5-2024

QUALITATIVE ASSESSMENT OF HUMAN EMBRYONIC- AND INDUCED PLURIPOTENT STEM CELL DERIVED NEURAL STEM CELLS UNDER CGMP METHODS

Michelle Hernandez

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A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Biology

by

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Approved by:

Nicole Bournias-Vardiabasis, Ph.D., Committee Chair, Biology

Brian J. Cummings, Ph.D., Committee Member

Laura Newcom, Ph.D., Committee Member

ABSTRACT

Neural stem cell therapies represent a promising tool for the development of regenerative medicine and are being tested in clinical trials for several neurological disorders. However, the clinical applicability of stem cell therapies is dependent on the implementation of current good manufacturing practices (cGMPs) to ensure the quality, safety, and consistency that stem cell products need to meet FDA regulatory requirements. As such, there is a need for a shift to xeno-free methodologies so experimental conditions are cGMP compliant. The purpose of this study is to test a GMP compatible production method to generate multipotent neural stem cells (NSCs) from embryonic and pluripotent stem cells. Comparability of NSCs is dependent on quality controls such as safety, stability, purity, and multipotent neural stem cell differentiation. Accordingly, we will test Shef6 hESC and ADRC76 hiPSC lines for their suitability to produce CD133-positive neural stem cells. Due to potential epigenetic differences found in hiPSCs, such as aberrations in DNA, we predict that hESCs will produce higher quality NSCs compared to hiPSCs. To test this, both cell lines will be cultured under identical reagents and methods for expansion and neuralization. For NSC characterization, trophic factor and cytokine secretion and neural differentiation will be compared between the processing runs. General Biological Product Standards will be applied throughout the downstream processing such as safety, stability, potency and purity validations. This manufacturing protocol will be compared with a prior derivation of Shef6 hNSCs. The adoption of cGMP-compliant methods early in the research environment will improve the replication

of results across cell lines and may increase translatability of preclinical studies as the protocols are transferred to GMP facilities.

ACKNOWLEDGEMENTS

I would like to thank Dr. Nicole Bournias-Vardiabasis for her guidance and support throughout my time as an undergraduate, graduate student and CIRM Bridges Scholar at CSUSB. Special thanks to Dr. Laura Newcomb for her guidance and insight on my thesis writing from proposal to defense. Deepest gratitude to Dr. Brian Cummings for allowing me to conduct research in his laboratory during my CIRM internship and for continuous guidance in my future career goals. Special thanks to the Andermings Lab for listening to my endless grievances and mad scientific rants as well as assisting me throughout my imposter syndrome. Thank you to CIRM for funding my education as a graduate student that allowed me the opportunity to network and gain research skills. Lastly, I would like to express my deepest appreciation to Daniel and Susana Hernandez, that without their emotional support I would have given up. This is for you, mom and dad. We did it.

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CHAPTER ONE:

INTRODUCTION

Stem Cell Research

Brain tumors, neurodegenerative disorders, and traumatic brain injuries are within a wide range of disorders that currently have no effective therapies due to a lack of FDA approval. Thus, many conditions with no treatment have given rise to the field of stem cell research. This rise over years has attracted researchers to regenerative medicine. As a result of the expanding field of stem cell research, there has been development towards stem cell products in recent years, propelled by the recognized potential for treating a wide range of medical conditions. Commonly used stem cells for research and development include mesenchymal, hematopoietic and neural stem cells. These stem cells can be derived from different sources, primarily Embryonic tissue or stem cells (ESC) and induced Pluripotent Stem cells (iPSC). ESCs and iPSCs are two types of pluripotent stem cells that hold significant potential in the field of regenerative medicine (Kingham et al., 2013; Zakrewski et. al., 2019).

Embryonic Stem Cells (ESCs)

Embryonic stem cells are pluripotent cells derived from the inner cell mass of blastocysts, which are early-stage embryos typically obtained from in vitro fertilization clinics. ESCs possess the extensive ability to differentiate into cells of all three germ layers: endoderm, mesoderm, and ectoderm (Eguizabal et al., 2019). The discovery of embryonic stem cells dates back to the early 1980s

when scientists first isolated them from mouse embryos. However, it was not until 1998 that researchers successfully derived embryonic stem cells from human embryos (Eguizabal et al., 2019). This characteristic, known as pluripotency, makes ESCs a valuable tool for regenerative medicine, developmental biology research, and drug discovery. ESCs have opened new avenues for understanding early human development, modeling diseases, and potentially treating a wide range of medical conditions. However, their use has raised ethical concerns due to the destruction of human embryos in the process of obtaining them (Lo & Parham, 2009).

Induced-Pluripotent Stem Cells (iPSCs)

On the other hand, in 2006, Shinya Yamanaka and his team made a remarkable discovery when they successfully reprogrammed adult mouse fibroblast cells into a pluripotent state, essentially turning them into embryonic-like stem cells. The following year, in 2007, human cells were successfully reprogrammed into induced-pluripotent stem cells (iPSCs), marking a milestone in regenerative medicine (Takahashi & Yamanaka, 2006). This allowed adult cells such as skin cells to revert back to a pluripotent state due to Yamanaka's efforts. This breakthrough technology allows for the generation of patient-specific stem cells, overcoming ethical concerns associated with ESCs. iPSCs can differentiate into various cell types and serve as a valuable tool for disease modeling, drug screening, and personalized medicine. However, iPSCs are not without their concerns, as they have the potential to form tumors and introduce genomic instability post reprogramming (Yoshihara et al., 2017).

Despite controversies, the history of stem cells bookmark their pivotal role in advancing our understanding of cell biology and their potential therapeutic applications. Both ESCs and iPSCs offer advantages and challenges, and ongoing research aims to harness their potential for therapeutic applications in a safe and effective manner.

Neural Stem Cells (NSCs)

Neural stem cells (NSCs) are multipotent stem cells that can differentiate into specific cell types within the nervous system. NSCs can also secrete a variety of molecules and factors that play important roles in neurodevelopment and tissue repair. These secreted factors can have paracrine effects, influencing the behavior of endogenous cells (Zhang et al., 2020; Teng et al., 2011). Some of the key molecules produced or secreted by neural stem cells include: Growth Factors, to promote the survival, proliferation, and differentiation of neural stem cells. Cytokines and Chemokines that are involved in immune regulation, cell migration, and inflammation. Extracellular Matrix Components (ECM) to provide structural support and influence cell adhesion, migration, and differentiation. Neurotransmitters that play essential roles in neural communication and modulating neural activity. Lastly, Trophic Factors also promote cell survival, axonal growth, and synaptic plasticity (Zhang et al., 2020; Teng et al., 2011). It is important to note that the specific molecules secreted by NSCs can vary depending on their developmental stage, microenvironmental cues, and experimental conditions (Jiao et al., 2023). The aforementioned secretions

represent some of the common factors associated with NSCs, but the field of NSC research is continuously evolving, and new discoveries are made regularly.

A major identifier of neural stem cells is their capacity for self-renewal and multipotency through the biomarker, CD133. CD133, also known as Prominin-1, is a surface marker that has been specifically used to identify and isolate neural stem cells from various sources, including the developing brain, adult brain, and stem cell cultures (Holmberg et al., 2011). These cells possess the ability to divide symmetrically and generate more neural stem cells, ensuring a sustainable population for research and therapeutic applications. Additionally, CD133 positive neural stem cells are ependymal cells found in the forebrain that can differentiate into cell types of the Central Nervous System, including neurons, astrocytes, and oligodendrocytes through the generation of neurospheres (Holmberg et al., 2011; Liu et al, 2023). This multilineage potential makes them valuable tools for studying neural development, modeling some neurological disorders, and exploring potential regenerative therapies. This regenerative potential makes CD133 positive neural stem cells a promising candidate for cell replacement therapies aimed at treating conditions such as traumatic brain injury, stroke, and neurodegenerative disorders.

Neural Stem Cell Therapies

Neural stem cell therapies represent a promising tool for revolutionizing the treatment of various neurological disorders and injuries (Takagi, Y., 2016). These therapies involve the use of neural stem cells, which possess the ability to self-renew and differentiate into different cell types within the central nervous system. The concept of neural stem cells (NSCs) as a possible therapy emerged in the 1960s, challenging the belief that the adult brain could not generate new neurons (Takagi, Y., 2016). Researchers discovered that specialized regions within the adult brain contained a population of undifferentiated cells capable of generating neurons and glial cells throughout an individual's life (Maldonado-Soto et al., 2014). This discovery laid the foundation for investigating the therapeutic potential of neural stem cells in various neurological conditions. The development and implementation of all neural stem cell therapies necessitate extensive research, careful consideration of safety and ethical concerns, and most importantly, adherence to regulatory guidelines to ensure their effectiveness and safety (Mousaei et al., 2022). However, the translation of neural stem cell therapies from the laboratory to clinical practice faces numerous challenges.

Therapeutic Efficacy

One of these primary challenges is evaluating therapeutic efficacy through preclinical studies for neural stem cells (NSCs) before advancing to clinical trials (Tang et al., 2017). To obtain efficacy these studies involve rigorous experimentation in animal models of neurological disorders or injuries to assess

various aspects of NSC behavior and functionality. Key objectives of preclinical studies for NSCs include determining their survival, migration, integration, and differentiation following transplantation into the host tissue. Additionally, it is necessary to evaluate the impact of NSC transplantation on neurological function, such as motor skills, cognition, and sensory function, through behavioral assessments (De Gioia et al., 2020; Baker et al., 2019). Currently, the idea that NSC transplantation may promote recovery of only selected, less complex aspects of motor control is still possible while on the other hand, preclinical studies have reported only partial recovery (Fischer et al., 2020). Preclinical studies also focus on investigating potential adverse effects, including tumor formation, immunogenicity, and inflammatory responses.

The possibility that NSCs can make new neurons, astrocytes, or oligodendrocytes is important for their therapeutic potential in neurodegenerative conditions. This potential has been exploited in many preclinical models, for example stroke. Unfortunately, in this study, after the stroke, only few neural stem cells survive, differentiate, and migrate to form novel neural circuits (Lindvall & Kokaia, 2015); these were not numerous enough to recover neurologic functions. Therefore, a treatment that encompasses the enhancement of proliferation, survival, and neuronal maturation of endogenous or transplanted NSCs is important for neuro trauma and disease. While in animal models of neurodegenerative diseases like stroke, epilepsy, Alzheimer's, Huntington's, and Parkinson's diseases, cell proliferation and neurogenesis can occur, but in humans, this is still particularly difficult. In another study for spinal cord injury

(SCI) of human NSC transplantation in non-human primates (rhesus monkeys), subjects were evaluated for limb motor function (Trusts, P.C., 2021). Although the authors reported that transplantation revealed significant improvements in “an overall measure of motor function that combines all measures as compared to monkeys without surviving grafts” (Trusts, P.C., 2021).

Since FDA preclinical standards were made for efficacy and safety, clinical trials are also no easy feat. Table 1 summarizes the non-pediatric clinical trials selected on clinicaltrials.gov by using the key terms “neural stem cells”. Around 26 clinical trials reporting on transplant of NSCs have been listed that are either withdrawn, terminated, unknown or recruiting status for multiple neurological conditions. Some report a withdrawn or unknown status due to ethical considerations, logistical challenges, financial constraints, and changes in the scientific landscape. In the context of therapeutic efficacy, the studies are mostly withdrawn or terminated due to concerns about participant safety. If unexpected or serious adverse events occur during the trial, the researchers and regulatory authorities may decide to withdraw the trial to protect the health and well-being of participants. These adverse events could range from mild side effects to severe complications that were not anticipated during the planning stages of the trial. Additionally, if the trial fails to demonstrate the anticipated benefits of the intervention or if the results indicate potential harm outweighing the benefits, it may be halted or withdrawn to prevent further exposure of participants to ineffective or unsafe treatments.

