**Danah F Ashour**

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**Danah F Ashour**

**Differentiation of Neural Stem Cells (NSCs) into Astrocytes using Specific Growth Factors**

1. **Introduction**
2. **Importance of Astrocytes**
	1. **Features**
	2. **Why these growth factors are special & what are their functions?**
3. **Brief history on stem cells bioengineering**
4. **Morphology and function of different astrocytes**
5. **Astrocytes differentiation process**
	1. **From cord-blood to iPSCs**
		1. **iPSCs conversion to NSCs and subtypes**
			1. **Astrocytes differentiation and growth factors role in development**
6. **Conclusion**
7. **Terminology**
8. **References**

**I. Introduction**

Most stars you see, only when it is dark clear from your window or from a telescope. You might not see all stars, because they are hidden deep in space, also they must be so big than what you think. Another type of stars could not be seen without a microscope in a laboratory. This time they are so tiny for someone who eye ball them without magnification. These types of stars are usually hidden in your brain and protected by your skull. In other words, these stars are called astrocytes, type of neural cells that occupy about more than 50% of the brain mass.

Astrocytes represent an important primary cell type of the brain’s architecture that function in maintaining the central nervous system (CNS) homeostasis, trophic support of neurons, detoxification, and immunosurveillance (Malik et al., 2014). These cells are considered as the guards of brain tissue and the spinal cord, after blood brain barrier (BBB). Therefore, growing astrocytes in labs have become necessary to cure neurodegenerative disorders, and can be utilized in several brain disorder research applications. I write this review to present the research advances in stem cell progression regarding astrocyte differentiation from neural stem cells (NSCs).

II. **Importance of Astrocytes**

Propagating reprogrammed hiPSCs and derivating them into neural stem cells (NSCs), which can produce all of the major central nervous system (CNS) cell lineages (neurons, astrocytes, and oligodendrocytes) *in-vitro* (Zeng et al., 2014). A range of methodologies of the induction of neural stem cells development has improved during the last few years. Therefore, scientists are debating the accuracy and efficacy of individual differentiation protocols to produce the optimized neural cell subtypes growth *in-vitro* at higher quality in an attempt to attain the transplantable organ grade (Zeng et al., 2014). Astrocytes retain a major role in the neuronal connectivity and nourishment; they also occupy the majority of the CNS system. This review will be focusing on different growth factors presented in selected literature; discussed here for the purpose of seeking the appropriate method for differentiating human derived NSCs towards functional mature astrocytes.

1. **Brief history on stem cells bioengineering**

When the field of human iPSCs began to develop, amongst stem cells professional, numerous cell lineages became more accessible to clinical and therapeutic applications. The use of research behind stem cells has obvious benefits and could revolutionize certain fields of medicine and to promote human health and survival. (Singh et al., 2015). As a summation of outcomes from scientific innovation within the field of stem cell productivity, multiple cell lines were created and registered at the National Institute of Health (NIH). At the NIH where Center of Regenerative Medicine reside, scientists attempt to validate derived cell lines from the iPSCs, to certify and perpetuate cells for research and clinical testing. Considerable cell lines were produced and preserved at the NIH: Mesenchymal Stem Cells, Neuronal Stem Cells (NSCs), Endothelial Stem Cells, and Adipose-Derived Stem cells, are made obtainable for conducting clinical and academic research.

Reprogramming is defined as the conversion of adult somatic cells to embryonic stem cell–like (ES-like) pluripotent state (Malik & Rao, 2013). The purpose of reprogramming is to transform non self-renewal cells onto human induced pluripotent stem cells (hiPSCs) equivalent to ESCs in morphology, proliferation, gene expression, and epigenetic qualities. ESCs and hiPSCs are both comprise the capability of self-renewal and to generate all three germ layers: ectoderm, endoderm, and mesoderm (Welstead et al., 2008).

Some researchers anticipated their stem cell studies would undergo treatment for degenerative brain diseases and psychological disorders. By any mean, to attain a conventional patient specific cure, research advances has been progressing by inducing the hiPSCs to neural stem cells, which could then be used for transplantation therapy treating such conditions as Parkinson’s, Huntington’s, tremors, dementia, schizophrenia, Alzheimer’s, and a wide range of other neurological diseases (Zeng et al., 2014).

In this review, we are presenting a range of methodologies of induction of neural stem cells have developed and improved during the last few years to produce specifically the specialized brain cell: Astrocyte.

