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Challenges and Advances in the Use of Induced Pluripotent Stem Cells for Modeling and Treating Hypoplastic Left Heart Syndrome: A Comprehensive Review

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ABSTRACT

Hypoplastic Left Heart Syndrome (HLHS) is a severe and rare congenital heart disease characterized by the underdevelopment of the left side of the heart, which is responsible for pumping oxygenated blood throughout the body. This underdevelopment results in an insufficient supply of oxygenated blood to vital organs and tissues. Current treatments for HLHS face significant challenges and typically involve complex surgical interventions that can only extend patient survival to a limited degree. Heart transplantation is another potential option, but it is constrained by the scarcity of donors and the risk of immune rejection. Induced pluripotent stem cells (iPSCs), derived from somatic cells, possess the remarkable ability to differentiate into any cell type within the three primary germ layers, making them a promising tool for tissue regeneration, disease modeling, and repair. However, despite their potential, the clinical application of iPSCs remains limited, primarily due to the risk of tumorigenesis. This review explores the role of iPSCs in advancing the understanding and treatment of HLHS, examining their cellular and molecular mechanisms, historical development, and their application in cardiovascular research. Additionally, we consider the major challenges and limitations associated with the use of iPSCs in the context of HLHS, underscoring the critical need for further research to enhance their therapeutic efficacy and safety.

Keywords: Induced pluripotent stem cells, tumorigenesis.

INTRODUCTION

Hypoplastic Left Heart Syndrome (HLHS) is a rare but severe congenital heart defect, affecting approximately 2 in every 10,000 births (Gobergs *et al.*, 2016). It is characterized by the underdevelopment of the left side of the heart, including the left ventricle, left atrium, aorta, and associated valves. This structural abnormality impairs the heart's ability to pump oxygenated blood effectively to the rest of the body, making HLHS one of the congenital heart defects with the highest mortality rate without timely surgical

intervention (Lopez *et al.*, 2020). The current standard of care for HLHS involves a series of surgical procedures designed to reconstruct the heart and establish a functional circulatory system. These surgeries, which must be performed within the first few years of life, are critical because, as the ductus arteriosus closes, the heart becomes unable to maintain proper circulation due to the inability of the underdeveloped left ventricle to pump oxygenated blood efficiently (Norwood Jr., 2000). The introduction of surgeries such as the Norwood, Glenn, and Fontan procedures has significantly improved survival rates for HLHS patients, from approximately 0% to over 90% (Fruitman, 2000). However, these procedures come with limitations, including the need for additional surgeries, lifelong monitoring, and the potential development of complications such as arrhythmia (Fredenburg *et al.*, 2011).

Heart transplantation is another treatment option, but it is constrained by a limited supply of donors and the risk of immune rejection (Rai *et al.*, 2019). In 2006, the advent of stem cell therapy, particularly using induced pluripotent stem cells (iPSCs), emerged as a promising approach in regenerative medicine. iPSCs are somatic cells reprogrammed to attain pluripotency, allowing them to differentiate into any cell type, including cardiomyocytes (Romito and Cobellis, 2016). This capability can potentially repair and regenerate heart tissues in HLHS patients, offering a more permanent and less invasive solution than current treatments. The first clinical application of iPSCs occurred in 2014, marking a pivotal moment in cell therapy. Since then, the scope of iPSC-based clinical trials has expanded, with over 30 trials worldwide focusing on therapeutic applications (Kim *et al.*, 2022), (Yasui *et al.*, 2022), (Yamanaka, 2020). iPSCs have also been successfully employed to model HLHS, enabling researchers to identify crucial gene markers implicated in the development of the condition. Despite these advancements, significant challenges remain. The clinical application of iPSCs is still hindered by tumorigenicity, immune rejection, and high treatment costs (Zhao *et al.*, 2011), (Jiang *et al.*, 2014), (Yang *et al.*, 2019), (Doss and Sachinidis, 2019), (Huang *et al.*, 2019), (Danter, 2019). Current research highlights the inefficiency in deriving iPSCs from HLHS patients, with iPSCs from these patients frequently tending to differentiate into smooth muscle cells rather than cardiomyocytes. Moreover, the efficiency of deriving iPSCs from HLHS patients is as low as 0.0002%, which is markedly lower than that of healthy patients (Yang *et al.*, 2019), (Jiang *et al.*, 2014). Furthermore, iPSCs derived from HLHS patients demonstrate reduced expression of key gene markers and abnormalities in cellular structures, significantly limiting their potential clinical application in HLHS (Yang *et al.*, 2019), (Jiang *et al.*, 2014), (Miao *et al.*, 2019). Additionally, the cellular and molecular interactions underlying HLHS are not yet fully understood, further emphasizing the need for continued investigation in this area (Bejjani *et al.*, 2021). According to (Saraf *et al.*, 2019), (Soma *et al.*, 2024), (Tani *et al.*, 2022), iPSCs have been widely utilized for disease modeling and experimental applications in various cardiovascular diseases, including HLHS. However, these studies are predominantly limited to clinical data and outcomes from human trials, which constrains a comprehensive understanding of the potential use of iPSC-derived cardiomyocytes in human patients. Furthermore, the challenges associated with differentiating cardiomyocytes from iPSCs, as well as the subsequent structure and physiological function of these cells post-differentiation, have not been thoroughly explored. Additionally, while these articles discuss the potential of iPSCs for disease modeling, they provide limited information on completed or ongoing practical applications in clinical settings. This review aims to explore the role of iPSCs in understanding and potentially treating HLHS, focusing on their applications in cardiovascular research across both animal models and human patients, as well as the underlying molecular mechanisms. Furthermore, it examines the tumorigenic and immunogenic risks associated with iPSCs, highlighting the importance of developing safer reprogramming techniques and more precise gene-editing technologies to mitigate these risks. This review also aims to contribute to the goal of making regenerative medicine, particularly iPSCs, a viable and less invasive alternative to current palliative treatments, thus improving the quality of life for HLHS patients. This study did not involve any clinical trial, therefore, the clinical trial number is not applicable.

II. OVERVIEW OF HYPOPLASTIC LEFT HEART SYNDROME

A. Hypoplastic Left Heart Syndrome HLHS is a congenital heart defect that occurs in approximately 2 per 10,000 live births (Gobergs *et al.*, 2016). It is recognized as the most common form of congenital cardiac malformation, characterized by the

underdevelopment of the left side of the heart. Patients with HLHS typically have only one fully developed ventricle (Norwood Jr., 2000). Key anatomical features include hypoplasia of the left ventricle and the ascending aorta, with the mitral and aortic valves often exhibiting stenosis or atresia (Hinton *et al.*, 2007). These defects result in the inability of the left side of the heart to adequately support systemic circulation. Before birth, the ductus arteriosus, a fetal blood vessel, allows for the temporary viability of this condition. However, once infants are born and begin breathing independently, the ductus arteriosus closes, preventing oxygenated blood from being circulated to the body. Therefore, medical intervention is required within the first few days of life to maintain ductal patency and ensure survival (Norwood Jr., 2000), (Barron *et al.*, 2009).

A 2015 study indicated that survival rates for infants with HLHS improved significantly over time, increasing from 0% between 1979 and 1984 to 42% between 1999 and 2005 (Siffel *et al.*, 2015).