Therapies Gone Wrong

Stem cell therapies that have not received approval from the U.S. Food and Drug Administration (FDA) exists in a realm of controversy and empty promise. These treatments often fall into the category of experimental or unproven interventions, bypassing the rigorous regulatory scrutiny that FDA approval entails. As of 2021, more than 700 clinics in the U.S. offer unapproved stem cell and regenerative treatments for many neurological conditions (Kuriyan et al, 2017). While some clinics and practitioners may offer these therapies with claims of potential benefits for a wide range of medical conditions, it's crucial to approach them with caution. Many self-proclaimed stem cell clinics have inflicted harm to the public with one case reported vision loss when patients received stem cell-based orbital injections (Turner, L., 2021). These are often private clinics or facilities that offer stem cell treatments without proper oversight, regulation, or scientific evidence to support their claims. Patients may receive injections of stem cells derived from various sources without knowing the potential risks or benefits. Some clinics offer amniotic stem cell therapies claiming they contain potent stem cells. However, evidence suggests that these products often contain minimal to no live stem cells and are more likely to be a mix of proteins and other unknowns (Ul Hassan et al., 2009).

In some other cases, clinics may extract a patient's own stem cells (usually from fat tissue or bone marrow) and transplant them back into the body, often intravenously or by injection, as a treatment for neurological disorders (FDA, 2022). The efficacy and safety of these procedures remain largely

unproven. Without proper oversight, there is a risk of unverified efficacy, safety concerns, and potential harm to patients. As a result, individuals considering such treatments should seek out comprehensive information, consult with qualified medical professionals, and carefully weigh the potential risks and benefits before making any decisions about pursuing non-FDA approved stem cell therapies (Hoang et al., 2022). By developing proper regulatory standards for stem cell-based therapies, patients that will receive these treatments are protected along with the industry that provides the treatment.

FDA Guidelines

From Benchside to Bedside

Since NSCs are living cells, they pose unique obstacles when it comes to manufacturing and quality control. The development and translation of stem cell products from benchside research to clinical applications are subject to rigorous regulatory oversight by the FDA. The FDA provides comprehensive guidelines to ensure the safety, efficacy, and quality of stem cell-based therapies throughout the entire development process, from initial laboratory research to clinical trials and eventual commercialization (Commissioner, 2020; FDA, 2019). These guidelines emphasize the need for well-controlled preclinical studies, including characterization of the stem cell product, assessment of potential risks and benefits, and demonstration of therapeutic efficacy in relevant preclinical models.

Additionally, the FDA mandates adherence to current good manufacturing

practices (cGMP) for the production, testing, and quality control of stem cell products intended for clinical use (Commissioner, 2019). This entails establishing robust manufacturing processes, implementing quality control measures, and maintaining comprehensive documentation of all manufacturing steps to ensure consistency and reproducibility of the final product. Furthermore, the FDA requires sponsors to conduct well-designed clinical trials to evaluate the safety and effectiveness of stem cell therapies in human subjects, with particular emphasis on patient safety and informed consent (Bauer, S. R., 2004). Compliance with FDA guidelines is essential to ensure the successful translation of stem cell therapies from benchside research to bedside clinical practice.

General Biological Product Standards

The General Biological Product Standards (GBPS) for stem cell therapies encompass a set of regulatory requirements established by FDA to ensure the safety, efficacy, and quality of stem cell products intended for clinical use (George B., 2011; Jha et al., 2021). These standards outline comprehensive guidelines covering various aspects of product development, manufacturing, characterization, and testing to mitigate risks associated with stem cell therapies and protect patient welfare. Key components of these standards include requirements for the selection and characterization of cell sources, establishment of robust manufacturing processes compliant with current good manufacturing practices (cGMP), implementation of rigorous quality control measures, and validation of analytical methods to assess product identity, stability, purity, and safety (George B., 2011; Jha et al., 2021).

Stem cell characterization allows researchers to confirm the identity and purity of the stem cell population being used in a therapy. This ensures that the intended cell type is being employed, minimizing the risk of unintended consequences or adverse reactions (Abubakar et al., 2023). Thorough characterization enables manufacturers to maintain consistent production of stem cell products. This is critical for ensuring that each batch of cells used in a therapy meets predefined specifications, reducing variability and increasing the reliability of treatment outcomes. Characterization helps identify and eliminate any potentially harmful contaminants or impurities that may be present in the stem cell population (O'Brien et al., 2015). This prevents adverse reactions or unintended side effects in patients receiving the therapy. It helps establish the stability and viability of stem cells over time and under various storage conditions. This information is crucial for determining the shelf-life of the product and ensuring that it remains effective when administered to patients. Assesses the genetic stability of stem cells to ensure that they do not carry any harmful mutations or abnormalities. This helps mitigate the risk of unintended genetic changes or malignancies in patients. Lastly, when seeking FDA approval, detailed characterization data provides regulatory agencies with the necessary information to evaluate the safety and efficacy of the stem cell therapy. This data is crucial for making informed decisions about whether to grant approval for clinical use (Abubakar et al., 2023; O'Brien et al., 2015). Therefore, manufacturing a pure population of neural stem cells could provide possible advancement in neural regenerative medicine.

Current Good Manufacturing Process

Stem cell products seeking FDA approval must adhere to Current Good Manufacturing Practice (cGMP) requirements (George B., 2011; Mousaei et al., 2022). cGMP requirements for stem cell products involve several key steps within the GBPS using Quality Control Attributes (QCA). These guidelines establish a comprehensive framework for the production, testing, and control of stem cell therapies (FDA, 2023). They also ensure that the entire process, from the isolation of stem cells to their differentiation into neural stem cells, is performed in a consistent and controlled manner, using validated methods and equipment. This helps to secure the safety and quality of the resulting differentiated cells, which is crucial for their successful use in clinical applications (Giancola et al., 2012; Sart et al., 2014). Using a current Good Manufacturing Practice (cGMP) compliant protocol for both embryonic stem cell (ESC) and induced pluripotent stem cell (iPSC) derived lines is crucial as it is essential when considering clinical translation.

Another important aspect of cGMP guidelines for stem cell products is ensuring that the raw materials used in the manufacturing process are of high quality, xeno-free and properly tested (Baghbaderani et al., 2015). This includes everything from the culture media used to grow the cells to the reagents and chemicals used in the processing and purification steps. By following these rigorous standards, developers and manufacturers of stem cell products can ensure consistent product quality and minimize the risk of contamination or adverse effects, ultimately safeguarding patient well-being and promoting the

advancement of regenerative medicine (FDA, 2023). Moreover, cGMP compliance facilitates the scalability and commercial viability of cell-based products. By implementing standardized manufacturing processes, it becomes easier to scale up production while maintaining consistent cell quality (Hoang et al., 2023). This is crucial for meeting the demands of large-scale clinical trials or commercial distribution, where robust manufacturing practices are required to ensure uniformity and reproducibility. Overall, using a cGMP-compliant protocol for both ESC and iPSC line generation is important to ensure the quality, safety, consistency, regulatory compliance, scalability, and commercial viability of the cells, ultimately advancing their potential in research and therapeutic applications.

“No One-Size Fits All”

The lack of specific FDA guidelines for stem cell therapies and weak translatability is primarily due to the dynamic and rapidly evolving nature of the field (Hoang et al., 2023; Bashor et al., 2022). Stem cell therapies encompass a wide variety of approaches, including those using embryonic stem cells, induced pluripotent stem cells (iPSCs), fetal stem cells, and more. Each type presents unique challenges and considerations, making it difficult to create one-size-fits-all regulations. They can be derived from different sources, such as embryos, adult tissues, or induced from specialized cells. Thus the characteristics, behavior, and potential applications of these stem cells vary significantly, necessitating nuanced regulatory approaches.

Stem cells exist in various states of differentiation, ranging from pluripotent cells capable of forming any cell type in the body, to more specialized progenitor cells (Zakrewski et al., 2019). Guidelines need to account for these distinctions in terms of safety, efficacy, and intended therapeutic applications. Our understanding of stem cell biology and the capabilities of various stem cell types continues to evolve. This means that regulatory frameworks must adapt to keep pace with advancing scientific knowledge. Stem cell therapies have the potential to treat numerous medical conditions, from neurodegenerative diseases to injuries, cancer, and more. Each application requires specific considerations regarding safety, efficacy, and ethical considerations.

Additionally stem cell research and therapies often involve ethical considerations, particularly when embryonic stem cells are involved. Balancing scientific progress with ethical concerns adds an additional layer of complexity to regulatory decision-making. Lastly, the emergence of unregulated stem cell clinics and practices has complicated the regulatory landscape. These clinics often operate outside established medical and scientific standards, necessitating regulatory responses to protect patient safety. While the FDA has issued guidance documents and taken steps to regulate specific aspects of stem cell therapies, such as those involving human cells, creating precise, all-encompassing guidelines for stem cell therapies remains a challenging task due to the multifaceted nature of the field (Heathman et al., 2015). It requires ongoing collaboration between scientists, clinicians, ethicists, and regulatory bodies to

develop comprehensive and adaptable methods that ensure the safety and efficacy of stem cell-based treatments.

In summary, cGMP compliant guidelines for stem cell products are critical to ensuring that these products are safe and effective. cGMP compliance is essential when considering clinical translation. If the goal is to use ESC or iPSC-derived cells in human therapies, regulatory authorities require adherence to cGMP standards. Establishing cGMP-compliant protocols from the early stages of cell line generation could ensure smoother transition and regulatory approval, as the necessary quality and safety controls are already in place. In the end there is no “one-size-fits-all” set of guidelines for assessing whether a stem cell-based product is ready for clinical trials (Heathman et al., 2015). Let alone a standard cGMP compatible method for generating neural stem cells.

Disease	NCT Number	Phase	Status
Neuronal Ceroid Lipofuscinosis	NCT01238315	1	Withdrawn
Parkinson's	NCT01329926	N/A	Withdrawn
Glioblastoma	NCT03072134	1	Completed
Spinal Cord Injury	NCT02163876	2	Terminated
Spinal Cord Injury	NCT01772810	1	Unknown
Glioblastoma	NCT02177578	2	Recruiting
Glioblastoma	NCT05139056	1	Recruiting
Retinitis Pigmentosa	NCT04284293	1	Recruiting
Glioblastoma	NCT01478854	N/A	Completed
Amyotrophic Lateral Sclerosis	NCT01730716	2	Unknown
Glioblastoma	NCT02055196	1	Withdrawn
Ischemic Stroke	NCT03629275	2	Terminated
Amyotrophic Lateral Sclerosis	NCT02943850	1	Completed
Macular Degeneration	NCT01632527	2	Completed
Glioblastoma	NCT02015819	1	Completed
Glioblastoma	NCT02192359	1	Active
Multiple Sclerosis	NCT03355365	2	Completed
Cancer Treatment	NCT00581113	3	Terminated
Amyotrophic Lateral Sclerosis	NCT01348451	1	Unknown
Epilepsy	NCT05135091	2	Recruiting
Multiple Sclerosis	NCT01933802	1	Completed

Amyotrophic Lateral Sclerosis	**NCT05306457**	1	Recruiting
Glioblastoma	**NCT01172964**	1	Completed
Spinal Cord Injury	**NCT02302157**	2	Completed
Ischemic Stroke	**NCT04631406**	2	Recruiting

Table 1. List of Clinical Trials Using NSC in the United States as of 2024

CHAPTER TWO:

MATERIALS AND METHODS

hPSC Culture

Culture of hESC line Shef6 (University of Sheffield, UK) and hiPSC line ADRC76 (UC Irvine) was established at UC Irvine in accordance with all appropriate hSCRO and IBC protocols. The Shef6 hESC line was previously transitioned to xeno-free (XF) conditions (Haus et al. 2014). To transition ADRC76 cells to XF culture conditions, all non-human animal- based components (ie, MEFs) were removed and replaced with cGMP compliant products that are human-based or recombinant including CELLstart CTS (Life Technologies) for the substrate and Nutristem (Sartorius) as the XF Growth Medium. The Shef6 hESC line was expanded from starting passage 8 till passage 11. ADRC76 iPSC line was expanded from passage 20 till passage 22. For cryopreservation, all hPSC cell lines were frozen while passaged using NutriFreez D10 (Sartorius).

