Stem Cell Biology and Regenerative Medicine

Mayana Zatz
Keith Okamoto *Editors*

# Stem Cells in Modeling Human Genetic Diseases

Humana Press

# Stem Cell Biology and Regenerative Medicine

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Editors

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# Preface

The cloning of the sheep Dolly in 1996, demonstrating for the first time that it was possible to reprogram a differentiated mammalian cell to a pluripotent state, opened a new and very promising field of research in regenerative medicine. Although the technology of nuclear transfer from a somatic cell to an enucleated egg was banned for human reproductive cloning, it looked very promising for therapeutic cloning and research purposes. However, in practice, it was shown that this technology was extremely challenging and the results were very disappointing and frustrating. The groundbreaking discovery of induced pluripotent stem cells (iPSCs) in mice by Dr. Yamanaka's group in 2006 came as a surprising alternative to therapeutic cloning approach for reprogramming differentiated cells to an embryonic stem cell (ESC)-like state. The report of the first human iPSCs just a year later was received with high enthusiasm by the scientific community, since most technical and ethical issues involving access to human ESCs could then be circumvented, promoting therapeutic applications. Since then, iPSC research became a fast-growing field that quickly dominated most of the scientific publication in stem cell biology. Interestingly, progress in iPSC research has been pushed by scientists interested in the mechanisms of pluripotency (re)programming, maintenance of the pluripotent state, differentiation to defined cell types, and consequences of genetic/epigenetic abnormalities to cell ontogeny and function. While applications of iPSCs in cell therapy are envisioned but still in a premature stage of development, the use of iPSCs as tools to study human genetic diseases boomed in the last few years. With the contribution of experts in the field, this book provides to readers a glimpse of this effervescent scenario, emphasizing the concept of "patients in a petri dish" model. Chapters 1 and 2 provide excellent examples of how iPSCs have been used to model inherited disorders affecting brain and heart function, as well as advantages and limitations compared to other experimental models. Chapters 3 and 4 present the perspectives that iPSCs bring to better understand and treat severe forms of neurodegenerative disorders for which there are no effective therapy available. Applications of iPSC technologies to address common diseases that are

leading cause of mortality and morbidity worldwide are covered in Chaps. 5 and 6. Finally, Chaps. 7 and 8 discuss how stem cells in general, not only iPSCs, have been instrumental in the study of common neurodevelopmental disorders as well as complex multifactorial diseases such as cancer. Readers will find in the forthcoming text enlightening issues of this rapidly developing field of research within the broader context of regenerative medicine.

São Paulo, Brazil

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# Chapter 1
# Modeling Fragile X Syndrome in Human Pluripotent Cells

**Tomer Halevy and Nissim Benvenisty**

## Background

Fragile X Syndrome (FXS) was first identified as a distinct form of inherited intellectual disability in 1969 by Lubs, who described the physical constriction on the long arm of the X chromosome in lymphocytes isolated from FXS patients [1, 2]. This constriction was referred to as a fragile site, giving the syndrome its name. Apart from the cytogenic phenotype, FXS is recognized by mild to severe intellectual disability, with some patients displaying autistic behavior. This neurological symptom is accompanied by developmental delay, susceptibility to seizures, and dense and immature dendritic spines in affected neurons. Other, non-neurological symptoms include macroorchidism and distinct facial features such as a long narrow face with prominent ears, joint laxity and flat feet [3].

## Genetic Variance Underling the Syndrome

It is now known that FXS is the leading cause of inherited intellectual disability in males and is the major monogenic cause of autism, affecting approximately one in every 4,000 boys and one in 8,000 girls worldwide [4–7]. In 1991, the syndrome was found to be caused by a single gene on the X chromosome, exhibiting length variation in patients [8]. The gene was named Fragile X mental retardation

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gene 1 (*FMR1*) [8] and its position correlated precisely to the location in which the physical constriction was first found [9]. The gene's variation in length was found to stems from a CGG repeat located at the 5′ untranslated region (UTR) of *FMR1*. This CGG expansion at the 5′ UTR of the gene leads to CpG methylation of the region, which spreads to the *FMR1* promoter [10]. This methylation is accompanied by hypoacetylation of histones and heterochromatization. These epigenetic changes result in the silencing of *FMR1* and therefore the Fragile X mental retardation protein (FMRP) is not produced [11, 12].

In a healthy population, CGG repeats vary from 6 to 54 repeats [13]. If the number of repeats is between 55 and 200, the individual is referred to as a carrier with a permutation allele [13]. Premutation alleles do not cause FXS but they do cause other distinct disorders and are prone to major expansion of the repeats, especially during the female meiosis [13]. When the CGG repeat length exceeds 200, the allele is considered as having a full mutation and results in FXS [13]. Due to the location of the affected gene, FXS has almost complete penetrance in males while only 50 % of females show FXS symptoms. In some cases, deletions or missense mutations in the *FMR1* gene can also cause FXS. In other rare cases, males with a full mutation allele show no symptoms of the syndrome, implying that the epigenetic changes at the expansion region are the cause of the disease and not the CGG repeats themselves [14, 15].

## FMRP Function

FMRP is a highly conserved protein found in animals such as fruit flies, mice, and humans [1, 8]. It is expressed in most mammalian tissues but is most abundant in the brain and testes [16]. Most of its functions are known from studies of brain tissues, as the neural phenotype has the greatest effect on the patient's quality of life. In the brain, FMRP is found primarily in neurons [16], where it plays an important role in synaptic plasticity. It is an RNA-binding protein that can selectively bind as much as 4 % of the mRNA in the mammalian brain [1, 17]. As FMRP is also present in the nucleus and can move to the cytoplasm, it may act as a cargo protein [18], shuttling to the periphery many of its targets mRNAs, known to localize at dendrites [19]. Much work has been done on the interaction of FMRP with its target transcripts and it is now known that FMRP regulates translation by acting as a translational repressor. It does so by associating with polyribosomes [20–22] and inhibiting translation initiation [23], or by stalling ribosomes during translation elongation [22]. Several works have also shown that FMRP interacts with the microRNA (miRNA) pathway as both the *Drosophila melanogaster* ortholog (*dFMR1*) and the mammalian FMRP associate with components of the RNA-induced silencing complex (RISC) and several miRNAs [1, 6, 24–27].

## Animal Models

Since *FMR1* is highly conserved across species, much work has been done on animal models (Fig. 1.1a). Null mutations of *dFmr1* produce flies with abnormal neuronal architecture and synaptic function [28–30], impairment of long-term memory [31], and reduced courtship interest [32]. The advantage of the fruit fly model is the ability to manipulate gene expression in different tissues using a variety of techniques, and the robust ability to study changes in behavior.

The mouse ortholog of *FMR1*, *Fmr1*, is also located on the X chromosome and shares 97 % homology in the amino acid sequence with the human gene [33]. KO mice exhibit many of the phenotypes seen in FXS patients. Like in affected humans, dendrites of KO mice are denser and have immature spines [34]. Other FXS characteristics such as learning and memory disability, increased susceptibility to seizures, and macroorchidism are also seen in KO mice [35].

These animal models are critical for our understanding of the various functions of *FMR1*, its importance in different tissues and cell types, and the conserved pathways underlying FXS in which it plays a part. However, there is no animal model

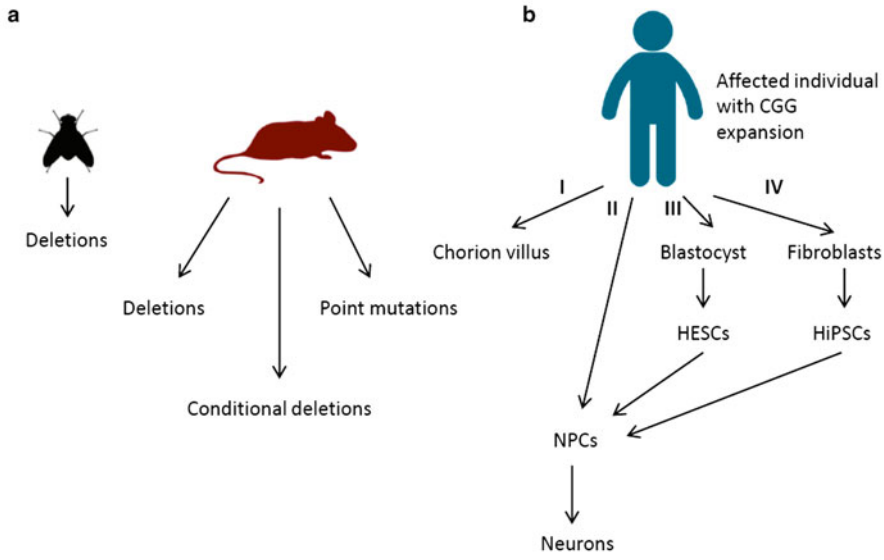


**Fig. 1.1** Modeling fragile X syndrome in animals and humans. (**a**) To model the syndrome in animals, null mutations are created either by deletions or point mutations in flies or mice. (**b**) Different tissues from affected individuals are used to study the syndrome in humans. (*I*) Chorion villus samples are taken from the affected fetus. (*II*) Neural progenitor cells (NPCs) are taken from postmortem individuals. (*III*) Human embryonic stem cells (HESCs) are created from affected blastocysts. (*IV*) Human-induced pluripotent stem cells (HiPSCs) are generated by reprogramming of somatic cells (fibroblasts). The pluripotent cells can be differentiated into NPCs and then further differentiated into neurons

for the study of the CGG repeat expansion, nor the epigenetic silencing which causes the syndrome in humans. Even genetically engineered mice, with an extended number of CGG repeats, do not undergo methylation of the promoter and thus the gene remains active [36]. Understanding the mechanism and the timing of *FMR1* silencing during development is a key step in the search for treatment. Moreover, as it appears that *FMR1* plays a crucial role in differentiation and maturation of neurons [7, 37], it is of great importance to study these processes in humans as to better understand the role of FMRP in brain development.