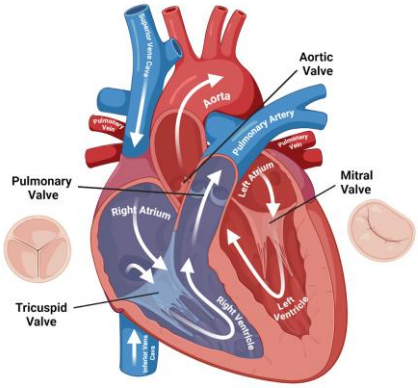

Fig. 1: Detailed anatomical diagram of a normal human heart, illustrating the flow of blood through its four chambers and associated structures. Deoxygenated blood enters the heart through the superior and inferior vena cava into the right atrium (RA). From there, it flows through the tricuspid valve into the right ventricle (RV), which pumps it through the pulmonary valve into the pulmonary artery and towards the lungs for oxygenation. The oxygen-rich blood returns to the left atrium (LA) via the pulmonary veins and then passes through the mitral valve into the left ventricle (LV). The left ventricle pumps this oxygenated blood through the aortic valve into the aorta, distributing it throughout the body. The heart's key valves (tricuspid, pulmonary, mitral, and aortic) ensure unidirectional blood flow.

The survival rates for preterm infants born in high-poverty neighborhoods were 66% during the first week, 27% during the first year, and 24% during the first ten years. For infants who underwent surgical intervention, the overall survival rate was 52%, with 31% of preterm infants and 56% of term infants surviving the procedures (Siffel *et al.*, 2015). Among those who survived the first year, the survival rate improved to approximately 90%. A 2021 study provided updated estimates of survival, reporting 84.4% survival at one week of age, 76.2% at one month, 63.5% at one year, 58.6% at five years, 54.6% at ten years, and 32.6% at fifteen years (Best *et al.*, 2015). According to the Centers for Disease Control and Prevention, an estimated 1 in 3,955 live births in the United States is affected by HLHS, with approximately 929 babies diagnosed annually (CDC, 2024).

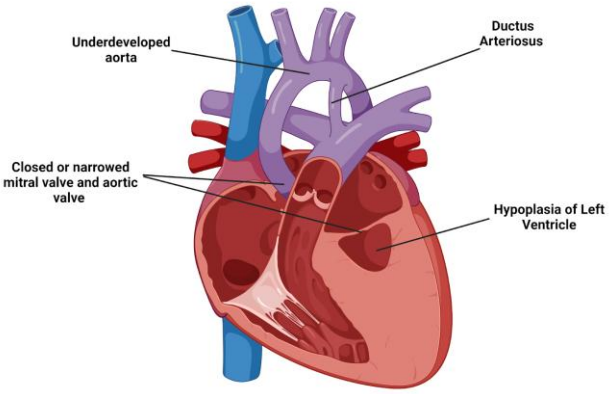

Fig. 2: Diagram of a heart with hypoplastic left heart syndrome. The left ventricle is severely underdeveloped (hypoplasia), leading to impaired pumping capacity. The mitral and aortic valves are either closed or narrowed, restricting blood flow between the left atrium and ventricle, as well as from the heart to the body. The aorta is underdeveloped, limiting its ability to deliver oxygenated blood to the body. The ductus arteriosus, a temporary fetal blood vessel, is shown as critical for bypassing the malformations and maintaining some blood flow to the body.

B. History of HLHS

HLHS was first described by the German physician Dr. von Bardeleben in 1851. Although the initial published cases of aortic atresia were reported earlier by Romberg in 1846 and Canton in 1849, neither report

explored the pathophysiologic or pathogenetic aspects of the condition (Gehrmann *et al.*, 2024). It was not until 1952 that Lev became noteworthy for his detailed description of the abnormalities associated with HLHS, using the term ”Hypoplasia of the aortic tract” in his work (Fruitman, 2000). However, it was not until 1958 that Noonan and Nadas introduced the term ”Hypoplastic Left Heart Syndrome” to describe the condition more comprehensively (Connor and Thiagarajan, 2007).

C. Surgical Intervention

The Norwood procedure is the first of three stages in the palliative treatment for HLHS and is typically performed during the neonatal period. The procedure focuses on utilizing the right ventricle (RV) as the primary pumping chamber for the entire circulatory system (Rai *et al.*, 2019). Prior to the operation, prostaglandin E1 is administered to maintain ductal patency and balance systemic and pulmonary blood flow. During surgery, the patient’s body temperature is maintained at 20°C. The aortic arch is dissected, and a pulmonary homograft patch is applied to enlarge the pathway for blood flow from the heart to the body (Norwood Jr., 2000). This process involves anastomosis of the pulmonary artery to the neoaorta, effectively reconstructing the underdeveloped aorta. Additionally, an atrial septectomy is performed to improve blood flow between the atria (Warnes *et al.*, 2008). A shunt is then created to connect the aortic arch and the pulmonary arteries, establishing outflow from the RV. This shunt may either be a systemic-to-pulmonary arterial shunt, connecting the brachiocephalic artery to the right pulmonary artery, or a Sano modification, which connects the RV directly to the pulmonary artery (Warnes *et al.*, 2008), (Norwood Jr., 2000).

The second stage of the palliative treatment is the Glenn procedure, also known as the hemi-Fontan procedure, typically performed when the child is between 4 and 6 months old (Rai *et al.*, 2019). This procedure involves redirecting the superior vena cava to the pulmonary arteries, bypassing the right atrium (RA), and sending blood directly to the lungs (Cleveland Clinic, 2022). This adjustment reduces the workload on the left ventricle (LV). The Glenn procedure serves as a preparatory step for the final stage of treatment, the Fontan procedure (Rai *et al.*, 2019).

The Fontan procedure is the third and final stage of palliative treatment for HLHS and is usually performed when the child is between 24 and 48 months of age (Rai *et al.*, 2019). This procedure links the inferior vena cava to the pulmonary artery using a conduit. The conduit can either pass through the RA and into the pulmonary artery (internal conduit) or bypass the heart entirely (external conduit). For the internal conduit, part of the lateral wall of the RA is used, whereas synthetic material is used for the external conduit. In the interatrial tunnel method, the conduit directs blood from the inferior vena cava to the pulmonary arteries. This method is typically employed in 1-year-old infants, as the conduit grows with the child; however, it may result in atrial arrhythmias (Fredenburg *et al.*, 2011).

The second approach, known as the extracardiac conduit, is often used for children over the age of three. This method involves using a polytetrafluoroethylene tube graft to link the inferior vena cava and the pulmonary arteries, bypassing the RA. While this method reduces the pressure on the RA, it does not grow with the patient. Surgeons may also create a small fenestration between the conduit and the RA to allow some blood flow into the RA, preventing excessive pressure buildup in the pulmonary circulation and reducing the risk of pleural effusions, thereby increasing cardiac output (Fredenburg *et al.*, 2011).

Heart transplantation is an available and critical treatment option for newborns with HLHS. However, organ rejection, infection, or tumor chances are inevitable. Moreover, heart donors are also limited. For infants, heart transplants can come from non-compliant donors. Despite these challenges, studies suggest that patients who undergo heart transplantation generally experience a higher quality of life compared to those who receive palliative treatments alone (Rai *et al.*, 2019).