Neural Stem Cell Derivation

For neuralization, an adapted version of a previously published “EZ-sphere” based neuralization protocol (Ebert et al., 2013) was utilized where hPSC colonies were chemically detached using ReLeSR (Stem Cell Technologies) and cultured as floating spheres in Ultra Low Cell Culture Flasks (Corning Inc.). The growth media consisted of X-Vivo 15 (Lonza Group), 1× N2, 100 ng/mL bFGF, and 100 ng/mL EGF (Life Technologies). Spheres were split

approximately every 7 days or when the size reached ~1000 μm via mechanical trituration slowly using a P1000 pipette tip to avoid dissociation to single cells up until passage E3. 5 days prior to adherent monolayer culture, 10 ng/mL LIF (EMD Millipore) was added to the culture media (Xeno-Free Neural Stem Media, or XF-NSM). To develop a monolayer culture, spheres were plated onto CELLstart coated plates in XF-NSM. Upon reaching 80–90% confluence, cells were dissociated using TrypLE Select CTS (Life Technologies) for cell sorting.

Cell Sorting for CD133+/CD34-

Fluorescence-Activated Cell Sorting (FACS) was performed using an FACS Aria Fusion (BD Biosciences) according to protocols via positive selection of CD133+ cells and negative selection for CD34+ cells. Human serum albumin (HSA, Octapharma USA Inc.) was used in place of bovine serum albumin. Antibodies listed in table 2 were used. All cells were grown prior to sorting as well as post-sorting on CELLstart coated plates in XF-NSM. TrypLE Select CTS was used to dissociate cells prior to sorting.

Magnetic-Activated Cell Sorting (MACS, Miltenyi Biotec) was performed using an autoMACS Pro Separator (Miltenyi Biotec) according to manufacturer-provided protocols via positive selection of CD133+ cells to retain, followed one passage later a negative selection for CD34 + cells, in order to obtain a CD133 +/ CD34− enriched cell population. 25% Bovine serum albumin was used in place of human serum albumin (HSA, Octapharma USA Inc.) for cost purposes. Magnetic microbead kits Indirect CD133 (130-050-801, Miltenyi Biotec) and CD34 (130-046-702, Miltenyi Biotec) were used. All cells were grown in monolayer after

neuralization prior to sorting as well as post-sorting on 1:100 CELLstart coated plates in XF-NSM. TrypLE Select CTS was used to dissociate cells prior to sorting.

For flow cytometric analysis, antibodies listed in table 2 were used. Surface marker staining was performed according to antibody manufacturing protocols. Briefly, cells were resuspended in a 180 µL X MACS buffer. 10 µL of FcR Blocking Reagent (Miltenyi Biotec) and 5 µL of each respective antibody were then added to the cell suspension. Compensation control samples were also included among the samples. The suspension was then mixed and incubated on ice for 30 min. Following incubation the cells were washed 3 times and analyses were performed using a BD Fortessa and FlowJo (ver. 10.10) software.

Genomic Stability

2 samples of hNSCs and 2 samples of hPSCs for ADRC76 and Shef6 were produced at UC Irvine were assessed for genome stability by Agilent 60K Standard aCGH microarray. Samples were analyzed by Cell Line Genetics Inc. (CLG) Madison, Wi.

hNSCs lines were plated on T75 flasks and hPSC lines were plated on 6-well plates till ~80% confluency was reached. TrypLE Select CTS was used to dissociate hNSCs and ReLeSR was used to lift the hPSC colonies. Each cell line was cryopreserved into cryovials. 2 samples of hNSCs and hESC, ADRC76 and Shef6 were produced at UC Irvine. The samples consisted of human neural stem cells both at passage 7 (P7) and human pluripotent stem cells ADRC76 at

passage 20 and Shef6 at passage 10. Samples were coded as CLG-51042,-51043, -51044,-51045. Alliance of Genome Resources database was used to interpret results.

Microbiological Safety Testing

Each cell line was allowed to grow post-passage for 48 hours with no media change. The media was collected for testing using the Lonza MycoAlert™ Mycoplasma Detection Kit (LT07-418) and Assay Control Set (LT07-518) to obtain contamination status. The assay was conducted in accordance with the manufacturer's protocol. Initially, aliquots of cell culture supernatants were collected and centrifuged @ .3 rcf for 5 mins to eliminate cellular debris. On a 96-well black plate, samples were conducted in duplicate and ran on a BioTek Cytation 5. Results were interpreted according to the manufacturer's guidelines.

Luminex Intelliflex Assay

At P8, both hNSC lines were grown for ~48 hrs in XF-NSM. An equivalent of 500 μL of the culture supernatant was collected after centrifugation at 0.3rcf for 10 min for each hNSC line. The concentrations and Median Fluorescence Intensities (MFIs) of growth factors, trophic factors, cytokines, and chemokines in the culture supernatants were measured using the Luminex Assay kits: 8-plex, 18-plex, and 42-plex (LXSAHM, R&D Systems, Emoryville, CA, USA). In total, 68 analytes would be measured between the supernatants of each cell line. 5 samples of each cell line along with a growth media control (n=11) were processed in duplicate on a 384-well plate and assessed on the Luminex xMAP INTELLIFLEX system.

Differentiation Assay

For neural differentiation, TrypLE Select dissociated single cells at P7 were seeded 7000 cells/well for 7-days and 5000 cells/well for 14 days onto CELLstart coated Lab-Tek Permanox chamber slides (Thermo Fisher Scientific/ Nunc) in XF-NSM. 24h after attachment, the media was changed to differentiation media (DM) consisting of X-Vivo 15, 10 ng/mL BDNF (Peprotech), 10 ng/mL GDNF (Peprotech), 1 × N2, 1 × B27 (Life Technologies), 2 ng/mL Heparin (Sigma-Aldrich), 63 µg/mL NAC (Sigma-Aldrich), 0.1 ng/mL bFGF, and 10 µg/mL Ciprofloxacin (Mediatech, Inc.). 50 uL of fresh differentiation media was added every 3 days. Differentiation was carried out for a total of 1-2 weeks before cells were fixed, permeabilized and immunostained.

Cells were fixed with 4% paraformaldehyde for 15 min. The cells were permeabilized in PBS containing 0.3% Triton X-100 (Sigma-Aldrich) for 10 min and blocked for 1 hr at room temperature in 10% donkey serum (Jackson ImmunoResearch). Primary antibody incubation in PBS-T buffer overnight in +4C Fridge. Primary antibodies included anti-ßIII Tubulin, anti-GFAP, and anti-hOlig2. (**Table 2.**)

Following overnight primary antibody incubation and washes, cells were incubated in corresponding secondary antibodies for 1hr at room temperature on a shaker and then mounted with Fluoromount-G (SouthernBiotech) for imaging. Secondary antibodies included: Alexa Fluor 488 Donkey Anti-Mouse, Alexa Fluor 555 Donkey Anti-Rabbit, and Alexa Fluor 647 Donkey Anti-Goat. Hoechst 33342 (H1399) was used for nuclear identification. All secondary antibodies and

Hoechst were from MolecularProbes/Life Technologies and used at a 1:500 dilution. All secondary antibodies were tested for cross-reactivity and non-specific binding. Imaging was performed using a Zeiss Axio Imager.M2 Apotome System (Carl Zeiss) and Imaris (MBF) was employed for quantification.

Statistical Analysis

Data was analyzed using Microsoft Excel and Graphpad PRISM software programs. Errors are the standard error (SEM) of averaged results. P values < 0.05 were deemed significant. Growth media was used to normalize the MFIs of analytes of interest.

Antibody	Species	Dilution	Catalog No.	Manufacturer
Immunocytochemistry				
B-Tub III	Mouse	1:2000	801213	Biolegend
GFAP	Rabbit	1:2000	Z0334	Dako
Olig2	Goat	1:250	AF2418	R&D Systems
Flow Cytometry				
CD133/2-PE	Mouse	1:50	130-113-186	Miltenyi Biotec
CD34-FITC	Mouse	1:50	130-113-178	Miltenyi Biotec

Table 2. Antibodies Used for Immunocytochemistry and Flow Cytometry

CHAPTER THREE:

RESULTS

Xeno-Free Transition and Neuralization of Human Pluripotent Stem Cell Lines

Culture of both human pluripotent stem cell lines Shef6 and ADRC76 were initially established in research conditions that contain animal components. In order to enhance the clinical translatability of these human stem cell lines, both hPSC lines were transitioned to Xeno-Free (XF) conditions in a single step conversion of both chemically defined serum-free media and a human based substrate.(Fig. 1, B, F) The XF transition required a stabilization period for the ADRC76 for 1-2 passages with chemical removal of spontaneously differentiated cells via ReleSR (Stem Cell Technologies).

After 2-3 passages in XF conditions, both cell lines were subjected to neural induction via EGF/FGF supplementation to generate embryoid bodies (EBs)(Fig. 1, C, G). Further induction of spheres with LIF supplement produced neurospheres in a similar morphology to the EBs. Sphere attachment on CELLstart generated a monolayer culture that was observed to be a heterogeneous population.(Fig. 1, D, H) As heterogeneous populations tend to have a mix of unwanted or undifferentiated cells, the identity of these cells are still unclear.

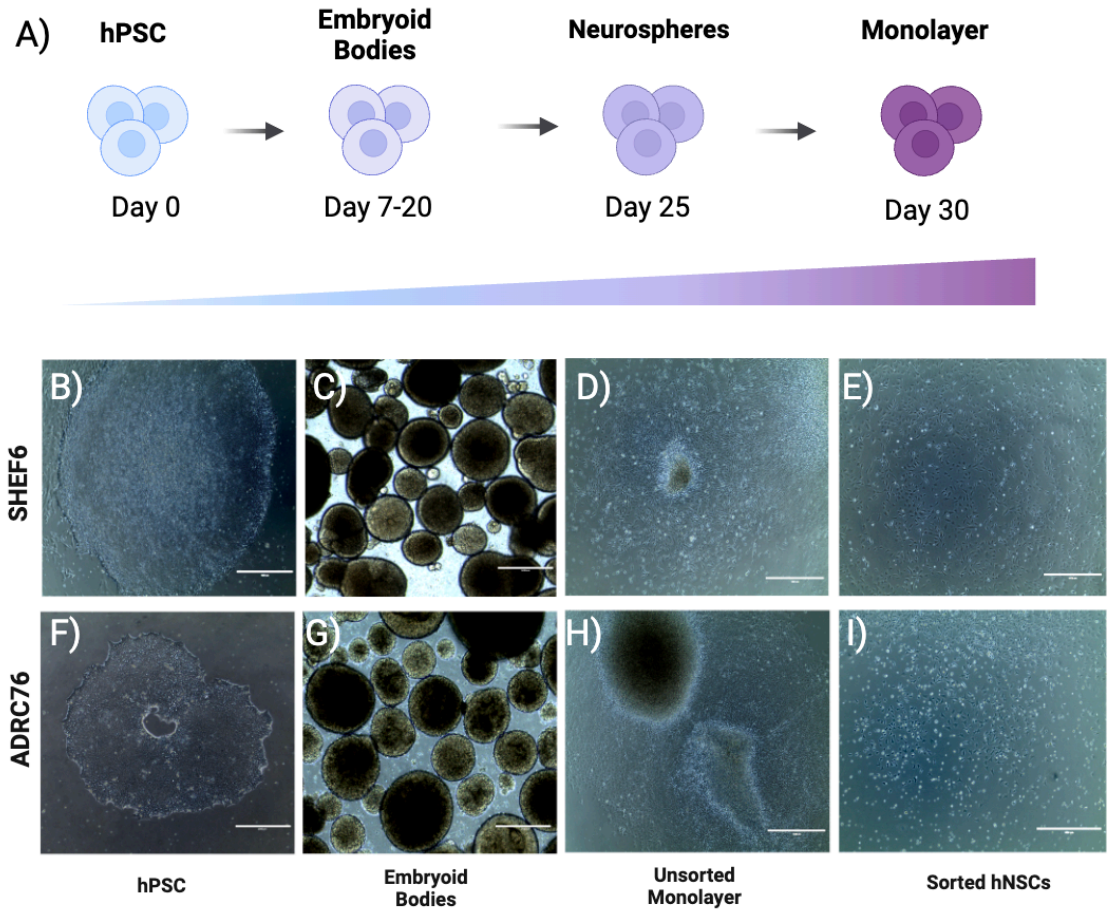


Figure 1. Xeno-Free Neuralization of Human Pluripotent Stem Cell Lines **A)** Diagram for the neuralization of hPSCs into neural stem/progenitor-like cells via a modified sphere-based protocol **B & F)** Undifferentiated hPSCs were originally maintained on animal-based substrates and then transitioned to XF conditions utilizing XF substrate CELLstart and media. **B-E)** **Shef6** and **F-I)** **ADRC76** hPSCs were neuralized identically under XF conditions to form neural morphologies. Scale: 1000um, 4x phase

