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Introduction to iPSCs

Human induced pluripotent stem cells (iPSCs) are now at the forefront of human disease and development research, facilitating highly representative modeling and the reliable development of new therapies. Rapidly advancing stem cell resources and technologies have enabled the investigation of disease in tissue- and genetics-specific contexts, in models from patient-derived sources, and in the context of development that was not previously possible. Adoption of iPSC culture in the laboratory requires education on appropriate cell sources, characteristics, maintenance, quality control, and study design. However, advances in reagents, resources, and methods have made iPSC culture simpler and more accessible, making this an opportune time to incorporate iPSCs into laboratory workflows.

Development of iPSCs as a research tool

Stem cells are undifferentiated cells that can give rise to multiple cell types in an organism and have the capacity for self-renewal.[1] While totipotent stem cells can differentiate into any cell of an organism, pluripotent stem cells (PSCs) can produce all germ layers of an organism except for extraembryonic structures, e.g. the placenta. Once PSCs differentiate into one of the germ layers, they are considered "multipotent" and can only become cells that make up that germ layer. Pluripotent and, subsequently, multipotent cells then differentiate into specialized cells under specific physiological or cell culture conditions. Potency, or the range of cell types a PSC can differentiate into, is therefore reduced with each step of differentiation.

Embryonic stem cells (ESCs), derived from the inner cell mass of a blastocyst, fall within the PSC category. The isolation and culture of ESCs from mice was first reported in the early 1980s.[2,3] Over the next 20 years, methods for the differentiation of ESCs into multiple lineages were developed. In 1998, the isolation and culture of human ESCs inspired great interest in their potential use as a source of tissues for transplantation and cell-based therapies.[4,5] Early methods for the differentiation of ESCs into hematopoietic, vascular, and cardiac cells included embryoid body formation, growth on stromal cells, and growth on extracellular matrix.[4] However, the undefined nature of differentiation factors involved in these methods led to the generation of undesired cell types. The use of fetal calf serum in cultures, which contains undefined factors, was replaced by the use of defined growth factors for lineage-specific differentiation.[4] The continued development of ESC culture and differentiation protocols included the use of animal-free culture conditions.

ESCs have been used for organoid development to study embryonic and fetal development.[6] Advances in ESC culture have also led to the derivation of cloned embryonic stem cells using somatic cell nuclear transfer for potential use in cell therapy and research (humans) and reproductive cloning (non-human animals).[6] Because of ethical concerns regarding embryonic sources of human ESCs, the potential for abnormal development, and immune reactivity to allogenic ESCs, ESC-based cell therapy clinical trials and the development of ESC-based models and therapies have proven difficult.[7] As a result, alternative technologies, such as iPSCs, have been developed that provide human PSCs from somatic sources and allow for the derivation of stem cell cultures from patients with known diseases.

Human iPSCs are generated artificially from somatic cells and exhibit functions that are similar to ESCs.[1] The development and differentiation of iPSCs derived from patients has provided

many *in vitro* disease models that previously could not be achieved. The ability to prepare iPSCs directly from individual patients makes them amenable to and useful for medical applications and disease models, including models of diseases arising from developmental or germline genetic causes. Other diseases, such as neurological or cardiac disorders, were not previously amenable to *in vitro* modeling using traditional techniques because of a lack of access to diseased tissues from living patients and difficulty in culturing post-mortem tissues.[8] For these and other diseases, iPSC-based modeling has become fundamental in research and drug development. Subsequently, several new drug candidates that were discovered using iPSC-based screening are now in development and clinical pipelines.[9] Models derived from iPSCs can be highly representative of human disease pathobiology compared to primary cells or non-human models.[9] In addition, iPSC models highly predictive of drug response may reduce the need for animal use. iPSC culture has also become increasingly accessible to research and drug development laboratories because of simplified access and the availability of validated and standardized iPSCs, reagents, and resources. As a result, iPSCs have become a key platform for research and drug testing.

Tissue sources for iPSC reprogramming can include skin biopsies, peripheral blood, hair follicles, and urine, which provide donor fibroblasts, lymphocytes, keratinocytes, and renal tubular cells, respectively.[7] These tissue and cell sources can vary in terms of the speed and efficiency with which they can be reprogrammed into iPSCs. The process of reprogramming terminally differentiated cells into iPSCs involves the transient expression of transcription factors, resulting in the inadvertent downstream repression of genes that can regulate genomic stability.[1] The transcription factors OCT3/4, SOX2, KLF4, and c-MYC (OSKM) are considered to be sufficient for reprogramming into iPSCs with a morphology and function that is similar to ESCs.[10] Delivery technologies for the reprogramming factors include retroviral, lentiviral, adenoviral, plasmid, transposon, synthetic RNA, and recombinant protein modalities.[10] Additional considerations, such as the preparation, delivery, and the removal of exogenous factors after reprogramming, must also be taken into account. While reprogramming efficiency varies among tissue sources and methods, a fraction of cells typically undergo complete reprogramming. These multiple experimental methods highlight the importance of standardized reprogramming methods and thorough characterization of the resulting iPSCs. When developing iPSC lines, important considerations include choosing appropriate sources, isolating appropriate cells, efficient reprogramming, isolating pluripotent cells, eliminating exogenous factors, quality control, ensuring genetic fidelity to source tissue, and controlling variability between clones/lines.

The variability between clones and cell lines in iPSC-based models goes to the heart of reproducibility and the generation of meaningful results. Factors that can affect variability include the genetic background and sex of the donor, culture conditions, and passage.[11] While representation of diverse genetic backgrounds and genders is an important feature of iPSC-derived models, minimization of undesired variability between clones can avoid effects that can overwhelm biological variables of interest.[11] Validated iPSC lines created using proven protocols and with comprehensive quality control information are becoming increasingly available, lending improved consistency and interpretability to iPSC-derived models.

iPSC culture for human disease modeling

Model systems for research and drug development require the reproduction of both pathogenic factors and disease context, with gold standard models allowing for the prediction of disease pathology and human treatment response. In particular, human models derived from patients are advantageous as they carry disease-relevant genes, mutations, and phenotypes. As iPSCs are derived directly from patients, libraries of cell types can be produced that match the genome of the patient. Therefore, iPSC-derived models can be created that recapitulate the microenvironment as well as the genetic and phenotypic variables of the disease, which is not possible in primary cell lines or animal models.

Using iPSCs, complex patient-derived models such as organoids can be generated that undergo differentiation and developmental programs that are implicit in diseases including cancer.[12] These models have the ability to recapitulate the intratumoral heterogeneity and three-dimensional interaction with other cells and the extracellular matrix, contributing to the highly disease-representative nature of these models.[12] The development of brain organoids from iPSCs has enhanced the ability to study diseases, including but not limited to genetic lissencephaly (smooth brain), Alzheimer's disease, and Parkinson's disease. This has been accomplished through the modeling of cellular function and development within the brain in the contexts of causal mutations and familial genetics.[13] For these and similar diseases, such representative modeling was not possible prior to the development of iPSC culture.