1. **Astrocytes differentiation process**
	1. **From cord-blood to iPSCs**

A widely used induction method to produce NSCs involves the formation of spherical cells aggregate (i,e.,embryoid bodies or EBs) in culture plates using either iPSCs or ESCs (Chambers et al., 2009a). Some studies showed that NSCs are directly inducible from somatic cells avoiding the pluripotency step using direct reprogramming of fibroblasts or cord blood cells CD34+. Liao et al. (2015) found a technique that led to improved direct reprogramming of somatic cord-blood CD-34+ cells (CB cells) and reprogrammed using non-integrating episomal plasmid system into iPSCs, which induced them into NSCs In this method, CD34+ isolated CB cells transfected with EBNV1-based pCEP4-OCT4 expression plasmid by electroporation using a human CD34 cell nucleofector kit (Liao et al., 2015).

* 1. **iPSCs conversion to NSCs**

Two experts of neural stem cells investigated that EB cell culture format, and with the combination or manipulation of signaling molecules or growth factors (GFs), could generate an efficient and expandable technique to grow NSCs *in vitro*. One of the investigators have attempted utilizing Retinoic acid (RA) in the tissue culture, a pure neural cell population that have the capability to differentiate into other cells (i.e., astrocytes and oligodendrocytes) (Sartore et al., 2011). The other study made EB culture in Dual-SMAD inhibition growth media is the least complicated method to induce NSCs (Chambers et al., 2009b). They also clamed: the yields of NSCs have the advantage to cryopreserve and re-culture, along with preserving cells genetic identity, and to expand further and derive all CNS cells (Vemuri, 2015).

Matter-of fact, these methods are efficient for producing NSCs, as in bioengineered cord blood cells, CD34+ cells to stem cell like. In many applications, scientists showed considerable cell culture techniques, that are more feasible to generate all brain sub-cell lineages. A well-acquainted CNS works of neurodevelopmental biologists are seeking an ideal NSCs differentiation protocol into neurons as an initial stage, then as differentiation and growth transform into astrocytes and or oligodendrocytes. Our emphasis here is to review some peer-reviewed articles that are investigating various methodologies on astrocytes differentiation from iPSCs.

1. **Astrocytes differentiation methodologies and growth factors selection**

Stem cell culture experts started the trial and error method by selectively adding and withdrawing different GFs that were found essential to induce CNS cells subtypes. Here we are focusing on one-cell subtype derivation methods and kinds of GFs that induce astrocytes differentiation, which are capable of orchestrating and maintaining CNS network circuitry, functionality, and nourishment for the brain and the spinal cord (Jones & Bouvier, 2014).

Few selected papers have been reported the importance of different growth factors that are required and are essential for the induction of astrocytes in their media formulations or composition. Most commonly used growth factors are fetal bovine serum (FBS), Fibroblast growth factor-2 (FGF-2) or basic-fibroblast growth factor (b-FGF), epidermal growth factor (EGF) are all basic growth factors inducing and maintaining cell differentiation, cytokine signaling, and proliferation (Duan, Peng, Pan, & Kessler, 2015).

Therefor, the complexity of the differentiation media and variability are relatively controversial, as well as these growth factors are also highly expensive in the growing new field of stem cell research materials and equipment industry. Accordingly, astrocyte differentiation media optimization advancement is under intensive investigation by both scientific research in academia and scientific manufacturers that need further evaluation, for efficacy and reproducibility.

A group of stem cell research investigators have proposed that addition and the removal of growth factors or (also called morphogens) to the differentiation media at certain time points of the differentiation period facilitated the NSCs to generate region-specific astrocyte cells at higher percentage outcome. For example, Krencik & Zang (2011) demonstrated that human-derived NSCs from iPSCs not only differentiate to neurons but into astrocytes as well. He also described a differentiation protocol in great detail on how astrocytes need to be derived *in-vitro* for an extended period of up to 180 days: producing GFAP+ cells and other astrocytes' distinctive markers (Malik et al., 2014).

Kondo et al. (2013) highly recommends Krencik's (2011) protocol for the production of functional astrocytes in vitro which enabled them to use the Krencik’s differentiation approach to their Alzheimer’s disease model research. Krencik’s protocol of differentiation involved three sequential main stages, starting from: 1) PSCs to neural epithelial cells (NE), 2) NE to astroglial progenitors (AP), and 3) the last stage from AP to immature astrocytes expressing over 90% GFAP+ cells. The key GFs of astrocyte differentiation stage includes the addition of ciliary neurotrophic factor (CTNF) which is used to induce the gliogenesis at the second and the third stages (Krencik & Zhang, 2011). The CNTF is a major cytokine member of the Neurotrophic family (NF), described to play a role in developing brain motoneurons and aid in the survival of these cells in tissue culture dishes (*Neuroscience in the 21st Century: From Basic to Clinical*, 2013). Similarly, Emdad et al. (2012) showed that CNTF induces astrocytes expressing GFAP+ cells phenotype specifically by gradually removing combined other GFs with CNTF followed by CNTF culture treatment alone. Emdad et al. (2012) was able to generate GFAP+ cell population efficiently at an incremental increase from ~18% to ~78% GFAP+ cells in five weeks.