## Modeling FXS in Human Embryonic Stem Cells

A few studies on FXS were performed on human tissue samples, including chorion villus samples and tissues of affected fetuses [10, 38] (Fig. 1.1b). However, these models give only a snapshot of the syndrome status. To study the neural phenotype, neural progenitor cells (NPCs) were derived from a fetal FXS human brain [37] (Fig. 1.1b). These NPCs were used to generate neurons, which displayed fewer and shorter neuritis with smaller cell body and volume. Studies such as these are extremely valuable in terms of data yet access to human tissues is restricted and hard to come by and so comparison between different affected individuals is also scarce. Therefore, in order to better understand the different aspects of the disease such as CGG expansion, timing of *FMR1* silencing, and the function of *FMR1* in different tissues and cell types, all of which occur at different points in time during development and differentiation, a new model was needed. In 2007, human embryonic stem cells (ESCs) from FXS affected embryos (FXS-ESCs) were derived [39] and enabled the study of all of the above issues (Figs. 1.1b and 1.2). In one study [39], FXS-derived ES cell line was established from a blastocyst obtained from a preimplantation genetic diagnosis (PGD). The embryo was a male who inherited a full mutation allele from a carrier mother. Interestingly, although they carried the full mutation, FXS-ESCs showed both *FMR1* mRNA expression and the presence of FMRP. This finding showed that the transcriptional silencing of *FMR1* is a developmentally regulated process. To further understand the mechanism controlling the silencing of *FMR1*, FXS-ESCs were differentiated by injecting them into immunodeficient mice. The injected cells produced teratomas which were dissociated and analyzed. Analysis of *FMR1* mRNA levels in the teratomas showed a clear reduction in transcript levels, indicating that *FMR1* inactivation is indeed dependent upon differentiation. Methylation status and histone modifications were also analyzed and showed that while methylation of the *FMR1* promoter does increase early in the differentiation process, it does so in a very modest way. In contrast, histone modifications such as H3-tail acetylation and H3K9 methylation, which correlate with positive and negative regulation of transcription, respectively, show marked differences upon differentiation. H3-tail acetylation falls dramatically upon FXS-ESCs differentiation, whereas H3K9 methylation increases. The importance of this study, apart from the generation of a new model system, is a clear indication that *FMR1* is

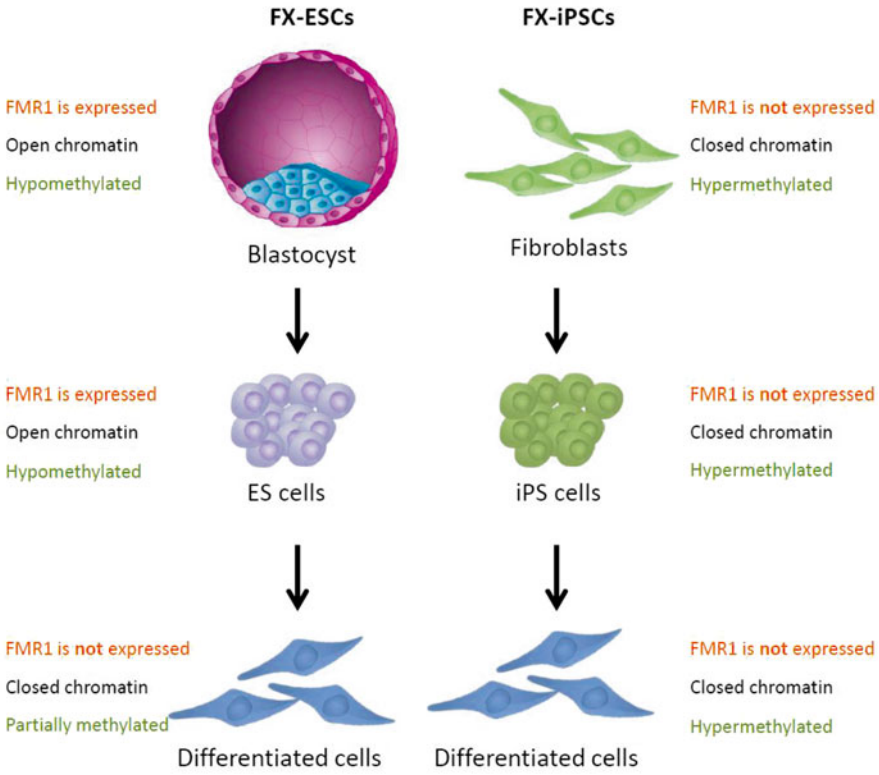


**Fig. 1.2** Comparison of embryonic and induced pluripotent stem cell-derived models for fragile X syndrome

silenced in FXS embryos only during development and that the inactivation is initiated by chromatin modifications prior to DNA methylation.

In further studies, additional FXS-ESCs lines were derived from spare IVF-derived embryos diagnosed by PGD [40, 41]. In one of them, the association between early neural differentiation and the silencing of *FMR1* was examined, by producing active neurons from FXS-ESCs which were able to create neuronal networks [41]. This study showed that differentiation into neurospheres induced a steady increase in *FMR1* expression in control lines while neural differentiation of the FXS-ESCs lines did not. This difference was accompanied by aberrant expression of several early neural genes in FXS-ESCs-derived neurons, while other neural genes and pluripotency genes behaved in a normal manner. This observation further asserts that *FMR1* plays an important role in early stages of neurogenesis. Further analysis indicated that FXS-ESCs neurite formation and elongation did not show significant differences from control lines. FXS-ESCs were further differentiated to produce mature neurons, which lacked FMRP, displaying full silencing of *FMR1* during the 50 days of the differentiation process. Neurogenic potential of the

FXS-ESCs was lower compared to control and had a bias towards glial lineage. This may be linked to the aberrant neural gene expression seen at the early stages of the differentiation process. FXS-ESCs derived neurons were also analyzed for functional qualities. Functional analysis demonstrated that while FXS-ESCs can differentiate into viable neurons with passive properties similar to control cells, they hardly developed functional synaptic connections.

Although human stem cells derived from embryos hold great potential as a tool for understanding the basic mechanism of the disease, there are limitations to this model. First, in many countries, the generation of human stem cells from discarded IVF embryos presents an ethical challenge. Second, FXS is represented by profound variability in patients, ranging from the varying length of the repeats, through the methylation levels, and to the neurological phenotype itself. The degree of intellectual impairment also varies between different individuals, as only about 30 % of full mutation carriers display autistic behavior [42, 43]. Additionally, some carriers of the full mutation allele do not display any of the syndrome's phenotypes [14, 15]. As this variability is not inherited from the parents and is detected only after PGD analysis, the probability of acquiring numerous human embryonic stem cells displaying the entire spectrum of genetic and epigenetic differences is quite small and may span over many years.

## Modeling FXS in Human-Induced Pluripotent Stem Cells

Recent advances in induced pluripotent stem cells (iPSCs) technology may resolve all of the aforementioned obstacles. Nowadays, creating iPSCs from somatic cells of any given individual is easy and reproducible. Recently, iPSCs from FXS patients (FXS-iPSCs) have been derived [44–46] (Fig. 1.2). Although iPSCs resemble human embryonic stem cells in most aspects, FXS-iPSCs differ in one critical manner. Despite successful reprogramming, it seems that the *FMR1* gene is resistant to the process and remains silent, maintaining both methylation status and repressive histone modifications [44] (Fig. 1.2). In this study [44], 11 FXS-iPSCs clones were derived from two different tissues of four affected male patients. These clones were then compared to multiple cell types including FXS-ESCs and wild-type iPSCs. The *FMR1* gene was found to be silent in all of FXS-iPSCs in contrast to all control cells. Comparison of DNA methylation revealed that the FXS-iPSCs retained the high methylation levels seen in their fibroblasts of origin. Further analysis of histone modifications in both FXS-iPSCs and FXS-ESCs and wild type cells revealed that the FXS-iPSCs are enriched for repressive chromatin markers in contrast to the control cells which are enriched for transcriptionally active chromatin markers. Thus, while the FXS model in human ESCs demonstrated the temporal silencing of *FMR1*, in FXS-iPSCs, *FMR1* was already inactive in the undifferentiated state (Fig. 1.2). FXS-iPSCs are still an important model as they are very useful for the analysis of derived neurons lacking the expression of *FMR1*. Another issue that must be addressed when creating iPSCs from individuals carrying the full mutation

allele is the stability or instability of the CGG repeats. It was observed that during reprogramming, or during the cells expansion following the reprogramming process, the CGG repeats can become unstable and either decrease or increase in length compared to the fibroblasts of origin. This change in repeat length may also lead to changes in the methylation status of the promoter and reactivation of the gene [45]. In the same study [45], two iPSCs subclones derived from the same FXS mosaic donor, one with a permutation allele and the other with a full mutation allele, were tested for their ability to differentiate into neurons. Although both subclones differentiated into neurons, the clone harboring the full mutation allele produced much shorter and fewer dendrites, despite the common genetic background.

## Drug Screening in FXS-iPS Cells

Although FXS-iPSCs may not always be a suitable model to study the silencing mechanism of the syndrome, they constitute a unique model system for the study of the disease phenotype in diverse subpopulations of FXS-affected individuals, and for drug screening. A study aimed to evaluate the reactivation of *FMR1* in FXS-iPSCs and their neuronal derivatives through epigenetic modulation drugs showed not only that reactivation is possible but also uncovered additional layers of epigenetic control on *FMR1* [47]. The drug of choice in this study was a demethylating agent called 5-azacytidine (5-azaC), an FDA-approved drug previously shown to reactivate *FMR1* in immortalized FXS lymphoblastoid cell lines [11]. The 5-azaC concentrations used in the above study [47] were in accordance with physiological levels found in plasma of cancer patients treated with the drug. As a result of 5-azaC treatment on FXS-iPSCs, *FMR1* was robustly reactivated with levels of expression ranging between 15 and 45 % of control cells. The ability of 5-azaC to restore *FMR1* expression was further tested on neurons derived from FXS-iPSCs. Like the results observed in FXS-iPSCs, their neuronal derivatives also reactivated *FMR1*. In both the FXS-iPSCs and the derived neurons, reactivation occurred at the translational level, as detected by the presence of FMRP. Expression of the gene persisted at similar levels even after drug withdrawal. In addition to re-expression, epigenetic modifications such as DNA methylation, histone methylation, and acetylation of the *FMR1* locus were also examined after 5-azaC treatment. While DNA methylation of the promoter in untreated cells was very high, the treated iPSCs and neurons showed a marked reduction in DNA methylation. Histone modifications indicative of transcriptionally active genes revealed almost complete restoration of both modifications analyzed compared to the control iPSCs, which express *FMR1*. However, the histone modification indicative of repressed chromatin was not affected by 5-azaC. This observation may explain why expression levels of *FMR1* remained lower than those of the control cells and suggests the existence of additional layers of epigenetic control on the *FMR1* gene. While 5-azaC has a global effect on methylation and is not an ideal drug when it comes to treating infants or children, this study showed the potential in using iPSCs to produce relevant cell

types for drug screening in order to not only reactivate *FMR1* but also broaden our understanding of the epigenetic regulation controlling and underlying the disease.

Due to their availability, iPSCs can now be used to study associated syndromes for which human ESCs are not available, such as Fragile X-associated "Tremor/Ataxia Syndrome" (FXTAS). FXTAS is a neurodegenerative disorder affecting carriers of the permutation allele. Production of iPSCs from affected FXTAS individuals revealed that the permutation allele is enough to impair synaptic density, neurite length, and the overall function of iPSCs-derived affected neurons [48].