By reprogramming cells from patients with specific diseases, cultured iPSCs are changing the standard of disease modeling by providing access to disease-specific models that can include both pathologic and normal tissues.[14,15] Examples include models of type 1 diabetes, schizophrenia, Duchenne muscular dystrophy, and polycystic kidney disease, among others.[14] These disease-specific iPSC cultures can be differentiated into the tissue type of interest and typically exhibit high fidelity to the mutational backgrounds, genotypes, phenotypes, and morphologies of their representative diseases.[16–18] Increasingly accessible and standardized iPSC lines of known genetic composition, data, protocols, and cell culture reagents are democratizing iPSC disease models as they become prominent methods for research and drug development.

Research and drug development problems that are addressed by iPSCs

The use of human iPSCs in research and drug development is improving our understanding of disease and its effective treatment. Previous limitations in how closely models have represented the pathobiology, genetics, and progression of disease are being minimized through the use of patient-derived human iPSC models. Further, the power of disease modeling to discriminate true mechanisms of disease and the clinical relevance of therapeutic targets have been strengthened by human iPSCs because of their complementarity to primary cell culture and animal models.

Relevance to human disease

Many primary cell culture and animal models differ from human disease states in ways that prevent clinical extrapolation of results. For example, primary tissue culture models of neurological diseases, such as Alzheimer's disease (AD), present issues with accurate representation of the disease process and pathology. Tissues that are obtained post-mortem do not allow the study of disease progression or therapeutic intervention.[19] Proliferating human and rodent cells also do not accurately model neurodegenerative processes or represent neurons, which are post-mitotic.[19] Moreover, the heterogeneity in AD etiology among patients and the spectrum of multiple small genetic or epigenetic changes contributing to disease cannot be feasibly recapitulated through genetic manipulation of non-diseased cells.[20] There are also concerns regarding the disease relevance of animal models, as mammalian models are not ideally matched to human evolution or genetics. As an example, sporadic AD risk-associated gene products, including CD33, TREM2, MS4A6A, and CR1 microglial surface proteins, only share approximately 50% identity between humans and mice.[19,21]

Human iPSCs allow the generation of cultures from patients without reliance on post-mortem tissues, exogenous expression of disease drivers, proliferation issues, or use of non-human models. For example, derivation of neurons from human patients with AD pathology can overcome the lack of disease relevance in conventional models. Microglia, astrocytes, and multiple neuronal subtypes can be generated using iPSCs from either sporadic or familial AD patients and healthy individuals as controls.[19] Patient-specific pathogenic alterations can be studied using patient-derived iPSCs, which can be generated using biopsies from living patients. For example, Van der Kant et al. recently demonstrated the use of iPSC-derived hippocampal neurons to elucidate a connection between ApoE variant regulation of lipid metabolism and inflammatory signaling in AD.[21] In addition, age-dependent pathological changes can be more accurately studied over long timecourses using iPSC-derived cells.[19]

In another example, primary cell line models of polycystic kidney disease (PKD) can also face issues of poor representation of disease. Since they are derived from nephrectomy tissues, they represent advanced disease, and their phenotypes may either be causally related to disease or secondary effects of kidney injury.[18] As previously mentioned, there are also functional and physiological differences between human and non-human systems. Mouse models of PKD that incorporate the most common mutation in the disease, that of the *NPHP1* gene, do not demonstrate altered kidney phenotypes.[22] This may be due to functional differences in the gene or genetic backgrounds between species. In contrast, patient-derived iPSCs can carry both causal mutations and the disease-associated genetic backgrounds of PKD and other diseases with complex etiologies. Patient-derived iPSC models can also facilitate cell-based functional genomic, proteomic, and expression screening to identify genes and factors involved in physiologic and pathologic processes, such as cilium assembly and maintenance in PKD.[18,23]

Conventional models of cardiac disease have also faced concerns regarding disease representation. Since primary human cardiomyocytes are not easily obtained or cultured, traditional *in vitro* models have relied on neonatal rodent cardiomyocytes and animal models to represent some types of heart disease, including cardiomyopathy.[24] These models have not been highly predictive of drug response in humans.[24] Transformed cell lines that can be propagated in culture are typically murine or derived from human cardiac sarcomas.[25] These are not representative of cardiac cells, which are not proliferative in culture.[25] Primary cardiac cells, while representative, are post-mitotic and difficult to obtain.[24,25] They are, therefore, limited in their utility for high-throughput or population-based study. As such, there has been a gap in the accurate representation of cardiovascular disease and cardiomyopathies. However, there has been significant advancement in the use of iPSCs for generating patient-derived models of cardiac disease, which are now being used to study disease mechanisms and test pharmacologic candidates.[26] Goktas et al. used tachycardia patient-derived cardiomyocytes to discover a causal pathogenic mechanism of an *SCN5A* gene variant.[27] Zhou et al. discovered potential mechanistic targets of GLP-1 therapy for diabetic cardiomyopathy using iPSC-derived cardiomyocytes.[28] These models are creating the opportunity for research, personalized medicine, and high-throughput screening for drug discovery.[29] Electrophysiologic measures in iPSC-derived cardiomyocytes have been found to correspond to human ECGs, demonstrating their reliability for studying arrhythmias and assessing adverse drug responses, such as drug-induced arrhythmogenic potential.[30] High-throughput, population-based studies using human iPSC-derived cardiomyocytes have also been shown to be feasible, as demonstrated by a recently published screen for drug-induced cardiotoxicity and neurotoxicity using panels of iPSC-derived cells that are representative of common global HLA haplotypes.[31]

These examples illustrate the improved fidelity and relevance to human disease that

iPSCs offer. The use of this technology supplemented with more traditional primary cell culture and animal models has the potential to focus research on disease mechanisms and therapeutic targets with higher likelihood of clinical impact.

Complementarity to other models

The use of multiple test systems, including human and animal models, has increased the success of efforts to discover disease mechanisms and test therapies. While there is a need to study neurodegenerative disease in *in vivo* systems, the difficulty of accurate modeling in animals presents the need for appropriate human-derived models. iPSC-derived models can be used to identify and study a range of human neurodegenerative disease phenotypes prior to validation and further study in animal models.[32,33] For example, cell lines and three-dimensional cultures derived from iPSCs have been used to discover mechanisms of protein aggregate pathologies as well as vascular (blood-brain barrier) and immunologic phenotypes in AD.[32,34,35] Laperle et al. recently discovered potential therapeutic targets for Parkinson's disease using patient-derived iPSCs and dopaminergic iPSC-derived cultures with validation in murine models.[33] Ayabe et al. used both iPSC-derived neuronal cells and mouse cells to mechanistically implicate the modulation of mitochondrial function in beta-lactolin preventive therapy for AD.[36] Patient-derived iPSCs are a particularly useful addition to programs investigating neurodevelopment, as stem cell differentiation programs can replicate pathways engaged in embryogenesis and organismal development.[32] Mechanisms identified using these models can be further studied using genetic manipulation and treatment *in vitro* and *in vivo*.

