem cell of in vitro difference process as an alternative therapy of neurodegenerative disease

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Abstract
Neurodegenerative is diseases which occur with age but no therapeutic approach is efficient enough to inhibit neurodegenerative progression which results from aging of the cells themselves. PSC is a stem cell which has the potential to differentiate into various types of cells in the body. In the expansion process, PSC encountered problems, one of the common obstacles. This research was conducted using a literature study method that aims to obtain information about the appropriate differentiation method so that it can be used as an alternative treatment for neurodegenerative disease in the future. The result showed that 3D technique is neuronal differentiation technique that has a good output and can resemble the conditions of its development in vivo. The use of growth factors such as FGF2, PDGF, IGF1, T3, FGF, EGF, and CNTF can also overcome the problem of differentiation of several target cells in neurodegenerative therapy such as neurons, astrocytes, and oligodendrocytes.

Keywords: Neuronal stem cell; pluripotent stem cell; differentiation technique

Metodologi proses diferensiasi neuronal pluripotent stem cell in vitro sebagai alternatif terapi penyakit neurodegeneratif

Abstrak
Penyakit neurodegeneratif merupakan salah satu penyakit yang muncul seiring dengan bertambahnya usia, tetapi belum ada pendekatan efisien untuk menghambat kerusakan sel yang diakibatkan oleh sel itu sendiri. Pluripotent *Stem Cells* (PSC) merupakan salah satu alternatif pendekatan terapi untuk menghambat progresi neurodegeneratif, namun dalam proses ekspansinya PSC mengalami kendala pada teknik diferensiasinya yang dianggap kurang optimal. Hasil pencarian di PubMed, Reasearch Guide, Elsevier menemukan 500 artikel terkait *Pluripotent Stem Cells* (PSC). Dua puluh artikel memenuhi kriteria inklusi dan digunakan untuk literature review ini. Hasil review menunjukan bahwa teknik 3D merupakan teknik diferensiasi neuron yang memiliki output yang baik dan mampu menyerupai kondisi perkembangannya secara in vivo. Penggunaan faktor pertumbuhan seperti FGF2, PDGF, IGF1, T3, FGF, EGF dan CNTF juga dapat mengatasi permasalahan diferensiasi beberapa sel target dalam terapi neurodegeneratif seperti neuron, astrosit, serta oligodendrosit

Kata-kata Kunci : *Neuronal stem cell*; *pluripotent stem cell*; teknik diferensiasi

1. Introduction

Neurodegenerative disease is a disease that frequently appears with age. The development of this disease can also be induced by genetic factors, tumors, stroke, physical trauma, viral infections, and others. Several types of diseases that are grouped under this disease include Parkinson's disease, Huntington's disease, Alzheimer's disease, amyotrophic lateral disease, Friedreich's ataxia, and spinal muscular atrophy. This group of diseases is caused by a fundamental mechanism that causes changes in the structure, function, and death of neurons (Hung et al., 2010). Although several mechanisms related to this disease are identified, there is no pharmacological or surgical approach to neuronal

therapy that is efficient enough to inhibit neurodegenerative progression caused by cell aging itself (Sakthiswary & Raymond, 2012).

Stem cell technology is a new alternative that is being developed to regenerate neuron networks in the brain. One method that allows for this therapy is to use adult stem cells derived from neuronal progenitor cells, also known as adult Neuronal Stem Cells (NSC). However, the extraction of progenitor neurons from brain tissue is not accessible enough and carries a high enough risk that it creates problems in terms of bioethics (Yap et al., 2015).

Several researchers then developed alternative treatments using stem cell therapy to reduce the risk of taking NSCs from brain tissue. Pluripotent Stem Cell (PSC) is a type of stem cell that has the ability to differentiate into various types of cells. PSC is also a stem cell that was developed in the study of tissue regeneration, especially neuronal tissue. However, in the expansion process, NSC encountered several problems, one of which was the existing differentiation technique that was not optimal. Existing literature discusses the role of autophagy in PSC differentiation, advantages and disadvantages of traditional cell culture to 3D systems and their application to investigate neurodegenerative diseases (Aurélie de Rus Jacquet, 2021; Chang et al., 2018; Jimenez-Moreno et al., 2017; Slanzi et al., 2020). Recently, there is still a little research that focuses on the differentiation technique of PSC. These problems make this literature study necessary, so that it can provide alternative solutions in overcoming obstacles related to existing differentiation techniques.

2. Research Method

This study employed a descriptive analysis with a literature study method that focuses on the biochemical and physical induction treatment method of neuron cell differentiation as well as the main components that play a role in the differentiation process. Literature searches were performed through several websites and sites such as Researchgate, Google Scholar, and Elsevier. The article search process was conducted according to keywords, that were “pluripotent stem cells”, “neuronal stem cells”, “neurogenesis”, “neuron differentiation techniques”, “monolayer techniques”, “three-dimensional culture techniques”. The inclusion criteria for scientific articles used are: scientific articles and review articles with a time span from 2000 to 2020 which discuss the development of stem cell technology in neuron regeneration, pluripotent stem cells, neuronal stem cells, neurogenesis, neuron differentiation techniques, monolayer techniques, and three-dimensional culture techniques; Indonesian and English articles. The articles obtained from the search results are then analyzed and processed through the stages of organizing, synthesizing, identifying and further analysis to find answers to the formulation of the problem.

We identified 500 articles obtained from the search results and removed the same 35 articles. Selection of titles and abstracts was performed on the remaining 465 articles and obtained 78 articles that met the inclusion criteria for full text article selection. The results of the full text article selection are 20 articles which are complete and worthy of a critical appraisal. Seven articles were excluded after the critical appraisal so that there were 13 articles used in this literature review on **Table 1.** The article selection process can be seen on **Figure 1.**