MAC Sorting for CD133+/CD34- Increases Purity and Identification of Cell Population

Previous studies have found MACS to be less efficient and more viable than FACS when sorting hNSCs (Cheng et al., 2017; Muratore et al., 2014), although these studies used different cell surface markers and sorted cells directly following rosette-based protocol. Therefore to compare our MACS protocol with FACS using CD133-1/PE as the cell surface markers in the Shef6 hNSC line generated from the sphere-based protocol and with variable proportions of non-NSC populations. The yield of cells following MACS was consistently higher in all replicates in the Shef6 cell line in comparison to FACS. Following MACS and FACS, flow cytometry for CD133 and CD34 was shown to quantify the efficiency and accuracy for isolating the desired cells. A trend towards the reduced proportion of CD34+ cells following MACS depletion for both Shef6 and ADRC76 (Fig. 2, C-F) was observed, and importantly the variance between methods was significantly different. Both Shef6 and ADRC76 hNSC yield 95–98% CD133+ and CD34- cells compared to 22% in the unsorted NSCs(Fig. 2, A, B). When comparing the CD133+/CD34- populations of unsorted NSCs and FACS Shef6 NSC lines (mean = 65%), FACS increased the proportion of CD133+/CD34- NSCs by 43% when compared to unsorted cell line. There was difference in the proportion of CD133+/CD34- cells between MACS and FACS populations (mean CD133+/CD34- cells following MACS = 98%) suggesting that MACS is as more efficient than FACS in enrichment of CD133+/CD34- NSCs. All cultures for both Shef6 and ADRC76 were sorted magnetically going forward.

Interestingly, a heterogeneous to homogeneous population can be observed between unsorted and sorted for both cell lines. (Fig.2. E, I)

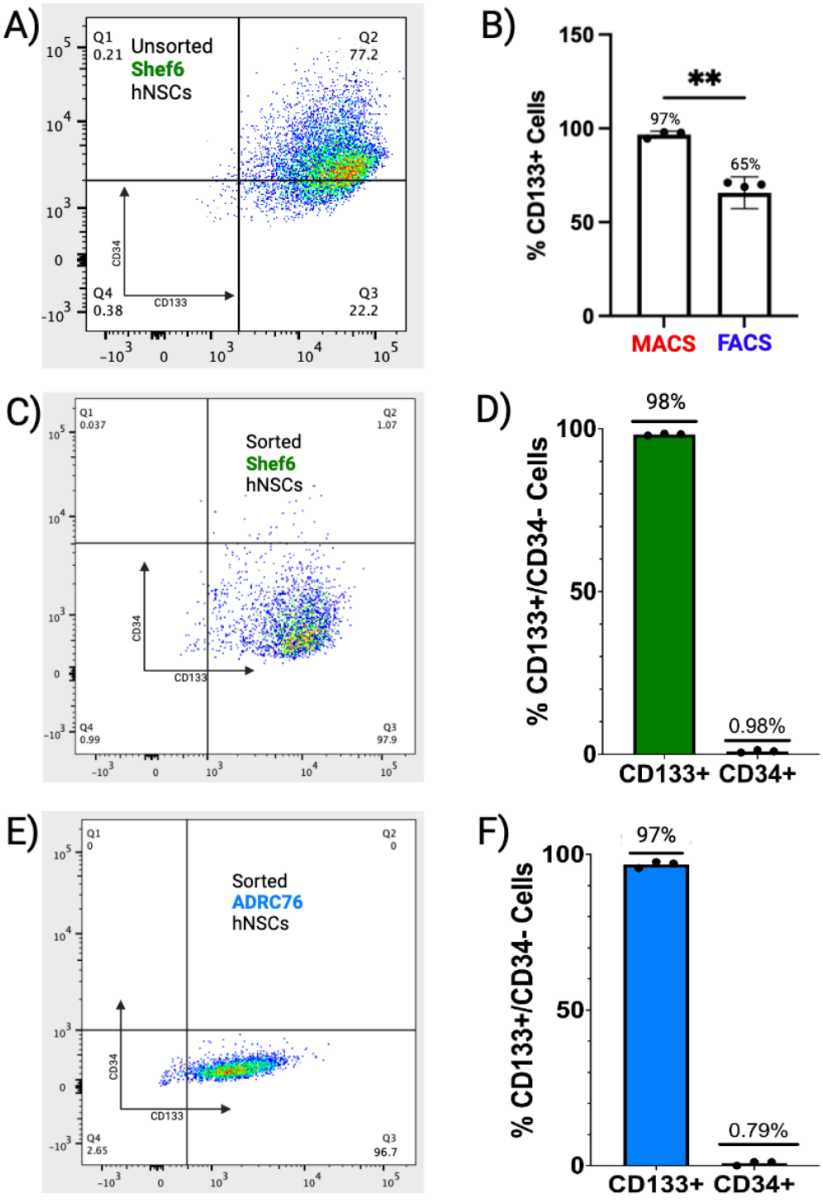


Figure 2. Flow Cytometry Analysis Confirmation of Unsorted and Sorted hNSCs Population.

A) Scatterplot of unsorted **Shef6** hNSCs **B)** Barplot comparing **Shef6** hNSC populations post FACS and MACS method using an unpaired t-test ($p<0.0018$) to determine the best cell sorting option to conduct endpoint experiments **C-D)** **Shef6** hNSC CD133+/CD34- population post-MACS **E-F)** **ADRC76** hNSC CD133+/CD34- population post-MACS. All experiments were run in 3 technical replicates with compensation controls for each to determine gating.

Microbiological Safety Test of Primary Cell Source and End Product

Two major sources for possible mycoplasma contamination include animal-based products used in culture and the lab environment. FDA recommends that a mycoplasma testing should be performed on the product at the manufacturing stage when the test is most likely to detect contamination, such as passaging (FDA, 2019). Testing should be conducted on both cells and supernatants.

Mycoplasma test results utilizing the Lonza MycoAlert™ Mycoplasma Detection Kit (LT07-418) are presented in the table below (Table 3). The testing revealed absence of mycoplasma contamination across the experimental samples required by the FDA General Biological Product Standards. Samples were assessed for mycoplasma contamination based on the enzymatic activity of mycoplasma-derived enzymes, which catalyze the hydrolysis of a specific substrate included in the kit. The presence of mycoplasma contamination was indicated by an increase in luminescence, measured as B/A ratio, compared to the negative control. Conversely, samples with B/A ratio below the predefined threshold of <0.9 were considered negative for mycoplasma contamination. The results were interpreted according to the manufacturer's instructions and confirmed through appropriate controls using Lonza MycoAlert™ Assay Control Set (LT07-518).

Sample	Results (< 0.9)
(+) Control	152.28
(-) Control	0.702
ADRC76 iPSC	0.417
ADRC76 hNSC	0.666
Shef6 hESC	0.564
Shef6 hNSC	0.627

Table 3. Mycoplasma Detection Using Lonza MycoALERT Assay
Duplicate samples were measured by the level of ATP in a sample before (Reading A) and after the addition of the MycoAlert Substrate (Reading B), a ration can be determined that indicated the presence or absence of mycoplasma. B/A Ratios less than 0.9 are consider free of mycosplasma.

Genomic Stability Between Cell Source and Product

Microarray assay results were obtained 3-weeks after samples were sent. The aCGH analysis aimed to detect genomic copy number variants (CNVs) across the genome of the cell lines, providing insights into potential genetic aberrations associated with the studied conditions or treatments. Copy number amplification or deletions were determined based on predefined thresholds through a matching sex pooled DNA sample control. The identified CNVs were further characterized in terms of their genomic locations, sizes, and frequency across the samples. Alliance of Genome Resources (AGR) database was utilized to interpret the genomic variants annotated in the Figure 3, A-D.

No aberrations much larger than 1Mb are not detected as this could indicate a genetic syndrome. Several discrete sub karyotypic aberrations were identified by the array. These include a 112.3 kb deletion at 1q21.2 that contains no genes and a larger 120 kb deletion at 2q37.3 containing 4 genes in Shef6

hESC (Fig. 3, A). Same deletion of 2q37.2 containing 4 genes showed in Shef6 hNSC (Fig. 3, B). A 457 kb amplification on chromosome 8p22 was detected and carried over from Shef6 hESC to Shef6 hNSC including 2 genes. (Fig. 3, A-B) In ADRC76 hiPSC, 112kb deletion on chromosome 1q21.2, similar to Shef6 hESC and a 278kb deletion on chromosome 10q21.3 were detected (Fig. 3,C). The deletion on chromosome 10 also carried over to ADRC76 hNSC including the CTNNA3 gene. According to AGR, this gene has been associated with arrhythmogenic right ventricular dysplasia and intellectual disabilities (Herriges et al., 2019; Bacchelli et al., 2014).

