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Unlocking the Potential of Induced Pluripotent Stem Cells for Neonatal Disease Modeling and Drug Development

Ziyi Liu[1,2], **Bonny Lami**[1,2], **Laertis Ikonomou**[3,4,5,#,**], **Mingxia Gu**[1,2,6,#,*]

[1]Perinatal Institute, Division of Pulmonary Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH

[2]Center for Stem Cell and Organoid Medicine, CuSTOM, Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH

[3]Department of Oral Biology. University at Buffalo, The State University of New York, Buffalo, NY, USA

[4]Division of Pulmonary, Critical Care and Sleep Medicine, Department of Medicine, University at Buffalo, The State University of New York, Buffalo, NY, USA

[5]Cell, Gene and Tissue Engineering Center, University at Buffalo, The State University of New York, Buffalo, NY, USA

[6]University of Cincinnati School of Medicine, Cincinnati, OH

Abstract

Neonatal lung and heart diseases, albeit rare, can result in poor quality of life, often require long-term management and/or organ transplantation. For example, Congenital Heart Disease (CHD) is the most common type of congenital disability, affecting nearly 1% of the newborns, and has complex and multifactorial causes, including genetic predisposition and environmental influences. To develop new strategies for heart and lung regeneration in CHD and neonatal lung disease, human induced pluripotent stem cells (hiPSCs) provide a unique and personalized platform for future cell replacement therapy and high-throughput drug screening. Additionally, given the differentiation potential of iPSCs, cardiac cell types such as cardiomyocytes, endothelial cells, and fibroblasts and lung cell types such Type II alveolar epithelial cells can be derived in a dish to study the fundamental pathology during disease progression. In this review, we discuss the applications of hiPSCs in understanding the molecular mechanisms and cellular phenotypes of CHD (e.g., structural heart defect, congenital valve disease, and congenital channelopathies)

[*]**Corresponding Authors:** Mingxia Gu, Cincinnati Children's Hospital Medical Center, Room R4.464; MLC 7029, 3333 Burnet Ave, Cincinnati, OH 45229, mingxia.gu@cchmc.org. [**]Laertis Ikonomou, University at Buffalo, The State University of New York, Department of Oral Biology, 619 Biomedical Research Building (BRB), South Campus, Buffalo, NY 14214-8024, laertisi@buffalo.edu.

[#]These authors have contributed equally to this work and share senior authorship

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and congenital lung diseases, such as surfactant deficiencies and Brain-Lung-Thyroid syndrome. We also provide future directions for generating mature cell types from iPSCs, and more complex hiPSC-based systems using three-dimensional (3D) organoids and tissue-engineering. With these potential advancements, the promise that hiPSCs will deliver new CHD and neonatal lung disease treatments may soon be fulfilled.

INTRODUCTION

Neonatal diseases, such as congenital heart disease (CHD) and lung disease, often result in short life spans for neonates and significant emotional and financial distress for parents and caregivers. Although certain conditions, such as tracheoesophageal fistula, can be surgically treated and may generally have favorable outcomes, the treatment options for several other conditions are limited to organ transplantation or palliative care. Therefore, new therapeutic modalities would have a profound impact on clinical symptomatology and management. The rapidly evolving technology of induced pluripotent stem cells (iPSCs)[1–3] has opened uncharted possibilities for regenerative medicine, including potential cures for congenital diseases.

CHD is a broad range of structural cardiac abnormalities present before birth and attributable to abnormal fetal cardiac development[4]. CHD was considered one of the leading causes of infant mortality, affecting 10–75 of every 1000 live births[5, 6]. Considering the diversity of cell types, structures, and functions impacted by the disease, congenital heart defects can present with a structural anomaly (i.e., atrial/ventricular defect, valvular heart disease) and/or congenital aortopathy (i.e., aorta or pulmonary vessel defect). CHD can also present with conduction anomalies, such as congenital transition defects (long-QT syndrome (LQTS), Brugada syndrome, and Wolff-Parkinson-White syndrome), caused by ion-channel defects.

The causes of CHD are complex and multifactorial, including genetic predispositions and environmental influences. One of the challenges for understanding the cause of CHD is the lack of animal models that represent human physiology and pathology. Increasing evidence finds discrepancies between human and mouse hearts in many aspects, including the conduction and contraction system, genetic backgrounds, and cellular function. Additionally, creating and characterizing a new transgenic mouse model might take 6–12 months, which remains a practical bottleneck. Thirdly, many animal models lack the resolution to study the cellular interactions and simulate the unique genetic environment that is the foundation of organ development, although some models have their ability to study the pathological processes that cause human congenital heart defects[7].

Current therapies for CHD are largely supportive in nature—they do not prevent the development of the structural abnormality, and do not result in repair nor regeneration of the damaged heart. Thus, a significant number of CHD patients still develop heart failure after surgical repair, which necessitates heart transplantation with high morbidity and mortality.