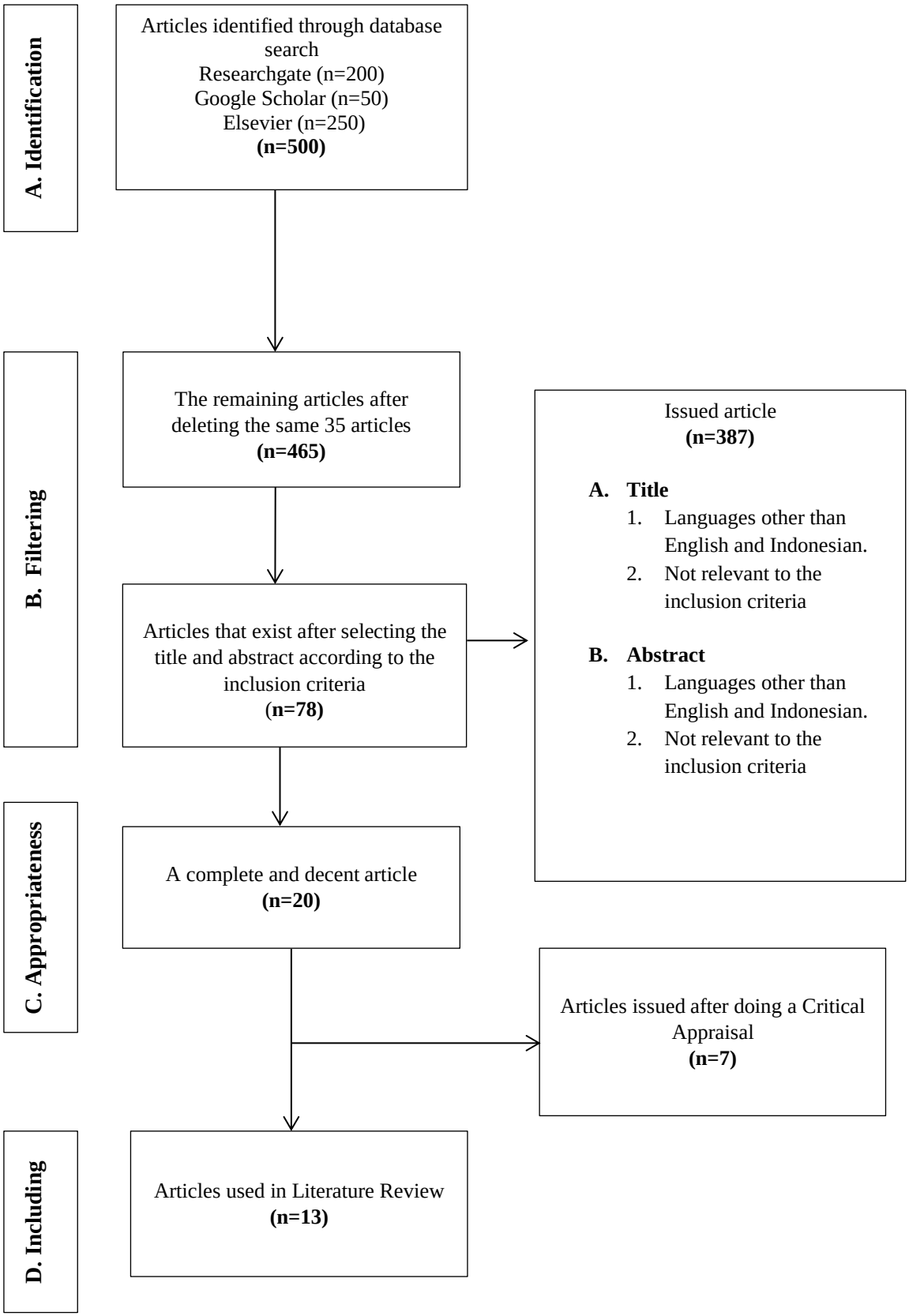

Figure 1. Article selection process

Table 1. Article on neuron differentiation technique

Author	Method	Neuron Cell Type	Result
(Shi et al., 2012)	Inhibition of SMAD	Neuron	It takes about 15 days to induce stem cells to become cortical stem cells or progenitor neurons.
(Chambers et al., 2009)	Inhibition of SMAD	Neuron	The formation of dopaminergic neurons is initiated by growth factor BDNF, ascorbic acid, sonic hedgehog (SHH) and FGF8 on N2 media.
(Kirkeby et al., 2012; Nolbrant et al., 2017)	Dual Inhibition of SMAD	Neuron	Producing high cell count in small scale
(Li et al., 2005)	Induction with RA, SHH and cAMP and Expansion with BDNF, GDNG and IFG1	Neuron	Neuron differentiation is characterized by the presence of columnar cells that form rosettes in cell colonies on days 8-10 after embryonic stem cells are removed from feeder cells.
(Krencik & Zhang, 2011)	EB	Astrocyte	The protocol was conducted by removing mitogens and adding CNTF for approximately 90 days. This method produces a dense population of astrocytes.
(Emdad et al., 2012)	EB	Astrocyte	Cell lines of hESC and hiPSC presented differentiation variability in producing astrocyte cells. Both of these cells show the same cell properties called migration and tropism towards Human high-grade gliomas (hHGG).
(Lafaille et al., 2012)	Inhibition of SMAD and EB	Astrocyte	The astrocyte cell differentiation process uses several growth factors such as EGF, FGF2.
(Serio et al., 2013)	Inhibition of SMAD dan EB	Astrocyte	The astrocyte cell differentiation process uses several growth factors such as EGF, LIF, FGF2 (suspension)
(Stacpoole et al., 2013)	EB	Oligodendrocyte	OPC cell differentiation into oligodendrocytes was conducted by adding growth factors SAG, PDGF, NT3, IGFI T3, cAMP
(Wang et al., 2013)	Inhibition of SMAD (Monolayer)	Oligodendrocyte	OPC cell differentiation into oligodendrocytes was performed by adding growth factors PDGF, IGFI, NT3, B27 BDNF.
(Douvaras et al., 2014)	Inhibition of SMAD, EB	Oligodendrocyte	OPC cell differentiation into oligodendrocytes was administered by adding growth factors Biotin, cAMP Insulin, T3, Biotin cAMP, AA
(Gorris et al., 2015)	Inhibition of	Oligodendrocyte	OPC cell differentiation into

Author	Method	Neuron Cell Type	Result
	SMAD		oligodendrocytes was performed by adding growth factors T3, AA, laminin

From several articles that have been analyzed in this literature review, it was found the characteristics of the articles, which were the technique of differentiation of neuron cells and types of neurons. Several cell differentiation methods focus on the differentiation process of neuron cells, astrocytes and oligodendrocytes. The technique used in the process of differentiation of neuron cells has many similarities, including using the SMAD inhibition method and the formation of EB. This method is considered effective in accelerating the differentiation process of target neuron cells and overcoming obstacles in the differentiation process. Meanwhile, neurons, astrocytes and oligodendrocytes are part of cells that play a role in the emergence of symptoms of neurodegenerative diseases when the cell's work system is disrupted. Hence, the differentiation protocol can be used in drug development models and detection of neurodegenerative diseases. There are 4 themes identified in this review, including: the basic concept of pluripotency; the process of developing neuron cells in the embryonic period; adaptation mechanisms of neurogenesis stages in PSC neuronal differentiation; and differentiation techniques.

2.1. The basic concept of pluripotency

Stem cells are cells that have not yet differentiated into specific functional body cells. Stem cells have the ability to proliferate and regenerate high and can also differentiate into somatic cells that form certain body tissues (Shirazi et al., 2012; Zakrzewski et al., 2019). Stem cells have 2 types of cell types, which are ESC and Adult Stem Cell (ASC). ESC is a type of stem cell that can differentiate into all types of cells that belong to 3 types of cell mass layers (Ectoderm, Mesoderm, and Endoderm) (Amira Ragab El Barky, 2017). ASC is a type of stem cell taken from adult tissue and has limited differentiation ability. Furthermore, the use of ASC as a source of stem cells can avoid ethical problems from using ESC (Ulrich et al., 2013). Stem cells have the ability to differentiate depending on the nature of the cell. Some of the characteristics of stem cells include totipotent, pluripotent, multipotent, and unipotent. Totipotent is the ability of cells to form completely new individuals, while pluripotency is the ability of stem cells to differentiate into cells in the ectoderm, mesoderm, and endoderm layers. The ability of stem cells will be limited if they are multipotent and unipotent because these stem cells can only differentiate into several types of cells (Larijani et al., 2012). According to PSC has the potential to become several types of somatic cells such as cardiomyocytes, smooth muscle cells, endothelial cells, neuron cells and hepatocytes cells (Doss & Sachinidis, 2019). This makes PSC an alternative therapy that can overcome degenerative diseases such as Parkinson's disease, Alzheimer's disease, diabetes, and bone injuries (Oh & Jang, 2019).

ESC and iPSC are two types of stem cells that are classified as PSC. Several ESCs can be generated from humans and animals. hESC is an embryonic stem cell produced from human cells. Embryonic stem cells can also be produced from animals and generally come from mice. These cells are known as Murine Embryonic Stem Cells (mESC). ESCs are pluripotent and are stem cells isolated from the Inner Cell Mass (ICM) structure in blastocysts in the early stages of embryonic formation which are about 4 to 5 days after fertilization (Liu et al., 2020).

iPSCs are PSCs made by genetic engineering through a reprogramming process by including pluripotent transcription factors (OCT4, SOX2, Klf4, and cMyc) so that they have the same characteristics as ESCs. iPSCs can be taken from animal or human cells. iPSCs taken from human cells are called hiPSCs. Generally, iPSCs taken from animals come from mouse cells, so these cells

are called Mouse Induced Pluripotent Stem Cells (MIPSC). In addition to using transcription factors, iPSCs can be obtained from somatic cells using the Somatic Cell Nuclear Transfer (SNCT) method, cell fusion, transcription factor transduction and small molecule induction (Singh et al., 2015). Moreover, iPSCs can be induced from fibroblast cells because of their easy access by simple biopsy of skin tissue. In addition to fibroblast tissue, iPSCs can also be generated from other cell types such as peripheral blood cells, keratinocytes, and epithelial cells (Zakrzewski et al., 2019).