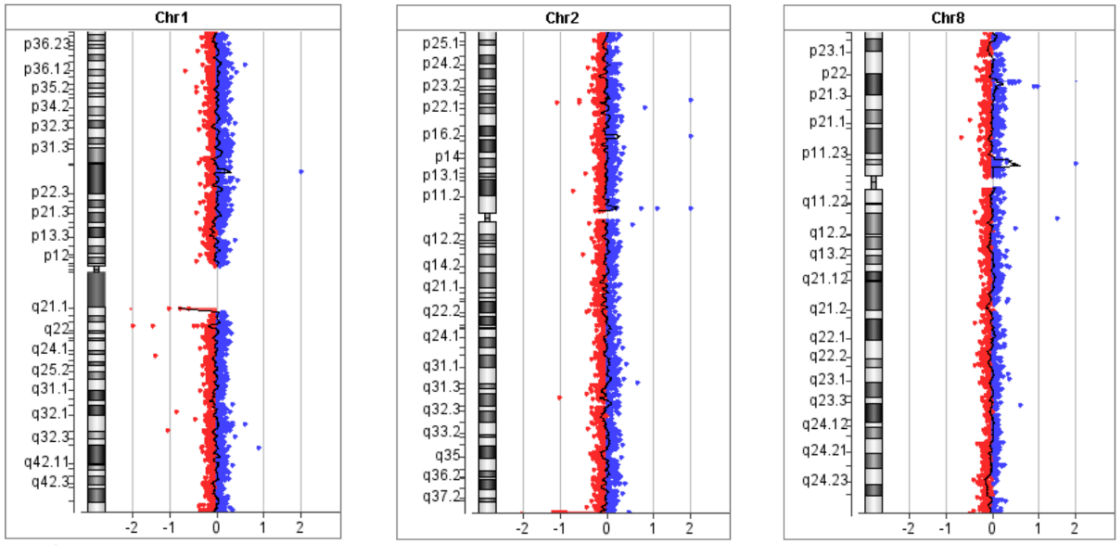
Although amplification at 20q11.21 was indicated in Shef6 hNSC line, it was also observed in ADRC76 hiPSC line (Fig. 3, B-C). Amplification of 20q11.21 is a mutation common spot in ESCs and has been reported for several different stem cell lines after long-term culture (Maitra et al., 2005; Wu et al., 2008; Spits et al., 2008). Studies suggest this commonly observed amplification in ESCs results in healthy growth, larger colonies, increased cell survival and decreased spontaneous differentiation (Spit et al., 2008). Moreover, amplification of 20q11 is frequently associated with cancer (Scotto et al., 2008; Guled et al., 2008). Several genes located in this region are thought to confer an advantage to the cells, particularly *BCL2L1* and *ID1*. These genes have the capability to enhance cell proliferation and inhibit differentiation in many cell types (Nguyen et al., 2014; Amps et al., 2011). Therefore it is plausible a mix up in the samples occurred as the Shef6 hESC showed no indication of this gene.

Amplification of chromosome 21q22.3 was detected and carried over to the ADRC76 hNSC line. Within this chromosome, PDE9A gene annotation is linked to congestive heart failure according to the AGR database. The aberration is associated with pediatric acute lymphoblastic leukemia (ALL) but is still not completely understood as not all genes on this chromosome have been linked to this diagnosis (Kudalkar et al., 2022).

A)Shef6 hESC

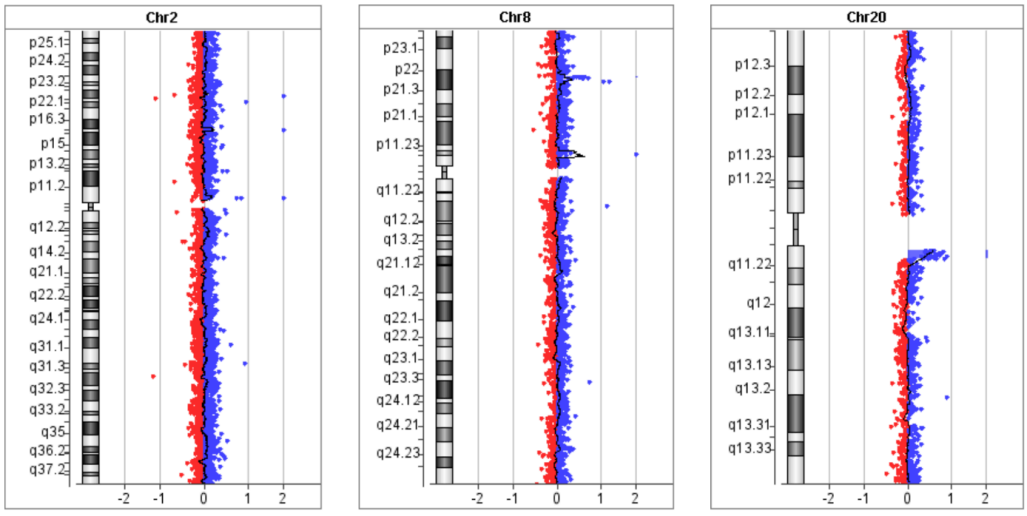
Chr	Start-Stop(bp)	Cytoband	Size(kb)	#Probes	Amp/Gain/ Loss/Del	Annotations
chr1	143585084-143697454	q21.1	112.371	3	-0.799	
chr2	241944235-242065208	q37.3	120.974	5	-1.022	LINC01237, LINC01238, LOC285097, LINC01880
chr8	14714802-15172544	p22	457.743	13	0.427	SGCZ, MIR383

Amp=Amplification Del=Deletion



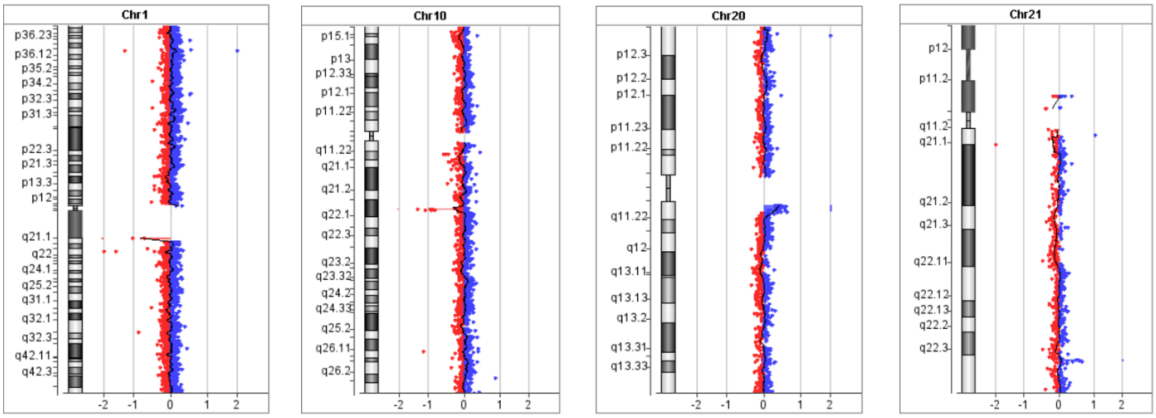
B)Shef6 hNSC

Chr	Start-Stop(bp)	Cytoband	Size(kb)	#Probes	Amp/Gain/ Loss/Del	Annotations
chr2	241944235-242065208	q37.3	120.974	5	-1.034	LINC01237, LINC01238, LOC285097, LINC01880
chr8	14714802-15172544	p22	457.743	13	0.530	SGCZ, MIR383
chr20	31300674-32474742	q11.21	1,174.069	35	0.562	DEFB116, DEFB118, DEFB119, DEFB121, DEFB122, DEFB123, DEFB124, REM1, LINC00028, HM13, HM13-AS1, ID1, MIR3193, COX4I2, BCL2L1...



C) ADRC76 hiPSC

Chr	Start-Stop(bp)	Cytoband	Size(kb)	#Probes	Amp/Gain/ Loss/Del	Annotations
chr1	143585084-143697454	q21.1	112.371	3	-0.809	
chr10	66405787-66684136	q21.3	278.35	7	-0.973	CTNNA3
chr20	31300674-32533660	q11.21	1,232.987	36	0.404	DEFB116, DEFB118, DEFB119, DEFB121, DEFB122, DEFB123, DEFB124, REM1, LINC00028, HM13, HM13-AS1, ID1, MIR3193, COX4I2, BCL2L1...
chr21	42539595-42771891	q22.3	232.297	7	0.541	SLC37A1, LINC01671, PDE9A



D)ADRC76 hNSC

Chr	Start-Stop(bp)	Cytoband	Size(kb)	#Probes	Amp/Gain/ Loss/Del	Annotations
chr10	66405787-66684136	q21.3	278.35	7	-0.975	CTNNA3
chr21	42539595-42771891	q22.3	232.297	7	0.483	SLC37A1, LINC01671, PDE9A

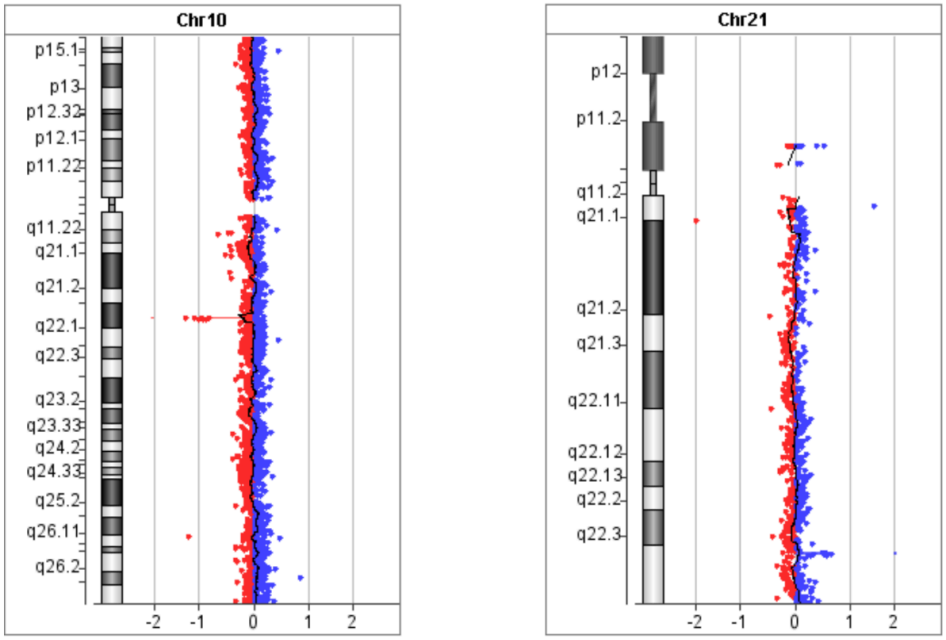


Figure 3. Microarray-Based Comparative Genomic Hybridization (aCGH) Analysis Conducted Through Cell Line Genetics (CLG).
The CGH analysis shows Amplifications (blue) and Deletions (Red) in: **A) Shef6** hESC line: 2 deletions and 1 amplification containing 6 genes **B) Shef6** hNSC line: 1 deletion and 2 amplifications containing 21 genes, including stemness genes *ID1* and *BCL2L1* **C) ADRC76** hiPSC line: 2 deletions and 1 amplification containing 19 genes, including stemness genes *ID1* and *BCL2L1* **D) ADRC76** hNSC line: 1 deletion and 1 amplification containing 4 genes *Annotations are genes located within cytoband region

Statistical Analysis of MFIs vs Concentrations Do Not Yield Identical Significance for Cell Characterization

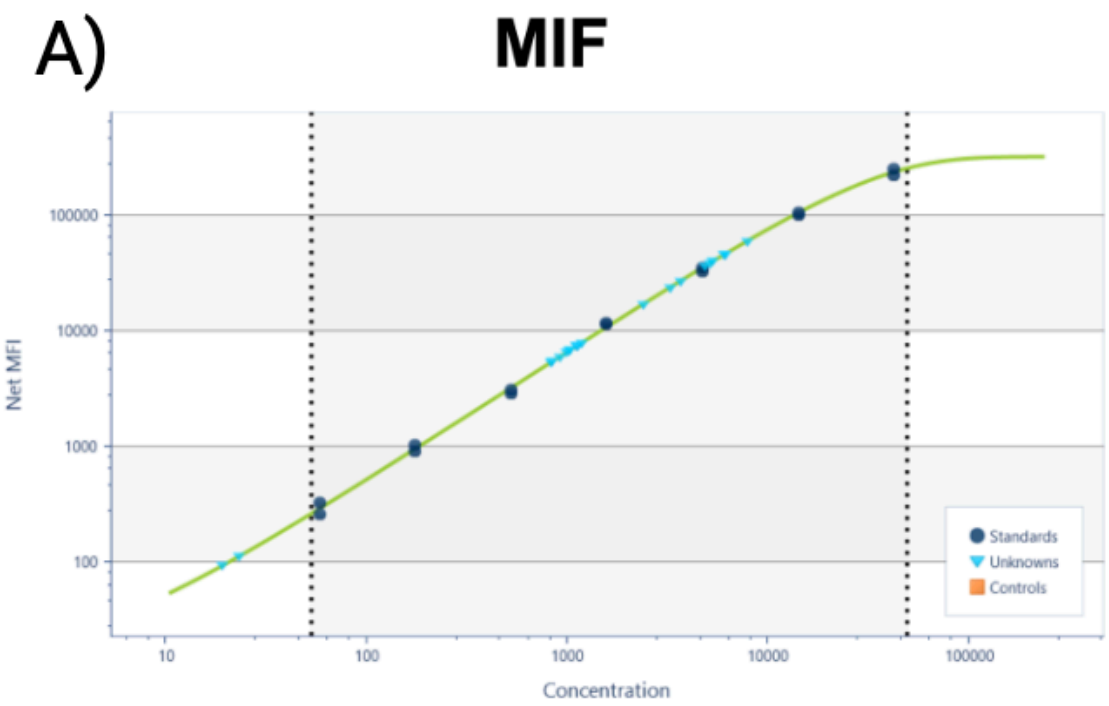
Reportedly, Luminex has determined that the Median Fluorescence Intensity (MFI) for analytes are best for analysis and reproducibility (Breen et al., 2015). A big issue is producing reliable standard concentration curves, a common problem is selecting the appropriate dilution series (Breen et al., 2015). If the dilution series is below (LLOQ) or above (ULOQ) the standard range, then the unknowns will be out of range when using the standard curve, and this can generate unbalanced data sets. This can mean that entire analytes or even samples will need to be ignored and removed from the data set. For this study, statistical analysis were conducted separately on the concentrations and Median Fluorescence Intensity (MFI) data obtained from the Luminex assay to determine significant differences among experimental groups.