A better understanding of the etiology of CHD and lung neonatal diseases could lead to the development of novel therapies for such devastating conditions. Increasing evidence shows

that iPSCs can be reprogrammed from patient somatic cells and differentiated into different cardiac cell types including cardiomyocytes, fibroblasts, endocardial cells, endothelial cells, smooth muscle cells, and Type II alveolar epithelial cells (AT2s) for modeling the disease progression and investigating the molecular mechanisms in a dish. Differences in heart pathophysiology and alveolar dysfunction between human and mouse models could also be circumvented using human iPSC (hiPSC)-derived systems. Additionally, iPSC-derived cardiac cells and engineered heart tissue have great potential in cardiac regenerative therapy, as demonstrated by multiple preclinical studies, and they are currently being evaluated in several clinical trials[8, 9].In this Mini-review, we explore the current status of iPSC-based models for neonatal disease with emphasis on conditions of the cardiovascular and respiratory systems. We will discuss the applications of iPSCs in understanding the molecular mechanisms and cellular phenotypes of CHD and lung neonatal diseases in a dish. We will also critically explore the limitations and future clinical and research opportunities the use of iPSCs derived heart and lung organoids and engineered heart tissue opens for disease modeling and drug development.

Directed differentiation of pluripotent stem cells

Pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs), had traditionally been used for generation of genetically modified, such as knock-in/knock-out, mice[10]. Major breakthroughs such as the generation of human ESC lines[11] and the development of iPSCs, first from mouse[2] and then from human[1, 3] somatic cells, have opened the possibility of regenerative therapies using PSCs as cellular source. The PSC fundamental potential of giving rise to any somatic cell type is realized through the application of directed differentiation i.e., the in vitro recapitulation of key developmental stages leading to the derivation of specific cell lineages. This approach is based on the concept of embryonic germ layer separation (ectoderm, endoderm, mesoderm) and requires precise multistage addition of soluble activators, such as growth factors, and/or inhibitors of developmental signaling pathways. This approach has proven particularly fruitful as PSCs are now routinely used to model various human pathologies in vitro and in tens of clinical trials worldwide[12, 13].

Modeling congenital valve disease using iPSCs

Congenital valve disease is complex anatomically, which might occur alongside other complications such as heart failure, pulmonary hypertension, and sudden death. Bicuspid Aortic Valve (BAV) is considered the most prevalent congenital abnormally, with an estimated prevalence of 4.6 per 1000 live births[14] or 1–2%[4]. Some other forms of abnormalities such as pulmonary valve stenosis (PVS) or mitral valve abnormality were less commonly reported as common features in diseases such as Williams syndrome and Jacobsen syndrome. Due to complexity in phenotypes, little is known about how different gene mutations might interfere with the valval developmental process.

Normal cardiac valves are composed of valve endothelial cells (VECs) and valve interstitial cells (VICs). VICs are derived from embryonic endocardial lineage through endothelial-to-mesenchymal transition (Endo-MT), followed by remodeling and maturation into valve leaflets. Disruption of this process could result in valve malformation[15]. Primary VECs

have been isolated from both human and mouse to understand their identity, function, and their ability to undergo Endo-MT[16]. Additionally, with the development of gene-editing and lineage tracing tools, mouse and zebrafish models have emerged as valuable animal models for studying cardiac valve development[17]. However, access to primary VECs and VICs, especially from patients with valvular diseases, is still very limited.

More recently, iPSCs derived from patients with congenital valvular disease provided a new platform to study the progression of the disease. iPSCs were used as a tool for functional assessment in abnormal Endo-MT in bicuspid valve development. Yang et al. generated iPSCs from a donor with a normal tri-leaflet aortic valve[18]. Mutation of *GATA4* was induced by gene editing, followed by differentiation and analysis of endothelial cells. Under their investigation, endo-MT was interrupted by inducing *GATA4* disruption, which could result in development of a bicuspid aortic valve[18]. In another investigation from Neri et al., they generated a protocol of inducing human iPSCs into endocardial cells (or Human Pre-Valvular Cells, HPVCs); the endo-MT process was observed in HPVCs after BMP2 incubation. Patient-derived hiPSCs recapitulated features of mitral valve prolapse with dysregulation of the SHH pathway[19]. Another study from Theodoris et al. revealed the relationship between *NOTCH1* mutations and the progressive calcification of valvular interstitial cells. Analysis of endothelial cells derived from hiPSCs from patients with familial history of calcific aortic valve disease revealed that a mutation in *NOTCH1* might result in dysregulation of osteogenic and inflammatory gene networks[20]. Increasing data has demonstrated that patient iPSCs or hiPSCs with targeted gene editing might be a more useful tool for analyzing disease phenotype in vitro. However, iPSC-ECs still do not fully recapitulate in vivo endocardial lineage, which eventually gives rise to VECs and VICs.

Modeling congenital channelopathies using iPSCs

Congenital channelopathies are other major causes of sudden cardiac death (SCD)[21]. Brugada syndrome, LQTS, and Wolff-Parkinson-White syndrome are some of the leading causes of SCD[22].