D. Genetic and Molecular Mechanisms of HLHS

Although a complete understanding of the cellular and molecular interactions contributing to HLHS has not yet been fully elucidated, current research suggests that HLHS is a multifactorial heart defect resulting from genetic mutations, irregular mechanical stimulations, and disrupted cell signaling pathways (Bejjani *et al.*, 2021). HLHS has a high heritability and is considered a digenic inheritance disorder. Despite

ongoing research, the genetic mechanisms behind HLHS remain partially understood. However, several genes, including NOTCH1, RBFOX2, MYH6, NKX2.5, and LRP2, have been identified as being linked to HLHS (Gabriel *et al.*, 2022). Among these, NKX2.5 and HAND1 are critical for the development of the heart chambers, specifically the left ventricle, and mutations in these genes have been found to result in hypoplasia in patients with HLHS. NOTCH1 also plays a key role in activating other heart-related genes, further implicating it in the pathogenesis of HLHS.

In animal models, the combination of the Sap130 and Pcdha9 genes has been shown to induce heart defects similar to those seen in HLHS. Although gene mutations affecting cardiomyocyte proliferation are common, the combination of these two genes in mice leads to heart chamber hypoplasia and defects (Bejjani *et al.*, 2021).

Patients with HLHS also exhibit mutations in other genes, including MYRF, MACF1, and LRP6, all of which are associated with the Wnt signaling pathway—a critical regulator of heart development. Additionally, genes such as GATA5 and HOXB9 are upregulated in these patients. Multihit gene families associated with other signaling pathways, such as Hedgehog, FGF, and Notch, have also been implicated, alongside histone modifications, particularly H3K4me and H3K27me. Dysregulation of the transition from the G1 to the S phase, involving the E2F pathway and the mitotic checkpoint PH3, has been observed in HLHS, indicating premature cell cycle exit and increased cardiomyocyte proliferation (Krane *et al.*, 2022).

NOTCH signaling is an essential pathway in early heart development and valve formation. Specifically, NOTCH1 is involved in the endocardial-to-mesenchymal transition (EMT), a process critical to heart formation. This pathway activates the transcription factor Snail, which plays a key role in embryonic development, cancer progression, and the regulation of epithelial markers, including E-cadherin.

TABLE I: Key Gene Markers and Their Roles in the Formation of the Heart

Gene Marker	Function	References
NOTCH1	Involved in valve formation and early heart development .	(McBride *et al.*, 2008)
RBFOX2	Regulates RNA splicing vital for normal heart development.	(Verma *et al.*, 2022)
MYH6	Encodes for alpha-myosin heavy chain (α-MHC)crucial for heart contraction .	(Anfinson *et al.*, 2022)
NKX2-5	cells into cardiomyocytes.	(Cao *et al.*, 2022)
LRP2	Maintains Sonic Hedgehog (SHH)-dependent cardiac progenitor cells prior to differentiation into cardiomyocytes and facilitates SHH signaling to ensure proper heart structure formation	(Christ *et al.*, 2020)
HAND1	Critical for the early development of the left ventricle and certain heart tubes	(George & Firulli, 2019
Sap130	Regulates heart cell proliferation	(Bejjani *et al.*, 2021)
Pcdha9	Involved in the formation of proper heart valves	(Bejjani *et al.*, 2021)
MYRF	Critical for proper heart morphogenesis, including the separation of chambers	(Calonga-Solís *et al.*, 2022)
MACF1	Regulate microtubule organization and cardiomyocyte adaptation to stress.	(Fassett *et al.*, 2013)
LRP6	Regulates Wnt/β-catenin signaling pathway, crucial for cardiac development	(Wu *et al.*, 2021)
GATA5	Plays a role in valve and vessel development .	(Krane *et al.*, 2022)
HOXB9	Regulates heart structure development	(Krane *et al.*, 2022)
GJA1	Encodes connexin 43, essential for electrical impulses among the heart. Critical for the development of the outflow tract.	(Huang *et al.*, 2011)
ETS-1	Involved in angiogenesis. Control cardiomyocyte proliferation in different layers of the heart.	(Wang *et al.*, 2022)

Mutations in NOTCH1, such as G661S and A683T, have been shown to inhibit the receptor's ability to initiate EMT (McBride *et al.*, 2008), (Wang *et al.*, 2013). There is substantial evidence linking NOTCH signaling to HLHS, as mutations in all four NOTCH receptors (NOTCH1-4) have been identified in HLHS patients. Additional genetic variants have been found in HLHS patients, including mutations in Calcium Voltage-Gated Channel Auxiliary Subunits (CACN) and myosin heavy chain 6 (MYH6) genes (Datta *et al.*, 2024), (Yang *et al.*, 2019), (Bejjani *et al.*, 2021). Furthermore, HLHS patients exhibit reduced levels of connexin 43 (GJA1) which disrupts heart cell development and alignment. In addition to these gene mutations, deletions of the distal end of chromosome 11q are often associated with HLHS. The removal of the gene ETS-1 from this chromosomal segment has been shown to cause heart defects similar to HLHS (Bejjani *et al.*, 2021).

III. OVERVIEW OF INDUCED PLURIPOTENT STEM CELLS

A. Induced Pluripotent Stem Cell

Pluripotent stem cells (PSCs) are characterized by their ability to differentiate into all cell types in the body and their capacity for self-renewal (Zakrzewski *et al.*, 2019). A notable example of PSCs is embryonic stem cells, which are derived from the inner cell mass of mammalian blastocysts (Takahashi and Yamanaka, 2006). PSCs are capable of differentiating into cell types from all three primary germ layers: the ectoderm, mesoderm, and endoderm (Romito and Cobellis, 2016). However, they are unable to form extraembryonic structures, such as the placenta (Zakrzewski *et al.*, 2019).

Induced pluripotent stem cells (iPSCs) are somatic cells that can be reprogrammed to a pluripotent state either by transferring their nuclei into oocytes or by fusing them with embryonic stem cells. The process is facilitated by four essential transcription factors: octamer-binding transcription factor 4 (Oct4), sex-determining region Y-box 2 (Sox2), Kruppellike factor 4 (Klf4), and the oncogene c-MYC. These factors bind to specific DNA regions to activate genes critical for maintaining pluripotency (Takahashi and Yamanaka, 2006). Collectively referred to as the OSKM factors or Yamanaka factors, they are integral to inducing pluripotency in somatic cells.

The reprogramming process itself involves significant remodeling of chromatin structure and the epigenome and occurs in two distinct phases: an early phase and a late phase. In the early phase, somatic genes are silenced, while early pluripotency-associated genes begin to activate. The late phase involves the full activation of pluripotency genes. The early phase is highly stochastic due to the closed and compact chromatin structure, which limits the binding of OSKM factors to target DNA. To improve reprogramming efficiency during this phase, histone deacetylase inhibitors are employed. These chemical compounds inhibit histone deacetylases, enzymes that remove acetyl groups from histones, thereby loosening chromatin and enhancing the accessibility of OSKM factors to activate pluripotency-specific genes (Cerneckis *et al.*, 2024). In the late phase, the process becomes more deterministic as the chromatin structure is fully open. Sox2 overcomes repressive chromatin structures and induces DNA demethylation, while Oct4 maintains pluripotency, Klf4 facilitates enhancer-promoter connectivity necessary for reprogramming, and c-Myc enables rapid cell division Takahashi and Yamanaka. 2016), Park and Kim, 2020), (Cerneckis *et al.*, 2024).