2.2. The process of developing neuron cells in the embryonic period

The development of neurons in mammals is transient and begins as a collection of neuronal cells that proliferate and differentiate into more specific cell types (Ming & Song, 2011). Some of these cells are classified as Neuronal Stem Cells (NSC) which have the ability to produce NSC derivatives through asymmetric division. The process of asymmetric division can produce two stem cells that have different differentiation potentials. These NSCs can also differentiate into several types of brain cells such as neurons, astrocytes and oligodendrocytes. Under physiological conditions, the process of neurogenesis is confined to niche areas located in 2 parts of the brain. The first part is in the subventricular zone which is where new neurons start and migrate to the olfactory bulb, while the second part is the sub granular zone in the dentate gyrus and hippocampus (Feliciano et al., 2015).

The process of neurogenesis is a complex process and is divided into 6 stages (Kempermann, 2004). The first stage occurs 1 to 3 days after birth and is referred to as the proliferative phase. In this phase, NSCs can proliferate and differentiate into several cells, but NSCs cannot carry out self-renewal in their cells. The second to fourth stages last for 1 week after birth and are referred to as the differentiation phase. In this phase, NSCs exit the cell cycle and are ready to differentiate into a network of neuron cells. After this process occurs, the immature neurons enter phase 5 or what is known as the migration phase. This phase occurs for 2 to 3 weeks after birth. In the mitotic phase the neurons begin to elongate their axonal projections and dendritic growth begins. Stage 6 in the neurogenesis process takes place after 4 weeks of birth, in this phase the integration of new neuron synapses begins to appear (de la Torre-Ubieta & Bonni, 2011; Kristiansen & Ham, 2014).

Several studies have reported that several intrinsic factors are required in the regulation of neurogenesis (Avila et al., 2014; de la Torre-Ubieta & Bonni, 2011; Secondo et al., 2018; Stappert et al., 2018)**.** Some of these factors are transcription factors, epigenetics and metabolic pathways (Mira & Lie, 2017; Stappert et al., 2018). Epigenetic factors consist of DNA methylation, post-translational modifications in histones, and structural changes in chromatin. Several transcription factors that play a role in the process of neurogenesis, one of which comes from the Sox transcription factor group. Sox2 is a transcription factor that is often studied by several scientists. The presence of Sox2 expression in neurons and glial cells plays an important role in the formation of morphology and connectivity in neuronal cells (Mercurio et al., 2019). The role of metabolic pathways cannot be separated from the process of forming neurons in mammals. One of the metabolic pathways that play a role in the differentiation and degeneration of neurons is oxidative phosphorylation. This metabolic process can produce ATP which is needed in the process of forming neurons (Bourgognon et al., 2018).

2.3. Adaptation mechanisms of neurogenesis stages in PSC neuronal differentiation

The development of the neuronal system in vertebrates begins in the gastrula phase when the ectoderm tissue specializes into a network of neuron cells through a neural induction. This process can arise due to the presence of a cell from the ectoderm tissue that can release BMP inhibitor molecules and activate the Fibroblast Growth Factor (FGF) pathway (Tao & Zhang, 2016). The mechanism of neuron differentiation basically follows the principle of neuronal induction. There are several types of derivatives of neuron cells that are often used in the process of differentiation of neuron cells including Neuron Progenitor (NPC), neurons, astrocytes, and oligodendrocytes. Several

differentiation processes in neuronal cells are initiated by converting hPSCs into more specific neuronal cell types, which is Neural Ectoderm (NE) through embryoid body formation and SMAD inhibition. The second stage is to change the NE until the process of gliogenesis occurs. The process of gliogenesis will produce glial progenitor cells that express markers NF1A, S100b and CD44. The third step is to convert glial progenitor cells into astrocytes in the presence of Bone Morphogenic Protein (BMP) and Ciliary Neurotrophic Factor (CNTF). BMP and CNTF activate the STAT3 pathway so that glial progenitor cells can differentiate into astrocytes (Tao & Zhang, 2016).

2.3.1. *Neuron Progenitor Cell* (NPC)

NPC or progenitor neuron cell is one of the cells that can differentiate into neuron cells, astrocytes and oligodendrocytes. Several processes of differentiation of hPSC into NPC were conducted using monolayer induction and the formation of an embryo body in a suspension. In addition to these methods, several induction factors such as Retinoid Acid (RA), FGF-2, Epidermal Growth Factor (EGF) and Sonic Hedgehog (SHH) are also used in the NPC differentiation process (Li et al., 2014). Currently, synergistic induction using two inhibitors of SMAD signaling, that is noggin and SB431642 is an efficient method in the process of differentiation of NPCs derived from hPSC cultures (Gordeeva, 2019; Stover et al., 2013). SMAD is an intracellular protein that transduces extracellular signals from the TGF- ligand to the nucleus which can activate transcriptional genes for neuronal development. These NPC cells can later be differentiated into dopaminergic cells when induced with SHH and FGF8, while they will become motor neuron cells when induced using Brain-derived Neurotrophic Factor (BDNF), ascorbic acid, SHH and RA (Li et al., 2014).

2.3.2. Neuron differentiation

Neurons are the main cellular components that make up the neuron system in humans. These cells function to convey stimuli through signals in the form of electrical excitation that are delivered to and from the tissue. The development of the neuronal system is controlled by several signaling complexes. Before forming a specific type of cell type, there are several stages that must be passed. PSC including ESC and iPSC will first turn into ectoderm tissue. The tissue will then turn into neuroepithelium through the induction of FGF and WNT, then the neuroepithelial cells will multiply into neural plates. In the rostral neural plate or the forebrain, it is the first part to form after caudalization. This occurs due to the presence of a growth factor including WNT, BMP, FGF and RA (Dhara & Stice, 2008). Several parts formed due to the induction of these growth factors consist of the forebrain, midbrain, hindbrain and spinal cord. After the formation of a network of neurons in the rostro-caudal region is complete, the formation of the dorso-ventral portion of the neural tube is controlled by two types of signaling. BMP signaling dorsally from the roof plate and SHH signaling ventrally from the notochord. Cells located at specific locations along the rostro-caudal and dorso-ventral axial sections respond to specific morphogens and produce several types of specific neurons and glial cells**.** Many protocols have been developed in performing neuronal cell differentiation techniques, some of these protocols can be seen in **Table 2.**

Table 2. Neuron differentiation protocol

References	Duration (Emdad et al.)	Marker	Method	Growth Factor
(Pankratz et al., 2007; Zhang, 2011)	80	PAX6, OTX2, FOXG1	EB	FGF2, Insulin, Transferrin, Progesterone, Heparin
(Chambers et al., 2009)	28-35	PAX6, OTX2, FOXG1	Inhibition of SMAD (*Monolayer*)	FGF2, FGF8, BDNF, GDNF, TGFb1, cAMP
(Eiraku et al., 2008; Watanabe et al., 2005)	180	FOXG1, EMX1, Nestin	EB	BMP4, FGF2 (Suspension)
(Shi et al., 2012)	80	Tbr1 CTIP2	Inhibition of SMAD	N2, B27, FGF2
(Kirkeby et al., 2012; Nolbrant et al., 2017; Osborn et al., 2016)	40	TH, LMX1, FOXA2, EN1, MAP2	Dual Inhibition of SMAD	FGF8b, BDNF, AA GDNF, DAPT db-cAMP
(Li et al., 2005)	50	HB9, HoxC8, ChAT, VAChT	Induced with RA, SHH, and cAMP and expanded with BDNF, GDNG and IGF1.	FGF2, BDNF, GDNF, IGF1