As the generation of an action potential is the key fundamental element in the cardiac conduction system, inward (Na^+ and Ca^{2+}) and repolarizing, outward (K^+) currents play an essential role in conducting electric signals among cardiomyocytes. Mutations of major pore-forming a-subunits in the sodium channel (*SCN5A, SCN10A*), calcium channel (*CACNA1C, CACNA1G*), as well as potassium channel (*KCND2, KCND3, KCNA4, KCNA5, KCHN2*), may result in dysfunctional action potential, which leads to different subtypes of LQTS, sick sinus syndrome and Brugada syndrome[23]. Little is known about how these mutations could result in different phenotypes due to a lack of suitable modeling systems. For instance, mouse hearts beat 8 times faster than humans with a relatively short action potential, suggesting a huge physiological gap between the two systems[24]. As a result, patient-derived iPSCs could provide a powerful tool for functional studies and potential drug screening.

Functional studies—To evaluate the electrophysiology of iPSC-derived cardiomyocytes (iPSC-CMs), multiple pediatric cardiomyocyte cell lines with variant mutations were

generated from patient-specific iPSCs (Table 1). Patch clamp, multielectrode arrays, fluorescence imaging, and calcium imaging were used as experimental approaches to assess the function of iPSC-CMs. Isogenic hiPSC lines with gene gain- or loss-of-function studies using gene editing approaches were also tested to validate the relationship between specific gene mutations and their role in phenotypes. The first hiPSC cardiac disease modeling system was established for investigating LQTS. iPSC-CMs derived from an 8-year-old LQTS patient with *KCNQ1* R190Q mutation showed prolonged action potential and abnormal potassium current due to altered I_{ks} activity[25]. Novak et al. generated iPSC-CMs from a 12-year-old patient with *CASQ2* D307H mutation to model catecholaminergic polymorphic ventricular tachycardia (CPVT). They discovered that isoproterenol caused delayed afterdepolarization and oscillatory arrhythmic prepotentials. They also discovered CPVT iPSC-CMs displayed an immature phenotype with less organized myofibrils, and reduced number of caveolae[32]. For Jervell & Lange-Nielsen syndrome (JLNS), another rare congenital LQTS syndrome, Zhang et al. generated two sets of JLNS iPSC models with *KCNQ1* mutations. They observed pronounced action potential prolongation and reduction of I_{ks}. They also found that JLNS-iPSC-CMs could be corrected using proarrhythmic drugs[33]. Immaturity of hiPSC-CMs, induced alterations during passaging, and differentiation-dependent heterogeneity of iPSC-CMs are some of the limitations for disease modeling of channelopathies using hiPSCs[35].

Drug screening—It is now known that certain drugs, such as RAF and MEK inhibitors and sodium-channel blockers may induce Brugada syndromes[36, 37]. Patients with LQTS syndrome may have different responses to beta-blocker therapy. With patient derived PSCs, it is possible to discover new treatments and avoid drug induced cardiotoxicity in individual patients using their own iPSC-CMs as a platform for drug screening. As LQTS syndrome type 2 is caused by mutation in *KCNH2* mutation, they can be classified into four different categories based on separate mechanisms by which they lose Kv11.1 channel function. Class 1 *KCNH* mutations disrupt synthesis of a-subunits and class 2 *KCNH* mutations reduce intracellular transport of the Kv11.1 channel. About 90% of LQTS2 mutations were considered as class 1 and class 2[38, 39]. As lumacaftor - a drug which corrects misfolded ATP-gated Cl^- channel proteins (CFTRs) is clinically used for the treatment of cystic fibrosis by improving trafficking of delta-F508-CFTR protein, Mehta et al. hypothesized that lumacaftor could also improve the activity of Kv11.1 channel in class 1 and 2 LQTS patients[40]. Using hiPSC-CMs derived from LQTS2 syndrome patients they discovered Lumacaftor could increase expression of Kv11.1 level on the cell membrane, increase maturation of mutant Kv11.1, shortened potential duration and increased I_{kr} density. However, their data demonstrated that phenotypical changes by Lumacaftor only appears in iPSC-CMs with class 2 mutation. Together, this data, based on human-induced PSC-CMs, showed that Lumacaftor could be used clinically in patients with LQT2-class 2 mutations, which represents a significant advancement in precision medicine using drug screening[41]. Compared to mouse models for drug screening, Lumacaftor's discovery utilizing patient's iPSC-CM verified that iPSCs are a more accessible and efficient way of drug discovery for congenital channelopathies.

Modeling structural heart disease using iPSCs

Congenital structural heart disease is a more complicated disease category compared to congenital valve disease and channelopathies. Interaction of endocardial and neural crest cells in a three-dimensional structure is crucial for cardiac development. Cell differentiation and migration could be regulated by NODAL, WNT and BMP signals. Genetics and environmental factors also contribute to the development of structural heart disease.