In psychiatric disease, mechanisms employed within inhibitory and excitatory neurons can also be revealed using neurons derived from iPSCs and further tested in animal systems. While behavioral changes associated with disease are best tested in animal models, human cells, such as iPSC-derived neurons, can add human relevance and complement these models through the correlation of molecular and cellular phenotypes to these behaviors. The human translatability of these models has been shown through the demonstration of correlation of human EEG findings to electrophysiologic functions of iPSC-derived neurons.[37] Drug response, toxicity, and mechanistic insight into psychiatric drugs can be accomplished using iPSC-derived cells prior to animal or clinical testing. Mechanistic insights into drug-induced effects can be gained using patient-derived *in vitro* modeling, as exemplified by recent studies by Vadodaria et al., delineating mechanisms of SSRI resistance in major depressive disorder using iPSC-derived serotonergic neurons.[38,39] A complementary approach using iPSC-derived and animal models has also been used in the study of schizophrenia. Shao et al. implicated differential expression of *PCDHA2* in familial cases of schizophrenia using iPSC-derived neurons from affected and unaffected individuals.[40] This was followed by phenotypic findings of decreased inhibitory neuron development and function in *Pcdha* knockout mice.

Because animal models of cardiomyopathy are poorly predictive in regard to clinical efficacy but representative of whole-body effects, there is a need for complementary *in vitro* modeling to better define clinically relevant pathobiological mechanisms, drug targets, and drug responses. Given the limitations of primary culture models of cardiomyocytes, iPSCs have the potential to meet this challenge. Models of various cardiac pathologies have been developed using gene editing. For example, iPSC-derived cardiomyocytes have been edited with *KCNQ1* and *KCNH2* dominant negative mutations, conferring long QT syndrome phenotypes that are responsive to ion channel-regulating drugs for the treatment of this condition.[41] While the study cited here did not include animal models itself, it illustrates the ability to use iPSC-derived models to recapitulate human phenotypes for predictive drug testing. Such capability is likely to help select drug candidates with responses in animal models that are most likely to translate to activity in humans. Another example is modeling diabetic cardiomyopathy using human iPSC-derived cardiomyocytes that can be shifted metabolically in response to media substrates or genetically induced to exhibit insulin resistance.[24] Such models can be used to validate drug targets and responses observed in animal models in a manner that is specific to human cardiomyocytes. These examples illustrate that human- and cardiomyocyte-specific iPSC-derived systems can provide a way to feed validated pathophysiologic, molecular, and cellular mechanisms of cardiac disease into animal models and thereby improve the reliability and output of research and drug development.

Reduction of animal use

High cost, time consumption, ethical considerations, and suboptimal translation to humans have led to efforts to replace or reduce animal use in toxicology and preclinical testing of drugs. Since 2007, the National Research Council and the National Academies of Science, Engineering and Medicine have encouraged a transition from animal testing to mechanisms of action models to assess the risks associated with experimental drugs. More recently, the US Food and Drug Administration (FDA) has outlined plans for advancing novel techniques using *in vitro* human-derived and *in silico* platforms for discovery and toxicology through its Alternative Methods Working Group.[42] The stated goal of the FDA is to "replace, reduce and/or refine" animal use in preclinical testing. Patient-derived iPSC models for hepatotoxicity, neurotoxicity, cardiotoxicity, and airway epithelial toxicity/carcinogenicity are examples of alternative methods given in recent FDA reports.[42] These methodologies are recognized by the FDA as having the potential for obtaining better predictive and mechanistic insights from preclinical studies. In late 2022, the FDA Modernization Act 2.0 was signed into federal law, which turns away from the previous requirement for animal testing of all drugs and calls for increased reliance on non-animal testing.[43] These moves by federal regulators ensure that advanced modeling methods, including human iPSC-derived cultures, will be increasingly utilized in regulatory studies.

Strategies for successful iPSC and iPSC-derived culture

While there are clear benefits of implementing human iPSC culture in research and drug development, the generation of meaningful data requires attention to specific quality control and experimental design considerations. These include ensuring genetic heterogeneity and stability, sufficient sample size, thorough characterization, and appropriate maturation of cell lines. These aspects of iPSC-based research are discussed herein.

Minimizing clonal variation and genetic instability

Phenotypic and genetic variation between cell lines and controls is a critical issue in any cell-based model and applies to iPSC culture similarly. While the traditional use of family- and gender-matched healthy control subjects can be susceptible to heterogeneity, the use of isogenic iPSC cell lines, which are generated using gene editing of well-characterized iPSCs from healthy controls or from the patient line with genetic correction, can minimize the problems associated with cell line variation.[7] However, genetic instability acquired during culture remains a risk with iPSCs. This risk also exists with established cultures derived from primary cells, but is particularly important with PSCs because of their self-renewing capacity. With cell-based therapy, genetic instability can present the risk of introducing pathogenic changes. To mitigate this risk, therapies with engineered suicide genes exist that can eliminate cells with chromosomal abnormalities.[7,44] An example of a suicide gene is an inducible thymidine kinase that can be activated using ganciclovir and is linked to cyclin D1, which is activated upon cell cycle progression to ablate proliferative cells.[40] Therefore, suicide genes in cell-based therapies can effectively eliminate the evolution of tumor-initiating cells after transplantation, facilitating the safety of these treatment modalities.

Increasing sample size

As a wide variety of genetic backgrounds and causal genetic variations underlie certain diseases, large cohorts are required to elucidate disease etiology and clinical translation, especially when the underlying genetics are unknown. Interpretation of results obtained from studies that incorporate only a few cell lines derived from iPSCs can be confounded by genetic, epigenetic, and clonal variation between lines.[7] Increasing the number of iPSC lines can minimize confounding "noise" in order to be sensitive to disease-relevant signals.[7] High cost, low donor availability, and insufficient resources can be obstacles to in-house development of sufficiently large and well-characterized panels of human iPSCs to study a particular disease. However, large numbers of well-characterized iPSC lines derived from patients with a variety of diseases and healthy controls are growing in availability. These resources increase confidence in results obtained from panels of iPSC lines and can be accessed through biobanks and repositories with searchable databases of available iPSC lines, such as the ones indicated in the Further reading and resources section.

Maturity

iPSC-derived cells can exhibit relatively immature phenotypes compared to adult tissues, even after differentiation.[7] While immature phenotypes are amenable to the study of developmental or early-onset diseases, the representative study of late-onset diseases can be difficult using immature cells. Likewise, long-term disease-related alterations in terminally differentiated cells may be difficult to recapitulate longitudinally when the model is undergoing maturation over a time scale that is not representative of the disease process. However, maturation protocols and specialized maturation media are being developed to address this issue. For example, strategies for maturation of the metabolic characteristics of iPSC-derived cardiomyocytes, such as modulation of fatty acid oxidation and metabolic maturation media, have been shown to improve the metabolic maturity of these cells.[25] A combination of approaches that combine substrate availability and electrophysical stimulation has also been shown to improve the maturation of iPSC-derived cardiomyocytes.[45] Specialized media kits for culture and maturation are also available for neurons, microglia, and astrocytes, among other lineages.