2.3.3. Astrocytes

Astrocytes are cells that are generally found in the mammalian brain, these cells function to maintain homeostatic conditions in neuronal cells by maintaining ion balance, neurotransmitter turnover and the release of growth factors in the formation of cell membranes (McComish & Caldwell, 2018). Several studies reported that astrocytes are part of cells that play a role in the emergence of symptoms of neurodegenerative diseases, so astrocyte cell expansion was performed to determine the role of astrocytes in the occurrence of neurodegenerative diseases in cells (Liddelow et al., 2017; Lobsiger & Cleveland, 2007; Osborn et al., 2016). Several protocols have been performed to differentiate astrocytes, including taking into account the density of cells to be cultured, type of substrate, media composition, concentration of growth factors, morphogens and the dimensions of the culture (monolayer or embryoid body) (Chandrasekaran et al., 2016). One of the most important factors in the differentiation process is the presence of growth factors. Several growth factors that play a role in the astrocyte differentiation process include FGF-2, RA, cAMP, hIGF, and BDNF (Araki et

al., 2021; Carpenter et al., 2001). Several astrocyte differentiation protocols from PSC can be presented in **Table 3.**

Table 3. Astrocyte differentiation protocol

References	Duration (Day)	Marker	Method	Growth Factor
(Mormone et al., 2014)	28-35	GFAP, A2B6	EB	FGF2, EGF, (FGF+EGF+CNTH), Noggin
(Krencik & Zhang, 2011)	180	GFAP, S100β, CD44, NF1A	EB	EGF, FGF
(Emdad et al., 2012)	35	GFAP, A2B5, S100β	EB	N2, FGF, EGF, CNTF
(Gupta et al., 2012)	70	GFAP, S100β, EAAT1, EAAT2	EB	EGF, FGF2 Heparin
(Lafaille et al., 2012)	90	GFAP	Inhibition of SMAD and EB	EGF, FGF2
(Serio et al., 2013)	>70	Vimentin, NF1A, GFAP, S100β, EAAT1	Inhibition of SMAD and EB	EGF, LIF, FGF2 (Suspension)

2.3.4. Oligodendrocyte

Oligodendrocytes are myelination cells from the process of forming neuron cells. Disruption and damage to these cells will cause various neurodegenerative diseases such as Multiple Sclerosis (MS) and amyotrophic lateral sclerosis (Livesey et al., 2016). Like astrocytes, the presence or absence of oligodendrocytes in neuronal cells can cause degenerative disease symptoms, so the development of oligodendrocyte cells derived from iPSC is an alternative in detecting and knowing several disease mechanisms that occur in neurodegenerative diseases. According to Merten and colleagues, the myelination process will occur if there is sufficient oligodendrocyte implantation, so an effective differentiation protocol is needed in the study of myelination disorders that occur in MS and ALS. Several protocols have been developed to differentiate oligodendrocytes using the method of embryonic body formation, suspension culture and monolayer culture, while growth factors used include PDGF, IGF, and FGF (Ehrlich et al., 2017; Livesey et al., 2016; Wang et al., 2013). Several protocols in the process of oligodendrocyte differentiation can be presented in Fig **Table 4.**

Table 4. Protocols in the process of oligodendrocyte differentiation

Reference	Duration (day)	Marker	Method	Growth Factor		
				***Pre-Oligodendrocyte Progenitor Cell* (OPC)**	**Pre-OPC to OPC Transition**	**OPC to Oligodendrocyte**
(Keirstead et al., 2005)	42	OLIG, SOX10, A2B5, NG2, PDGFRα, GalC, RIP, O4	EB	EGF, FGF (Suspension)	EGF	-
(Izrael et al., 2007)	>45	OLIG 1/2, SOX10, NKX2.2, PDGFRα, O4	EB	EGF, FGF, Noggin	EGF, FGF, Noggin	Noggin
(Hu et al., 2009)	112	OLIG2, NKX2.2, PDGFRα, O4	EB with low oxygen levels	SHH, B27 RA, FGF2	PDGF, IGFI, NT3, T3	PDGF, IGFI, NT3
(Stacpoole et al., 2013)	100	OLIG2, NKX2.2, NG2, PDGFRα, O4	EB	SAG, FGF, RA (pMN, SAG, FGF	PDGF, T3, purmorpham ine/SAGRA (pMN), PDGF, FGF, purmorpham ine/ SAG, T3	SAG, PDGF, NT3, IGFI T3, cAMP
(Wang et al., 2013)	140	OLIG2, SOX10, NKX2.2, MBP PDGFRa, O4	Inhibitio n of SMAD (*Monola yer*)	RA, B27, FGF, Purmorphamine	PDGF, IGFI, T3, NT3, purmorpham ine	PDGF, IGFI, NT3, B27 BDNF
(Douvaras et al., 2014)	95	OLIG2, SOX10, NKX2.2, MBP, O4	Inhibitio n of SMAD and EB	RA, SAG	PDGF, HGF, IGFI, NT3, Insulin, T3 Biotin, cAMP	Insulin, T3, Biotin cAMP, AA

Reference	Duration (day)	Marker	Method	Growth Factor		
				***Pre-Oligodendrocyte Progenitor Cell* (OPC)**	**Pre-OPC to OPC Transition**	**OPC to Oligodendrocyte**
(Gorris et al., 2015)	>90	OLIG 1/2, NKX6.2, NKX2.2, NG2, MBP SOX1	Inhibition of SMAD and EB	PDGF, EGF, forskolin	PDGF, T3, AA, Noggin	T3, AA, laminin
(Jinghua Piao, 2015)	70	OLIG2, NKX2, SOX10, O1, MBP, O4	EB	purmorphamine, AA, BDNF, FGF8	PDGF, IGFI, T3, cAMP	BDNF, AA, T3, cAMP

There are several growth factors that play a significant role in the PSC differentiation process into several types of derivatives including FGF2, PDGF, IGF1, T3, FGF, EGF and CNTF. These growth factors play a role in the process of forming neurons, astrocytes and oligodendrocytes. In general, the function of growth factors is to increase the proliferation of neurons and other central nervous cells. Growth factors also have other functions such as wound healing angiogenesis, increased expression of NMDA receptors and antioxidant enzymes such as SOD or GSH reductase (Cabezas et al., 2016).

2.4. Differentiation technique

2.4.1. Adherent culture/monolayer

The 2D culture method is performed by culturing cells on a plate containing a substrate. The substrate consists of Extracellular Matrix (ECM) which can increase the proliferation, adhesion, and differentiation of a desired cultured cell. Some of the substrates commonly used in stem cell culture include laminin, poly-ornithine, poly-lysine and fibronectin (Hopkins et al., 2013). These substrates function to increase cell adhesion through integrin receptors, contribute to NSC differentiation, facilitate cell attachment through electrostatic attraction on the cell surface, coordinate synaptic activity and synaptogenesis, and regulate neural cell migration (Centeno et al., 2018). The purpose of doing this monolayer culture is to determine the biological components of stem cells and provide a source of cell therapy in regeneration medicine. Stem cell expansion in a short time is also performed using monolayer culture to produce several types of neuron cells and also the neurosphere (Kim et al., 2013; Rosenzweig et al., 2018)

2.4.2. 3D culture

In 3-dimensional culture, it is generally divided into two types of techniques, which are Scaffold-free and Scaffold-based. Scaffold-free includes spheroids, embryonic bodies, neurospheres, organoids and microtissues that can be cultured by growing cells in suspension culture, microwells, gel matrices and a variety of other methods (Ko & Frampton, 2016). This technique does not require the addition of an Extracellular Matrix (ECM) because once the cells have reached sufficient density, they can produce ECM by themselves. It is a scaffold-free culture strategy in forming a 3-dimensional

structure. One of them is by using a combination of chondrocytes and growth factors given at the culture site (Miyazaki et al., 2010).