## Concluding Remarks

Because of the variability in FXS patients, the potential embodied in human ESCs and FXS-iPSCs is vast. Future experiments will try to address open questions concerning FXS such as the basic molecular mechanisms underlying the syndrome and the cause of the CGG expansion during the female meiosis. The main advantage of using iPSCs to study FXS lies in the availability and the ease of generating pluripotent cells and their derivatives from a wide range of affected individuals and carriers. FXS-iPSCs and their derivatives will enable us to broaden our search for new drugs; these will either reactivate *FMR1* itself or its key downstream targets which display aberrant activity in affected cells. In using FXS-iPSCs, there are neither ethical concerns nor obtainable restrictions, allowing us to study all aspects of the syndrome, screen for drugs, examine response variation in the population and better tailor the treatment to each case and individual.

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# Chapter 2
# Induced Pluripotent Stem Cells in Familial Dilated Cardiomyopathy

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## Introduction

Cardiomyopathies typically refer to the conditions that the cardiac muscles exhibit structural and functional abnormalities in the absence of any identifiable hemodynamic cause. Among various types of cardiomyopathies, dilated cardiomyopathy (DCM) is the most common type of disease. It has been estimated that the prevalence of DCM could be as high as 1 in 250 individuals and is about double to the prevalence of hypertrophic cardiomyopathy (HCM) [1].

As a matter of fact, DCM is the major cause of heart failure and accounts for over 50 % of the cardiac transplantation cases worldwide [2–4]. Regardless of the genetic contribution, DCM is characterized by the presence of left ventricular dilation followed by systolic dysfunction [5]. As the disease progresses, the condition may spread to the right ventricle and the atria, and leads to heart failure [5].

Currently, the classification of cardiomyopathy is based on the differences in pedigree assessments. Sporadic DCM refers to the case that a single member of a family is affected, and viral infection accounts for most of the known causes. On the other hand, when two or more family members are affected, the incidence is assigned as familial DCM [6]. Although by definition, genetic abnormality is not necessary a prerequisite for familial DCM, it is now generally accepted that genetic

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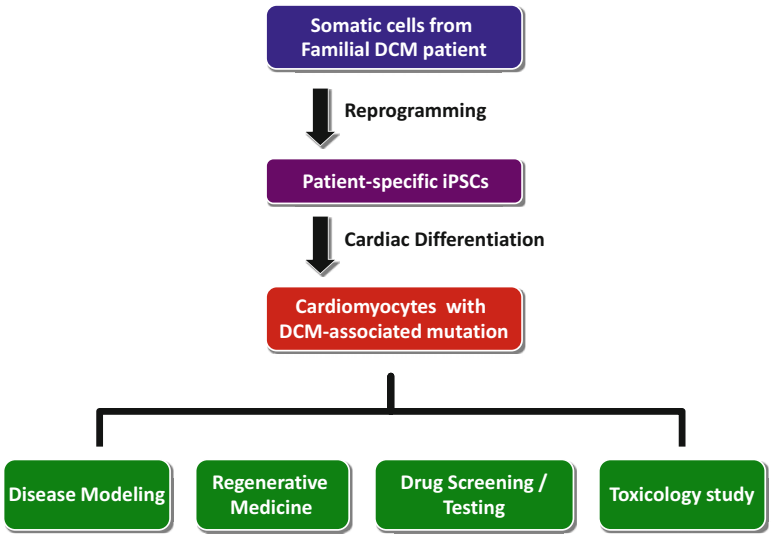


**Fig. 2.1** Clinical application of iPSCs derived from patients carrying dilated cardiomyopathy (DCM)-associated mutations. With the latest development of the iPSCs generation methods, somatic cells from patients carrying specific DCM-associated mutation can be reprogrammed into the iPSCs for the production of cardiomyocytes. The resultant patient-specific iPSCs-derived cardiomyocytes offer an exclusive human cardiomyocytes-based platform for disease modeling, regenerative medicine, drug development, and toxicology studies

basis is an essential element contributing to the development of DMC. For that reason, the term familial DCM is considered synonymous with genetically caused DCM in general.

Owing to the lack of appropriate experimental model, the studies of familial DCM have been difficult tasks to clinicians and researchers. Until several years ago, Yamanaka and colleagues demonstrated that terminally differentiated somatic cells could be reprogrammed into induced pluripotent stem cells (iPSCs) with the introduction of four transcription factors. This discovery offers a brand new opportunity to the study of genetic diseases with the application of authentic patient-specific iPSCs. As outlined in Fig. 2.1, patient-specific iPSCs not only could be used in disease modeling but also provided new ways for drug screening and toxicology testing. In this review, the potential applications of patient-specific iPSCs are discussed.

## Genetic Basis of Familial DCM

Dating back to a few decades ago, sporadic DCM was considered to be the majority of cardiomyopathies. With the increasing information from pedigree analysis and the improved knowledge in molecular genetics, it is now evidenced that up to 50 % of the idiopathic cardiomyopathies are of inherited causes [7–10]. Actually, this figure

**Table 2.1** Genes associated with familial dilated cardiomyopathy

| Gene symbol | Gene product | Primary function | Reported mode of inheritance | Reference |
|---|---|---|---|---|
| *Sarcomere* | | | | |
| *ACTC1* | Actin, alpha cardiac muscle 1 | Muscle contraction | Autosomal dominant | [46, 47] |
| *ACTN2* | Actinin, alpha 2 | Myofibrillar actin anchoring | Autosomal dominant | [48, 49] |
| *ANKRD1* | Ankyrin repeat domain-containing protein 1 | Complex with titin and myopalladin | Autosomal dominant | [50] |
| *MYBPC3* | Cardiac Myosin binding protein-C | Muscle contraction | Autosomal dominant | [51] |
| *MYH6* | Myosin-6 | Muscle contraction | Autosomal dominant | [52, 53] |
| *MYH7* | Myosin-7 | Muscle contraction | Autosomal dominant | [46, 51, 54] |
| *MYPN* | Myopalladin | Z-disc component | Autosomal dominant | [55, 56] |
| *TCAP* | Telethonin | Z-disc component | Autosomal dominant | [54, 57] |
| *TNNC1* | Cardiac Troponin C | Muscle contraction | Autosomal dominant | [58, 59] |
| *TNNI3* | Cardiac Troponin I | Muscle contraction | Autosomal dominant/ recessive | [47, 60, 61] |
| *TNNT2* | Cardiac Troponin T | Muscle contraction | Autosomal dominant | [46, 47, 54, 58, 62] |
| *TPM1* | Tropomyosin alpha-1 chain | Muscle contraction | Autosomal dominant | [54, 63] |
| *TTN* | Titin | Connecting Z-line and M-line | Autosomal dominant | [64, 65] |
| *Sarcoplasmic reticulum* | | | | |
| *PLN* | Phospholamban | SERCA2a inhibitor | Autosomal dominant | [66–68] |
| *RYR2* | Ryanodine receptor 2 | Calcium channel on sarcomeric reticulum | Autosomal dominant | [49] |
| *Ion channel* | | | | |
| *ABCC9* | ATP-binding cassette, subfamily C member 9 | Drug-binding channel-modulating subunit of the extrapancreatic ATP-sensitive potassium channels. | Autosomal dominant | [69] |
| *SCN5A* | Sodium channel protein type 5 subunit alpha | Controls Na + influx | Autosomal dominant | [70, 71] |
| *Cytoskeleton* | | | | |
| *DES* | Desmin | Transduces contractile forces | Autosomal dominant | [25, 72, 73] |
| *DMD* | Dystrophin | Transduces contractile forces | X-linked dominant | [74–77] |

(continued)

**Table 2.1** (continued)

| Gene symbol | Gene product | Primary function | Reported mode of inheritance | Reference |
|---|---|---|---|---|
| *MVCL* | Metavinculin | Anchoring actin filaments to plasma membrane | Autosomal dominant | [78, 79] |
| *SGCD* | Delta-sarcoglycan | Formation of dystrophin-glycoprotein complex | Autosomal dominant or recessive | [80–82] |
| *Nuclear membrane* | | | | |
| *LMNA* | Lamin-A/C | Maintenance of nuclear envelope integrity | Autosomal dominant | [54, 62, 83, 84] |
| *Mitochondrial* | | | | |
| *TAZ* | Tafazzin | Function as phospholipid-lysophospholipid transacylase | X-linked recessive | [85–87] |
| *Desmosomal* | | | | |
| *DSC2* | Desmocollin-2 | Desmosome component | Autosomal dominant | [88, 89] |
| *DSG2* | Desmoglein-2 | Desmosome component | Autosomal dominant | [89, 90] |
| *DSP* | Desmoplakin | Desmosome component | Autosomal dominant | [91] |
| *Lysosomal* | | | | |
| *LAMP2* | Lysosome-associated membrane protein 2 | Mediating lysosomal clearance of autophagosome | X-linked dominant | [92, 93] |
| *Others* | | | | |
| *BAG3* | BAG family molecular chaperone regulator 3 | Mediating chaperone-assisted selective autophagy | Autosomal dominant | [94] |
| *CRYAB* | Alpha-crystallin B chain | Molecular chaperones | Autosomal dominant | [95, 96] |
| *PSEN1* | Presenilin-1 | Regulating γ-secretase activity | Autosomal dominant | [97] |
| *PSEN2* | Presenilin-2 | Regulating γ-secretase activity | Autosomal dominant | [97] |
| *MLP* | Muscle LIM protein | Interacting with actinin | Autosomal dominant | [48] |

could be an underestimation, given that many of the mutations are of low penetrance and highly variable expressivity.

Familial DCM is a highly heterogeneous disease; depending upon the genes involved, the pattern of inheritance could be autosomal dominant, autosomal recessive as well as X-linked [11]. To date, over 40 genes have been implicated in the

development of familial DCM. As outlined in Table 2.1, the reported candidate genes are highly diverse in functions. In addition to the genes encoding sarcomeric proteins, mutations in the genes encoding nuclear or lysosomal membrane proteins could also be engaged. The identification of the genes associated with familial DCM is definitely critical to the understanding of the disease development. Nevertheless, the elucidation of the molecular mechanism behind remains challenging due to the wide divergence of phenotypic outcomes. This could be easily illustrated by the fact that many of the so-called DCM-causing genes, such as *ACTC1* and *DES*, are also associated with hypertrophic cardiomyopathy or other non-cardiac diseases [12, 13].