Yan's et al. (2013) designed a new defined media, using serum-free neural induction medium that induced hiPSCs into NSCs to produce region-specific neural cell subtypes efficiently. He generated CNS neurons, astrocytes, and oligodendrocytes using a commercially available culture medium that is simple and easy to mix without the need for additional growth factors (Vemuri, 2015). While Yan's study proposed the efficacy of the commercially and simply formulated media, in particular, to make astrocytes from both ESCs and iPSCs; there was no evidence of data representation on the purity of cell culture and the percentage of GFAP+ cells produced.

Likewise, Shaltouki, Peng, Liu, Rao, & Zeng, (2013) and collogues disagreed with some of Krencick’s published technique due to its time-consuming protocol and the complexity of the method to derive neurons and astrocytes from NSCs. Accordingly, they developed an efficient differentiation protocol to produce neurons, astrocytes, and oligodendrocytes using different NSC sources that gave similar differentiation results in defined culture conditions In this experiment, the NSC cells were grown for 35 days in a complex astrocyte differentiation medium (ADM) with the addition of specific growth factors to derive human astrocytes with shorter time and higher yield of GFAP+ cells (Shaltouki et al., 2013).

The use of the growth factors, showed to generate ~80% single positive population effectively for GFAP+ cells after day 35 from NSCs differentiation in ADM. Shaltouki's media composition of ADM showed more promising results in stem cell studies in the production of a good manufacturer product (GMP) cells for biomedical therapy (2013).

Satoh, Sugino, & Yoshida, (2000) designated three key GF ingredients promoted their ADM’s and elevated the percentage of GFAP+ cells in culture dishes, as data of the cell markers results were significantly high. They also proposed that the addition of Activin A and Hergulin-1β as two member of TGF-β transforming factor family that involve in promoting astrocyte development in the CNS (Sharif et al., 2006; Wang et al., 2007). In addition to the first two factors, is Insulin-like growth factor-1 (IGF-1) that belongs to the transforming factor cytokine (Wang et al., 2007). Together, the three GFs together contribute for the induction of astroglial progenitors to force the cells into the astrocyte-dominant cell culture in 35 days (Shaltouki et al., 2013).

Most recently Rao, Mujtaba, Wu, & Liu, (2014) registered a patent project that was not published in the press yet; it advocated that attaining a pure culture of astrocytes from either human ESCs or human iPSC is a fundamental requirement for the GMP to be employed in the transplantation clinical trials. The patent suggested for a newly invented media that is specifically meant to produce cells in large quantities of pure astrocytic tissue culture in-vitro using more GFs to what was used previously in Shaltouki’s and Malik’s published work as mentioned above. The main goal of the invention is to formulate cell culture to grow in "astrocytes promoting conditions" are only slight increase on the GFs list used previously such as glial cell-derived neurotrophic factor (GDNF), platelet-derived growth factor (PDGF), Bone morphogenic protein (BMP), as well as the addition of a higher concentration of serum in culture media. Mahendra claimed that these newly added supplements would promote the induction of more than 95-99% astrocytes purity in culture.

The use of the BMP growth factor was tested intensively by et al. (2001), Nakashima suggested that BMP have an inhibitory effect on the neuronal cell growth and allowing astrocytes with GFAP+ phenotype to grow more dominantly in-vitro. The se findings were supported by a later report showed the possibility of up-regulation of differentiated astrocytic cells with the treatment of BMP in the presence of the other neurotrophic factor GFs mentioned earlier in the ADM with the presence of 10% FBS (Chang, Son, Lee, & Lee, 2003).

FBS has been widely used in tissue culture protocols; its concentration varies from 1%(Yan et al., 2013), to 5%(Yuan et al., 2015), to 10% in culture media. However, an addition of 20% FBS in other studies showed satisfactory increase number of astrocytes differentiation in-vitro due to neuronal and microglial growth inhibition that usually contaminated astrocyte differentiation culture ([Halonen, Taylor, & Weiss, 2001](#_ENREF_7); Mahendra, Mujtaba, Wu, & Liu, 2014).

Certainly, most selected literature on NSC culture protocols were discussed above and defined the ultimate combination of growth factors to serve in optimizing a defined astrocyte differentiation media for producing a highly pure and GFAP+ specific cell type specifically.

**V. Conclusion and future remarks:**

Differentiation of astrocytes in a culture dish varies and depending on: the starting cell line, chosen methodology, set of growth factors, and purity percentage of cell differentiation culture are exclusively dependent on the research preference. Perhaps purity of astrocytes in some cases is not required based on the requirement of the conducted research.