Characterization of source materials

iPSCs can be obtained by reprogramming somatic cells in the lab, obtaining cells from other labs, or from cell banks providing validated iPSC lines and controls. However, there is variability between laboratories in terms of reprogramming protocols and the efficiency of reprogramming, which calls for strategies to maximize consistency, reproducibility, population representation, and standardization. The utilization of existing characterized and validated iPSC lines from commercial sources or cell banks may help mitigate these concerns. Nonetheless, it is important to understand each aspect of iPSC characterization.

Proper characterization of iPSCs, which is critical for assessing the fitness of cell lines for the representative study of disease, includes the identification of disease-relevant mutations, karyotyping, assurance of genomic integrity, and pluripotency assessment. Any new iPSC line should be evaluated for heterogeneity and percentage of pluripotent cells to ensure decreased variability between clones and a sufficient population of cells to expand and use for assays. While the expression of markers of the undifferentiated state (TRA-1-60, SSEA-3, SSEA-4, and TRA-1-81) is commonly checked, pluripotency is empirically determined using embryoid body formation, where differentiation into the three germ layers is assayed *in vitro*.[46] As these methods can be time-consuming and low-throughput, the determination of both pluripotency and heterogeneity may be improved using flow cytometric assays.[46]

Genomic integrity is typically evaluated using G-band karyotyping by a cytogeneticist, but can also be accomplished using SNP

microarray karyotyping.[46] While multiple methods exist for karyotyping, using a combination of methods offers the highest confidence. The detection of chromosomal rearrangement in iPSC subclones has presented a challenge, and SNP microarray karyotyping continues to be investigated for this purpose.[46] Genomic integrity, variants, and mutations in iPSCs have also been evaluated using targeted deep sequencing. Using this technology, the majority of mutations in iPSCs are found to be a result of pre-existing variants in clonal populations within source tissues, rather than those arising during the reprogramming process.[47] However, some mutation and genetic instability can still occur during culture and expansion. The complexity of iPSC line characterization often requires expensive commercial outsourcing, making a case for the procurement of iPSC lines that have been previously established, validated, and characterized.

Ease of culture

Either 1) media that is conditioned by feeder mouse embryonic fibroblasts (MEFs) or 2) co-culture of iPSCs with mitotically inactivated MEFs and the addition of fetal bovine serum were previously required to provide growth factors for the culture of iPSCs in the laboratory.[48] To increase culture efficiency and consistency, feeder-free media with serum replacement, such as the first defined, feeder-free medium mTeSR™1 (STEMCELL Technologies), have been developed and commercialized.[48–52]

The exogenous stem cell culture factor FGF2 can be unstable under culture conditions, requiring frequent feeding of cells (typically every 1 to 2 days) for their maintenance. FGF2 regulates the expression of BMP antagonists, which are required for stem cell maintenance.[48] It has been found that only with sustained release of FGF2, can the feeding of stem cell cultures be reduced to twice weekly.[53] A practical method to overcome these limitations is the use of media with stabilized growth factors, including FGF2, and increased buffering capacity to maintain pH with restrictive feeding, which allows for the extension of the interval between feeding to 72 hours. As these simplified media have become more commonly used, their compatibility with differentiation and genome editing protocols has been demonstrated.[49,50]

Aside from media, requirements for iPSC culture include extracellular matrix proteins and gentle, enzyme-free dissociation reagents for passage as cell aggregates or single-cell suspensions, depending on optimized protocols for specific cell types. The commercial availability of these stem cell-optimized culture reagents simplifies the routine growth and maintenance of iPSCs in the laboratory.

Guidelines for selecting appropriate iPSC lines

The selection of iPSC lines for research or drug testing should be undertaken with attention to their appropriateness for the particular research application, i.e. ethics (cloning, consent, etc.), use restriction, relevant disease/genetic background, and culture quality. Thorough quality control is a critical component that determines whether iPSC lines can yield relevant and meaningful results without complicating experimental procedures or introducing alterations that may confound results. Quality control consideration should be given to sterility, unique cell line identity as determined by STR profiling, clearance of reprogramming factors, pluripotency, and genetic fidelity to the originating tissues and donor. Identity analysis can help to avoid the use of lines that have inadvertently been switched or contaminated with other cell lines. Testing for residual reprogramming vectors can also help avoid the use of lines with integration of vectors, which may cause unintended or confounding genotypic or phenotypic effects.[1] Selection of lines that are disease-appropriate and of high quality can ensure that an iPSC line can be reliably implemented in the laboratory and that the data generated will be meaningful.

Stem cell biobanks and databanks

Several databanks exist for finding validated iPSC lines. Existing iPSC lines from a variety of disease backgrounds and from healthy controls can be searched for using global resources, such as the Integrated Collection of Stem Cell Bank (ICSCB-II)[54] and the Human Pluripotent Stem Cell Registry (hPSCreg)[55]. The ICSCB-II platform can search over 16,000 cell lines from European, Japanese, and United States registries, including hPSCreg (Germany), CIRM (US), and RIKEN BRC (Japan). Over 80% of the searchable cell lines are iPSCs, and roughly 40% are from individuals with disease, leaving around 60% from healthy donors. Search results provide basic information as guided by Minimum Information About a Cellular Assay for Regenerative Medicine (MIACARM) guidelines regarding ethical consent, approved use, source tissue, cell type, disease background, ethnicity, and gender. Links to the data source are provided, although some links have expired or their search platforms have been discontinued, e.g. eagle-i. Detailed information given through hPSCreg can include reprogramming methods, pluripotency testing results, karyotype stability, and genotyping information. Provider details and ordering information are also provided if the lines are commercially available, for example, the healthy donor-derived iPSCs described at https://hpscreg.eu/cell-line/SCTi003-A.

Standardization and reproducibility

The use of standard criteria for derivation, characterization, quality control, and maintenance of stem cell lines is critical for reproducible and reliable research using iPSCs. Without standardization, interpretation of basic scientific observations, extrapolation of clinically meaningful conclusions, reproducibility, and comparison to other studies becomes difficult. The International Stem Cell Banking Initiative (ISCBI)[56] and the European Bank for induced Pluripotent Stem Cells (EBiSC)[57] have established guidelines for the generation, qualification, banking, and distribution of stem cells including iPSCs. These databanks and guidelines address the challenges of clonal and genetic background variation between cell lines, standardization of methods and criteria for reprogramming, characterization, quality control, and accessibility of cell lines through biobanks. These guidelines and standardized information on iPSC lines based on MIACARM guidelines have been implemented by biobanks and databanks, such as the ICSCB-II, to improve the standardization of available iPSC cultures. Standardization of information requires consistent nomenclature and the inclusion of standard types of information regarding iPSC lines. Examples of standard characterization and quality control components for iPSCs include karyotyping, genotyping, RT-PCR for reprogramming factors, morphology description, sterility/mycoplasma testing, single-cell RNA-seq for heterogeneity, and functional assays for pluripotency using embryoid body formation or germ-layer differentiation.[58] Guidelines dictate that quality control should be performed from primary tissue acquisition through banking of iPSCs.[57] Using standard iPSC controls and reference panels may be a simple approach to ensuring robust experimental design and reproducibility when initiating research with iPSCs.