A critical step in iPSC reprogramming is the mesenchymalto- epithelial transition (MET), during which mesenchymal cells are converted into epithelial cells. This process is essential as most somatic cells used for reprogramming, such as fibroblasts, are in a mesenchymal state. Klf4 promotes the expression of Chd1, which encodes E-cadherin, a key marker of epithelial cells, while Oct4 and Sox2 suppress Snail, a transcription factor that inhibits MET. Additionally, Myc inhibits the TGFβ signaling pathway, known to promote epithelial-to-mesenchymal transition (EMT), thus facilitating MET (Berx *et al.*, 2001; Baulida *et al.*, 2001;, Hao *et al.*, 2019;, Cerneckis *et al.*, 2024).

There are two primary approaches to introducing reprogramming factors: integrating viral vector systems and nonintegrating methods (Singh *et al.*, 2015). Integrating methods involves the use of viral vectors, such as retroviruses or lentiviruses, to deliver reprogramming factors directly into the target cells' genome. This method has been shown to achieve high reprogramming efficiency, as well as stable expression of transgenes in a variety of somatic cell types (Patel and Yang, 2010;, Sommer *et al.*, 2012;, Kang *et al.*, 2015;, Wen *et al.*, 2017). However, the integrating method poses significant risks, including insertional mutagenesis, which can lead to genomic instability by

disrupting essential coding regions, promoters, and enhancers, potentially activating oncogenes. Although transgenes can be silenced after iPSCs are generated, there remains a risk of reactivation during differentiation or maturation (Belviso *et al.*, 2020), (Thanaskody *et al.*, 2022). Non-integrating methods, in contrast, introduce reprogramming factors without integrating them into the host genome, thereby reducing the risk of mutations or tumor formation. For example, the Sendai virus remains in the cytoplasm without integrating into the host genome, and the adenovirus achieves reprogramming without incorporating genetic material into the host cell's DNA. Other non-integrating methods, such as episomal vectors, minicircle vectors, PiggyBac transposons, and mRNA-based reprogramming, provide safer alternatives to integrating methods. However, these methods are often less efficient, require repeated administration of reprogramming factors, and may be limited to specific somatic cell types Kang, (Belviso *et al.*, 2020), (Thanaskody *et al.*, 2022).

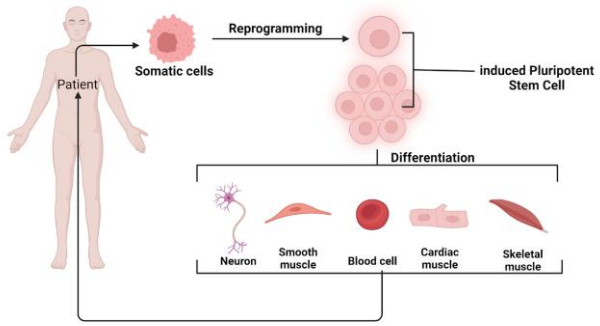

Fig. 3: Diagram illustrating the process of reprogramming patient-derived somatic cells into induced pluripotent stem cells for regenerative medicine. Somatic cells from a patient are reprogrammed into iPSCs, which can differentiate into various cell types, including neurons, smooth muscle, blood cells, cardiac muscle, and skeletal muscle. These differentiated cells can potentially be used for tissue repair or disease modeling.

B. History of PSCs

In 1961, Drs. James A. Till and Ernest A. McCulloch at the University of Toronto made a groundbreaking discovery when they identified stem cells extracted from mouse bone marrow. These cells exhibited the ability to differentiate into various cell types, laying the foundation for the modern concept of pluripotent stem cells (Liu *et al.*, 2020). Two decades later, in 1981, embryonic stem cells (ESCs) were successfully derived from mouse blastocysts by Martin Evans and Matthew Kaufman at Cambridge University (Evans & Kaufman, 1981) and independently by Gail Martin at the University of California, San Francisco (Martin,1981). This development marked a significant advance in stem cell research.

In 1996, another major milestone occurred when Keith Campbell, Ian Wilmut, and their colleagues at the Roslin Institute in Scotland cloned Dolly the sheep using somatic cell nuclear transfer, further advancing the field of regenerative medicine. Just two years later, in 1998, James Thomson and his team in the United States successfully derived the first human embryonic stem cells from embryos (Liu *et al.*, 2020).

Eight years after this breakthrough, in 2006, Shinya Yamanaka and Kazutoshi Takahashi at Kyoto University published a pivotal study demonstrating that mouse fibroblasts could be reprogrammed into iPSCs using four transcription factors: Oct4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006). The following year, Yamanaka, Takahashi, and their colleagues applied these same factors to adult human fibroblasts, successfully generating iPSCs from human somatic cells in both in vivo and in vitro settings (Takahashi *et al.*, 2007). Around the same time, James Thomson and his team also reported the creation of human iPSCs, using a different set of transcription factors: Oct4, Sox2, Nanog, and Lin28. Thomson's team discovered that the use of c-Myc could lead to cell death and unwanted differentiation, making it less ideal for reprogramming. They also identified Oct4 and Sox2 as essential for the process, noting that without these factors, iPSC formation could not occur. Although Nanog and Lin28 were found to be beneficial, they were not strictly required for pluripotency. Nevertheless, the experiment was successful, as the iPSCs exhibited characteristics similar to human embryonic stem cells (Yu *et al.*, 2007).

In recognition of their contributions to the field, Shinya Yamanaka and John Gurdon were jointly awarded the Nobel Prize in Physiology or Medicine in 2012 for their discoveries related to reprogramming somatic cells into pluripotent states (Liu *et al.*, 2020). In 2014, the first clinical application of iPSCs was realized in a patient with age-related macular degeneration. Since then, the number of iPSC-related clinical trials has expanded significantly, with more than 30 trials currently underway worldwide. These trials address a variety of therapeutic areas, including

neurodegenerative disorders, eye diseases, and cardiovascular conditions (Kim *et al.*, 2022) (Yasui *et al.*, 2022) (Yamanaka, 2020).

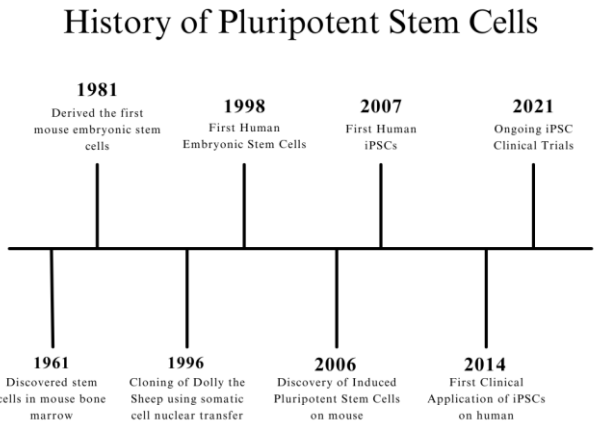

Fig. 4: Timeline highlighting key milestones in the history of pluripotent stem cells, from the discovery of stem cells in 1961 to the ongoing clinical trials of iPSCs as of 2021. Major breakthroughs include the derivation of embryonic stem cells, the cloning of Dolly the sheep, and the creation of human iPSCs.