Although monolayer culture is still being conducted today, there are some drawbacks in expanding using this method. Some of them are monolayer cultures that cannot represent the actual state of the brain organs and have limited interactions between cells. The limitations of cell culture using the monolayer method can affect cell morphology, the level of cell resistance, differentiation and proliferation processes so that a more complex expansion model is needed and can represent the situation in vivo, one of which is by using 3-dimensional culture (Antoni et al., 2015).

Currently, there is one type of differentiation technique that is being developed, that is using a 3-dimensional organoid. Organoid is a differentiation technique that belongs to the scaffold-free technique. Organoid is a mass of tissue consisting of several types of cell types that are organized spontaneously (Simunovic & Brivanlou, 2017). Most organoids are spherical and irregular in shape in suspension or in several different types of matrices (Lou & Leung, 2018). Organoids can be generated from iPSCs and ASCs by mimicking the chemical and physical substances of the developed target tissue and their homeostatic conditions (Lancaster & Knoblich, 2014). In the process of organoid formation, several growth factors are required so that these cells can differentiate and undergo self-renewal. These growth factors play a role in the process of cell defense, proliferation of self-renewal and in the process of differentiation into more specific cells (Yin et al., 2016). Organoids are considered to be a model system that has system stability and can also represent physiological conditions in vivo (Yin et al., 2016). Several studies have also reported that cerebral organoids have been used to study early stages of brain development and disorders of neuronal development. Therefore, organoids are considered to have a better picture in the process of modeling the central nervous system compared to using a 2-dimensional culture model (Lee et al., 2016; Yin et al., 2016).

Scaffold-based technique is a differentiation technique that requires the addition of an external biomaterial to produce several types of neural cells. The matrix used in this differentiation technique usually has a loose structure so that oxygen and nutrients can be absorbed into the cell. Some of the synthetic biomaterials used in this technique include polycaprolactone, polyethylene glycol and polystyrene (Lou & Leung, 2018; Oliva et al., 2019). Another method of scaffold-based technique is the addition of ECM such as laminin and collagen to neural cell cultures. ECM in this technique functions to increase tissue regeneration, structure, rigidity and elasticity (Alghuwainem et al., 2019). Another scaffold-based technique that is applied in the process of neural differentiation is Self-assembling peptides (SAPS). This technique is one of the methods used in forming 3D scaffolds to increase the proliferation and differentiation of NSC (Cunha et al., 2011). Studies in the use of 3D scaffolds from the synthesis of collagen and hyaluronic acid are also considered as an efficient method in the process of transdifferentiating mesenchymal stem cells into neuronal cells and glial cells. This method is implemented by inducing stiffness and porosity of the 3-dimensional structure of the scaffold (Her et al., 2013).

In the application of differentiation techniques, both techniques have advantages and limitations. The advantages and limitations of 2-dimensional and 3-dimensional differentiation techniques can be seen in **Table 5.**

Table 5. Advantages and limitations of 2D and 3D differentiation techniques

	2D	3D
Advantages	Easy to apply	Has more complex cell interactions
	Does not cost a lot	Has interactions between cells and ECM
	Homogeneous culture	Has a higher complexity and is relevant in in vivo modeling
	Easy to apply in downstream applications and cell visualization.	Requires high cost
Limitations	Unable to represent the physiological environment in vivo	Slightly difficult to visualize using a microscope and other visualization methods
	Limited interactions between cultured cells	Slightly difficult to distribute homogeneous components (oxygen and nutrients) so that it will lead to the process of necrosis, heterogeneity and death.
	Limited interaction between cells and ECM.	Requires special equipment and expert handling
	Can cause changes in morphology and gene expression.	Scaffold-based systems must consider several things such as biodegradability, pore size, and chemical composition of the substance.

3. Conclusion

In the development of stem cells in neuronal regenerative therapy, a good differentiation technique is needed. In vitro, 2D and 3D culture techniques are differentiation techniques that are currently used in general. The 3D technique is a technique that is known to have good output in neuronal differentiation because it is able to provide a good artificial microenvironment of cells and can mimic the conditions of their development in vivo. The use of growth factors such as FGF2, PDGF, IGF1, T3, FGF, EGF and CNTF can also overcome the problem of differentiation of several target cells in neurodegenerative therapy such as neurons, astrocytes, and oligodendrocytes. Further research is required on efficient methods and protocols in performing the NSC differentiation process. The existence of this protocol is expected to be applied in many countries, especially in Indonesia so that it can overcome problems in implementing NSC differentiation culture techniques.

References

Alghuwainem, A., Alshareeda, A. T., & Alsowayan, B. (2019, Oct 4). Scaffold-Free 3-D Cell Sheet Technique Bridges the Gap between 2-D Cell Culture and Animal Models. *Int J Mol Sci, 20*(19). https://doi.org/10.3390/ijms20194926

Amira Ragab El Barky, E. A., Tarek M Mohamed. (2017). Stem Cells, Classifications and their Clinical Applications. . *Stem cells and development*. https://www.researchgate.net/publication/319277041_Stem_Cells_Classifications_and_their_Clinical_Applications

Antoni, D., Burckel, H., Josset, E., & Noel, G. (2015, Mar 11). Three-dimensional cell culture: a breakthrough in vivo. *Int J Mol Sci, 16*(3), 5517-5527. https://doi.org/10.3390/ijms16035517

Araki, T., Ikegaya, Y., & Koyama, R. (2021, Sep). The effects of microglia- and astrocyte-derived factors on neurogenesis in health and disease. *Eur J Neurosci, 54*(5), 5880-5901. https://doi.org/10.1111/ejn.14969

Aurélie de Rus Jacquet, H. L. D., Francesca Cicchetti, Melanie Alpaugh. (2021). Current and future applications of induced pluripotent stem cell-based models to study pathological proteins in neurodegenerative disorders. *Molecular Psychiatry, 26, pages2685–2706*. https://www.nature.com/articles/s41380-020-00999-7

Avila, A., Vidal, P. M., Tielens, S., Morelli, G., Laguesse, S., Harvey, R. J., Rigo, J. M., & Nguyen, L. (2014, Nov). Glycine receptors control the generation of projection neurons in the developing cerebral cortex. *Cell Death Differ, 21*(11), 1696-1708. https://doi.org/10.1038/cdd.2014.75

Bourgognon, J. M., Spiers, J. G., Scheiblich, H., Antonov, A., Bradley, S. J., Tobin, A. B., & Steinert, J. R. (2018, Aug). Alterations in neuronal metabolism contribute to the pathogenesis of prion disease. *Cell Death Differ, 25*(8), 1408-1425. https://doi.org/10.1038/s41418-018-0148-x

Cabezas, R., Avila-Rodriguez, M., Vega-Vela, N. E., Echeverria, V., Gonzalez, J., Hidalgo, O. A., Santos, A. B., Aliev, G., & Barreto, G. E. (2016). Growth Factors and Astrocytes Metabolism: Possible Roles for Platelet Derived Growth Factor. *Med Chem, 12*(3), 204-210. https://doi.org/10.2174/1573406411666151019120444

Carpenter, M. K., Inokuma, M. S., Denham, J., Mujtaba, T., Chiu, C. P., & Rao, M. S. (2001, Dec). Enrichment of neurons and neural precursors from human embryonic stem cells. *Exp Neurol, 172*(2), 383-397. https://doi.org/10.1006/exnr.2001.7832

Centeno, E. G. Z., Cimarosti, H., & Bithell, A. (2018, May 22). 2D versus 3D human induced pluripotent stem cell-derived cultures for neurodegenerative disease modelling. *Mol Neurodegener, 13*(1), 27. https://doi.org/10.1186/s13024-018-0258-4

Chambers, S. M., Fasano, C. A., Papapetrou, E. P., Tomishima, M., Sadelain, M., & Studer, L. (2009, Mar). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol, 27*(3), 275-280. https://doi.org/10.1038/nbt.1529

Chandrasekaran, A., Avci, H. X., Leist, M., Kobolak, J., & Dinnyes, A. (2016). Astrocyte Differentiation of Human Pluripotent Stem Cells: New Tools for Neurological Disorder Research. *Front Cell Neurosci, 10*, 215. https://doi.org/10.3389/fncel.2016.00215