Investigations focusing on structural heart disease are still minimal, with few focused on Hypoplastic Left Heart Syndrome (HLHS). Functional studies of iPSCs derived from neonatal patients with HLHS revealed a strong connection with NOTCH signaling pathway. Impaired function of the NOTCH1 signaling pathway in patient-derived iPSC-CM could result in impaired differentiation, sarcomeric function as well as myofiber organization that might be essential for heart development as well as cell migration[42–44]. Other studies demonstrated the relationship between Notch signaling and *MYH6*, *NKX2–5*, and *HAND1*[45, 46]. Kobayashi et al. generated iPSCs from cardiac progenitor cells from HLHS patients. Using RT-PCR, they discovered lower differentiation potential of HLHS-derived iPSC-CM, and showed transcriptional depression of *NKX2–5*, *TBX2*, NOTCH signaling, which could contribute to cardiac malformation in HLHS[45]. Tomita-Mitchell et al. performed next-generation sequencing on a family with a high prevalence of HLHS/CHD, identifying a rare mutation in *MYH6* and discovering a high enrichment of *MYH6* damaging variants on 190 unrelated HLHS patients compared to the control cohort. Using iPSC derived from HLHS patients with *MYH6* variants, they discovered an increased expression level of *MYH7*, and unorganized sarcomere(s) which could result in defective differentiation[46]. Similar investigations were conducted from Theis et al., where after whole genome sequencing on a family with HLHS/CHD, they reported two rare mutations of *NOTCH*, P1964L and P1256L mutation. A functional study was also conducted comparing proband (HLHS patient carrying both *NOTCH* P1964L and P1256L mutation) derived-iPSC-CMs and iPSC-CMs from the father (*NOTCH1* P1256L) and mother (bicuspid pulmonary valve, *NOTCH1* P1264L). They discovered a lower expression level of *NOTCH1*, decreased cardiogenesis, and higher proportion of myofibrils in HLHS-iPSC-CM compared to iPSC-CMs from the parents[43].

A recent publication from Miao et al. identified a developmentally impaired endocardial population in HLHS using single-cell RNA profiling, hiPSCs, and human fetal heart tissue[47] . Using iPSC-ECs from HLHS patients and single-cell analysis of the differentiated HLHS iPSC-EC, they discovered suppression of several signaling pathways related to endocardial function and valvular structural remodeling and development including VEGF and NOTCH signaling pathways. More importantly, using a transwell co-culture system to culture normal iPSC-derived cardiomyocytes with HLHS iPSC-ECs, dysfunctional iPSC-CMs showed a less proliferative and mature profile. All together, these patient-derived iPSC-CM and iPSC-EC studies provided comprehensive insights into functional defects and aberrant endocardium-myocardium crosstalk in HLHS. Translational research of HLHS using patient-derived iPSCs clearly demonstrates that using iPSC technology, it is possible to draw a more precise picture of the functions of each subgroup of cells, and their interactions during the developmental process.

Limitations/Future Directions in modeling of CHD with human iPSCs

Human iPSCs have been widely used for disease modeling and drug screening in cardiovascular research of CHD. However, several key questions remain for further investigations. Firstly, growing evidence suggests that there could be differences structurally and functionally between iPSC-CM and adult cardiomyocytes. Increasing methodology research was conducted to improve the maturity of iPSC-CMs. However, capturing abnormalities during the development is essential in pediatric CHD pathogenesis. Additionally, functional/structural/metabolic differences between cells in embryonic, prenatal, pediatric, and adult hearts are critical and whether iPSC-derived cells can recapitulate cardiac cell abnormalities during prenatal or pediatric stages remains largely unknown. Secondly, abnormalities in interactions, transformations between cell types, and migration of cells in a three-dimensional are crucial in the pathogenesis of structural heart disease. The culture of a single type of iPSC-derived cells in 2D might not be enough for such analysis.

With the development of 3D models come new opportunities for a new era of higher complexity culture systems hoping to capture more mature phenotypes. There are few types of cardiac organoids (COs; precardiac CO, developmental CO, chamber CO) currently in use that can model early development of the heart and begin to model congenital heart defects[48]. Specifically, precardiac COs show primitive patterning and organization showing a first heart field (FHF) and second heart field (SHF) consistent with current development theory. To generate this model, BMP and Activin A are used for mesoderm and cardiac differentiation in suspension culture[49]. When producing a developmental CO, the final goal is to recapitulate the germ-layer definition and also cardiogenesis using a more complex differentiation protocol. This protocol involves Wnt activation/inactivation (using/withdrawing CHIR99021, a GSK-3β inhibitor) to induce the mesoderm induction followed by addition of cardiogenic factors such as Ascorbic Acid, bFGF (FGF2), and VEGF[50]. For chamber COs, researchers have now shifted the focus to further mature previous developmental COs and observe early chamber formation. Manipulation of Wnt, BMP, FGF, Activin A, and retinoic acid was necessary to balance the effects of differentiation since it was found that cavity formation and cardiac specification were not as connected as previously thought[51].

With the added complexity of 3D culture systems, difficulties such as an apoptotic/hypoxic core could arise without proper nutrient diffusion when organoids reach a certain size. However, 3D organoid cultures have revolutionized the way we study cell interactions. Physiologically relevant structures were made possible which led to a better understanding of development and drug responses that could be translated directly to patient care[52]. With the continuing combination of biology and technology, we can ensure reproducibility while minimizing variability and ultimately, further increase the translational potential of the field.