Differentiation protocols

Since there is variability in protocols and cells derived from differentiation programming, optimization and standardization of the methods of differentiation is critical for the generation of reproducible and meaningful data. To that end, standardized reagents, such as commercially available differentiation kits, and protocols for differentiation into various cell types now exist. Such media and protocols include those for trilineage (endoderm, mesoderm, and ectoderm), neuron, glial cell, hematopoietic, immune, cardiac, skeletal muscle, and lung progenitor lineages. There are also off-the-shelf culture medium kits for differentiation into brain, lung, and kidney organoids. With accumulating peer-reviewed literature demonstrating the effectiveness of these media and protocols, differentiation using defined factors has become more reliable, consistent, and readily accessible.[59–64]

Identification of potential drug targets and biomarkers

Frataxin deficiency alters gene expression in Friedreich ataxia-derived iPSC-neurons and cardiomyocytes[65]

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Friedreich's ataxia (FRDA) is a multisystem disorder affecting the heart, nervous system, musculoskeletal system, and pancreas. FRDA is inherited through an autosomal recessive GAA trinucleotide repeat expansion in the frataxin (*FXN*) gene causing its transcriptional repression. Carried by one in every 60–100 people, FRDA is the most common inherited ataxia. The majority of patients with FRDA succumb to heart disease rather than to the neurodegenerative manifestations of the disease. Frataxin has been proposed to have various functions, including iron and heme metabolism and cellular redox regulation. However, no prior study has definitively identified the mechanism by which frataxin deficiency results in pathophysiological effects. There is also a lack of effective therapies or preventive interventions for FRDA, presenting a clear need for the identification of mechanistically implicated therapeutic targets and theranostic markers for this devastating disease. This knowledge gap and unmet need are likely to be due in part to a lack of accessible models and the historical unavailability of intact neurons and cardiomyocytes from patients. In this study, iPSC resources were utilized to generate patient-derived induced neurons and cardiomyocytes for the study of FRDA. These experiments served to overcome the previous technical obstacles to the investigation of FRDA pathobiology and the discovery of targetable mechanisms for its treatment.

For this study, a patient with FRDA in the previous round was recruited at a neurology clinic for the collection of peripheral blood mononuclear cells and their subsequent reprogramming into iPSCs. Reprogramming, quality assurance and characterization of this single iPSC line required enough effort and data to justify its own publication, which is cited in the methods.[65] These iPSCs were differentiated into neurons and cardiomyocytes using commercially available differentiation media, e.g. STEMdiff™ Cardiomyocyte Differentiation Kit (STEMCELL Technologies) and characterized using neuronal and cardiomyocyte markers (DCX/MAP and α-actinin/tropomyosin, respectively) (Figure 1). The presence of the inherited GAA repeat expansion in the *FXN* gene and repressed frataxin expression was confirmed in these cells. Induced neurons demonstrated

Figure 1

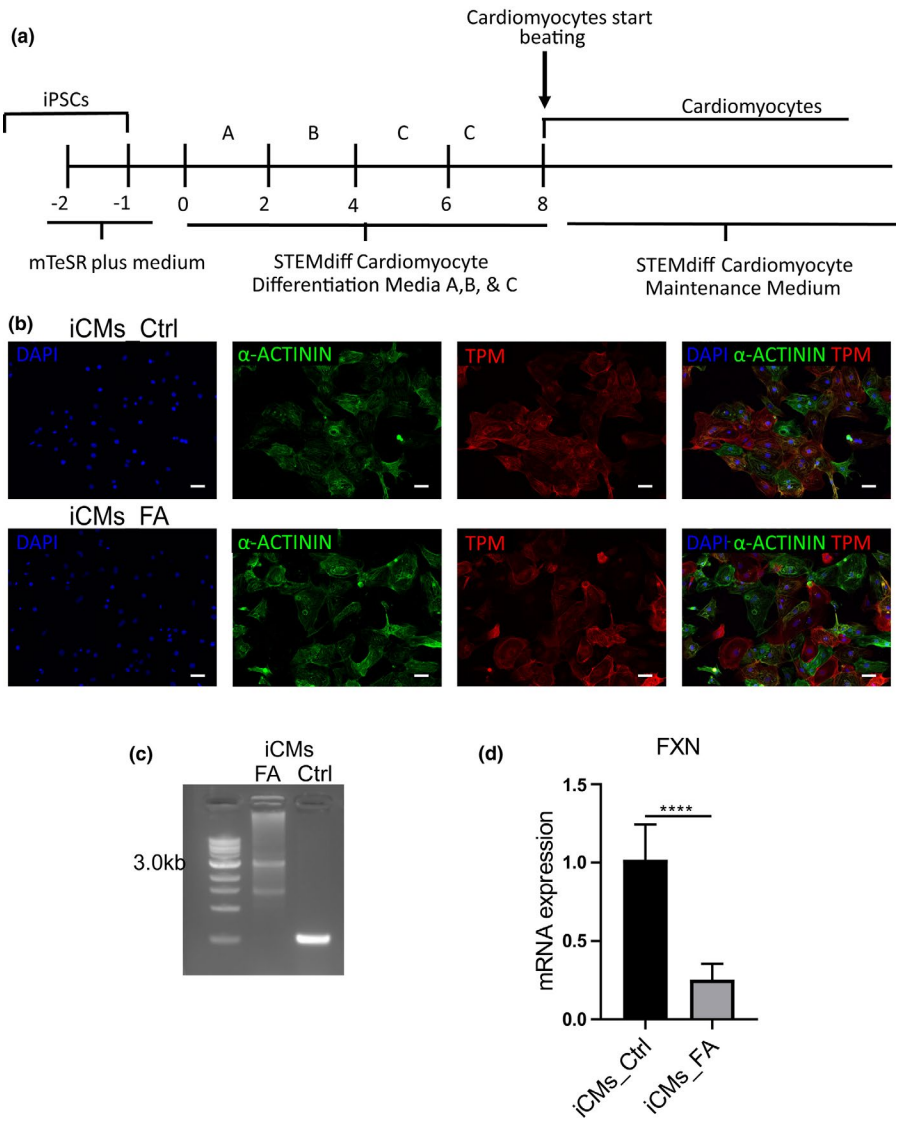

Fig. 1: *iCMs characterization. (a) Protocol diagram for cardiomyocyte differentiation, using mTeSR™ Plus Medium, STEMdiff™ Cardiomyocyte Differentiation Media A, B, & C, and STEMdiff™ Cardiomyocyte Maintenance Medium. (b) Immunostaining of control and patient iCMs showing expression markers α-actinin (green) and tropomyosin (TPM) (red). Nuclei were stained with DAPI (blue). Scale bar 50 µm. (c) PCR analysis of the GAA repeat region in the FXN gene using genomic DNA extracted from iCMs from control and patient. (d) qPCR quantification of FXN mRNA levels in iCMs from control and FRDA patient. Data are expressed as means ± SD of three independent experiments. *p < 0.05. *FA means FRDA. Molec Gen & Gen Med, Volume: 11, Issue: 1, First published: 11 November 2022, DOI: (10.1002/mgg3.2093)*

typical neuronal morphology, and induced cardiomyocytes exhibited spontaneous contractions.