## Human-Induced Pluripotent Stem Cells as a Tool for Modeling Familial DCM

The study of the pathophysiological mechanisms underlying a specific gene mutation is crucial for the development of therapeutics targeting inherited disorders. Traditionally, the study is accompanied by

1. The direct examination of the tissue biopsy, or
2. The use of primary or immortalized cell lines carrying the gene mutation of interest, or
3. The creation of a transgenic animal model

Unfortunately, with respect to familial DCM, the application of these approaches was either technically difficult or functionally compromised by various factors. Regardless to the difficulty in obtaining the cardiac biopsy samples, cardiomyocytes are terminally differentiated. As such, isolated cardiomyocytes do not further proliferate and only survive for 1–2 days; thus, the conductance of in vitro examinations is largely limited.

Until recently, the generation of transgenic mouse lines is the best approach available for modeling genetic disorders and therapeutic development. In fact, in the cases of familial DCM, transgenic mouse model is somehow useful. This could be appreciated by the studies of the transgenic mouse carrying the mutation of *TNNT2* gene that encodes the cardiac troponin T [14, 15]. Such transgenic animals recapitulated the human DCM phenotypes and revealed the importance of calcium sensitivity in the development of DCM [16]. However, owing to the substantial differences in the electrophysiological properties and metabolic needs between human and mouse hearts, the applicability of such transgenic mouse model is controversial. And this is especially important when one considers the mutations in ion channels, such as *SCN5A*.

In 2006, Yamanaka and colleagues demonstrated the first time that mouse adult somatic cells can be reprogrammed into pluripotent stem cells with defined factors [17]. Such iPSCs retain the ability of proliferation and further differentiate into various cell types including cardiomyocytes. This technology was subsequently proven to be applicable in human somatic cells as well [18, 19]. These findings not

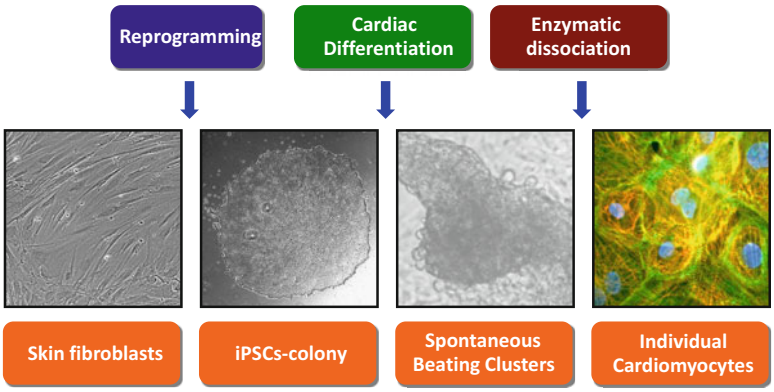


**Fig. 2.2** Cardiomyocytes derived from patient-specific iPSCs. After reprogramming, the iPSCs colonies can be differentiated into functional cardiomyocytes using various methods. In the traditional protocol, dissociated iPSCs aggregates will be cultured in suspension to allow the formation of spontaneous beating embryoid body. Depending on the study of interest, individual cardiomyocytes can be dissociated from the embryoid body using various enzymes, such as collagenases

only created a huge impact to biological science, but also offered a new paradigm for modeling human genetic disorders. With the advent of iPSC technology, now, somatic cells, such as skin fibroblasts from patients carrying a disease-causing gene, could be reprogrammed into iPSCs for the generation of cardiomyocytes [20] (Fig. 2.2). Evidences from various studies demonstrated that, when comparing to mouse cardiomyocytes, the cardiomyocytes derived from human iPSCs possess more comparable electrophysiological properties and gene expression profiles to normal human cardiomyocytes [21, 22], reflecting the potential of these cells in modeling human cardiac defects. With the help of this approach, the enormous resources required for generating transgenic animals can be saved. More importantly, such human cardiomyocyte-based platform may eliminate the ambiguities raised from the difference of species.

In 2011, Gepstein and colleagues demonstrated the first time that the cardiomyocytes derived for the patient-specific iPSCs carrying a mutation in the *KCNH2* (potassium channel) gene reproduced the disease phenotype of congenital long QT syndrome [23]. This study strongly confirmed the possibility of using the cardiomyocytes derived from patient-specific iPSCs in modeling genetic-based cardiac defects.

With the light from this pioneering study, Wu and colleagues have created the first patient-specific iPSC-based model for familial DCM [24]. In their study, family members with a DCM-associated point mutation in the *TNNT2* (cardiac troponin T) gene were recruited. The sequencing analysis showed that such mutation causes the 173rd amino acid residues of the cardiac troponin T changed from arginine (R) to tryptophan (W). Clinically, individuals carrying that mutation show the typical DCM symptoms including left ventricle dilation and decreased ejection fraction. As such, the iPSCs generated from these patients appear to be an ideal tool for modeling

the effect of $TNNT2_{R173W}$ mutation on DCM pathogenesis. To minimize the ambiguity from individual differences, skin biopsy samples were collected from both affected and normal (wild-type) individuals of three generations of a single family. The skin fibroblasts derived from the biopsy samples were reprogrammed into iPSCs utilizing the well-reported 4 factors-mediated approach, and allowed to differentiate into cardiomyocytes for structural ad functional analyses. Compare to the control, the cardiomyocytes derived from the mutation containing-iPSCs showed with increased incidence of abnormal sarcomeric alpha actinin distribution. Functionally, although there was not much difference in the electrophysiological property between the normal and mutant groups, the mutant groups exhibited a significant reduction in contractility and impairment in calcium handling ability under β-adrenergic stimulation. These observations indicated that the increased susceptibility to inotropic stress may be a mechanism underlying the effect of *TNNT2* mutation on DCM development.

The association between *TNNT2* mutations and DCM development has been previously studied using a transgenic mouse model [14, 15]. The transgenic mice with both *Tnnt2* alleles knocked down exhibited more severe DCM symptoms, and the isolated cardiomyocytes showed reduced contractility, sarcomeric disorganization, and diminished calcium sensitivity. These observations are quite consistent with the later findings observed in the cardiomyocytes derived from patient-specific iPSCs. However, for the heterozygous transgenic mice that carried one normal *Tnnt2* allele, their hearts appeared grossly normal. Such appearance was obviously contradicted with the case in human, in which *TNNT2* mutation caused a dominant transmission of the disease phenotype. Although such difference could be explained by the different nature of the mutations; yet, it also demonstrated clearly the weakness of using a mouse model for the study of human cardiac disorders.

## Application of Patient-Specific iPSCs in the Validation of the DCM Candidate Genes

With the advanced sequencing technology, large-scale screening of mutations becomes more cost effective since large number of the so-called disease-associated mutations can be identified in a very short period of time.

For instance, very recently, via employing the whole exome-sequencing analysis, our laboratory has identified a novel *DES* mutation in a patient with left ventricular dilation and impaired left ventricular ejection function [25]. The *DES* gene encodes the type III intermediate filament protein, desmin. In this *DES* mutation, a single nucleotide substitution was identified and leads to the change of alanine residue to valine at the 285th amino acid position. Although the exact function of desmin is not known, its interaction with sarcometric components and nuclear membrane implicates that desmin plays critical roles in the maintenance of cellular architecture and the mediation of excitation–contraction coupling [26, 27]. The association between DES mutations and various cardiac defects, including familial

DCM, has been documented [13]. In a transgenic mouse model, homozygous *Des* null animals developed hypertrophic and dilated cardiomyopathy [28]. Further investigations demonstrated that abnormalities such as the occurrence of extensive myocyte death, calcific fibrosis and disruption of sarcolemma are believed to contribute to the development of the DCM phenotype.

However, unlike the case in the transgenic mouse model, in which no immunoreactive desmin is produced, the patient with the $DES_{A285V}$ mutation produces a desmin mutant resembling the wild-type desmin protein in terms of molecular weight and immune reactivity. This observation has complicated the interpretation of the diagnosis. Although this specific desmin mutation has been observed in various cases of familial DCM, with the lack of family history data of the patient, more experimental evidence is required to validate the association between the newly identified mutation and the observed disease phenotype. Actually, an important issue is raised here. In a single run of large-scale screening, one can observe thousand gene mutations; thus, the exact association among any of these mutations and the disease phenotype yet remains questionable.

In order to evaluate the putative pathogenic effects of the novel $DES_{A285V}$ mutation, we generated the iPSCs from the skin fibroblasts derived from the patient. These iPSCs were subsequently differentiated into cardiomyocytes for structural and functional characterization. Compare to the normal iPSCs-derived cardiomyocytes, the cardiomyocytes carrying *DES* mutation exhibited abnormal protein aggregations in the sarcomere and Z-disc streaming (Fig. 2.3). In addition, contraction failure was observed when the mutant cardiomyocytes were subjected to the stress induced by isoproterenol. These observations not only provided an explanation in the pathogenic mechanism underlying the $DES_{A285V}$ mutation but also validated the causal ion relationship between the *DES* mutation and the DCM phenotype observed in the patient.

## Application of iPSCs-Derived Cardiomyocytes in Regenerative Medicine

In addition to the potential importance of iPSCs in the applications mentioned above, the therapeutic applications of iPSCs in familial DCM represent an equally important issue to be addressed. As DCM progresses, irrecoverable death of cardiomyocytes occurs, this directs the patient to heart failure. As such, replacing the loss of functional cardiomyocytes is an obvious therapeutic strategy. Transplantation of human embryonic stem cells (ESCs)-derived cardiomyocytes into the infarcted myocardium of an immunodeficient rodent appeared to improve the cardiac functions of the implanted animal [29, 30]. With the homologous genomic composition and immunological profile, the patient-specific iPSCs-derived cardiomyocytes are definitely a better source for cell replacement therapies. To date, various technologies have been developed to correct mutated genes [31, 32]. In this regard, the

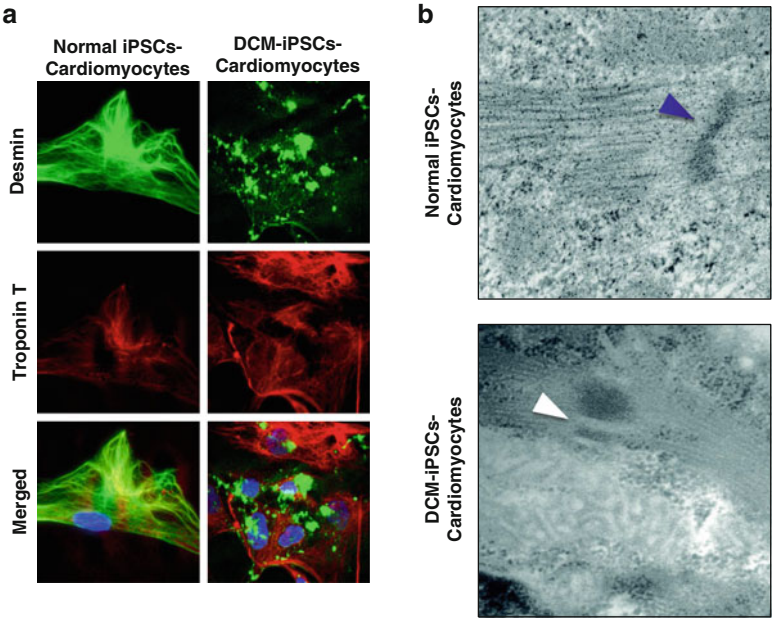


**Fig. 2.3** Cardiomyocytes derived from iPSCs with *DES* mutation recapitulated DCM phenotypes. (**a**) Immunofluorescence staining demonstrated the abnormal accumulation of desmin-positive aggregates in the iPSCs cardiomyocytes carrying the $DES_{A285V}$ mutation. (**b**) Electron microscopy analysis reveals the disruption of Z-disc in the iPSCs cardiomyocytes carrying the $DES_{A285V}$ mutation

mutated genes in the patient-specific iPSCs could be corrected in vitro and used to replace the affected cells. However, due to various limitations (to be discussed in the next section), the direct application of the patient-specific iPSCs-derived cardiomyocytes in cell replacement therapy remains questionable.