Derivation of lung lineages from hiPSCs

As it is evident from the previous sections, several congenital heart pathologies can be effectively modeled with the use of hiPSCs as directed differentiation protocols for iPSC-CMs and other cardiac cell types are quite developed. In the case of the respiratory

system, the last ten years have also witnessed impressive progress in the derivation of lung epithelial[53–56] and most recently mesenchymal[57, 58] lineages from PSCs. We now have at our disposal PSC-based experimental tools, including organoids, for the modeling of neonatal lung disease in vitro and drug screening. In particular, the robust derivation of AT2-like cells from human iPSCs (iAT2s)[56, 59] offer a powerful platform for modeling neonatal diseases that mostly affect the alveolar epithelium. These cells possess a transcriptome similar to fetal-like AT2s, have been shown to possess lamellar bodies for surfactant packaging and secretion, and most importantly can be maintained as long-term self-renewing alveolospheres in culture. Furthermore, the widespread deployment of gene-editing techniques allows for the correction of disease-causing mutations in patient-specific iPSCs thereby providing a tractable system to make functional and molecular comparisons between diseased and gene-corrected iAT2s[60].

Modeling surfactant deficiencies using iPSCs

Children's Interstitial Lung Disease (ChILD) disorders are a heterogeneous group of rare lung pathologies that include bronchiolitis obliterans, neuroendocrine cell hyperplasia of infancy (NEHI) and disorders due to surfactant deficiency, among others. It has been recognized that pediatric lung disorders cannot be adequately described using adult classifications due to their different course, histology patterns and outcomes[61]; hence, a new classification scheme has been established[62]. The surfactant dysfunction disorders belong to disorders more prevalent in infancy and are due to mutations in genes whose products are important for processing, storage and recycling of pulmonary surfactant, such as the surfactant proteins *SFTPB, SFTPC*[63, 64], and the lipid transport protein *ABCA3*[65]. Mortality is high in cases due to *SFTPB* and *ABCA3* mutations due to neonatal respiratory distress syndrome and life prolongation can be achieved only by lung transplantation[65–67]. Even for milder manifestations of the disease, there is currently no cure and the only therapeutic option is supportive care. Thus, new research approaches that may lead to novel effective therapies for surfactant deficiency disorders are urgently needed.

Mouse models have been invaluable in the study of surfactant deficiencies and appear to recapitulate key features of the human disease[68–72]. For example, $Abca3^{-/-}$ and $Sftpb^{-/-}$ mice display perinatal lethality due to lung atelectasis[68, 72] whereas $Sftpc^{-/-}$ mice exhibit emphysema-like disease with immune infiltrates in a 129/Sv background[70]. Surfactant deficiencies have also been studied in established cell lines, such as HEK and A549, which nevertheless lack the machinery for surfactant packaging and most importantly do not express the key lung epithelial transcription factor, *NKX2–1* (reviewed in[73]). Therefore, patient-derived iPSCs and their iAT2 progeny offer an attractive alternative to these model systems.

iPSCs from patients expressing the most pathogenic *SFTPB* variant (121ins2 mutation)[73] have been used to study surfactant deficiency in vitro[56, 74, 75] (Table 3). In the very first study, Jacob and coworkers used CRISPR/Cas9 to correct the mutation in patient-derived iPSCs and the two syngeneic lines, diseased and gene-corrected (SP212 and SP212corr, respectively) were used for the derivation of iAT2 alveolospheres. SP212 iAT2s had low levels of *SFTPB* transcript, possessed neither detectable SFTPB protein nor

lamellar bodies, and misprocessed pro-SFTPC. On the contrary, *SFTPB* transcript levels were restored, and ensuing defects were reversed in the SP21corr line, demonstrating a strong causal link between the *SFTPB* mutation and disease phenotype in vitro[56]. In another study, pro-*SFTPB* was expressed in undifferentiated patient-derived iPSC following lentiviral transduction with a vector encoding for *SFTPB* and antibiotic selection[75]. Directed differentiation to lung progenitors and subsequent derivation of lung organoids containing alveolar and airway lineages resulted in different phenotypes for the parental and transduced lines. The parental line (hiPro133) had decreased transcript levels for *SFTPB* and *SFTPC*, lacked organized lamellar bodies and the functional 8-KDa SFTPB protein, whereas the transduced line (hiPro133 + SFTPB-GFP) exhibited organized lamellar bodies and had significantly higher secretion of surfactant in the culture media. In a follow-up study, metabolomic differences between normal iPSC lines and the hiPro133 line were investigated during the stages leading to lung progenitor specification[74]. Different metabolites were upregulated in the various stages leading from undifferentiated iPSCs to lung progenitors, including phosphatidylcholine acyl-alkyl C36:3, sphingomyelin C24:0 (SM 18:1/24:0), octadecenoylcarnitine, and tyrosine. Although lysophosphatidylcholine and phosphatidylcholine diacyl C26:0 were upregulated in only hiPro133 lung progenitor cells in that stage, there were not statistically significant differences in up- or downregulated metabolomic pathways between the normal lines and hiPro133. Future studies that focus on more advanced stages of lung differentiation using lines with the same genetic background (e.g., diseased and CRISPR/Cas9 corrected) may shed light on the metabolomic changes that underlie the inception of surfactant deficiency disorders.