Chang, C. Y., Ting, H. C., Liu, C. A., Su, H. L., Chiou, T. W., Harn, H. J., & Lin, S. Z. (2018, Nov). Induced Pluripotent Stem Cells: A Powerful Neurodegenerative Disease Modeling Tool for Mechanism Study and Drug Discovery. *Cell Transplant, 27*(11), 1588-1602. https://doi.org/10.1177/0963689718775406

Cunha, C., Panseri, S., & Antonini, S. (2011, Feb). Emerging nanotechnology approaches in tissue engineering for peripheral nerve regeneration. *Nanomedicine, 7*(1), 50-59. https://doi.org/10.1016/j.nano.2010.07.004

de la Torre-Ubieta, L., & Bonni, A. (2011, Oct 6). Transcriptional regulation of neuronal polarity and morphogenesis in the mammalian brain. *Neuron, 72*(1), 22-40. https://doi.org/10.1016/j.neuron.2011.09.018

Dhara, S. K., & Stice, S. L. (2008, Oct 15). Neural differentiation of human embryonic stem cells. *J Cell Biochem, 105*(3), 633-640. https://doi.org/10.1002/jcb.21891

Doss, M. X., & Sachinidis, A. (2019, Apr 30). Current Challenges of iPSC-Based Disease Modeling and Therapeutic Implications. *Cells, 8*(5). https://doi.org/10.3390/cells8050403

Douvaras, P., Wang, J., Zimmer, M., Hanchuk, S., O'Bara, M. A., Sadiq, S., Sim, F. J., Goldman, J., & Fossati, V. (2014, Aug 12). Efficient generation of myelinating oligodendrocytes from

primary progressive multiple sclerosis patients by induced pluripotent stem cells. *Stem Cell Reports, 3*(2), 250-259. https://doi.org/10.1016/j.stemcr.2014.06.012

Ehrlich, M., Mozafari, S., Glatza, M., Starost, L., Velychko, S., Hallmann, A. L., Cui, Q. L., Schambach, A., Kim, K. P., Bachelin, C., Marteyn, A., Hargus, G., Johnson, R. M., Antel, J., Sterneckert, J., Zaehres, H., Scholer, H. R., Baron-Van Evercooren, A., & Kuhlmann, T. (2017, Mar 14). Rapid and efficient generation of oligodendrocytes from human induced pluripotent stem cells using transcription factors. *Proc Natl Acad Sci U S A, 114*(11), E2243-E2252. https://doi.org/10.1073/pnas.1614412114

Eiraku, M., Watanabe, K., Matsuo-Takasaki, M., Kawada, M., Yonemura, S., Matsumura, M., Wataya, T., Nishiyama, A., Muguruma, K., & Sasai, Y. (2008, Nov 6). Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. *Cell Stem Cell, 3*(5), 519-532. https://doi.org/10.1016/j.stem.2008.09.002

Emdad, L., D'Souza, S. L., Kothari, H. P., Qadeer, Z. A., & Germano, I. M. (2012, Feb 10). Efficient differentiation of human embryonic and induced pluripotent stem cells into functional astrocytes. *Stem Cells Dev, 21*(3), 404-410. https://doi.org/10.1089/scd.2010.0560

Feliciano, D. M., Bordey, A., & Bonfanti, L. (2015, Sep 18). Noncanonical Sites of Adult Neurogenesis in the Mammalian Brain. *Cold Spring Harb Perspect Biol, 7*(10), a018846. https://doi.org/10.1101/cshperspect.a018846

Gordeeva, O. (2019, Nov 23). TGFbeta Family Signaling Pathways in Pluripotent and Teratocarcinoma Stem Cells' Fate Decisions: Balancing Between Self-Renewal, Differentiation, and Cancer. *Cells, 8*(12). https://doi.org/10.3390/cells8121500

Gorris, R., Fischer, J., Erwes, K. L., Kesavan, J., Peterson, D. A., Alexander, M., Nothen, M. M., Peitz, M., Quandel, T., Karus, M., & Brustle, O. (2015, Dec). Pluripotent stem cell-derived radial glia-like cells as stable intermediate for efficient generation of human oligodendrocytes. *Glia, 63*(12), 2152-2167. https://doi.org/10.1002/glia.22882

Gupta, P. K., Das, A. K., Chullikana, A., & Majumdar, A. S. (2012, Jul 9). Mesenchymal stem cells for cartilage repair in osteoarthritis. *Stem Cell Res Ther, 3*(4), 25. https://doi.org/10.1186/scrt116

Her, G. J., Wu, H. C., Chen, M. H., Chen, M. Y., Chang, S. C., & Wang, T. W. (2013, Feb). Control of three-dimensional substrate stiffness to manipulate mesenchymal stem cell fate toward neuronal or glial lineages. *Acta Biomater, 9*(2), 5170-5180. https://doi.org/10.1016/j.actbio.2012.10.012

Hopkins, A. M., De Laporte, L., Tortelli, F., Spedden, E., Staii, C., Atherton, T. J., Hubbell, J. A., & Kaplan, D. L. (2013). Silk Hydrogels as Soft Substrates for Neural Tissue Engineering. *Advanced Functional Materials, 23*(41), 5140-5149. https://doi.org/10.1002/adfm.201300435

Hu, B. Y., Du, Z. W., & Zhang, S. C. (2009). Differentiation of human oligodendrocytes from pluripotent stem cells. *Nat Protoc, 4*(11), 1614-1622. https://doi.org/10.1038/nprot.2009.186

Hung, C. W., Liou, Y. J., Lu, S. W., Tseng, L. M., Kao, C. L., Chen, S. J., Chiou, S. H., & Chang, C. J. (2010, May 5). Stem cell-based neuroprotective and neurorestorative strategies. *Int J Mol Sci, 11*(5), 2039-2055. https://doi.org/10.3390/ijms11052039

Izrael, M., Zhang, P., Kaufman, R., Shinder, V., Ella, R., Amit, M., Itskovitz-Eldor, J., Chebath, J., & Revel, M. (2007, Mar). Human oligodendrocytes derived from embryonic stem cells: Effect of noggin on phenotypic differentiation in vitro and on myelination in vivo. *Mol Cell Neurosci, 34*(3), 310-323. https://doi.org/10.1016/j.mcn.2006.11.008

Jimenez-Moreno, N., Stathakos, P., Caldwell, M. A., & Lane, J. D. (2017, Aug 11). Induced Pluripotent Stem Cell Neuronal Models for the Study of Autophagy Pathways in Human Neurodegenerative Disease. *Cells, 6*(3). https://doi.org/10.3390/cells6030024

Jinghua Piao, T. M., Gordon Auyeung, Edelweiss Policarpio, Jayanthi Menon, Leif Droms, Philip Gutin, Kunihiro Uryu, Jason Tchieu, Denis Soulet, Viviane Tabar. (2015). Human Embryonic Stem Cell-Derived Oligodendrocyte Progenitors Remyelinate the Brain and Rescue Behavioral Deficits following Radiation. *Cell Stem Cell, Pages 198-210*. https://www.sciencedirect.com/science/article/pii/S1934590915000053https://www.sciencedirect.com/science/article/pii/S1934590915000053

Keirstead, H. S., Nistor, G., Bernal, G., Totoiu, M., Cloutier, F., Sharp, K., & Steward, O. (2005, May 11). Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. *J Neurosci, 25*(19), 4694-4705. https://doi.org/10.1523/JNEUROSCI.0311-05.2005

Kempermann, G., Jessberger, S., Steiner, B., & Kronenberg, G. (2004). Milestones of neuronal development in the adult hippocampus. *Trends in Neurosciences, 27(8), 447–452*. https://doi.org/10.1016/j.tins.2004.05.013