IV. APPLICATIONS OF IPSCS ON THE CARDIOVASCULAR SYSTEM

A. Disease Modeling

Disease modeling with iPSCs enables scientists to investigate diseases with unknown pathogenesis. iPSCs can be derived from patients with specific diseases, retaining the genetic mutations and phenotypic characteristics of the condition, thereby allowing researchers to study disease mechanisms and develop potential treatments (Kavyasudha *et al.*, 2018). Advances in protocols now enable iPSCs to be converted into heart cells (hiPSC-CMs) using monolayer or suspension culture-based techniques. To replicate the physiological environment of the heart, hiPSCCMs can be cultured in 2D monolayers, organoids, or 3D cultures. However, studies suggest that hiPSC-CMs do not fully mature to the level of adult cardiomyocytes, resulting in cells that do not entirely replicate the functional and structural properties of adult heart cells Deicher & Seeger, 2021).

Various techniques have been employed to enhance the maturity of hiPSC-CMs, including prolonged culture, Triiodothyronine (T3) hormone treatment, mechanical and electrical stimulation, Glycogen Synthase Kinase-3α (GSK-3α) inhibition, Wnt pathway modulators, and specific microRNAs (Sacchetto *et al.*, 2020), (Lundy *et al.*, 2019). When cultured for prolonged periods, particularly for 80 to 120 days, iPSCs undergo significant morphological changes, such as an increase in cell size and elongation, accompanied by greater anisotropy. Additionally, the sarcomere becomes more organized, aligned, and elongated, leading to denser myofibril formation, which is crucial for effective contraction and force generation. Furthermore, prolonged culture results in faster calcium release and reuptake kinetics, as well as more mature electrophysiological properties, such as hyperpolarized maximum diastolic potential and increased action potential amplitude. These changes indicate improved ion channel function and a closer resemblance to adult cardiomyocytes. Notably, gene expression analysis reveals an 18-fold increase in MYH7 and a 15-fold increase in MYH6, both of which are critical for contractile function. Intercellular communication is also enhanced by the upregulation of Connexin-43. Transmission electron microscopy further demonstrates denser mitochondria and well-defined Z-disks, A-bands, and I-bands within iPSCCMs (Sacchetto *et al.*, 2020), (Lundy *et al.*, 2013).

T3, a growth hormone essential for heart development, significantly influences iPSC-CM maturation. Following T3 treatment, iPSC-CMs show marked increases in size and anisotropy, as well as longer sarcomeres. Additionally, an upregulation of p21 expression suggests that T3 reduces cell cycle activity in cardiomyocytes. The force per beat of iPSCCMs doubles, and calcium transient kinetics improve. Moreover, mitochondrial respiratory capacity and reserve increase substantially (Yang *et al.*, 2014), (Parikh *et al.*, 2017).

Once the reprogramming process is complete, cells are cultured under conditions that promote the formation of iPSC colonies. These colonies are selected based on morphology, expression, and surface markers, and can be expanded for further use (Ghaedi & Niklason, 2019) . For purification, a lactate supplement is applied to iPSC-CMs (Reilly *et al.*, 2022).

Cardiomyopathy is a heterogeneous group of diseases that frequently leads to heart failure (Wexler *et al.*, 2009). It can be classified into hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM), both of which are characterized by ventricular

dilation, impaired systolic function, and eventual progression to heart failure (Kavyasudha *et al.*, 2018). Somatic cells from patients with DCM can be reprogrammed into iPSCs, with these patients often possessing specific point mutations in exon 12 of the TNNT2 gene, which encodes Troponin T2, a protein essential for heart contraction. iPSC-derived cardiomyocytes from DCM patients exhibit an increase in sarcomere heterogeneity early in the differentiation process (Kavyasudha *et al.*, 2018).

For HCM, after the introduction of OSKM factors, iPSCs are differentiated into cardiomyocytes using various approaches, such as co-culture with END-2 cells, embryoid body (EB)-based differentiation, and monolayer cell culture. END-2 cells function similarly to visceral endoderm, releasing signals that promote iPSC differentiation. EB-based differentiation allows iPSCs to form embryoid bodies, which can later differentiate into cell types from all three germ layers (Li *et al.*, 2022). Furthermore, genome editing techniques such as CRISPRCas9, CPF1, and TALENs are used to ensure that the observed phenotypes in iPSCs are related to specific mutations. Similar to DCM, iPSC-CMs derived from HCM patients exhibit disorganized cardiomyopathy. These iPSC-CMs also display hypertrophy and elevated basal levels of intracellular Ca2+ due to mutations in MYH7 (R663H), which impair sarcoplasmic reticulum function and contribute to hypercontractility. Mutations in the MYBPC3 (R502W) gene are also associated with HCM (Li *et al.*, 2022).

B. Regenerative medicine

iPSCs have emerged as a groundbreaking technology for regenerative medicine, particularly in the cardiovascular system. The ability to reprogram somatic cells into a pluripotent state allows cells to differentiate into various types of heart cells. An experiment on infarcted rat hearts used human iPSCs (hiPSCs) derived from a donor via OSKM factors. Cardiomyocyte differentiation was initiated with CHIR-99021, activating the Wnt/β-catenin pathway, followed by inhibition using IWR- 1 to replicate natural cardiac development. The resulting cardiomyocytes spontaneously beat for 8-10 days, confirming functionality. After purification, the cells were cryopreserved and transplanted into rats 10 days post-myocardial infarction to avoid severe inflammation. Transplantation of the iPSCderived cardiomyocytes significantly improved heart function, with enhanced ejection fraction and fractional shortening, while also reducing myocardial fibrosis. The transplanted cells survived in the myocardium for at least 28 days without migrating to other organs, indicating their potential to reduce scarring and enhance cardiac repair (Guan *et al.*, 2020), (Horitani and Shiojima, 2024).

iPSCs have also been tested on larger animals to repair heart damage. The hiPSC-CMs were seeded onto dishes with thermoresponsive polymer, resulting in the formation of a thin, cohesive sheet of cells called cell sheets at low temperatures. Four weeks after inducing myocardial infarction, the cell sheets were transplanted into the infarcted myocardium.

Tacrolimus is also used to inhibit T-cell activation and avoid immune rejection. Similar to the study using rats, there was also a significant improvement in the left ventricle ejection fraction and a reduction in ventricular dilation in pigs after receiving hiPSC-CM sheets. Moreover, the study suggested that there is a decrease in hypertrophy and fibrosis in the heart, and the capillary density increases, overall improving cardiac function (Kawamura *et al.*, 2012).