Alveolar dysfunction caused by the most common non-BRICHOS *SFTPC* disease-associated variant, p.I73T, was investigated thoroughly using syngeneic diseased ($SFTPC^{I73T/tdT}$) and gene-corrected ($SFTPC^{tdT/WT}$) lines carrying an *SFTPC* fluorescent reporter[76]. After establishing stable, long-term iAT2 cultures from both lines the authors witnessed clear morphological and functional differences between the diseased and corrected lines in a time-dependent manner. Despite similar levels of *SFTPC* expression, $SFTPC^{I73T/tdT}$ iAT2s were less proliferative, displayed reduced apical-basal polarity and accumulation of mistrafficked and misprocessed SFTPC. Importantly, diseased cells exhibited perturbed autophagosome dynamics with increased autophagosome initially followed by a late block in autophagy. Additionally, diseased cells upregulated the NF-kB pathway compared to gene-corrected cells, suggesting a pro-inflammatory phenotype. Lastly, the potential of this line as a drug screening platform was tested as treatment with hydroxychloroquine, a drug used in pediatric patients with surfactant deficiency disorders, exacerbated the autophagic perturbations and further reduced the proliferative capacity of diseased iAT2s.

Taken together, data from iPSC-based studies of surfactant deficiencies indicate that these in vitro platforms can efficiently complement or even substitute studies using transformed cell lines and mouse strains. It is to be expected that established iAT2 lines from a wide variety of patients will be a valuable tool in the preclinical screening of drugs aiming to alleviate disease manifestations in patients with surfactant deficiency disorders.

Modeling of other lung congenital defects using iPSCs

a) Brain-Lung-Thyroid Syndrome—The homeodomain transcription factor *NKX2–1* plays an overarching role in the regulation of essential genes for lung development and function, and it is the earliest known marker of the lung epithelial primordium[81, 82]. Thus, dysregulation of *NKX2–1* function is likely to result in a broad spectrum of lung disease phenotypes. Since NKX2–1 also controls the expression of several genes in the forebrain and thyroid[83, 84], the emergence of a ChILD entity known as "Brain-Lung-Thyroid" syndrome has been documented in cases of *NKX2–1* haploinsufficiency[85, 86] and is characterized by neurological defects[87–89], hypothyroidism, pulmonary infections and interstitial lung disease[85, 90–94]. Following the first report of an infant with hypothyroidism and respiratory failure[95], it has been gradually recognized that *NKX2–1* haploinsufficiency due to de novo mutations[85, 86, 96, 97] or autosomal dominant transmission[98, 99] can affect several organs since patients present with neurological defects such as chorea, congenital hypothyroidism and respiratory problems such as atelectasis and recurrent infections. In the lung, the dysregulation of *NKX2–1* function can result in several disease phenotypes resembling surfactant dysfunction mutations[100]. This is not surprising since *Nkx2–1* is important in lung development[84, 101] and *Nkx2–1* null mouse demonstrates impaired lung branching morphogenesis, thyroid and pituitary agenesis and forebrain defects[102–106]. In humans, the resulting phenotype from *NKX2–1* haploinsufficiency is not easy to predict and depends on the effects of the NKX2–1 mutated protein (dominant negative or positive) on the production of surfactant-associated proteins[85]. Thus, the overall mechanisms that control the differential expression of surfactant proteins in *NKX2–1* haploinsufficiency are currently unknown.

In a proof-of-principle study, somatic cells from three previously described pediatric patients with *NKX2–1* mutations or deletion[94] were reprogrammed to iPSCs[78]. Subsequent differentiation to thyroid progenitors through definitive endoderm and anterior foregut endoderm intermediates resulted in the emergence of NKX2–1^{+}/$PAX8^{+}$ cell clusters, denoting thyroid specification. On the contrary, there are no currently published data on derivation of forebrain and lung progenitors from Brain-Lung-Thyroid patient iPSCs.

Derivation and differentiation of iPSC lines from Brain-Lung-Thyroid syndrome patients opens exiting possibilities for in vitro modeling of the disease. The use of patient-specific iPSC lines and well-defined protocols of iPS cell differentiation to lung and thyroid epithelia as well as pituitary and other forebrain lineages can be used to study the emergence of divergent phenotypes at various developmental times and to characterize the underlying molecular and signaling pathways using state-of-the-art techniques, such as multimodal profiling[107].

b) Other pathologies—Congenital diaphragmatic hernia (CDH) is a rare congenital condition (1 case in every 2,500 – 3,000 live births) that is due to incomplete diaphragm closure during embryonic development and subsequent herniation of abdominal organs into the chest and close proximity to the lungs. This results in defective lung development (hypoplastic lungs) and pulmonary hypertension. Disease management involves surgical

correction of the diaphragm defect following stabilization of the newborn and lifetime monitoring of the patient.