To identify altered pathways in disease- and patient-derived induced neurons and cardiomyocytes, RNA-seq experiments were carried out on these cells and those with re-expression of frataxin using lentiviral vectors. The glycolysis and gluconeogenesis KEGG pathway was identified as the most significantly differentially expressed pathway in induced neurons, while pathways related to the extracellular matrix were the most significantly in induced cardiomyocytes. The phenotype ontology associations of these pathways and the differentially regulated genes within were sensory dysfunction, muscle weakness, peripheral neuropathy, and cardiac fibrosis, which are features of FRDA.

Comparison of FRDA patient-derived iPSC-induced neurons and induced cardiomyocytes to healthy control iPSC-derived equivalents showed alterations in glycolysis and Hif1 signaling pathways in induced neurons, which were also enriched in the previously mentioned frataxin reexpression experiments. Alterations in glycolysis and gluconeogenesis in induced cardiomyocytes were also confirmed in this comparison with control neurons. Specific sets of relevant glycolysis/gluconeogenesis and extracellular matrix-related genes that were differentially regulated were identified in induced neurons, including the energy metabolism-related genes *ENO1*, *PFKP*, *ALDOA*, *LDHA*, and *PKM* in induced neurons and *GDF15*, *HSPG2*, and *HMOX1* in induced cardiomyocytes. These factors are likely to play a role in meeting the high energy demands of neurons, subsequently affecting neuropathic symptoms, and extracellular matrix regulation deficiencies leading to cardiac fibrosis in FRDA. *GDF15* was identified as a potential serum biomarker for FRDA-associated heart disease.

The ability to study FRDA in a disease-relevant model incorporating the causal genetic alteration and associated genetic background of the disease resulted in a clean study that produced a focused set of differentially regulated genes and pathways that were reproducible across multiple experimental approaches. The use of iPSCs and reliable differentiation methods allowed the identification of potential drug targets and biomarkers in a highly translatable manner, highlighting that these methods are useful for the generation of clinically meaningful results.

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Delineation of key molecular pathways of neurodegenerative disease

Viral mediated knockdown of GATA6 in SMA iPSC-derived astrocytes prevents motor neuron loss and microglial activation[66]

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The genetic disorder spinal muscular atrophy (SMA) causes muscle atrophy due to spinal cord neuron loss and death due to respiratory distress in pediatric patients. SMA is a prevalent cause of infant mortality. SMA is caused by homozygous loss of function or mutation in the survival motor neuron 1 (*SMN1*) gene, although restoring SMN1 function to motor neurons does not significantly improve the pathogenesis of the disease. While astrocytes, which are glial cells that regulate central nervous system inflammation and homeostasis, have been implicated in SMA pathology, and GATA binding protein 6 (GATA6) has been shown to be upregulated in SMA astrocytes, the underlying mechanisms of disease pathophysiology are not completely elucidated.

Conventional modeling of SMA has been done in mice.[67] The genetic modification of SMN in these models results in embryonic lethality or relies on inducible mutant SMN expression postnatally.[68] These caveats render such models imperfect in the recapitulation of developmental and pathophysiological aspects of SMA in humans. In this study, the authors employed spinal cord astrocytes that were differentiated from iPSCs from SMA patients and healthy controls to identify a mechanism by which GATA6 upregulation may affect astrocyte phenotypes and drive SMA pathology. The iPSCs were differentiated into various cell types that interact and are relevant to SMA including astrocytes, microglia, and motor neurons. Differentiation was accomplished using multiple methods including 1) sequential incubation with a host of factors and 2) using commercial differentiation kits (STEMdiff™ Hematopoietic Kit and STEMdiff™ Microglia Differentiation Kit (STEMCELL Technologies)). The authors differentiated neural progenitors specifically into spinal cord-like astrocytes since there are slight differences among astrocyte subtypes. Differentiated cell lines were characterized by evaluation of the expression of markers that are specific for each cell type.

GATA6 and nuclear factor kappa B (NFκB) transcript and protein levels were confirmed to be upregulated in iPSC-derived SMA astrocyte cultures, which is consistent with previous findings in patients and SMA astrocytes. In addition, inflammatory cytokines and complement cascade factors were found to be transcriptionally upregulated in SMA astrocytes. Increased GATA6 expression in SMA astrocytes was found to be associated with increased NFκB expression, increased activation of SMA microglia, decreased neurotrophic support, and increased SMA motor neuron loss, and these effects were reversed upon knockdown of GATA6. Conversely, exogenous expression of GATA6 in induced astrocytes from healthy control patients resulted in the activation of microglia and motor neuron damage. The effects of astrocytes on other cell types were determined by incubating microglia or motor neurons with astrocyte-conditioned media.

Figure 2

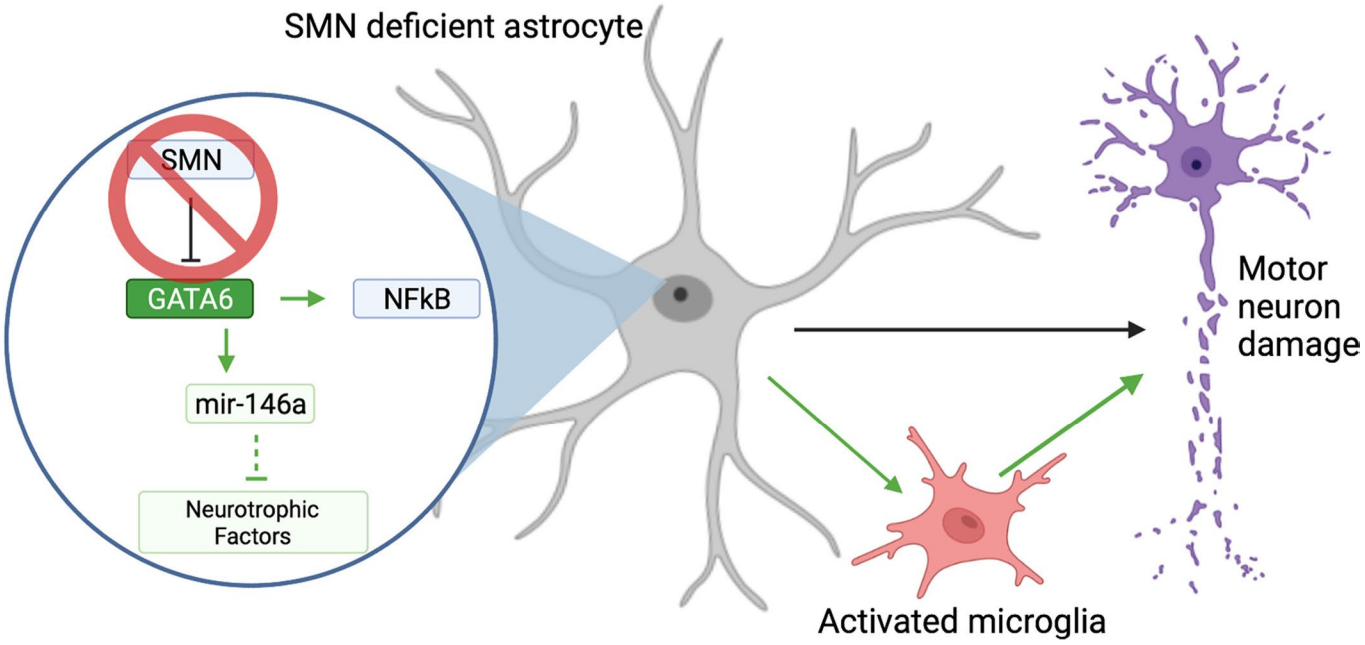

Fig. 2: *Schematic of proposed role of GATA6 in relation to NFκB-mediated astrocyte neurotoxicity. In SMA astrocytes, the loss of SMN allows for GATA6 overexpression through the loss of a negative regulator. GATA6 overexpression may contribute to NFκB overexpression leading to an increase in pro-inflammatory cytokine production. These secreted factors lead to motor neuron damage directly, as well as serve to activate microglia to drive additional neurotoxicity. Black lines represent accepted mechanisms in published literature; green lines represent relationships proposed by this manuscript. Created using biorender.com. Glia, vol. 70, no. 5, p. 989, May 2022, doi: 10.1002/glia.24153.*