Kim, C., Ho, D. H., Suk, J. E., You, S., Michael, S., Kang, J., Joong Lee, S., Masliah, E., Hwang, D., Lee, H. J., & Lee, S. J. (2013). Neuron-released oligomeric alpha-synuclein is an endogenous agonist of TLR2 for paracrine activation of microglia. *Nat Commun, 4*, 1562. https://doi.org/10.1038/ncomms2534

Kirkeby, A., Nelander, J., & Parmar, M. (2012). Generating regionalized neuronal cells from pluripotency, a step-by-step protocol. *Front Cell Neurosci, 6*, 64. https://doi.org/10.3389/fncel.2012.00064

Ko, K. R., & Frampton, J. P. (2016, Jul). Developments in 3D neural cell culture models: the future of neurotherapeutics testing? *Expert Rev Neurother, 16*(7), 739-741. https://doi.org/10.1586/14737175.2016.1166053

Krencik, R., & Zhang, S. C. (2011, Oct 13). Directed differentiation of functional astroglial subtypes from human pluripotent stem cells. *Nat Protoc, 6*(11), 1710-1717. https://doi.org/10.1038/nprot.2011.405

Kristiansen, M., & Ham, J. (2014, Jul). Programmed cell death during neuronal development: the sympathetic neuron model. *Cell Death Differ, 21*(7), 1025-1035. https://doi.org/10.1038/cdd.2014.47

Lafaille, F. G., Pessach, I. M., Zhang, S. Y., Ciancanelli, M. J., Herman, M., Abhyankar, A., Ying, S. W., Keros, S., Goldstein, P. A., Mostoslavsky, G., Ordovas-Montanes, J., Jouanguy, E., Plancoulaine, S., Tu, E., Elkabetz, Y., Al-Muhsen, S., Tardieu, M., Schlaeger, T. M., Daley, G. Q., Abel, L., Casanova, J. L., Studer, L., & Notarangelo, L. D. (2012, Nov 29). Impaired intrinsic immunity to HSV-1 in human iPSC-derived TLR3-deficient CNS cells. *Nature, 491*(7426), 769-773. https://doi.org/10.1038/nature11583

Lancaster, M. A., & Knoblich, J. A. (2014, Jul 18). Organogenesis in a dish: modeling development and disease using organoid technologies. *Science, 345*(6194), 1247125. https://doi.org/10.1126/science.1247125

Larijani, B., Esfahani, E. N., Amini, P., Nikbin, B., Alimoghaddam, K., Amiri, S., Malekzadeh, R., Yazdi, N. M., Ghodsi, M., Dowlati, Y., Sahraian, M. A., & Ghavamzadeh, A. (2012). Stem cell therapy in treatment of different diseases. *Acta Med Iran, 50*(2), 79-96. https://www.ncbi.nlm.nih.gov/pubmed/22359076

Lee, H. K., Velazquez Sanchez, C., Chen, M., Morin, P. J., Wells, J. M., Hanlon, E. B., & Xia, W. (2016). Three Dimensional Human Neuro-Spheroid Model of Alzheimer's Disease Based on

Differentiated Induced Pluripotent Stem Cells. *PLOS ONE, 11*(9), e0163072. https://doi.org/10.1371/journal.pone.0163072

Li, X. J., Du, Z. W., Zarnowska, E. D., Pankratz, M., Hansen, L. O., Pearce, R. A., & Zhang, S. C. (2005, Feb). Specification of motoneurons from human embryonic stem cells. *Nat Biotechnol, 23*(2), 215-221. https://doi.org/10.1038/nbt1063

Li, Y., Liu, M., Yan, Y., & Yang, S. T. (2014, Jan 26). Neural differentiation from pluripotent stem cells: The role of natural and synthetic extracellular matrix. *World J Stem Cells, 6*(1), 11-23. https://doi.org/10.4252/wjsc.v6.i1.11

Liddelow, S. A., Guttenplan, K. A., Clarke, L. E., Bennett, F. C., Bohlen, C. J., Schirmer, L., Bennett, M. L., Munch, A. E., Chung, W. S., Peterson, T. C., Wilton, D. K., Frouin, A., Napier, B. A., Panicker, N., Kumar, M., Buckwalter, M. S., Rowitch, D. H., Dawson, V. L., Dawson, T. M., Stevens, B., & Barres, B. A. (2017, Jan 26). Neurotoxic reactive astrocytes are induced by activated microglia. *Nature, 541*(7638), 481-487. https://doi.org/10.1038/nature21029

Liu, G., David, B. T., Trawczynski, M., & Fessler, R. G. (2020, Feb). Advances in Pluripotent Stem Cells: History, Mechanisms, Technologies, and Applications. *Stem Cell Rev Rep, 16*(1), 3-32. https://doi.org/10.1007/s12015-019-09935-x

Livesey, M. R., Magnani, D., Cleary, E. M., Vasistha, N. A., James, O. T., Selvaraj, B. T., Burr, K., Story, D., Shaw, C. E., Kind, P. C., Hardingham, G. E., Wyllie, D. J., & Chandran, S. (2016, Apr). Maturation and electrophysiological properties of human pluripotent stem cell-derived oligodendrocytes. *Stem Cells, 34*(4), 1040-1053. https://doi.org/10.1002/stem.2273

Lobsiger, C. S., & Cleveland, D. W. (2007, Nov). Glial cells as intrinsic components of non-cell-autonomous neurodegenerative disease. *Nat Neurosci, 10*(11), 1355-1360. https://doi.org/10.1038/nn1988

Lou, Y. R., & Leung, A. W. (2018, Jan - Feb). Next generation organoids for biomedical research and applications. *Biotechnol Adv, 36*(1), 132-149. https://doi.org/10.1016/j.biotechadv.2017.10.005

McComish, S. F., & Caldwell, M. A. (2018, Jul 5). Generation of defined neural populations from pluripotent stem cells. *Philos Trans R Soc Lond B Biol Sci, 373*(1750). https://doi.org/10.1098/rstb.2017.0214

Mercurio, S., Serra, L., & Nicolis, S. K. (2019, Sep 13). More than just Stem Cells: Functional Roles of the Transcription Factor Sox2 in Differentiated Glia and Neurons. *Int J Mol Sci, 20*(18). https://doi.org/10.3390/ijms20184540

Ming, G. L., & Song, H. (2011, May 26). Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron, 70*(4), 687-702. https://doi.org/10.1016/j.neuron.2011.05.001

Mira, H., & Lie, D. C. (2017, Nov 9). Regulation of Adult Neurogenesis 2.0 - Beyond Signaling Pathways and Transcriptional Regulators. *Brain Plast, 3*(1), 1-3. https://doi.org/10.3233/BPL-179001

Miyazaki, T., Miyauchi, S., Matsuzaka, S., Yamagishi, C., & Kobayashi, K. (2010, May). Formation of proteoglycan and collagen-rich scaffold-free stiff cartilaginous tissue using two-step culture methods with combinations of growth factors. *Tissue Eng Part A, 16*(5), 1575-1584. https://doi.org/10.1089/ten.TEA.2009.0443

Mormone, E., D'Sousa, S., Alexeeva, V., Bederson, M. M., & Germano, I. M. (2014, Nov 1). "Footprint-free" human induced pluripotent stem cell-derived astrocytes for in vivo cell-based therapy. *Stem Cells Dev, 23*(21), 2626-2636. https://doi.org/10.1089/scd.2014.0151

Nolbrant, S., Heuer, A., Parmar, M., & Kirkeby, A. (2017, Sep). Generation of high-purity human ventral midbrain dopaminergic progenitors for in vitro maturation and intracerebral transplantation. *Nat Protoc, 12*(9), 1962-1979. https://doi.org/10.1038/nprot.2017.078

Oh, Y., & Jang, J. (2019, Mar 31). Directed Differentiation of Pluripotent Stem Cells by Transcription Factors. *Mol Cells, 42*(3), 200-209. https://doi.org/10.14348/molcells.2019.2439