The etiology of CDH is complex and most probably involves a constellation of genetic, biomechanical, and environmental factors. Animal models of CDH have been developed and used to study the disease. For example, deletion of COUP-TFII (NR2F2) using an *Nkx3–2*^{Cre} driver (highly expressed in foregut mesentery) resulted in a phenotype reminiscent of left-sided CDH[108]. Interestingly, COUP-TFII was identified as one of the genes in a minimal deletion region for human CDH patients. In another study, deletion of Roundabout (Robo) receptors 1 and 2 in mice resulted in CDH phenotypes, due to defects in early foregut morphogenesis[109].

The possibility of derivation of several foregut lineages (mesenchymal and epithelial) from human iPSCs and formation of organotypic cultures offers the opportunity to model complex genetic diseases, such as CDH, in vitro. Indeed, a recent proof-of-principle study derived lung organoids containing both epithelial and mesenchymal cell types from hiPSCs[80]. The starting iPSC lines were either from non-diseased subjects or from fetuses and infants with Bochdalek congenital diaphragmatic hernia (CDH). CDH-patient lung organoids had lower *NKX2–1, SFTPB* and *SFTPC* expression as well as decreased expression of *PDGFRa* compared to non-disease controls. Interestingly, several ECM genes such as *HAPLN1*, *ACAN*, and *EMILIN1* were significantly upregulated in day 40 CDH-patient lung organoids. When organoids were subjected to increasing mechanical compression to model herniation of abdominal organs in the lung vicinity, there was significant downregulation of *PDPN1*, a marker of Type I alveolar epithelial cells in both types of organoids. Although the studied organoids did not undergo branching morphogenesis, this platform may provide the basis for future studies aiming to elucidate the multifactorial nature of CDH.

Another pathology affecting the respiratory system with oftentimes manifestation in childhood is dyskeratosis congenita, a rare genetic disorder characterized by premature onset of pulmonary fibrosis, among other symptoms. A well-characterized mutation (*DKC1* A386T) was engineered into a dual reporter line[56] allowing for the derivation of isogeneic lines (wild type (WT) and DKC1 A386T)[79] that were differentiated to *SFTPC*$^{+}$ iAT2s. The latter were cultured in previously described alveolarization media[56] but without addition of exogenous Wnt activators. DKC1 A386T iAT2s showed inability to self-renew with repeated passaging, had increasing expression of p21 protein, a hallmark of senescence, and were characterized by short telomeres. Genome-wide transcriptome analysis revealed upregulation of pathways associated with pulmonary fibrosis, such as mithochondrial dysfunction and autophagy and marked downregulation of the Wnt pathway compared to WT iAT2s. Subsequent treatment of DKC1 iAT2s with increasing concentrations of CHIR99021 reversed senescence and rescued both iAT2 growth and telomere defects. This suggests that the engineering of iPSC lines with dyskeratosis congenita-related mutations may offer in the future a tractable in vitro platform to model pulmonary manifestations of this devastating disease.

Conclusion

CHD describes a variety of structural cardiac abnormalities present before birth attributable to abnormal fetal cardiac development. Stem cells play a crucial role in disease modeling and cardiac regenerative therapy, and patient-iPSCs will be increasingly used to such ends. With the development of differential strategies for iPSC-derived cardiomyocytes, disease modeling of congenital valve disease, channelopathies, and structural heart disease have been investigated using iPSC-CMs from pediatric patients. Similarly, neonatal pulmonary diseases, especially the ones with well-defined monogenic drivers, are already being studied in vitro using hiPSCs. The combined use of gene editing tools, multilineage lung organoids, and microfluidic systems will certainly provide considerable impetus to the development of precision systems for drug screening and disease modeling

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Table 1.

studies of channelopathies using pediatric patient-derived iPSC-CMs.