While these experiments implicate a GATA6/NFκB/inflammatory cytokine axis in astrocytes as a key player in SMA pathogenesis (Figure 2), the study stopped short of mechanistically connecting the effect on microglia to motor neuron loss. This may be accomplished using mixed cultures of SMA microglia and SMA motor neurons. Alternatively, this could have been investigated by treating SMA motor neurons with SMA microglia-conditioned media. Nonetheless, the ability to generate the multiple cell types involved in SMA pathophysiology using patient-derived iPSCs facilitated the complex but elegant modeling of the disease in a representative manner and clarification of key factors and mechanisms of SMA pathology. More broadly, this study demonstrates the utility of iPSCs for recapitulating a disease process in complex tissues to delineate pathological mechanisms that are specific to cell types within those tissues. It also demonstrates the power of models using parallel lineages that are patient-derived and representative of the genetics behind disease in an individual.

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Testing multi-cell type disease mechanisms and drug response

Metabolic rescue ameliorates mitochondrial encephalo-cardiomyopathy in murine and human iPSC models of Leigh syndrome[69]

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The study of genetically complex disease affecting multiple systems is exemplified by Yoon et al., who utilized multiple complementary approaches, including human iPSCs and murine models, to reveal mechanisms of cardiac dysfunction and apoptosis in neurons in Leigh syndrome (LS). LS results in psychomotor regression in early life and cardiac involvement (cardiomyopathy, pericardial effusion, and abnormal conduction) in some patients, which is associated with poor prognosis. LS is commonly caused by mutations in NADH dehydrogenase complex I, which consists of several subunits. There are a variety of causal mutations in different subunits leading to a spectrum of LS variants. One such mutated subunit is NADH dehydrogenase [ubiquinone] iron–sulfur protein 4 (Ndufs4). Treatment options are limited for mitochondrial disorders, including LS. Here, iPSC-derived cardiomyocyte and neuron models are applied to identify mechanisms responsible for the cardiac manifestations of LS that may be translatable to better treatment.

This study incorporated human iPSC-derived models of multiple tissues that are involved in LS pathophysiology. Healthy human iPSCs from a validated and characterized collection were obtained for this study and modified using CRISPR/Cas9 to introduce homozygous *NDUFS4* deletion. Cardiomyocytes were differentiated with STEMdiff™ Cardiomyocyte Differentiation and Maintenance Kits (STEMCELL Technologies), and neural progenitors and embryoid bodies were differentiated using STEMdiff™ SMADi Neural Induction Kit (STEMCELL Technologies). Induction of neural progenitors was determined by the formation of neural rosettes. Neuron differentiation and maturation was accomplished using the STEMdiff™ Neuron Differentiation Kit and STEMdiff™ Neuron Maturation Kit (STEMCELL Technologies).

The authors first demonstrated that LS mice with Ndufs4 deficiency exhibited the LS phenotypes of metabolic derangement, runting, cardiomyopathy and bradyarrhythmia (promoted by $Na_V1.5$ sodium channel hyperacetylation). Acetylation of $Na_V1.5$ decreased inward polarizing Na^+ current in HEK293 cells. The manifestation of these phenotypes in human iPSC-derived cardiomyocytes and neurons with *NDUFS4* deletion were then examined. Decreased $Na_V1.5$ current was observed in Ndufs4-deficient induced cardiomyocytes, and increased p53 acetylation and apoptosis were observed in neural rosettes formed from induced neurons with Ndufs4 deletion. Metabolomics suggested impairment of NAD^+-dependent dehydrogenases and reduced glutathione as associated phenotypes

Figure 3

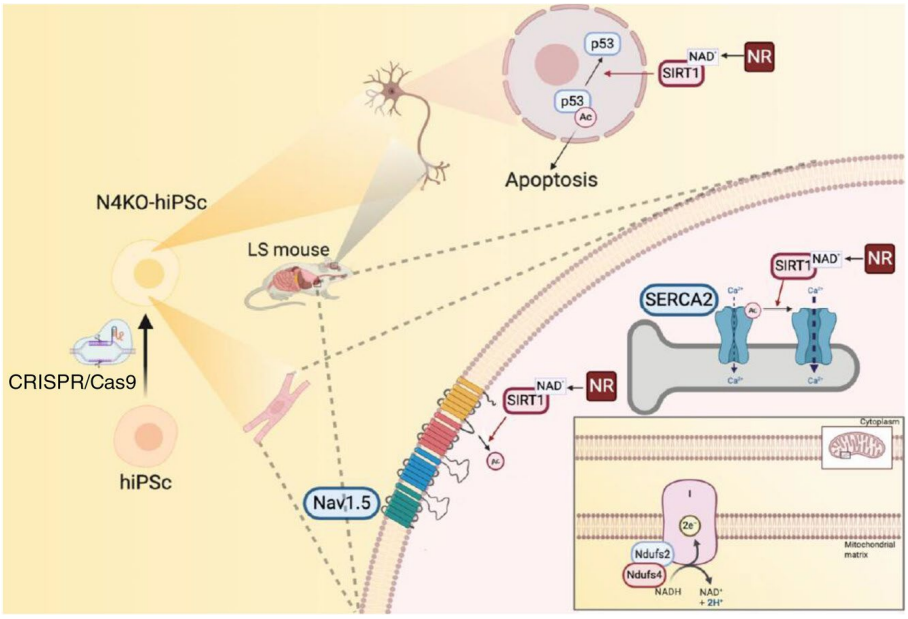

Fig. 3: *Schematic illustration of the molecular mechanism of cardio-encephalomyopathy in Ndufs4 KO mice and hiPSCs (created by Biorender). Clinical & Translational Med, Volume: 12, Issue: 7, First published: 25 July 2022, DOI: (10.1002/ctm2.954)*

in these cells. Metabolic rescue using nicotinamide riboside (NR) was found to mitigate neuronal apoptosis and current abnormalities in Ndufs4-deficient iPSC-derived neurons and cardiomyocytes, respectively. Likewise, NR treatment corrected impaired motor function, microgliosis, and neuronal apoptosis in Ndufs4-deficient LS mice. Together, these results created a mechanistic illustration of multi-cell type effects of Ndufs4 deficiency and drug-induced reversal of these effects in models that lend human relevance (Figure 3).