Oliva, J., Florentino, A., Bardag-Gorce, F., & Niihara, Y. (2019, Mar). Engineering, differentiation and harvesting of human adipose-derived stem cell multilayer cell sheets. *Regen Med, 14*(3), 151-163. https://doi.org/10.2217/rme-2018-0053

Osborn, L. M., Kamphuis, W., Wadman, W. J., & Hol, E. M. (2016, Sep). Astrogliosis: An integral player in the pathogenesis of Alzheimer's disease. *Prog Neurobiol, 144*, 121-141. https://doi.org/10.1016/j.pneurobio.2016.01.001

Pankratz, M. T., Li, X. J., Lavaute, T. M., Lyons, E. A., Chen, X., & Zhang, S. C. (2007, Jun). Directed neural differentiation of human embryonic stem cells via an obligated primitive anterior stage. *Stem Cells, 25*(6), 1511-1520. https://doi.org/10.1634/stemcells.2006-0707

Rosenzweig, E. S., Brock, J. H., Lu, P., Kumamaru, H., Salegio, E. A., Kadoya, K., Weber, J. L., Liang, J. J., Moseanko, R., Hawbecker, S., Huie, J. R., Havton, L. A., Nout-Lomas, Y. S., Ferguson, A. R., Beattie, M. S., Bresnahan, J. C., & Tuszynski, M. H. (2018, May). Restorative effects of human neural stem cell grafts on the primate spinal cord. *Nat Med, 24*(4), 484-490. https://doi.org/10.1038/nm.4502

Sakthiswary, R., & Raymond, A. A. (2012, Aug 15). Stem cell therapy in neurodegenerative diseases: From principles to practice. *Neural Regen Res, 7*(23), 1822-1831. https://doi.org/10.3969/j.issn.1673-5374.2012.23.009

Secondo, A., Esposito, A., Petrozziello, T., Boscia, F., Molinaro, P., Tedeschi, V., Pannaccione, A., Ciccone, R., Guida, N., Di Renzo, G., & Annunziato, L. (2018, Dec). Na(+)/Ca(2+) exchanger 1 on nuclear envelope controls PTEN/Akt pathway via nucleoplasmic Ca(2+) regulation during neuronal differentiation. *Cell Death Discov, 4*, 12. https://doi.org/10.1038/s41420-017-0018-1

Serio, A., Bilican, B., Barmada, S. J., Ando, D. M., Zhao, C., Siller, R., Burr, K., Haghi, G., Story, D., Nishimura, A. L., Carrasco, M. A., Phatnani, H. P., Shum, C., Wilmut, I., Maniatis, T., Shaw, C. E., Finkbeiner, S., & Chandran, S. (2013, Mar 19). Astrocyte pathology and the absence of non-cell autonomy in an induced pluripotent stem cell model of TDP-43 proteinopathy. *Proc Natl Acad Sci U S A, 110*(12), 4697-4702. https://doi.org/10.1073/pnas.1300398110

Shi, Y., Kirwan, P., & Livesey, F. J. (2012, Oct). Directed differentiation of human pluripotent stem cells to cerebral cortex neurons and neural networks. *Nat Protoc, 7*(10), 1836-1846. https://doi.org/10.1038/nprot.2012.116

Shirazi, R., Zarnani, A. H., Soleimani, M., Abdolvahabi, M. A., Nayernia, K., & Ragerdi Kashani, I. (2012). BMP4 can generate primordial germ cells from bone-marrow-derived pluripotent stem cells. *Cell Biol Int, 36*(12), 1185-1193. https://doi.org/10.1042/CBI20110651

Simunovic, M., & Brivanlou, A. H. (2017, Mar 15). Embryoids, organoids and gastruloids: new approaches to understanding embryogenesis. *Development, 144*(6), 976-985. https://doi.org/10.1242/dev.143529

Singh, V. K., Kumar, N., Kalsan, M., Saini, A., & Chandra, R. (2015). Mechanism of Induction: Induced Pluripotent Stem Cells (iPSCs). *J Stem Cells, 10*(1), 43-62. https://www.ncbi.nlm.nih.gov/pubmed/26665937

Slanzi, A., Iannoto, G., Rossi, B., Zenaro, E., & Constantin, G. (2020). In vitro Models of Neurodegenerative Diseases. *Front Cell Dev Biol, 8*, 328. https://doi.org/10.3389/fcell.2020.00328

Stacpoole, S. R., Spitzer, S., Bilican, B., Compston, A., Karadottir, R., Chandran, S., & Franklin, R. J. (2013). High yields of oligodendrocyte lineage cells from human embryonic stem cells at physiological oxygen tensions for evaluation of translational biology. *Stem Cell Reports, 1*(5), 437-450. https://doi.org/10.1016/j.stemcr.2013.09.006

Stappert, L., Klaus, F., & Brustle, O. (2018). MicroRNAs Engage in Complex Circuits Regulating Adult Neurogenesis. *Front Neurosci, 12*, 707. https://doi.org/10.3389/fnins.2018.00707

Stover, A. E., Brick, D. J., Nethercott, H. E., Banuelos, M. G., Sun, L., O'Dowd, D. K., & Schwartz, P. H. (2013, Oct). Process-based expansion and neural differentiation of human pluripotent stem cells for transplantation and disease modeling. *J Neurosci Res, 91*(10), 1247-1262. https://doi.org/10.1002/jnr.23245

Tao, Y., & Zhang, S. C. (2016, Nov 3). Neural Subtype Specification from Human Pluripotent Stem Cells. *Cell Stem Cell, 19*(5), 573-586. https://doi.org/10.1016/j.stem.2016.10.015

Ulrich, D., Muralitharan, R., & Gargett, C. E. (2013, Oct). Toward the use of endometrial and menstrual blood mesenchymal stem cells for cell-based therapies. *Expert Opin Biol Ther, 13*(10), 1387-1400. https://doi.org/10.1517/14712598.2013.826187

Wang, S., Bates, J., Li, X., Schanz, S., Chandler-Militello, D., Levine, C., Maherali, N., Studer, L., Hochedlinger, K., Windrem, M., & Goldman, S. A. (2013, Feb 7). Human iPSC-derived oligodendrocyte progenitor cells can myelinate and rescue a mouse model of congenital hypomyelination. *Cell Stem Cell, 12*(2), 252-264. https://doi.org/10.1016/j.stem.2012.12.002

Watanabe, K., Kamiya, D., Nishiyama, A., Katayama, T., Nozaki, S., Kawasaki, H., Watanabe, Y., Mizuseki, K., & Sasai, Y. (2005, Mar). Directed differentiation of telencephalic precursors from embryonic stem cells. *Nat Neurosci, 8*(3), 288-296. https://doi.org/10.1038/nn1402

Yap, M. S., Nathan, K. R., Yeo, Y., Lim, L. W., Poh, C. L., Richards, M., Lim, W. L., Othman, I., & Heng, B. C. (2015). Neural Differentiation of Human Pluripotent Stem Cells for Nontherapeutic Applications: Toxicology, Pharmacology, and In Vitro Disease Modeling. *Stem Cells Int, 2015*, 105172. https://doi.org/10.1155/2015/105172

Yin, X., Mead, B. E., Safaee, H., Langer, R., Karp, J. M., & Levy, O. (2016, Jan 7). Engineering Stem Cell Organoids. *Cell Stem Cell, 18*(1), 25-38. https://doi.org/10.1016/j.stem.2015.12.005

Zakrzewski, W., Dobrzynski, M., Szymonowicz, M., & Rybak, Z. (2019, Feb 26). Stem cells: past, present, and future. *Stem Cell Res Ther, 10*(1), 68. https://doi.org/10.1186/s13287-019-1165-5

Zhang, S. C., Wernig, M., Duncan, I. D., Brüstle, O., & Thomson, J. A. (2011). In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nature Biotechnology, 19(12), 1129–1133*. https://doi.org/10.1038/nbt1201-1129