PMID	Journal	Year	First Author	Disease	Phenotype	Patient description
20660394 [25]	N Engl J Med	2010	Alessandr a Moretti	Long-QT Syndrome type 1	Prolonged action potential, reduction in Ik current, altered channel activation	8-year-old boy with ADHD, R190Q in *KCNQ1*
22739119 [26]	Cardiovas c Res	2012	Toru Egashira	Long-QT syndrome	Downregulation of KCNQ1 at peripheral cite, smaller IKs peak and tail current	13-year-old boy, heterozygous deletion of *KCNQ1,* 1893delC
23277474 [27]	J Gen Physiol	2013	Cecile Terrenoire	Long-QT syndrome type 3	Dysfunctional inactivation of Na channel activity	4-year-old, *SCN5A* F1473Cmutation, *KCNH2*_K8 97T mutation
23998552 [28]	Int J Cardio	2013	Dongrui Ma	Long-QT syndrome type 3	Prolonged action potential duration or APD, increased TTX-sensitive late or persistent Na current	7-year-old Chinese girl, *SCN5A* V1763M mutation
28956012 [29]	Biochem Biophys Rep	2017	Yusuke Kuroda	Andersen-Tawil syndrome	Strong arrhythmic events, higher incidence of irregular Ca release	10-year-old male with a *KCNJ2* R218W mutation
28335032 [30]	Hum Mol Genet	2017	Yuta Yamamoto	Long-QT syndrome type 15	Significantly lower beating rates, prolonged duration, impaired inactivation of LTCC currents	A 12-year- old boy with LQTS carrying a heterozygou s *CALM2* N98S mutation
28158429 [31]	Cardiovasc Res	2017	Marcella Rocchetti	Long-QT syndrome	CALM-F142L prolonged repolarization, altered its rate-dependency and its response to isoproterenol	A 14-year- old male with *CALM1*-F142L mutation
22050625 [32]	J Cell Mol Med	2012	Atara Novak	Catechola minergic polymorphic ventricular tachycardia	Isoproterenol causes delayed afterdepolarizat ions, oscillary arrhythmic prepotentials, aftercontractions and diastolic Ca rises	A 12-year- old boy and a 30-year- old woman with *CASQ2* D307H mutation
25453094 [33]	Proc Natl Acad Sci U S A	2014	Miao Zhang	Jervell & Lange-Nielsen syndrome	Pronounced action and field potential prolongation and reduction or absence of Iks	Three pediatric patients with *KCNQ1* c.478–2A>T and c.1781G>A
21367833 [34]	Eur Heart J	2011	Elena Matsa	Long-iPSCQT syndrome type 2	Prolonged field/action potential duration, developed early after depolarization when challenged with isoprenaline	15-year-old LQT2 patient with *KCNH2* G1681A mutation

Table 2.

Studies of disease modeling of HLHS using pediatric patient-derived iPSCs.

PMID	Journal	Year	First Author	Patient description	Genetic Alternation	Findings
28521042 [44]	Hum Mol Genet	2017	Yang Chunbo	neonatal	NOTCH signaling pathway	Expression of NOTCH receptors was significantly downregulated in HLHS-iPSC-derived cardiomyocytes alongside NOTCH target genes, confirming the downregulation of NOTCH signaling activity
28142228 [42]	Stem Cells	2017	Sybil CL Hrstka	NA	NOTCH signaling pathway	The mutational burden in NOTCH1 is sufficient to cause dysregulated NO signaling during heart development
26164125 [43]	Hum Genet	2015	Jeanne L Theis Jeanne L	m f proband	*NOTCH1*	Patient-specific iPSCs exhibited diminished transcript levels of NOTCH1 signaling pathway components, impaired myocardiogenesis, and a higher prevalence of heterogeneous myofilament organization.
27789736 [46]	Physiol Genomics	2016	Aoy Tomita-Mitchell	probands and their parents	*MYH6*	increased expression of MYH7observed in vivo while revealing defective cardiomyogenic differentiation
25050861 [45]	PLoS One	2014	Junko Kobayashi	neonatal	*NKX2–5, HAND1,* and *NOTCH1*	Impaired differentiation and reduced notch signaling

Table 3.

Modeling of lung neonatal diseases with iPSCs

PMID	Journal	Year	First author	Disease	Gene and mutation(s)	Derived iPSC line(s)
28965766 [56]	Cell Stem Cell	2017	Anjali Jacob	Surfactant protein deficiency	*SFTPB* (c.397delCins GAA; p.Pro133Glnfs Ter95)	SP212, SP212Corr
31530844 [75]	Sci Rep	2019	Sandra L. Leibel	Surfactant protein deficiency	*SFTPB* (c.397delCins GAA; p.Pro133Glnfs Ter95)	hiPro133, hiPro133 + SFTPB-GFP
35198866 [74]	iScience	2022	Sandra L. Leibel	Surfactant protein deficiency	*SFTPB* (c.397delCins GAA; p.Pro133Glnfs Ter95)	hiPro133, hiPro133 + SFTPB-GFP
34469722 [76]	Cell Reports	2021	Konstanti nos-Dionysios Alysandratos	Surfactant protein deficiency	*SFTPC* (p.Ile73Thr, p.I73T)	SPC2 ($SFTPC^{tdT/WT}$; $SFTPC^{I73T/tdT}$), SPC6, SPC7
33839547 [77]	Stem Cell Res	2021	Xiaojuan Yin	Surfactant metabolism deficiency	ABCA3 (c.3997–3998del, p.R1333fs, and c.3137C > T, p.A1046V)	SMCPGHi0 01-A
26593959 [78]	Cell Stem Cell	2015	Anita A. Kurmann	Brain-Lung-Thyroid Syndrome	*NKX2–1* (c.384–391del8; entire *Nkx2–1* locus; c.552–556del5	T1, T3, T4
35559731 [79]	Elife	2022	Rafael J. Fernandez	Dyskeratosis congenita	*DKC1* (A386T)	BU3 NGST *DKC1* A386T
32949227 [80]	Stem Cells Transl Med	2021	Shaun M. Kunisaki	Congenital diaphragmatic hernia	Unknown	CDH-iPSCs