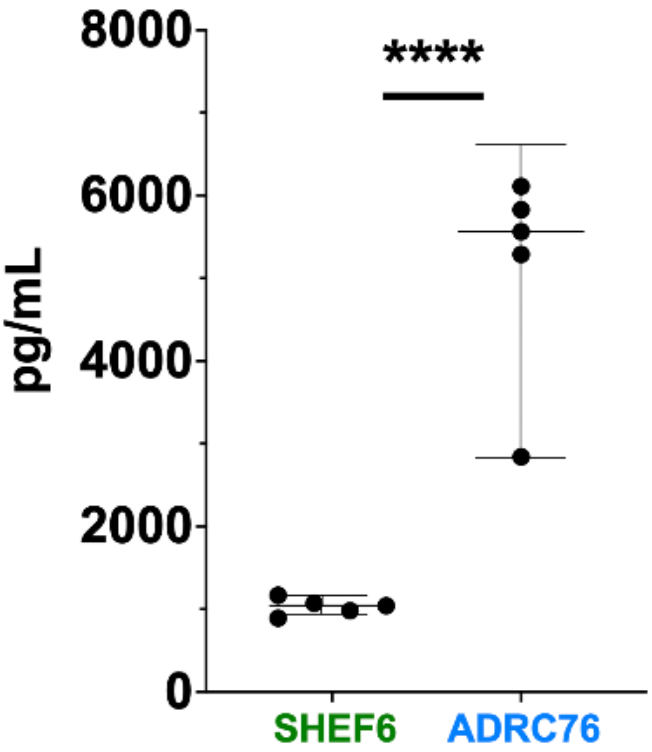
A total of 27 analytes with a minimum of 3 proper concentration readings were taken into consideration between the cell lines. The means were calculated for each analyte measured within the 6-8 standard curve range. Normality of the data was assessed using Shapiro-Wilk tests, and a two-way ANOVA (holm-sidak post-hoc) were employed to compare the different cell lines

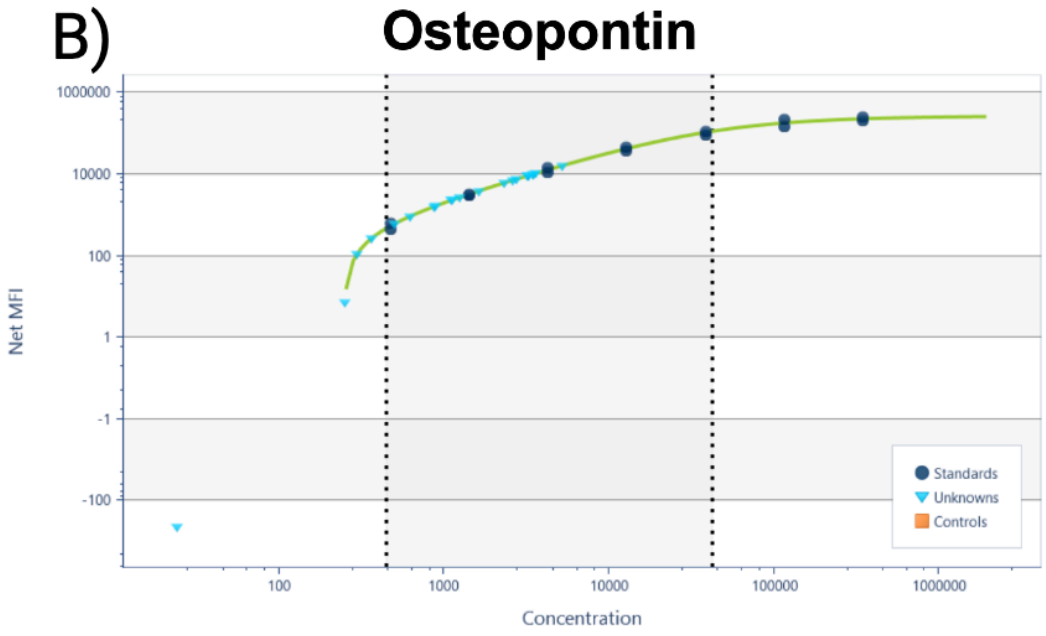
across 27 different analytes. Statistical significance was determined at the predetermined alpha level ($p < 0.05$). Among all 27 analytes, MIF and Osteopontin held significance between cell lines. ADRC76 mean concentration of MIF was 5126.4 pg/mL compared to Shef6 with a mean concentration of 1030.72 pg/mL.(Fig.4, A) For Osteopontin, ADRC76 expressed a concentration of 3194.32 pg/mL and Shef6 was 1015.5 pg/mL.(Fig.4, B)

When analyzing the MFI data obtained, all 68 analytes were compared between the cell lines' supernatant. The average MFIs for both cell lines were normalized to average MFIs of the control growth media using Log base 2 transformation for protein expression. Other analytes are highly expressed in Figure 5 but none are similar to the concentration data. High expression through MFIs can be observed in CCL2/JE/MCP-1 and Serpin E1/PAI-1 in ADRC76 hNSC compared to Shef6 hNSC.(Fig. 5, A-B)

A)


MIF
Net MFI
100000
10000
1000
100
10
100
1000
10000
100000
Concentration
Standards
Unknowns
Controls


8000
6000
4000
2000
0
pg/mL
****
SHEF6
ADRC76


B)
Osteopontin
Net MFI
1000000
10000
100
1
-1
-100
100
1000
10000
100000
1000000
Concentration
Standards
Unknowns
Controls

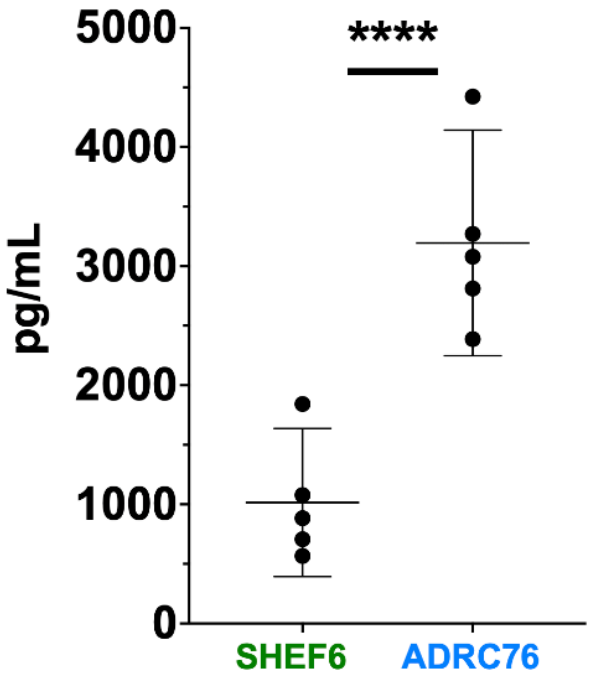

5000
4000
3000
2000
1000
0
pg/mL
****
SHEF6
ADRC76

Figure 4. The Average Concentration for Analytes Secreted Between Shef6 and ADRC76 hNSCs. (n=5 per cell line)
Significant concentration data shown among 2 out of 27 analytes analyzed using two-way ANOVA. **A)** MIF standard curve range with mean concentration (pg/mL) w/ SEM +/- comparison between **Shef6** and **ADRC76** hNSC lines **B)** Osteopontin standard curve range with mean concentration (pg/mL) w/ SEM +/- comparison between **Shef6** and **ADRC76** hNSC lines