Other studies focus on using iPSCs from skin fibroblasts in nonhuman primates iPSCs were reprogrammed using OCT4, SOX2, KLF4, LIN28, and p53 shRNA transcription factors by a non-integrating method. Initially, the fibroblasts were cultured with fibroblast growth factors, ROCK inhibitors to enhance cell survival, and penicillin-streptomycin to prevent bacterial contamination. Then, iPSCs are cultured on geltrexcoated dishes, allowing them to grow in colonies before being differentiated into cardiomyocytes (Stauske *et al.*, 2020). Though, non-human primate iPSCs require a different approach than hiPSCs such as the need for supplementation with CHIR99021 first for increase Wnt/β-catenin signaling pathway (Kerr, 2023), and later activin A and bone morphogenetic protein 4 (BMP4) were used for growth as well as promote the formation of mesoderm. Furthermore, iPSCs are exposed to IWR-1, a type of Wnt pathway inhibitor, similar to the study on rats. iPSC-CMs then undergo prolonged culture to ensure maturity and are later generated into functional cardiomyocytes (Stauske *et al.*, 2020). These studies on animals indicated a promising application of iPSC on the cardiovascular system.

Recent advancements in iPSC-derived cardiomyocyte (iPSC-CM) transplantation have demonstrated potential in treating advanced heart failure. A notable phase I/IIa clinical trial explored the epicardial injection of allogeneic human iPSC-CMs in patients with advanced heart failure caused by ischemic heart disease (IHD). This trial involved patients aged 35 to 75 receiving hiPSC-CMs derived from donor peripheral blood mononuclear cells produced under cGMP conditions. The cells were delivered during coronary artery bypass grafting (CABG) surgery, with ten injections targeting different regions of the epicardium. Post-surgical monitoring focused on detecting tumor formation, arrhythmias, and other complications. Additionally, immunosuppressive therapy was employed to prevent immune rejection. While the trial's protocol has been established, no results have yet been published as the study is ongoing (Zhang *et al.*, 2022). Complementing this, another study applied hiPSC-CM patches to a patient with ischemic cardiomyopathy whose condition had not improved despite prior medical interventions. In this case, allogeneic hiPSC-CMs were placed directly onto the anterior and lateral epicardium of the left ventricle, following rigorous preparation processes to clear cancer-related genes and ensure safety. Intra-aortic balloon pumping was employed to maintain stable cardiac output, and immunosuppressive therapy was used to prevent rejection (Miyagawa *et al.*, 2022). Over a six-month period, the patient exhibited significant improvements, with the regional wall motion of the left ventricle showing marked recovery, suggesting successful integration of the transplanted cells with heart tissue. There was also a notable reduction in both global and regional stress in the anterior and lateral walls of the left ventricle, improved myocardial blood flow, and importantly, no signs of arrhythmias or tumor formation (Miyagawa *et al.*, 2022). These findings underscore the therapeutic potential of iPSC-CMs in regenerative medicine for heart failure treatment.

V. APPLICATIONS OF IPSCS IN HLHS

Historically, the treatment of Hypoplastic Left Heart Syndrome (HLHS) has relied on a series of complex surgical procedures to ensure patient survival. iPSCs, however, offer the possibility of generating patient-specific cells that can be used to regenerate and repair damaged heart tissues. Despite this potential, iPSC therapy is not currently a viable option for the treatment of HLHS. Although iPSCs have been successfully used to model various cardiovascular diseases, their application in modeling congenital heart defects such as HLHS remains limited (Birla *et al.*, 2022).

Modeling HLHS using iPSCs has revealed several factors that contribute to the development of this condition, including hypoxia. iPSC-derived cardiomyocytes (iPSC-CMs) can be generated from peripheral blood mononuclear cells, and studies have shown that hypoxia induces the upregulation of hypoxia-inducible factor (HIF-1α), which results in oncogeneassociated cellular senescence. Additionally, hypoxia increases the activity of Transforming Growth Factor Beta 1 (TGF-β1), which hinders proper cardiac function (Birla *et al.*, 2022). These findings provide important insights into how hypoxic conditions during fetal development may exacerbate or contribute to the anatomical defects observed in HLHS.

iPSC technology also highlights the potential for modeling the pathophysiology of HLHS at the cellular level. Research using iPSC-derived cardiac progenitor cells has demonstrated that these cells can reveal transcriptional and epigenetic alterations associated with HLHS, particularly with respect to key cardiac transcription factors such as NKX2-5 and HAND1 (Junko *et al.*, 2014). Moreover, through the use of single-cell RNA sequencing (scRNA-seq), iPSCs derived from endothelial cells of HLHS patients showed significant reductions and absences in essential genes and signaling pathways that are critical for cardiovascular development (Mullen *et al.*, 2021), (Miao *et al.*, 2019). These insights are crucial as they may lead to the development of targeted strategies aimed at correcting the underlying defects in HLHS.

In another study, iPSC-CMs were successfully derived from dermal fibroblasts of HLHS patients. The cells were cultured in Iscove's Modified Dulbecco's Medium, supplemented with 10% fetal calf serum, L-glutamine, and nonessential amino acids to support cell growth, along with penicillin to prevent bacterial contamination. OSLM factors were introduced using a lentiviral vector under the control of an EF1α promoter. Polybrene was also utilized to improve the efficiency of the transcription factors. After transduction, the fibroblasts were disaggregated and plated onto feeder layers, which provided support and mimicked the natural conditions necessary for iPSC

colony expansion. The iPSCs derived from HLHS patients were then tested for pluripotency by observing teratoma formation in mice over a period of 6 to 12 weeks, and the results confirmed the presence of all three germ layers within the teratomas (Jiang *et al.*, 2014).

VI. CHALLENGES & LIMITATIONS OF IPSCS

A. Tumorigenicity

One of the major concerns associated with the application of iPSCs is their potential to form tumors. Both iPSCs and cancer cells share the ability to proliferate indefinitely by inhibiting apoptosis, unlike somatic cells which are limited in the number of divisions they can undergo. Additionally, c-Myc, a key marker of iPSC reprogramming, is also expressed in many cancer cells, contributing to the concern of tumorigenicity. While c-Myc is crucial for enhancing the proliferation of iPSCs, its pro-tumorigenic effects increase the risk of tumor formation (Doss and Sachinidis, 2019), (Qiao *et al.*, 2020), (Marión, *et al.*, 2009).

Beyond c-Myc, other markers such as OCT4, SOX2, and NANOG, which are expressed in iPSCs, have been linked to tumorigenesis. These markers are known to function as biomarkers for cancer stem cells, regulating their self-renewal and maintaining their undifferentiated state (Lee, *et al.*, 2013), (Riggs *et al.*, 2013), (Gu *et al.*, 2019). The ectopic activation of OCT4 has been shown to induce dysplastic cellular growth and structural changes characteristic of malignancy. Similarly, NANOG is involved in the maintenance and renewal of cancer cells, while SOX2 has been identified as an oncogene, promoting tumor survival and progression. KLF4, another transcription factor essential for pluripotency, has been shown to suppress p53, a critical protein involved in tumor suppression. This suppression allows cells to proliferate uncontrollably, even when pre-existing DNA damage is present (Lee, *et al.*, 2013). Interestingly, p53-null cells have demonstrated higher reprogramming efficiency in iPSC generation (Marión, *et al.*, 2009), (Nakagawa *et al.*, 2010).