## Application of iPSCs-Derived Cardiomyocytes in Efficacy Testing and Drugs Screening

Familial DCM is an extremely heterogeneous disease; instead of targeting the disease-causing genes, the current treatment for DCM is largely symptomatic. Based on the disease status, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers, beta-blockers, aldosterone antagonists, cardiac glycosides, diuretics, vasodilators, antiarrhythmic, and inotropic agents are being used as the mainstay DCM drugs. Conventionally, the effects of putative cardiac drug are tested in the well-established rabbit or canine Purkinje fiber model prior to clinical trials. However, such models often give false-positive or -negative results [33–35], so that many drugs that have passed the animal trial turn out to be

a failure in the clinical ones. Recent reports evidenced that human ESCs-derived cardiomyocytes showed excellent pharmacological response to various known antiarrhythmic agents, revealing the clinical potentials of human ESCs-therapies [36, 37]. However, due to the difference in genetic background, individuals carry the same gene mutation may response differently to a specific drug. For that reason, the patient-specific iPSCs-derived cardiomyocytes offer an exclusive platform for evaluating the efficacy of a particular drug or treatment strategy on the patient.

Based on the latest breakthrough in the cardiac differentiation method, a yield of more than 80 % of cardiomyocyte differentiation is no longer difficult to achieve [20]. These patient-specific iPSCs could be used in a MEA-based drug testing platform that allows a high throughput assay for evaluating effects of different drugs in terms of electrophysiological property of the cardiomyocytes. Obviously, DCM-causing mutation is not necessarily limited to ion channel-encoded genes. In fact, recent studies from our group and other investigators have pointed out that altered calcium handling could be an important pathogenic mechanism contributing to DCM progression [24, 25, 38, 39]. This implies that drugs, which can alter the cellular calcium transient properties, are of therapeutic potentials. To address this issue, Mercola and colleagues have recently developed a high throughput automated kinetic image cytometry system for the measurement of calcium ion dynamics. This advanced system allowed the authors to measure individual calcium transients from 100 human iPSCs-derived cardiomyocytes at a time [40]. Taking advantage of such system, high throughput screenings of calcium handling–enhancing properties of known or novel drugs can be performed on familial DCM-specific iPSCs-derived cardiomyocytes.

## Application of iPSCs-Derived Cardiomyocytes in Toxicology Test

Cardiac toxicity represents one of the most critical concerns to be addressed in the pharmaceutical industry. Due to the moral and technical issues raised from the use of human cardiomyocytes, alternatively, isolated canine cardiomyocytes is the most popular preclinical model for testing the cardiac safety of a developing drug. However, the reliability of such model remains questionable. In fact, many drugs that have passed animal tests turn out to show unanticipated cardiac toxicity when administered to patients [41], and such unexpected drug-induced cardiac side effects, notably ventricular arrhythmias, have led to the withdrawal of many drugs, including the famous nonsteroid anti-inflammatory drug-Vioxx, from the markets in the last few years [42]. It should be noted that when comparing to the normal cardiomyocytes, the heart with DCM is obviously more susceptible to toxic side effects; thus, a more predictive and reliable human cardiomyocyte-based model for toxicology test for DCM drugs is in an immediate demand. Increased evidences have demonstrated that the pharmacological sensitivities of

human ESCs and iPSCs-derived cardiomyocytes are much more advanced than any animal models [36, 37]. These information suggests that these cells are good detectors for undesired proarrhythmic side effects of a developing drug.

Recently, Mendenius and colleagues demonstrated the possibility of using human ESCs and iPSCs-derived cardiomyocytes to evaluate the drug-induced cardiac injury [43, 44]. In their study, the human ESCs- and iPSCs-derived cardiomyocytes were treated with doxorubicin, and the release of cardiac troponin T in culture medium was detected with a Biocore-based system and used as an indicator of cell injury. Compare to the conventional ELISA-based assay, the surface plasmon resonance-based method not only offers superior sensitivity and specificity but also allows simultaneous analysis of multiple samples. This study clearly proved the use of iPSCs-derived cardiomyocytes in toxicology testing is applicable.

## Limitations of iPSCs

The recent achievement in the patient-specific iPSC technology has created new opportunities in familial DCM modeling and personalized medication development. Nevertheless, like many other technologies, the clinical applications of iPSCs-derived cardiomyocytes are also hindered by various limitations. With the latest development in cardiac differentiation protocol, a high yield of over 80 % of cardiac differentiation is not a tough task to achieve; however, the quality of those iPSCs-derived cardiomyocytes becomes an important issue. As demonstrated earlier by our laboratory, compared to the human ESCs-derived cardiomyocytes, the human iPSCs-derived cardiomyocytes actually showed reduced calcium handling ability and impaired sarcoplasmic reticulum function [45]. Therefore, for modeling the DCM resulted from mutations of genes that regulate calcium transients, such as phospholamban, iPSCs may not be an appropriate approach.

In addition, it should be noted that a high yield of cardiac differentiation is not equivalent to high purity. In fact, the iPSCs-derived cardiomyocytes are always grown in a mixed population of atrial, ventricular, and nodal subtypes. These subtypes obviously possess different electrophysiology properties. Previous studies reported that injections of human ESCs- or iPSCs-derived cardiomyocytes into mice with cardiac defects improved their cardiac functions [29, 30]. Rodents have a much higher heart rate compared to human, so that the injection of human cardiomyocytes into rodent hearts may not create significant arrhythmia problems. However, the injection of mismatched subtypes of cardiomyocytes into patients' hearts is obviously a different issue. So far, there is no efficient way to sort the subtypes of iPSCs-derived cardiomyocytes into pure populations; thus the direct application of the patient-specific iPSCs cardiomyocytes in regenerative medicine remains a theoretic foundation. Similarly, the immature phenotype and mixed cardiomyocyte subtypes also limit the application of the iPSCs-derived cardiomyocytes in drug-screening experiments. Therefore, it is important to verify and validate the results obtained in the initial screening steps.

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# Chapter 3
# Induced Pluripotent Stem Cells and Amyotrophic Lateral Sclerosis

**Miguel Mitne-Neto**

## Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is the most common adult-onset motor neuron disease (MND) and affects 30,000 individuals in the United States at any given time [1, 2]. Clinical findings include muscular atrophy and weakness, accompanied by fasciculation and spasticity, and a fast progressive degeneration of motor neurons in the cortex, brainstem, and spinal cord [3]. Symptom onset occurs between the fourth and sixth living decade. Unfortunately, there is currently no cure for ALS, with patient's death occurring after 2–5 years of disease onset [4].

Approximately 90 % of all ALS cases are sporadic (SALS) and the remaining 10 % comprise the familial forms (FALS), most having an autosomal dominant pattern of inheritance [5]. The worldwide incidence ranges around 1–2 in 100,000 individuals, with a slightly higher frequency in men than in women.

The causes of sporadic ALS are still unclear, and it is speculated that genetic factors combined with environmental effects may contribute to their emergence. Exposure to neurotoxins, such as the one suffered by Gulf War veterans, contact with pesticides and electromagnetic field interference were proposed as ALS risk factors. Additionally, extenuating physical activities could be also involved with the disease, as indicated by an Italian study that showed an 8-time higher ALS frequency in former soccer players from that country [6].

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Although extensive data and knowledge accumulated over time with the discoveries of more than 30 ALS-causing genes, the field still lacks clear molecular mechanisms explaining the disease. The first ALS-related gene was mapped more than 20 years ago by Rosen et al. [7]. The discovery of ALS-causing mutations in the superoxide dismutase gene (*SOD1*) brought the first insights over the pathways leading to the motor neuron degeneration. Studies addressing the enzymatic function on free radical metabolism and its relationship with oxidative stress pathways established that the mutant forms act through a toxic gain of function. In this regard, mouse lineages overexpressing the SOD1 mutant enzyme became the main disease-modeling platform for drug discovery and therapy attempts.

In the following decade, a new FALS form was described and named ALS8, with the identification of a missense mutation in the *VAPB*/*C* gene in 13 Brazilian families [8]. As it is seen in other neurodegenerative disorders, overexpression of the mutant VAPB leads to cytoplasmic inclusions [8–10]. Despite the presence of VAPB cytoplasmic inclusions, transgenic mutant VAPB mice do not show any phenotype related to motor disturbance [11, 12]. Evaluations of VAPB availability in different systems have shown that the VAPB protein levels are reduced not only in ALS8 cases [10] but also in sporadic forms [9, 13, 14], establishing a relationship between these two ALS forms.

Previously to its identification as an ALS-causing gene by independent groups in 2008 [15, 16], the TDP-43 protein had been related to neurodegenerative disorders due to its presence as the main proteic component of cytoplasmic aggregates in post-mortem tissues from patients with frontotemporal dementia (FTD) and ALS [17].

Following TDP-43 discovery, Vance et al. [18] and Kwiatkowski et al. [19] showed that mutations in *FUS*/*TLS*, a gene with high identity domains to TDP-43, are the cause of another type of FALS. This discovery reinforced the possibility of diverse motor neuron death pathways, pushing forward the studies on RNA processing, since both TDP-43 and FUS hold conserved domains involved in mRNA transcription and splicing [20].

Until very recently, it was common to find in many scientific manuscripts and patient-directed materials the information that the most frequent familial form was caused by *SOD1* mutations, which would account for 20 % of those cases [21]. However, this concept rapidly changed after the identification of a hexanucleotide expansion in the *C9ORF72* gene [22]. Depending on the population, the GGGGCC expansion is responsible for 23–40 % of familial cases and it was identified in up to 8 % of sporadic forms [23, 24]. At the clinical level, a particularity of the *C9ORF72* gene expansion is a common overlap of motor neuron signs and FTD; at the molecular level, the main hallmark of this expansion is the formation of intranuclear RNA foci in the motor cortex and spinal cord [22].