Some research institutions desire to obtain an optimal pure and efficient cell culture product for particular clinical trial and their data analysis accuracy, drug discovery, and find a cure to treat brain degenerative disorders. Thus, transplantation achievability demands complete awareness of the complexity and architecture of the CNS system of cells network besides an intensive research understanding of what to use? Why? And when to deliver the paramount healthy cells required for treatment and repair as Dr. Rao concentrated in his recent 2014 patent in the discussion above. The growth factors discussed in this review focused on some of the unique GFs mentioned in; additional GFs were mentioned briefly but not explained here such as the PDGDF that play another role in astrocytes differentiation (Mahendra S. Rao et al., 2014).

Furthermore, other published literature introduced the advantage outcome of using cerebral spinal fluid (CSF) for NSCs differentiation research that produced more robust and functional astrocytes derived from human NSCs (Buddensiek et al., 2010; Kiiski et al., 2013; Lehtinen et al., 2011; Zhu et al., 2015). It possibility requires more attention and closer review in the future. The CSF use direction in ADM towards astrocyte differentiation criterion needs further investigation.

In conclusion, the growth and discipline of the development of iPSCs studies became more approachable and reproducible in laboratories in recent times. The battle of applying these mentioned findings in clinical studies approval would be the next absolute aim to solve various degenerative diseases in humans.

**Terminology**

Astrocytes:

A large star-shaped cell of the glia.

Cell explantation:

To remove (living tissue) especially to a medium for tissue culture.

Cell tissue culture:

The process or technique of making body tissue grow in a culture medium outside the organism.

Cytokines:

Any of a class of immunoregulatory proteins (such as interleukin or interferon) that are secreted by cells especially of the immune system.

Defined media:

A chemically **defined** medium is a growth medium suitable for the in vitro cell culture of human or animal cells in which all of the chemical components are known.

Differentiation:

The sum of the processes whereby apparently indifferent or unspecialized cells, tissues, and structures attain their adult form and function.

Epigenesis:

Development involving gradual diversification and differentiation of an initially undifferentiated entity.

Ectoderm:

The outermost of the three primary germ layers of an embryo that is the source of various tissues and structures (such as the epidermis, the nervous system, and the eyes and ears).

Endoderm:

The innermost of the three primary germ layers of an embryo that is the source of the epithelium of the digestive tract and its derivatives and of the lower respiratory tract.

Endothelial cells:

An epithelium of mesodermal origin composed of a single layer of thin flattened cells that lines internal body cavities and the lumens of vessels.

Embryoid body:

A mass of animal tissue that resembles an embryo.

Episome:

A genetic determinant, (such as the DNA of some bacteriophages) that can replicate autonomously in bacterial cytoplasm or as an integral part of the chromosomes.

GFAP:

A class-III intermediate filament, is a cell-specific marker that, during the development of the central nervous system, distinguishes astrocytes from other glial cells.

Homeostasis:

A relatively stable state of equilibrium or a tendency toward such a state between the different but interdependent elements or groups of elements of an organism, population, or group striving to achieve *homeostasis.*

Immunosurveillance:

A monitoring process of the immune system which detects and destroys neoplastic cells and which tends to break down in immunosuppressed individuals — called also *immunological surveillance.*

*In-vitro*:

Outside the living body and in an artificial environment.

Mesenchymal stem cells:

Loosely organized undifferentiated mostly mesodermal cells that give rise to such structures as connective tissues, blood, lymphatics, bone, and cartilage.

Mesoderm:

The middle of the three primary germ layers of an embryo that is the source of many bodily tissues and structures (such as bone, muscle, connective tissue, and dermis); *broadly* : tissue derived from this germ layer.

Morphogens:

Are signaling molecules that emanate from a restricted region of a tissue and spread away from their source to form a concentration gradient.

Neurogenesis:

Development of nerves, nervous tissue, or the nervous system

Neurospheres:

Are free-floating clusters of neuroprogenitor cells generated by neural stem cells (NSCs).

Oncogene:

A gene having the potential to cause a normal cell to become cancerous

Oligodendrocytes:

A glial cell that resembles an astrocyte but is smaller with few and slender processes having few branches and that forms the myelin sheath around axons in the central nervous system.

Plasmid:

An extrachromosomal ring of DNA especially of bacteria that replicates autonomously.

Pluripotent stem cells:

Not fixed as to developmental potentialities; *especially* : capable of differentiating into one of many cell types.

Somatic cells:

One of the cells of the body that compose the tissues, organs, and parts of that individual other than the germ cells.

Transcription factor:

Any of various proteins, that bind to DNA and play a role in the regulation of gene expression by promoting transcription.

Transfection:

Infection of a cell with isolated viral nucleic acid followed by production of the complete virus in the cell; *also* : the incorporation of exogenous DNA into a cell.

Upregulation:

The process of increasing the response to a stimulus; *specifically* : increase in a cellular response to a molecular stimulus due to increase in the number of receptors on the cell surface.

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