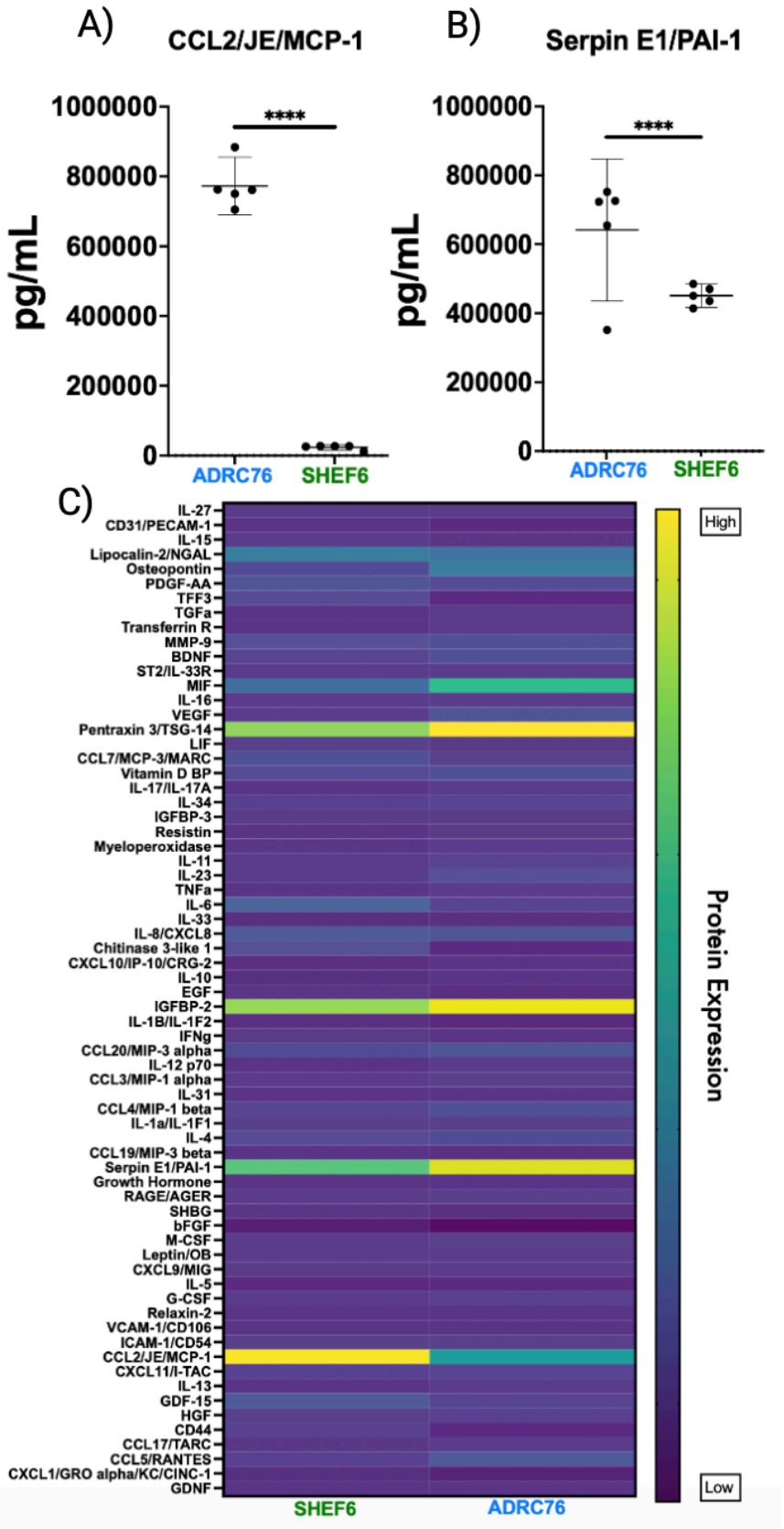

A)
CCL2/JE/MCP-1
pg/mL
1000000
800000
600000
400000
200000
0
****
ADRC76
SHEF6
B)
Serpin E1/PAI-1
pg/mL
1000000
800000
600000
400000
200000
0
****
ADRC76
SHEF6
C)
IL-27
CD31/PECAM-1
IL-15
Lipocalin-2/NGAL
Osteopontin
PDGF-AA
TFF3
TGFa
Transferrin R
MMP-9
BDNF
ST2/IL-33R
MIF
IL-16
VEGF
Pentraxin 3/TSG-14
LIF
CCL7/MCP-3/MARC
Vitamin D BP
IL-17/IL-17A
IL-34
IGFBP-3
Resistin
Myeloperoxidase
IL-11
IL-23
TNFa
IL-6
IL-33
IL-8/CXCL8
Chitinase 3-like 1
CXCL10/IP-10/CRG-2
IL-10
EGF
IGFBP-2
IL-1B/IL-1F2
IFNg
CCL20/MIP-3 alpha
IL-12 p70
CCL3/MIP-1 alpha
IL-31
CCL4/MIP-1 beta
IL-1a/IL-1F1
IL-4
CCL19/MIP-3 beta
Serpin E1/PAI-1
Growth Hormone
RAGE/AGER
SHBG
bFGF
M-CSF
Leptin/OB
CXCL9/MIG
IL-5
G-CSF
Relaxin-2
VCAM-1/CD106
ICAM-1/CD54
CCL2/JE/MCP-1
CXCL11/I-TAC
IL-13
GDF-15
HGF
CD44
CCL17/TARC
CCL5/RANTES
CXCL1/GRO alpha/KC/CINC-1
GDNF
SHEF6
ADRC76
High
Low
Protein Expression

Figure 5. Median Intensity Fluorescence (MFIs) for Analytes Secreted Between Shef6 and ADRC76 hNSCs. (n=5 per cell line)
Significant MFI data shown among 2 out of 68 analytes using two-way ANOVA. **A)**CCL2/JE/MCP-1 (pg/mL) w/ SEM +/- comparison between **Shef6** and **ADRC76** hNSC lines **B)** Serpin E1/PAI-1(pg/mL) w/ SEM +/- comparison between **Shef6** and **ADRC76** hNSC lines **C)** The heatmap was generated to visualize the log2-transformed median fluorescence intensity (MFI) results. Log2 transformation was applied to normalize the data and reduce skewness. Each row in the heatmap represents a specific analyte, while each column corresponds to the experimental groups. Intensity of coloration reflects the magnitude of log2-transformed MFIs, with lower expression represented by darker shades and higher expression by lighter shades. The heatmap provides a comprehensive overview of the expression levels of multiple analytes.

CD133+/CD34- Sorted hNSCs Respond Differently to Differentiation Conditions In Vitro

Immunocytochemistry staining showed that most of cells after 7 days in differentiation medium, Shef6 hNSCs could differentiate into β-tubulin III+ neurons, GFAP+ astrocytes and Olig2+ oligodendrocytes (Fig. 6, A) On day 14, Shef hNSC showed an increase in oligodendrocytes differentiation (Fig. 6, B). Quantification of Shef6 hNSCs from day 7 to day 14 showed the same results. A remarkable 66% of Shef6 hNSC preferred oligodendrocyte fate (Fig. 6, C). In the presence of differentiation medium, ADRC76 hNSCs differentiated in an abnormal behavior from growing into embryoid like bodies to overgrowth in the wells. Important to note, ADRC76 hNSC seemed to only differentiate into β-tubulin III+ neurons but many cells remain undifferentiated (Fig. 6, E, F).

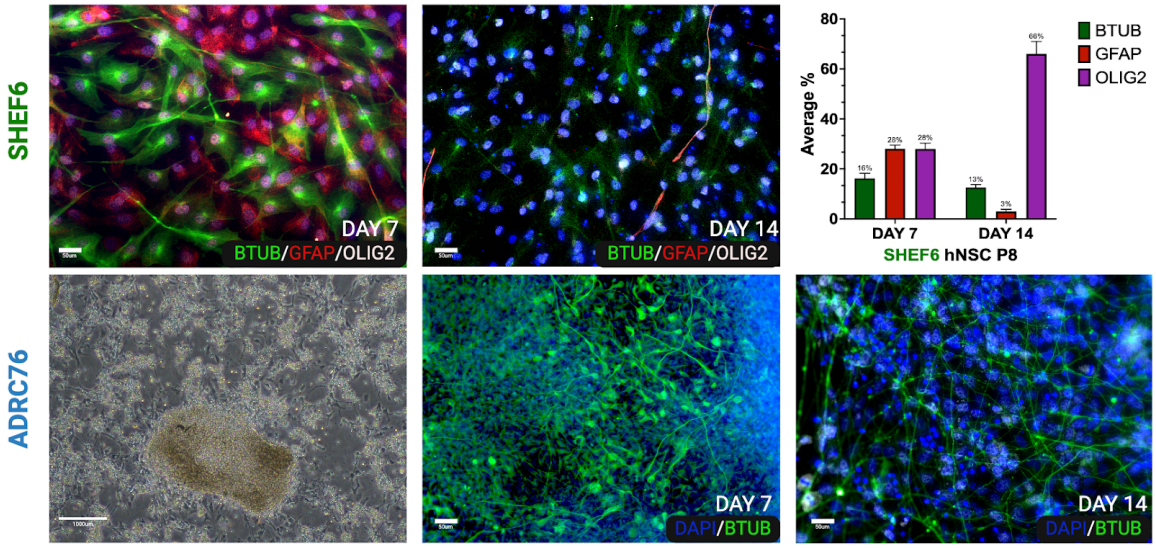


Figure 6. Morphology and Fate of NSCs for Both Shef6 and ADRC76 in Differentiation Conditions.

Representative microscopy images of differentiated neural stem cells (NSCs) labeled with specific markers: Beta III-tubulin, Olig2, and GFAP **(A-B, E-F)**. Hoescht was used to counterstain nuclei (blue). For quantification, 5 images (20x) were taken per well per timepoint. Manual quantification of neural cell types generated from NSCs over time, as analyzed by Imaris software. The 5 images were then averaged for statistical testing. Data are reported as percentages.**C**) Bar graph representing the mean percentage and standard error mean of neurons, astrocytes, and oligodendrocytes observed at day 7 and 14 time points for **Shef6** hNSC. **D)** Showcases abnormal growth (4x) response of **ADRC76** hNSC exposed to differentiation medium after 7 days in vitro. Scale bar is 50um.

CHAPTER FOUR:

DISCUSSION

Quality control and assessment are paramount to meet general biological product standards set forth by the FDA, especially by adhering to cGMP. This is particularly crucial when dealing with hESC- and iPSC-derived NSCs due to their potential therapeutic applications in regenerative medicine. These regulations are designed to ensure the safety, efficacy, and quality of therapeutic products, including those derived from stem cells. By implementing cGMP guidelines, manufacturers are required to establish and maintain rigorous quality control measures throughout the entire manufacturing process (George B., 2011).

Quality control starts from the procurement of source materials, ensuring that they meet predefined specifications and are obtained from reputable and traceable sources to establish XF conditions. In the case of hESC- and PSC-derived NSCs, the quality of the starting cell lines is critical as it impacts the characteristics and behavior of the final product (Galiakberova & Dashinimaev, 2020). Factors such as cell identity, purity, genetic stability, potency and absence of contaminants must be rigorously assessed and monitored.

To the field's knowledge, current therapies with the use of neural stem cells to address neurotrauma, disorders or diseases are still being thoroughly tested in pre-clinical studies. This is due to the need for standardized FDA guidelines for neuralization and characterization protocols, optimal yield, targeted delivery, and the importance of identifying the optimal cell source (FDA, 2019).

In this study, we have cultured and neuralized hESC and iPSC-derived neural stem cells under XF conditions to test their suitability to produce highly CD133-positive neural stem cells. The data presented here uses established protocols to transition and maintain multiple hPSC and hNSC lines long-term in a research setting under complete XF culture conditions, thus providing reliable cGMP methodologies and highlighting challenges to produce clinically translatable cell lines. The findings in this study present the benefits of early adoption of cGMP-like conditions to better prepare for clinical application.

An optimal method of cell sorting has been identified and characterized the population of neural stem cells in both cell lines which found the yield to be similar post-MACS. The MACS method has been shown to be a very efficient isolation technique for both *in vivo* and *in vitro* applications and without indications that the magnetic particles affect functionality of the isolated cells (Laghmouchi et al. 2020). MACS is extensively used for the enrichment of CD133-positive neural stem cells to increase yield and very gentle thus resulting in healthier and more robust cultures and will prevent costly delays in experimental planning and undertaking (Bowles et al., 2019).

Since purity, and identity can be influenced by sorting methods and culture conditions, a successful isolation of purified NSCs could aid in the reproducibility assessment of *in vitro* and *in vivo* assays for preclinical studies. Although no optimal range of cell purity exists, unwanted and unintended differentiated cell types could affect the behavior of a stem cell product (George B., 2011). For instance, CD34 is expressed on multiple cell types, including hematopoietic stem

cells, endothelial cells, macrophages, and microglia, and this possibility warrants an isolation of CD133+/CD34- cell population. In addition, could possibly be correlated with safety or efficacy outcomes, reproducibility of manufacturing conditions and clinical translatability from benchside to bedside can be assessed by this measurement (George B., 2011).

Although the functionality of the isolated cells seems not to be affected, the possible residuals of magnetic properties in isolated cell populations could make it difficult to translate clinically therefore requires additional in-process testing via flow cytometry for example. But the beads are composed of iron oxide and polysaccharide, it is advertised by the manufacturer (Miltenyi) that these magnetic nanoparticles are biodegradable, which implies a loss of magnetic properties over time in the MACS isolated cell populations (Laghmouchi et al., 2020).

Proteomics provides valuable insights into the potency of cell-based therapies by characterizing their protein expression profiles, elucidating signaling mechanisms, confirming cell identity and purity, assessing functional activity, and monitoring stability and quality attributes (Bravery et al., 2013). By integrating proteomic analyses into preclinical and clinical studies, researchers can generate comprehensive data sets that address FDA requirements for demonstrating the potency, safety, and efficacy of cell-based therapies. While NSCs themselves do not produce analytes in the traditional sense, they do secrete a variety of cytokines and trophic factors that play important roles in neurodevelopment and

tissue repair. These secreted factors can have paracrine effects, influencing the behavior of neighboring cells (Velikic et al., 2024).