The advent of new molecular tools, especially high-throughput sequencing, are speeding up the knowledge on ALS pathology, and an entire new field is being explored with the data achieved from new ALS gene studies, especially the relationship between motor neuron degeneration and RNA processing. Additionally, the recent data is rethinking the previous idea that ALS is purely a motor disease, since the clinical spectra may be presented with Parkinsonism, sensory abnormalities, autonomic dysfunction, and specially frontotemporal lobar degeneration.

Despite of the extensive efforts to find a cure, very little progress was achieved in ALS treatment during the last century. ALS patients are offered only palliative care which is mainly based on a multidisciplinary approach, involving motor and respiratory physiotherapy, nutritional and psychological support. Even though a number of drugs went to late clinical trials stages, Riluzol is still the only approved compound for ALS treatment. Marketed since the mid-1990s and acting in Glutamate metabolism, Riluzol presents very limited effects, since clinical tests showed a survival extension of only 4–6 months.

Due to the fast progression of ALS, conclusions on clinical trials are very limited, showing the need for alternative tools to evaluate new and more effective drugs. Additionally, diagnosis is still a critical area. With the lack of good biomarkers to define the disease, diagnosis is mainly based on clinical findings, and supported by the electroneuromyography. Considering a life expectancy of 5 years after the initial symptoms, the averaged 11 months needed to close the ALS diagnostic has an enormous impact on patients' lives.

## Modeling ALS

The fact that ALS is an incurable disease, with a poorly understood pathogenesis and necessitous of good resources for diagnosis, emphasizes the need for new tools that could improve this scenario. In this regard, disease modeling emerges as an alternative not only to better understand the disease but also (and especially) for drug screenings. In vivo and in vitro modeling have been the most common used alternatives for this purpose. Following the identification of metabolic alterations in patients' bodies and the reproduction of those in models, one could use a plethora of compounds and approaches in attempts to correct the phenotype. Animal and cellular models are of great interest since they can be studied in short period of time and be used in different approaches.

Despite their wide use in preclinical studies, a substantial number of successful drug tests made in ALS animals could not be translated to humans [25]. Animal models often do not recapitulate all aspects of complex human diseases. The difficulty of translating results from the overexpression models, especially the murine ones, to humans, may be due to the exacerbated effect of the target protein. In general, the protein levels in these models range from 4 to 20 copies of each gene, contrasting with the patient's double copy situation for autosomal genes. Additionally, for some researchers, the lack of success of such preclinical approaches may be caused by the use of a single model—SOD1 mice carrying a high transgene copy number [25, 26].

The inability to isolate populations of motor neurons from living subjects has hindered the progress toward studying the underlying mechanisms of many neurological diseases. Studies of cadaver tissue are often of limited use, especially for neurodegenerative disorders where the onset of disease usually precedes death by years, thus showing only the final stage of the disease. In addition, frozen tissue sections are of limited use for studying cellular physiology and neural networks.

In this regard, important aspects of a patient cellular model are (1) the avoidance of overexpression systems; (2) the analysis of the disease's affected cell population in the patient; and (3) the evaluation under a human genetic background. Moreover, this model could reveal altered molecular pathways before the disease onset, generating valuable diagnostic tools. The use of cellular models in conjunction with other available ones will certainly speed up the translation of such studies into clinical trials.

## Induced Pluripotent Stem Cells and ALS

The critical need for new human ALS models was the drive for many scientists searching alternatives to the limited available ones. Recent advances in stem cell biology have increased the prospect that perhaps the difficulty in unraveling the disease mechanisms that underpin ALS could finally be overcome with the availability of pluripotent stem cells.

The induced pluripotent stem cell (iPSC) technology provides a promising approach to this problem, as it allows the genomes of human subjects afflicted with ALS to be captured in a pluripotent stem cell line. Once the patient's cells are reprogrammed, they can be differentiated to human motor neurons or glial cells and evaluated for the physiological effects of a specific gene alteration, or a set of determined polymorphisms.

In spite of the great enthusiasm since the generation of the first ALS iPS cell lines, it is important to note that this is only the first step for modeling the disease. The development of faster and more robust protocols has turned the reprogramming process into a commodity. In this scenario, the differentiation to both glial or motor neuron cells and the identification of a phenotype in those lineages became the main challenge.

Additionally, it is noteworthy that having the ability to differentiate adult fibroblasts to any other cell type is a great advantage of this model, which allows the study of cell autonomous and non-cell autonomous mechanisms behind ALS.

## iPSC-Derived ALS Models

The generation of iPSCs [27] opened a new field in ALS studies, allowing the evaluation of patient-specific cells in vitro. Since most of ALS cases are classified as sporadic, it is currently difficult to point out the key players for motor neuron death. As it happens for other fields, most of the studies have directed their attention to the familial forms, since there is at least a first clue to uncover the degeneration pathways. Over the recent years, scientists have used iPSCs as a tool to evaluate specific lineages carrying ALS-causing mutations, as shown in the following sections.

## *SOD1*

Dimos et al. [28] were the first to show that human ALS fibroblasts could be reprogrammed to a pluripotent state and later differentiated to neurons. Although they didn't evaluate any phenotype on the L144F *SOD1* mutation, the fact that iPSCs were generated with cells obtained from a patient in her 80s, and that these cells normally differentiated into motor neurons, showed that patient age is not a limiting factor and that iPSCs from elder individuals can give rise to motor neurons.

iPSC-derived neurons carrying *SOD1* mutations were also developed in other studies to be used as a comparison with different ALS-related genes. Although no specific phenotypes were observed, Burkhardt et al. [29] could not find TDP-43 aggregates in SOD1 mutant cells, which is consistent with postmortem data and supports the hypothesis that TDP-43 and SOD1 undergo different degenerating pathways.

## *VAPB*

Based on our previous identification of a large ALS genealogy (ALS8) [8], we collected fibroblasts from two related Brazilian families carrying the same mutation (P56S) on the *VAPB* gene. In order to overcome the well-known heterogeneity of ALS, especially in ALS8, samples were taken from four patients and three normal siblings. It is expected that the use of samples from related individuals would reduce the genetic background variation, leading to a better comparison of mutant and wild type cells.

Cytoplasmic inclusions are a hallmark of ALS pathogenesis. In vitro and in vivo systems overexpressing VAPB also show aggregated protein clusters, which in mice are not sufficient to trigger the disease, even when they are 18 months old [11]. However, using a monoclonal antibody against VAPB, we found no obvious alterations in the VAPB distribution pattern of ALS8 fibroblasts, iPSC or differentiated motor neurons. This situation was also found when ALS8-derived motor neurons were kept in culture for 7–8 weeks (time required for neuronal maturation), or when the system was disturbed by a proteasome inhibitor.

Searching for a clear phenotype, we evaluated the VAPB protein levels in different lineages. A comparison between iPSC and embryonic stem cells led us to show for the first time that VAPB is expressed since early development. These results brought the question of why a mutation in a protein present since the embryonic stage will only promote a phenotype later in life. However, the main result of our work came with the identification of lower VAPB levels in patient's cell cultures, independently of the evaluated cell lineage. Additionally, during the differentiation process we observed a gradual upregulation of VAPB in wild-type cells, which fails to happen in the mutant ones [10].

The protein reduction levels and absence of cytoplasmic aggregates on motor neurons allowed us to hypothesize that the relationship between neurodegeneration and VAPB may not solely rely on the mutant protein's gain of function, as shown by other authors [30, 31], but also by a loss-of-function/haploinsufficiency mechanism.

Supporting this hypothesis, and increasing the importance of VAPB function on ALS pathogenesis, are recent works showing that there is a reduction of this protein and its smaller sub-products in spinal cord and in cerebrospinal fluid [9, 14].

## *TDP43*

ALS disease modeling on TDP43 was first shown by Bilican et al. [32], evaluating motor neuron derived iPSC susceptibility. Despite the increment of TDP43 in the soluble and insoluble fractions, cells carrying the M337V mutation did not show intracellular aggregates. Instead, they found a reduced survival under basal conditions and an increased vulnerability to a PI3K inhibitor, named LY294002 [32].

Complementary to that study, Egawa et al. [33] derived motor neurons from patients carrying three distinct mutations on *TARDBP* (G298S, M337V, Q343R). They found that, as shown for zebrafish models, mutation-carrying neurons have shorter neurites compared to wild-type ones. Additionally, the amount of detergent-insoluble TDP-43 was increased on patient cells. In order to evaluate cell death on motor neuron derived from iPSC, the authors exposed the cells to arsenite. It is known that inorganic arsenical is able to induce oxidative stress through the production of reactive oxygen species (ROS) and to increase the amount of TDP-43 in the insoluble fraction. Keeping in mind the involvement of ALS with RNA processing, they tested four drugs previously reported to act through histone modification on transcriptional process. Anacardiac acid was the only one able to recover the phenotype, since it produced an extension of neurite length of treated neurons and also reduced TDP-43 in the insoluble fraction [33].

By comparing a patient's sample carrying the A90V mutation with its normal sibling's, Zhang et al. [34] observed that, contrary to other studies, the levels of total TDP-43 were decreased in patient's cells. They also observed a reduction of microRNA9 in those cells lines and in another iPSC-derived motor neuron lineage carrying the M337V mutation. Although they could not explain the whole involvement between microRNA9 and TDP-43 in the ALS neurodegeneration, it is worth noting that microRNA9 downregulation was previously identified in Spinal Muscular Atrophy (SMA) mouse models with a potential role in the regulation of neurofilament subunits [35].

Another TDP-43 mutation (A315T) was studied by Burkhardt et al. [29], but they did not report any phenotype in those lines, highlighting the variability among different mutations in the same gene.

## *C9ORF72*

The normal number of GGGCC repeats in the *C9ORF72* region is of 23 or fewer. Studying samples from two subjects with more than 1,000 repeats, Almeida et al. [36] observed a repeat instability in fibroblast, iPSC, and neurons, with the

identification of GGGCC RNA-containing foci on the three lineages. Although these RNA foci do not sequester major RNA binding components, they found that the levels of p62 were elevated, and also that there was a higher sensitivity of mutant cells under autophagy inhibitors.

Also using mutant *C9ORF72* motor neurons, Donnelly et al. [24] found GGGGCC expanded RNA foci, which was confirmed by a comparison against postmortem tissue. According to the authors, the presence of those foci may suggest that the altered RNA may undergo a non-ATG translation, which would, ultimately, lead to an accumulation of high-molecular dipeptides based on the six nucleotide repeats [24].

Additionally, they compared the transcriptomes of iPSC *C9ORF72* cells to iPSC lineages carrying the SOD1 D90A mutation. Although a subset of altered genes were present on both lines, compared to controls, most of the differently expressed genes were particular to each lineage suggesting different disease acting pathways.