Another factor contributing to tumorigenicity is the presence of dysfunctional or shortened telomeres in cells undergoing reprogramming. Such cells are prone to chromosome end-to-end fusions, leading to genomic instability and tumor formation. The DNA damage response (DDR) system is activated early in the reprogramming process, marked by the presence of γH2ax and 53BP1 foci. DDR initiates a p53-dependent apoptosis mechanism, which eliminates damaged cells during reprogramming to prevent faulty stem cell formation (Marión, *et al.*, 2009). γH2ax identifies DNA damage sites, while 53BP1 facilitates repair. However, even after somatic cells are fully reprogrammed into iPSCs, DDR markers often persist, particularly in p53-deficient cells, indicating that genomic instability continues post-reprogramming (Marión, *et al.*, 2009). The absence of p53 increases the likelihood of damaged cells surviving the reprogramming process, thus raising the risk of tumorigenesis.

iPSCs are also known for their propensity to form teratomas, tumors containing differentiated tissues from all three germ layers. This tumorigenic potential is demonstrated in studies where iPSCs generated using OSKM factors were injected into immunocompromised mice, resulting in both benign and malignant teratomas(Riggs *et al.*, 2013). Another study involving MYCintegrated iPSCs injected into early mouse embryos found that reactivation of MYC led to the formation of somatic tumors(Lee, *et al.*, 2013). Furthermore, over 50% of chimeric mice derived from c-Myc-generated iPSCs developed tumors within the first year of life (Nakagawa *et al.*, 2010).

In addition to genetic factors, oxidative stress plays a significant role in promoting genomic instability and cellular damage in iPSCs. Reactive oxygen species (ROS), such as hydroxyl radicals (•OH), superoxide (O2-), and hydrogen peroxide (H2O2), are central to oxidative stress. While ROS are involved in various normal cellular processes, excessive ROS levels can overwhelm cellular antioxidant defenses, leading to toxicity and molecular damage(Wu *et al.*, 2013). Studies have shown that high concentrations of H2O2 decrease iPSC viability, triggering early apoptosis and G2/M cell cycle arrest. In extreme cases, this may result in cellular senescence or apoptosis, reducing iPSC efficiency and depleting cell populations (Liu *et al.*, 2018), (Wu *et al.*, 2013). Notably, the reprogramming process itself has been linked to an increased mutation rate, with a significant proportion of mutations arising due to the stress of reprogramming (Junfeng *et al.*, 2012).

B. Immune rejection

The immunogenicity of iPSCs presents significant challenges. An early study in mice demonstrated that undifferentiated iPSCs were rejected by the immune system despite being derived from the recipient, indicating that the immune system recognized the cells as foreign and attacked them. In contrast, differentiated cells derived from iPSCs showed little to no immune response in syngeneic recipients (Doss and Sachinidis, 2019). This phenomenon occurs because undifferentiated iPSCs express gamete-associated proteins, which are not tolerated by T cells, leading to immune rejection (Qiao *et al.*, 2020). However, in another study on mice, differentiated iPSCs also triggered a response mediated by T-cells, showing that iPSCs can still provoke an immune response even in their differentiated state. Additionally, in undifferentiated cells, the major histocompatibility complex (MHC), a group of genes essential for immune recognition, shows lower transcription levels of MHC-I, β2- microglobulin, and non-classical MHC-I molecules compared to mature somatic cells (Chen *et al.*, 2015). These findings suggest that both differentiated and undifferentiated iPSCs can provoke immune responses.

In an experiment using iPSCs derived from B6 mouse embryonic fibroblasts, researchers examined the cells' ability to form teratomas and assessed whether these teratomas would be immune-tolerated or rejected. The results revealed that the teratomas were often immune-rejected, with CD4+ T cells infiltrating the tissue, causing damage and severe necrosis. The study identified nine genes—Lce1f, Spt1, Cyp3a11, Zg16, Lce3a, Chi3L4, Olr1, Retn, and Hormad1—that were overexpressed in iPSC-derived teratomas, suggesting that these gene expressions may contribute to immune recognition of iPSCderived cells as foreign (Zhao *et al.*, 2011).

Another study found that cells differentiated from iPSCs exhibited an imbalance in ligand expression, triggering a strong response from natural killer (NK) cells, which led to degranulation and cytotoxicity (Bogomiakova *et al.*, 2023). Unlike T cells, NK cells target cells that lack sufficient expression of self-MHC class I molecules (Raulet, 2006). iPSC-derived cells displayed lower levels of Human Leukocyte Antigen 1 (HLA-I), an MHC class I molecule that inhibits activating ligands, and higher levels of activating ligands. This imbalance is a key factor in the NK cells' attack on iPSC-derived cells, which is thought to be due to the insufficient maturity of the cells and their failure to express inhibitory signals (Bogomiakova *et al.*, 2023).

Furthermore, studies suggest that the immunogenicity of iPSCs can vary significantly depending on the type of tissue from which the iPSCs are derived. For instance, cells derived from the skin or lungs tend to experience more severe rejection compared to cells from the kidneys or heart (Kamatani *et al.*, 2022). In humanized mouse models, iPSCs derived from smooth muscle cells were found to be highly immunogenic, whereas cells such as retinal pigment epithelium (RPE) cells were not. Each cell type has a different set of antigens, leading to varying immune responses (Qiao *et al.*, 2020). Additionally, cardiomyocytes derived from iPSCs tend to be highly immunogenic, whereas skin and bone marrow cells are less so, likely due to the absence of minor antigen expression in the latter (Cao *et al.*, 2024). The location of iPSC transplantation also influences immunogenicity. For example, iPSCs transplanted subcutaneously or intramuscularly are often rejected, possibly due to the absence of dendritic cells, which are responsible for presenting antigens to lymphocytes (Song *et al.*, 2018), (Qiao *et al.*, 2020).

C. High treatment cost

The cost of iPSCs presents significant challenges and limitations in their application for regenerative medicine and therapeutic interventions. The derivation of iPSCs is often a labor-intensive and expensive process, which can limit their widespread use in clinical settings. A process can take several months and requires major attention especially when multiple patient samples need to be reprogrammed [101], (Cefalo *et al.*, 2016), (Dzhoyashvili *et al.*, 2015). Moreover, this is followed by the inefficiencies in the reprogramming protocols, yielding low efficiencies from 0.001% to 1% depending on factors(Danter, 2019). It is also suggested that generating a clinical-grade iPSC line can cost up to $800,000 (Huang *et al.*, 2019), making it currently not affordable for the majority of patients and limiting access to these therapies (Lin *et al.*, 2017). In addition, the scalability of iPSC production is a large concern. While the potential for large-scale production exists, the current methodologies are not sufficiently optimized to achieve economies of scale, especially for autologous therapies, where iPSCs need to be derived from each

patient. The high costs and lengthy timelines associated with producing patient-specific iPSCs raise questions about the practicality of their use in clinical settings (Álvarez-Palomo *et al.*, 2017), (Madrid *et al.*, 2021). Furthermore, the fact that iPSCs can lead to tumor formation after transplant requires rigorous testing and monitoring, thereby increasing overall costs (Masumoto *et al.*, 2022).