Osteopontin (OPN) is a protein found in various tissues, including the brain, where it plays important roles in cell adhesion, migration, and signaling. In the context of NSCs, OPN has been implicated in several processes such as neurogenesis, migration, and neuroinflammation (Lund et al., 2009; Zhou et al, 2020). Understanding the role of OPN could explain the influence of the proliferation and differentiation responses of ADRC76 and Shef6 hNSCs whether this is up or down regulated. High expression of OPN has been linked to various pathologies including cancer metastasis and inflammation. Elevated levels are often associated with tumor aggressiveness and immune modulation indicating its significant role in disease progression (Lamort, A. S., 2019). The neuroinflammatory processes in the brain have also been known to be regulated through this protein as it can modulate the immune response and the activation of glial cells, which can in turn influence NSC behavior and function in pathological conditions (Zhou et al., 2020).

Similarly to OPN, Migration Inhibitory Factor (MIF) plays a role in regulating the migration behavior of neural stem cells (NSCs) during brain development and in response to injury or disease. MIF can influence the movement of NSCs by interacting with various signaling pathways involved in cell migration (Ohta et al., 2012). In addition to neuroinflammatory regulation, MIF was found to be expressed in neural stem cells (NSCs) and contribute to the proliferation of NSCs by increasing the self-renewal of NSCs (Ohta et al., 2012;

Grieb et al., 2010), revealing the functional role of MIF in neural stem cell proliferation.[60] High expression of MIF is associated with inflammatory responses, autoimmune diseases, and cancer progression(Grieb, G., et al., 2010).An overexpression for this protein is often correlated with exacerbated immune reactions and various pathological conditions.

When analyzing the MFI data between the cell lines, the previous mentioned proteins were not significant. Two different proteins, CCL2/JE/MCP-1 and Serpin E1/PAI-1, were present. CCL2 is a chemokine involved in immune cell recruitment and inflammation regulation. Elevated levels of CCL2 have been implicated in various diseases, particularly breast cancer, where it promotes tumor progression and metastasis through recruitment of immune cells and angiogenesis (Rogic, A., 2021). Serpin E1, is a serine protease inhibitor involved in regulating blood clotting. High expression of Serpin E1 has been associated with high proliferation and cancer metastasis as well (Chen, S.,2022).

Understanding the role of secreted proteins to determine NSCs behavior is important for elucidating the mechanisms underlying brain development, neurogenesis, and repair processes. It may also have implications for developing therapies for neurological disorders and injuries, where promoting or inhibiting NSC behavior response could be beneficial depending on the context. Therefore the presence of high expression of MIF, OPN, CCL2 and Serpin E1 using concentrations or MFIs could indicate the tumorigenicity potential of the ADRC76 hNSC line.

Possible genetic changes, such as aberrations, can occur in human stem cells during culture establishment (Steichen et al., 2019; Weissbein et al., 2017). Typically, these aberrations can provide growth advantages to the cells, similar to those observed during oncogenesis (Ronan A., 2018). But the potential use of neural stem cells in therapeutic medicine raises concern for possible tumorigenesis. To overcome this, assessing genetic stability is essential. Array comparative genomic hybridization (aCGH) is an advanced genetic diagnostic technique for uncovering chromosomal aberrations and genomic rearrangements not detected by G banding karyotype (Levy & Burnside, 2019). Shef6 human cell types (hPSC and hNSC) generated via the methods presented here appear to exhibit good genomic stability. The same cannot be confidently said for ADRC76.

As there are many genes not fully understood yet, currently no consensus among laboratories as to what genes/regions should or should not be targeted outside of well-documented and thoroughly researched syndromic genes/regions has been established (Levy & Burnside, 2019; Ronan A., 2018). Utilizing Alliance of Genome Resources (AGR), allowed for better understanding for the genes involved and their possible implications for cell behavior. Whether or not any amplifications and deletions reported are less likely to be oncogenic depends on the particular genes involved (Levy & Burnside, 2019; Ronan A., 2018).

As previously mentioned in results (Fig. 3D), ADRC76 hNSC product has a deletion on chromosome 21q22.3, which is a chromosome associated with a couple of diagnosis but none of the genes annotated were highlighted as oncogenic in the results (Kudalkar et al., 2022; Muenphon et al., 2006). PDE9A

gene has association with congestive heart failure but this cannot be definitive. For chromosome 10q21.3, the CTNNA3 gene has been identified using the AGR database to confirm the association with Arrhythmogenic right ventricular dysplasia. Some individuals grow and thrive with large sections of a chromosome missing, while a microdeletion of another chromosome can cause an intellectual disability. It depends on how many and exactly which genes are in the area affected, and as well as other effects on chromosome replication and function we still cannot completely predict (Elliot et al., 2010). *BCL2L1* and *ID1* genes found on Chromosome 20q11.21 are a prime example of an aberration that provides growth advantages to hPSC lines rather than an oncogenic nature. Although the same cannot be said conclusively for the Shef6 cell line due third-party sample mix up.

Advantages of using this approach is a strict profile of aberrations acquired during culture and the elimination of benign copy number variations found within the cell population (Ronan A., 2018). However, this approach will not detect any aberrations prior to reprogramming into hiPSC, which could be important to see if efficiency of the reprogramming was adequate.This could also be linked to the genomic instability generally associated with iPSC derived lines, as only 1% of publications address the genome integrity of hPSC lines (Steichen et al., 2019). Originally, all laboratories used to check for this by following up every anomaly found with microscopy using detailed fluorescent probes, but due to time and funding constraints, few now do this (Levy & Burnside, 2019).

Previous research has shown that human stem cells in long-term culture acquire chromosomal changes similar to those seen during tumorigenesis (Baker et al., 2007; Yang et al., 2008). As G-banding karyotype analysis can be beneficial as a rapid screening technique for large-scale abnormalities such as aneuploidy, it cannot detect the majority of aberrations obtained during prolonged culture. Therefore, it is essential that an assay used for genomic stability in stem cells has the ability to detect copy number variants between stemness and oncogenes as these aberrations can undoubtedly impact the survival and proliferative abilities of the cells for clinical translatability (Yang et al., 2008).

Stem cell-based therapies offer great potential for treating various diseases and injuries but pose unique challenges due to their ability to differentiate into diverse cell types. The FDA mandates differentiation assays for cell products primarily to ensure their safety, efficacy, potency, and quality. These assays play a critical role in confirming the desired differentiation status of cells intended for therapeutic use, mitigating risks associated with the administration of undifferentiated cells, such as tumorigenicity (Moradi et al., 2019). Moreover, they provide valuable insights into the therapeutic mechanism of action by demonstrating the cells' ability to differentiate into specific cell types relevant to the targeted disease or condition. By establishing rigorous quality control measures, differentiation assays contribute to the consistency and reproducibility of cell-based products, which are essential for regulatory approval and clinical translation.

In the case of hNSCs, their differentiation preferences and responses to environmental cues are critical considerations. Studies have shown that enrichment of hNSCs for cells expressing the surface marker CD133 using magnetic sorting can influence their fate preference (Haus et al., 2014). However, safety concerns regarding tumorigenicity inherent with pluripotent stem cell-derived hNSCs must be addressed. Evidence has been previously demonstrated that while these Shef6 hNSCs expand rapidly in culture, once they are transplanted into the brain there was no evidence of non-neural lineage tumors (Haus et al., 2014). The same cannot be said for the ADRC76 hNSCs.

Notably, while Shef6 hNSCs predominantly differentiate into Olig2+ oligodendrocytes, ADRC76 hNSCs exhibit a preference for β-tubulin III+ neurons possibly due to the regulation of OPN. This discrepancy may be influenced by the microenvironment, where NSCs tend to differentiate into neurons or oligodendrocytes under proper niche conditions (Biswas et al., 2019; Del Aguila et al., 2022; Ming & Song, 2011; Keung et al, 2012) but switch to astrocytes under harmful stimulation (Czéh & Lucassen, 2007; Encinas et al., 2011). The ability for Shef6 hNSC to have a preference for oligodendrocytes, could lead to re-myelination of axon to address Central Nervous System injuries such as spinal cord injury (Liu et al., 2000). Additionally, observations during differentiation reveal morphological changes in ADRC76 hNSCs, suggesting a transition to an embryoid body-like stage, highlighting the complexity of cellular responses to differentiation cues (Jiao et al., 2017). In return, ADRC76 hNSCs became unable to properly quantify for a comparison to Shef6 hNSCs. Which in turn could be

linked to the proteins secreted by ADRC76 hNSCs. This led to the realization that unabated in vitro cell differentiation can be problematic and could often create undesired results such as tumor formation in vivo. These findings underscore the importance of understanding cell signaling pathways and downstream effects in the context of therapeutic development.

Conclusions

The significance of maintaining cell identity and quality control becomes even more pronounced when considering the potential therapeutic applications of hESC- and iPSC-derived NSCs. These cells have the unique ability to differentiate into various neural cell types, including neurons, astrocytes, and oligodendrocytes, making them promising candidates for regenerative medicine approaches aimed at treating neurodegenerative diseases, spinal cord injuries, and other neurological disorders.

Pluripotent stem cells like embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have the capacity to differentiate into any human cell type. They also have a capacity to form teratomas, benign tumors, while undifferentiated. Therefore, the potency of such derived NSCs strongly relates to the intended differentiation status and their intended Mechanism of Action. For instance, NSCs could be used to replace damaged or lost neural cells, promote tissue repair and regeneration, and modulate the local microenvironment to support endogenous repair mechanisms. However, the success of these therapeutic strategies hinges on the ability to consistently produce high-quality

and functionally competent NSC products. Any variability or compromise in cell purity and quality could undermine the safety and efficacy of the therapeutic intervention, potentially leading to adverse outcomes for patients.

Qualitative assessment relies on visual interpretation, which can be subjective and prone to variability between different observers. This subjectivity may lead to inconsistencies in evaluating cell morphology, differentiation status, and purity, impacting the reproducibility and reliability of results. There is limited information provided about the molecular and functional characteristics of hESC-NSCs and iPSC-NSCs. While it can identify morphological features and basic cellular phenotypes, it may not capture subtle differences in gene expression, signaling pathways, or functional properties critical for therapeutic efficacy. Finally, differentiation of hESC-NSCs and iPSC-NSCs into neural lineages is a complex process influenced by various factors, including culture conditions, growth factors, and cell-cell interactions. Qualitative assessment may not adequately capture the heterogeneity and dynamics of differentiation, limiting the understanding of lineage specification and maturation stages.

Although this study may lack robust validation criteria to confirm the identity, purity, and potency of hESC-NSCs and iPSC-NSCs, without standardized protocols and well-defined criteria, the challenge still exists to ensure consistency and comparability across different batches or laboratories. Even meeting cGMP standards requires rigorous documentation, traceability, and quality control measures throughout the manufacturing process. The methods may not always fulfill regulatory requirements for demonstrating product

consistency, safety, and efficacy, necessitating the development of more standardized and quantitative assays. Not to mention are often labor-intensive, time-consuming, and difficult to scale up for large-scale production. Automation of qualitative assays to accommodate high-throughput screening and manufacturing demands remains a challenge, particularly in ensuring reproducibility and reliability. Ideally this qualitative assessment should complement quantitative analyses to provide a comprehensive characterization of hESC-NSCs and iPSC-NSCs. However, integrating qualitative observations with quantitative data, such as gene expression profiling, proteomics, and functional assays, poses challenges in data interpretation and correlation.

For future studies, more comparisons between and within cell sources will be implemented while addressing the challenges that occurred during the different stages of quality control of this study. In conclusion, quality control and the maintenance of cell viability are critical aspects of the manufacturing process, particularly in the context of producing hESC- and hiPSC-derived NSCs for therapeutic applications. Adhering to cGMP guidelines ensures that rigorous quality standards are upheld throughout the manufacturing process to allow or eliminate cell products, ultimately safeguarding the safety and efficacy of stem cell-based therapies in regenerative medicine.

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