Glutamate is a major component of excitatory synapses. Accumulation of glutamate in the synaptic cleft or an over activation of its receptors leads to a $Ca^{2+}$ release from the cell's storages, which may culminate in the production of free radicals [37]. The excess of glutamate signaling (excitotoxicity) has been, for many years, one of the main explanations for motor neuron degeneration and it is the target for the only approved substance for ALS treatment, named Riluzol, which acts through the glutamate synaptic recapture. Considering that patients with *C9ORF72* expansion express smaller levels of glutamate transporter 1 (GLT-1/EAAT), Donnelly et al. [24] inquired whether iPSC-derived neurons are more susceptible to glutamate excitotoxicity. Using propidium iodide cell viability experiments, they found the mutant cells to be 100-fold more sensitive to glutamate treatment, supporting the hypothesis of excitotoxic pathway involvement in motor neuron death. Ultimately, they used a series of designed antisense nucleotides (ASO) that could revert the mutant phenotype.

In a similar system, Sareen et al. [38] reprogrammed skin fibroblasts from 6 different patients and identified instability of the *C9ORF72* expansion during iPSC generation and motor neuron differentiation processes, as seen by Almeida et al. [36].

The *C9ORF72* generates two isoforms in which the transcripts differ in their 5′ noncoding region. The GGGGCC expansion occurs between exons 1a and 1b; i.e., for the isoform "b" the expansion relies on the promoter region. Using RNA-seq analysis, they found that the wild-type allele predominantly used exon 1b, while the mutant allele showed an increased use of exon 1a. A transcript expression level showed that there is no difference between cells carrying the *C9ORF72* expansion and the normal one, supporting the notion that the mutation leads to a gain of function. This data is also supported by the identification of a homozygote *C9ORF72* patient [39].

Their analysis of intracellular components also found RNA foci in mutant motor neurons, as seen in other studies, and an expression profile disturbance compared to wild-type cells [38]. Of special interest, they used ASO against *C9ORF72* transcripts and showed that knocking down the transcript to very low levels had no impact on neuronal survival and that gene expression profiles were improved by ASO.

The studies with *C9ORF72* modeling helped not only to consolidate the involvement of RNA processing in ALS pathogenesis, but the use of ASO may also come up as a new tool to be evaluated in clinical studies.

## *iPSC Lines Derived from Sporadic ALS Patients*

The lack of a deeper knowledge about the causes of sporadic forms of ALS turns its modeling into a difficult challenge and consequently delays the discovery of new drugs for this MND. Although it is not possible to point out a single cause for sporadic forms, it is thought that part of the motor neuron degeneration may have a genetic component that can be environmentally modulated.

In order to study whether a genetic background could influence motor neuron viability, Burkhardt et al. [29] studied a set of 16 iPSC lineages originated from different sporadic patients. Based on an intracellular analysis read out, they observed TDP-43 aggregates in 20 % of derived motor neurons. The identification of protein clusters in neuron lineages recapitulates ALS postmortem data, although it does not explain whether the aggregates are a primary effect, triggering the degeneration, or a secondary one, where it is generated as a consequence of cell death. Although a cause–effect relationship between TDP-43 clusters and sporadic ALS pathogenesis is not fully exploited, the authors of that manuscript evaluated three different cardiac glycosides and identified the previous approved drug Digoxin as a compound with the capacity to reduce the protein aggregates in a dose-dependent manner [29].

## *iAstrocytes*

Over the last years, a bulk of evidences demonstrated that ALS pathogenesis is a complex process. Even though it is still difficult to determine the weight of each variable, it is well accepted the idea that the motor neuron degeneration is composed by both cell autonomous and non-cell autonomous mechanisms.

Previous studies on non-cell-autonomous effects [40, 41] found that human embryonic stem cell-derived motor neurons had a reduced survival when co-cultured with astrocytes expressing mutant forms of SOD1. In that system, inflammatory pathways would be triggered in the presence of mutant SOD1. Recently, Meyer et al. [42] used reprogrammed fibroblasts from expanded C9ORF72, SOD1 A4V, and sporadic ALS patients to generate induced neuronal progenitor cells. In a similar approach as for iPSC generation, the authors used the four Yamanaka factors (*SOX2*, *OCT4*, *KLF4*, and *c-MYC*) to induce cell reprogramming. However, instead of creating a stem cell environment, 72 h after the transfection of the factor they moved the cells to a neuronal inducing media. Pushing the neuronal differentiation process early, they were able to obtain oligodendrocytes, neurons, and astrocytes in a period of 1 month [42].

Among them, each cell culture had its own particularity the production of induced Astrocytes (iAstrocytes) generated cells expressing high contents of astrocytic markers such as S100B.

Aiming the modeling of a complex system, the authors co-cultured the iAstrocytes with mouse motor neurons (MNs) and observed a reduced cell survival and shortened neurites in those astrocytic cells carrying mutant proteins. The results from

this single mutant allele system are in accordance with previous overexpression models [40, 41] and sustain the hypothesis of an astrocyte non-cell autonomous mechanism. Additionally, this data points out that a single mutant allele might be enough to generate a degenerative environment, without the need to exacerbate its expression.

Moreover, they asked whether the increased sensitivity was caused by a toxic mechanism produced by mutant cells, or by a lack of components that fail to be produced by mutation carrier astrocytes. Those analyses were made by a comparison between co-culture experiments (MNs + mutant iAstrocytes) and monocultures of mouse MNs. They observed a reduced survival in co-cultured cells, suggesting that external factors have a high impact in motor neuron metabolism. To further address the question of whether the reduced survival would be caused by a secreted component or by a straight cell-to-cell contact, they cultured mouse MNs solely in conditioned media obtained from mutant or wild-type iAstrocytes lineages. They analyzed the cell survival and found that the motor neuron death would be related to an astrocyte-neuron contact [42].

The reduced survival of motor neurons co-cultured with the *C9ORF72* iAstrocytes shows that the mutation exerts its effects from both mechanisms: non-autonomous [42] and autonomous [24, 36] (Sareen et al. 2013). Moreover, the non-autonomous effects are lineage-dependent since fibroblasts from the same donors failed to produce a neuronal cell death when compared to iAstrocytes [42].

## iPSC and Cell Therapy

Stem cell therapy is being one of the most discussed subjects in the biomedical science during these last years. Although it is already a reality for a couple of diseases (such as hematological ones), its application for neurodegenerative disorders is still under evaluation in clinical trials, especially in ALS.

It is evident that MN function largely depends on specific connections and environment in the motor cortex, brainstem, and spinal cord, and also on their spatial distribution on those sites. Additionally, since motor neurons are the biggest cells in the human body, with axons reaching up to 1 m (approximately 3 ft.) long, it seems clear that a stem cell therapy aiming MN substitution will encounter many physical barriers. While diagnostic tools are still limited, the above-described issues support the idea that a higher effectiveness of stem cell therapies on ALS will arise from strategies aiming to support the motor neurons while they are still alive—i.e., instead of a neuronal replacement strategy, one is looking for a therapy that is able to guarantee the survival of the remaining cells.

At this very moment, a dozen clinical trials are under course, with most of them injecting autologous cells or neuronal selected ones in the patient with the main objective of evaluating the safety and effectiveness of the procedures. Although the roaming processes are far from being understood, either through an intravenous or intrathecal injection it is expected that the injected cells will reach the affected region and improve motor neuron survival.

Despite the stem cells' plasticity, their expected action upon neuronal maintenance will probably depend on a first stimulus aiming the production of a specific product (protein/glycoprotein/lipid) or cell behavior that will be the main effector. For these reasons, a couple of trials are either culturing the cells in special media before the injection (autologous or heterologous) or using a more differentiated lineage in order to obtain better results.

Using the same rationale, in a preclinical study, Nizzardo et al. [43] reprogrammed fibroblasts from healthy subjects to iPSC using a non-viral protocol and induced their differentiation to neural stem cell, obtaining more than 90 % of neuroepithelial cells.

Later, using Fluorescence Assisted Cell Sorting (FACS) they picked up a specific cell fraction based on aldehyde dehydrogenase activity (ALDH) and selected cells expressing integrin VLA4 (VLA4+). The ALDH multipotent lineage was previously characterized as having a positive therapeutic effect and the VLA4+ NSC are able to cross the blood–brain barrier, especially when it is under inflammation process, as it is observed in ALS samples.

Using repeated intrathecal or systemic injection of the selected cells into SOD1G93A mouse models, the authors observed a survival extension of, respectively, 10 and 23 days. Also, there was an amelioration of neuromuscular function recorded by the motor neuron count on the spinal cord. In conjunction, the authors obtained a new neural stem cell lineage that is able to cross the blood–brain barrier, target the affected area and to produce a phenotype modulation that is translated to an increased cell survival.

The strategy presented by these authors brings many advantages. iPSCs were first generated by the overexpression of recombinant transcription factors, introduced in somatic cell lines through viral vectors. Although the recent engineered viral vectors were shown to be safe for human applications, the use of nonhuman components brings extra tests and validations. In this regard, the reprogramming process utilized by Nizzardo et al. [43], which lacks viral vectors, is of great interest since the translation of that strategy to the patients may happen in an accelerated pace. Additionally, purity and number of viable cells are the main factors for an effective cell injection approach. Since the differentiation process based on pluripotent stem cells will never reach the 100 % efficiency, the use of a *FACS*-based separation is critical, allowing a high "purity level" of the target cell line. Moreover, the translation of stem cell injections into clinical trials depends on a massively cell production. The data presented by the authors show that this strategy is feasible due to the scalability of the process.

## Challenges and Advantages of Using iPSC for ALS Studies

Although for some familial forms it is possible to indicate the disease-causing gene, most of ALS cases are classified as sporadic, since one cannot point a single villain component or situation. Until this moment, most of the in vitro iPSC models were based on cells that were carrying ALS-causing mutations, which may limit the conclusions of the studies to a restricted number of patients. On the other hand, a

clear phenotype, which can be evaluated and further corrected, was available only in mutation-carrying cells.

Phenotype recapitulation is one of the main challenges for modeling late-onset diseases in vitro. ALS patients affected by the familial forms carry the respective mutation since their birth. However, the symptoms will only emerge after the fifth or sixth life decade, which leads to the question of whether a couple of weeks in cell culture could recapitulate the patient's phenotype. In this regard, one could think about a long-term cell culture (year long), which is a laborious, difficult, and obviously time-consuming process, and thus incompatible for a model. Keeping in mind that a phenotype may arise only in specific circumstances, in order to challenge the cells, researchers appealed to metabolism and overall system stressors. The idea of such approach was to exacerbate a phenotype, aiming to check the model limits. Although some of the disturbed pathways may be restricted to a couple ALS forms, it has successfully identified a correcting component (Anacardiac Acid) for TDP-43 mutations carrying motor neurons [33].