VII. CHALLENGES & LIMITATIONS OF IPSCS ON HLHS

One of the significant challenges in applying iPSCs for treating HLHS is the efficiency of deriving iPSCs from HLHS patients, which is around 0.0002%, significantly lower compared to healthy patients. HLHS-iPSCs show a critical reduction in the expression of mesodermal (Brachyury) and cardiac progenitor markers (ISL1, NKX2.5) during the differentiation process. By the first week of differentiation, the percentage of cells expressing these markers in HLHS-iPSCs is notably lower than that of iPSCs derived from healthy patients. By weeks 2 and 3, HLHS-iPSCs continue to exhibit a reduction in cells expressing cardiac-specific markers such as HAND1, HAND2, cTnT, and SIRPA. It is suggested that HLHS-iPSCs have an impaired ability to follow the typical cardiac differentiation pathway, a phenomenon explained by a senescent phenotype in HLHS-derived cells (Jiang *et al.*, 2014)(Yang *et al.*, 2019).

Moreover, HLHS-iPSCs, instead of differentiating into cardiomyocytes, display an increased tendency to differentiate into smooth muscle cells due to elevated expression of the marker genes α-SMA (alpha-smooth muscle actin), CALDESMON, and CALPONIN. This is critical because the presence of smooth muscle cells in the cardiovascular system could result in structural and functional abnormalities, as they differ from cardiac muscle cells(Yang *et al.*, 2019). The impaired NOTCH signaling, associated with mutations in HLHS patients, further contributes to the condition. Similarly, cardiomyocytes derived from HLHS-iPSCs experience a significant reduction in the expression of NOTCH receptors and associated genes. The downregulation of NOTCH signaling disrupts cardiogenesis, the process of forming and developing cardiomyocytes, leading to lower efficiency in forming functional heart muscle cells (Yang *et al.*, 2019).

Furthermore, using iPSCs derived from HLHS patients often results in disorganized sarcomeres, with irregularly spaced Z-band formations (Jiang *et al.*, 2014), (Yang *et al.*, 2019). Sarcomeres are essential for muscle contractions and structural integrity, and their disorganization can result in cardiomyocytes that fail to contract or develop properly. This poses a significant challenge since the left side of the heart in HLHS patients is already underdeveloped. A study also demonstrated that when iPSCs derived from healthy cardiomyocytes were co-cultured with HLHSiPSC-derived endocardial cells, the heart muscle cells failed to mature properly, and the sarcomeres were disorganized. Additionally, the muscle cells from HLHS patients exhibited little to no contractions (Miao *et al.*, 2019). The sarcoplasmic reticulum, which regulates calcium handling and muscle contractions, in HLHSiPSC- derived cardiomyocytes was found to be impaired, likely due to the downregulation of genes such as CASQ2 and HRC(Yang *et al.*, 2019), (Jiang *et al.*, 2014).

Additionally, as HLHS is a rare congenital heart defect, affecting approximately 2 out of 10,000 births (Gobergs *et al.*, 2016), the availability of patient samples for deriving iPSCs for disease modeling and drug testing is limited.

VIII. CONCLUSION & FUTURE DIRECTION

In conclusion, iPSCs represent evolutionary potential for the treatment of HLHS. The ability of iPSCs to differentiate into various cell types, including cardiomyocytes, avails the singular opportunity of repairing and regenerating underdeveloped heart tissues characteristic of HLHS. This approach moves beyond the traditional palliative care options, which primarily focus on managing symptoms through complex surgical interventions and heart transplantation, both of which come with significant limitations such as the risk of complications, lifelong medical care, and limited donor availability.

Although this may sound promising, the application of iPSCs in HLHS therapy is still in its nascent stages and faces many challenges. One of the main difficulties for iPSC production from HLHS patients themselves is that generation alone is not very efficient, and the reduced expression of crucial 9 cardiac markers during differentiation. Additionally, there is a concerning

tendency for HLHS-derived iPSCs to differentiate into smooth muscle cells rather than cardiomyocytes, which could potentially exacerbate the structural and functional abnormalities already present in these patients. Furthermore, one major barrier to being translated into clinics is possibly the nature of iPSCs as somewhat behaving like tumorigenic cells since they can proliferate indefinitely. This is worrying for the potential of tumor formation, especially if it entails the usage of oncogenes such as c-Myc in the reprogramming process. What is more, is that early studies have shown that even iPSCs derived from an autologous source can be subjected to immune rejection. This paradoxical response underlines a need for further research that should be directed toward factors that modulate the immunogenicity of iPSC-derived cells and how to mitigate them. Moreover, the use of integrating viral vectors in the reprogramming process introduces additional risks, such as the potential for insertional mutagenesis, which could disrupt normal cellular function or activate oncogenes.

Looking ahead, several future directions could help overcome these challenges and bring iPSC-based therapies closer to clinical application for HLHS. First, enhancing the efficiency and accuracy of iPSC reprogramming, particularly with HLHS patient-derived cells, is essential. Moreover, advances in non-integrating reprogramming methods could reduce the risk of mutation or tumor formation, improving the safety profile of iPSC-derived cells. Refining differentiation protocols to yield higher proportions of functional cardiomyocytes and minimize the formation of unintended cell types, such as smooth muscle cells, is crucial. This might be achieved by adjusting the cultural conditions and growth factors. Furthermore, early activation of Wnt signaling has been shown to promote mesoderm formation in ESCs effectively. However, later activation may result in differentiation into non-cardiac lineages, producing various cell types instead of cardiomyocytes. Consequently, inhibiting Wnt signaling becomes necessary to differentiate mesodermal progenitors into cardiac precursors. (Buikema *et al.*, 2013), (Paige *et al.*, 2010). Moreover, strategies for the control of problems originating from tumorigenicity and immune rejection should be developed. According to (Castro-Vi˜nuelas *et al.*, 2010), transitioning iPSCs from a feeder-based system to a feeder-free culture system has demonstrated significant benefits, including reduced exposure to animal pathogens, minimized cellular stress, and a more controlled culture environment, which collectively decreases the risk of genetic mutations. Moreover, the implementation of controlled cryopreservation protocols for iPSCs has been instrumental in preserving cellular integrity while preventing genomic instability, as these protocols mitigate cellular stress during storage.

Additionally, it will be important to conduct rigorous preclinical studies to assess the safety and efficacy of iPSCbased treatments for HLHS. Such studies must focus on longterm outcomes, especially the potential for arrhythmias, heart failure, or other complications related to the use of iPSCs, to demonstrate that the benefits of iPSC therapy outweigh the risks. In summary, though the path to developing effective iPSC-based therapies for HLHS is pitted with challenges, their potential benefits are immense. By addressing the underlying causes of this congenital defect, iPSC treatments would mean permanent and less invasive alternative treatments, bringing the hope of improved life quality and long-term survival of the HLHS patient. Further research into iPSCs within cardiovascular studies and regenerative medicine holds the potential to revolutionize treatment options for HLHS and other congenital heart defects.

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Ethics approval & consent to participate

This study is a narrative review and did not involve human participants, human data, human material, or animals.

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The author was solely responsible for conceptualizing, researching, analyzing, and writing this manuscript. No additional contributors were involved.

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