The addition of iPSC-derived cardiomyocyte and neuron modeling to this study added translational relevance to the authors' findings in murine and heterologous cellular models of LS. Additionally, this study supports the idea that the use of iPSC modeling of LS has the potential to drive personalized medicine and test patient-specific drug responses. It is also reasonable to imagine building on this study with improved disease-relevance and representation of the genetic heterogeneity among LS cases using iPSCs derived from LS patients rather than healthy controls.

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Next steps

The ability to indefinitely expand iPSCs and differentiate them into many tissue and organ types is offering the opportunity for scalable and disease-representative drug screening platforms. An area of intense interest in research is the evaluation of how predictive these models are for drug response and toxicity compared to animal models. The feasibility of using human iPSC-derived cardiomyocytes and neurons for high-throughput population-based screening of drug cytotoxicity was recently demonstrated by Huang et al. Using several donors that represent high-frequency HLA haplotypes, cardio- and neuro-toxic compounds were identified in a 1536-well format and validated as having dose-dependent functional effects on iPSC-derived cardiomyocytes and neurons.[31] Results from validated compounds were reproducible in mouse models.

There is a need for better understanding of the value of iPSC-based versus animal-based model systems in predicting drug responses in human trials. Further standardization and optimization of validated iPSC sources, media, reagents, and protocols, as discussed above, continue to improve the prediction of drug response and toxicity in humans, ushering in a new paradigm for drug screening and preclinical testing. Deep phenotyping of panels of iPSCs and iPSC-derived cells using genomics, transcriptomics, proteomics, and microvesicle characterization can stratify patients according to expected drug response to facilitate the investigation of therapies with the greatest chances of success.[70]

Advancements in iPSC technology are leading to new capabilities for personalized medicine, organ, and multi-organ modeling, preclinical testing, and drug screening platforms. Even personalization of preclinical toxicity and drug response testing is becoming possible using iPSC-derived technologies. Organoids, organs-on-a-chip (OoC), and multi-organs-on-a-chip (MOC) can be complementary to other models and may represent ethical and informative alternatives to animal modeling as a gold standard for screening and preclinical testing. While organoids have structural organization, OoC technology has the potential to incorporate a more diverse system in terms of the cell and tissue types involved. Microfluidics in OoC platforms simulate circulation and allow for compartmentalization of various tissues, cells, and organoids.[14] OoC can therefore model interactions between tissues so that effects on tissues that are distal from the site of drug exposure can be studied.[14] MOC systems connect multiple OoCs or organoids to allow for the study of interactions between organs, such as those between glucose regulation in liver tissues and insulin regulation in the pancreas[71], and those between gut microbiome, brain, and liver OoCs in Parkinson's disease.[72] MOC systems have also been used to study the adverse effects and toxicity of drugs in the context of interaction between iPSC-derived liver and cardiac cells.[73] The use of iPSC-derived cells in these models allows for the study of known disease-modifying factors, incorporation of identical genetic backgrounds in all tissues, and recreation of known clinical manifestations, particularly in familial disease.[72] Standardization and improved accessibility of patient-derived three-dimensional models has the potential to revolutionize research and drug development.

Cardiomyopathies and central nervous system diseases have garnered the greatest impact from the use of iPSC-derived models thus far. However, the creation of cell lines and models for other diseases and new disease areas that currently lack sufficient representative research tools is also being made possible by iPSC technology. One attractive potential application for iPSCs is modeling of familial or syndromic cancers.[7] For example, an iPSC-derived model of familial breast cancer was recently used to demonstrate the effect of *BRCA1* haploinsufficiency on the tumor niche, including on angiogenesis.[74] Additionally, limits to available cellular models of liver disease are now being overcome using iPSC-derived hepatocytes and organoids.[75] These new technologies are facilitating disease modeling, gene editing studies, and drug testing for these diseases.

Additional considerations

The availability of iPSC lines to the broader research community is dependent on the ability to scale up production of cultures, which continues to be improved upon with technologies such as bioreactors.[76] Enhanced feeding methods and feeder-free culture are promoting the use of automated and bioreactor technology to expand iPSCs with reduced complexity of culture methods.[76–78]

A challenge in the use of patient-derived cell lines to model disease will be to ensure representation of the wider population and the inclusion of diverse demographic groups. Global studies that attempt to cover common HLA haplotypes, such as that conducted by Huang et al. using iPSC-derived cardiomyocytes and neurons[31], are a step in that direction. As with any model, patient-derived models require healthy controls that appropriately represent genetic backgrounds. In the application of iPSCs to personalized medicine, family and gender-matched controls are preferred.[7] Nonetheless, individual familial uniqueness in genetic and epigenetic background can confound results with such small sample sizes. To alleviate this issue, larger cohorts of healthy donor-derived iPSCs are becoming more available commercially and are more easily searchable using databanks. These cohorts are representative of diverse genetic backgrounds, reducing the potentially confounding impact of unique familial genetics.

The responsible use of iPSCs will require ethical reporting of results, appropriate donor consent, and consideration of potential legal factors affecting patient-derived or proprietary materials. Appropriate sources of patient-derived materials must acquire consent for research and commercial use as applicable. However, with novel potential uses of iPSCs, the ethical use of these materials may require updated regulation and guidelines that fully consider the possible uses of patient-derived material, such as cell-based therapies, germ cell or embryo development, and gene editing. Retraction of banked materials by a patient is typically given as an option on consent forms. However, dissemination and broad expansion of iPSC-derived materials creates a challenge in accounting for this material. A balance between the provision of relevant health information and the protection of confidential patient information must be struck in a way that ensures patient privacy, but is conducive to research. Perhaps the biggest challenge in protecting patient identity is the inevitable inclusion of genetic information that could be used to identify patients or their relatives. Some of this risk is being mitigated with proper consent and oversight. Lastly, avoidance of infringement on intellectual property rights is important and can apply to methods of generating iPSCs and the materials themselves. These ethical and legal risks may be avoided by acquiring already established and validated iPSC lines.

Conclusion

In summary, as a result of the efforts by regulatory agencies, banking and standardization initiatives, and companies focused on the advancement and democratization of iPSC culture, there has never been a better time to introduce this powerful technology to research and drug development laboratories. These advancements have provided standard and reliable iPSC lines and laboratory resources to simplify their culture and differentiation. There is an increasing number of examples of successes using iPSCs to advance scientific research in conjunction with other types of models and to open new areas of inquiry. Advances in iPSC culture have placed them among the most physiologically relevant model systems available and made them a potential new gold standard for research and drug development.

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Further reading and resources

EBiSC best practice: How to ensure optimal generation, qualification, and distribution of iPSC lines, Steeg et al. Stem Cell Reports, Volume 16, Issue 8, 2021, Pages 1853-1867, ISSN 2213-6711,
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ICSCB-II Integrated Collection of Stem Cell Bank data by MIACARM stem cell line search
https://icscb.stemcellinformatics.org

Human Pluripotent Stem Cell Registry
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