Despite their use in many different laboratories around the world, pluripotent stem cell cultures are laborious and costly. The maintenance of these cells' pluripotency increases the costs in terms of reagents and personnel (hands-on), since cultures are especially vulnerable to small environment variations, such as pH changes or physical shock, which can trigger spontaneous differentiation processes. For this reason, depending on each laboratory's infrastructure, researchers may spend a lot of time performing procedures to avoid this spontaneous differentiation as well as optimizing experiments.

In terms of modeling MNDs in vitro, the production of iPS cells is only the first step, and it may take up to several weeks before it can be evaluated. The differentiation of pluripotent stem cells to human motor neurons is a low-efficiency, tedious, and time-consuming process that may take up to 2 months [44] depending on the required cell maturity for each read out. The current procedures for generating MNs involve embryoid body formation in serum-free media and subsequent neural rosette formation in the presence of retinoic acid (RA) and sonic hedgehog (SHH). Respectively, they work in the caudalization and ventralization of motor neuron progenitors that will later be used for the read-outs.

Although different cell markers can be used for motor neuron identification, the main genetic-engineered tool used in iPSC-derived motor neuron studies is based on the *HB9* gene. This is a homeobox gene that produces a transcription factor which plays an essential role in motor neuron differentiation. Since antibodies against this factor are available from a variety of companies, it has been used as one of the main marker for the motor neuron lineages—other markers include Chat, Islet-1/2, and Olig2. Additionally, another important tool in this area is the plasmid construction *HB9*::GFP, which allows the green fluorescent protein (GFP) to be expressed under the control of *HB9* promoter sequence; i.e., only those cells with a complete expression repertoire to express HB9 factor will be fluorescent under UV light. In practical aspects, these tools allow not only the motor neuron identification on mixed cell culture but also its separation with a (Fluorescence Assisted Cell Sorting) FACS approach, showing the importance of different expertise for a correct and better exploration of iPSC-based models.

As it happens in embryonic stem cells, iPSC present an infinite cell division capacity, turning it into an almost endless cell source. This is a special characteristic that is highly desirable for drug screening purposes. Employing this tool permits the evaluation of thousands of molecules in high-throughput screenings, generating the possibility of reaching distinct read-outs in parallel.

The possibility of generating any tissue from the three embryonic germ layers bestows great value upon iPS cells, especially when it comes to increasing model complexity. The analysis of neuronal and non-neuronal lineages gives the perspective of studying both cell autonomous and cell non-autonomous hypotheses under the same genetic background. Also, different cell lineages from the same individual can be generated and co-cultured (motor neurons—astrocytes, for example) in order to approximate the cellular model to a patient situation. Additionally, co-cultures make it possible to test whether a specific cell type ameliorates or damages the system.

## Conclusions and Final Remarks

Since the first publication in 2006, the generation of iPS cells became a huge player in many scientific journals, bringing the hope of answering not only many of the stem cell-related questions but also those pertaining to disease pathways and their modeling. As previously mentioned, the lack of success in translating ALS preclinical data into good clinical trials may rely on a limited source of models. In this regard, the reprogramming of adult cells to a pluripotent stage appears as a prominent tool to overcome those issues.

Despite the distinct cell lineages that were reprogrammed, there is no report of any ALS-related mutation that has inhibited or has interfered in cell reprogramming. That is also true for the motor neuron differentiation, although studies from Almeida et al. [36] showed that the *C9ORF72* expansion is unstable during both reprogramming and differentiation.

Regarding the experimental design, it is interesting to note that the approaches used to study each different gene were based on distinct hypotheses (gain-of-function; loss-of-function; and/or haploinsufficiency), and most of the research groups used cell survival as a main read out. Additionally, the lack of a phenotype on cells carrying SOD1 mutations stands out, and contrasts with other mutant lineages and even with cells obtained from sporadic patients.

In terms of phenotype, different publications show that motor neurons derived from ALS reprogrammed cells recapitulate the patients' condition, with the reduction of VAPB protein in the soluble fraction [10]; the presence of TDP-43 aggregates [29]; and the identification of RNA foci originated from *C9ORF72* expanded transcripts [24, 36].

The iPSC ALS field is still new, but many advances were obtained since the first publication arose in 2008 [28]. With little more than a dozen studies, it was possible to identify phenotype modulators that, although still requiring further tests, will open new perspectives in terms of treatment. Previously evaluated compounds like

Anacardic Acid and Digoxin, which had a positive effect on mutated cells, may be translated in a faster pace to clinical trials. Other effectors like shRNAs against *C9ORF72* expanded transcripts, which was shown to reduce cell death, require additional studies not only to support the finding in other systems but also to improve shRNA delivery when in a complex organism.

The versatility of producing different cell types and their analysis in co-cultures allows the production of high-throughput systems that will definitely accelerate the ALS search for effective compounds. In this regard, the establishment of an open-access mutation-defined fibroblast bank aiming the generation of iPS cells [45] will be crucial for neurological disease research. The possibility of studying different clones from distinct patients will circumvent the variability issues that are typical from ALS studies. As an open-access initiative, it will also allow research groups from distinct specialties and with diverse knowledge to reach this invaluable tool.

Ultimately, the use of iPSCs as a source for injection therapy is noteworthy. The ability to select the cells according to specific characteristics (ability to cross the blood–brain barrier; production of cell survival factors) in a scalable manner is of great interest for ALS purposes. The recent stem cell-based clinical trials approved for ALS therapy point to the probable direction that the field will follow: a first differentiation of the stem cell lines (pluripotent or multipotent) to a lineage that express certain markers, which allow their purification and expansion for a further application. Additionally, it is important to note that the strategies may not solely rely on a cell action, per se. Based on the recent studies, one cannot rule out the possibility of using stem cells or stem cell derived lines as a vehicle for effective components.

Since ALS, at this moment, is an incurable disease, the contribution from a variety of laboratories is essential to reach the so expected treatment that could stop the disease's progression, or even cure this devastating disease.

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# Chapter 4
# iPS Cells and Spinocerebellar Ataxia

**Carole Shum and Agnes Lumi Nishimura**

## Introduction

Spinocerebellar ataxia (SCA, OMIM 164400) is a heterogeneous group of hereditary and progressive neurodegenerative disorders associated with ataxia and cerebellar degeneration. Ataxia refers to lack of coordination caused by dysfunction of the cerebellum and its neuronal connections [1]. The most common symptoms are: ataxia of the limbs, head, trunk, dysarthria, and cerebellar oculomotor disorders. Differential diagnosis such as retinal degeneration is observed in SCA7, tau aggregation is seen in SCA11, dentate calcification is observed in SCA20, and azoospermia is found in SCA32 (Table 4.1).

A typical observation of SCAs is a meiotic instability resulting in an increase of number of repeats in successive generations. As a result, anticipation is often observed in these repeat expansion diseases, i.e., tendency of disease to become more severe and to have a progressively earlier age of onset (SCA2, SCA7, and SCA8).

The current classification is based on the mode of inheritance: autosomal dominant (most common form), autosomal recessive (rare) and X-linked (rare) (Table 4.1). There are more than 30 types of autosomal dominant SCAs, with several genes linking different pathways leading to variable cerebellar dysfunction and atrophy [1–3] (Fig. 4.1). SCAs are characterized by an increase of number of nucleotide repeats located in the intron or exon including: CAG·CTG repeats (SCA8); ATTCT repeats (SCA10); CAA/CAG repeats (SCA17); TGGAA repeats

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**Table 4.1** Molecular genetics findings of hereditary spinocerebellar ataxia

| SCA type | *Gene or locus* | Inheritance | Type of mutation | Average age of onset (years) | Other distinguishing features (all types show ataxia) | Reference |
|---|---|---|---|---|---|---|
| SCA1 | *ATXN1* | AD | (CAG)n repeat | Adulthood | Active reflexes | [41, 42] |
| SCA2 | *ATXN2* | AD | (CAG)n repeat expansion | Adulthood | Slow eye movements, sometimes dementia | [30] |
| SCA3 | *ATXN3* | AD | (CAG)n repeat expansion | Adulthood | Muscle weakness and atrophy. Originally called Machado–Joseph disease | [36] |
| SCA4 | *16q22.1* | AD | – | Adulthood | Sensory loss | [43–45] |
| SCA5 | *SPTBN2* | AD | Non-repeat mutations | Adulthood | Early age of onset and slow worsening of symptoms | [46] |
| SCA6 | *CACNA1A* | AD | (CAG)n repeat expansion | Adulthood | Very slow worsening of symptoms, pure cerebellar symptoms | [47] |
| SCA7 | *ATXN7* | AD | (CAG)n repeat expansion | Adulthood | Visual loss, childhood onset with anticipation | [39] |
| SCA8 | *ATXN8 / ATXN80S* | AD | bidirectional (CAG·CTG)n repeat | Adulthood | Active reflexes and decreased sensation | [48] |
| SCA10 | *ATXN10* | AD | (ATTCT)n repeat expansion | Adulthood | Occasional seizures, pure cerebellar symptoms | [49] |
| SCA11 | *TTBK2* | AD | Non-repeat mutations | Adulthood | Very slow worsening of symptoms, pure cerebellar symptoms | [50] |
| SCA12 | *PPP2R2B* | AD | (CAG)n repeat expansion | Adulthood | Tremor, sometimes dementia, pure cerebellar symptoms | [51] |
| SCA13 | *KCNC3* | AD | Non-repeat mutations | Childhood–adulthood | Mild mental retardation, short stature | [52] |
| SCA14 | *PRKCG* | AD | Non-repeat mutations | Adulthood | Body tremor (rare) | [53] |
| SCA15/16 | *ITPR1* | AD | Non-repeat mutations | Adulthood | Very slow worsening of symptoms, head tremor | [54–56] |

| | | | | | | |
|---|---|---|---|---|---|---|
| SCA17 | *TBP* | AD | (CAA/CAG)n repeat expansion | Adulthood | Worsening of mental abilities, pigmentary retinopathy | [57] |
| SCA18 | *7q22-q32* | AD | – | Childhood–early adulthood | Early sensory/motor | [58, 59] |
| SCA19 | *1p21-q21* | AD | – | Adulthood | Slow worsening of symptoms | [60–65] |
| SCA20 | *11q12* | AD | – | Adulthood | Early dysarthria | [66, 67] |
| SCA21 | *7p21.3-p15.1* | AD | – | Childhood–adulthood | Mild cognitive impairment | [68] |
| SCA22 | *1p21-q23* | AD | – | Adulthood | | [61, 65] |
| SCA23 | *PDYN* | AD | Non-repeat mutations | Adulthood | Slow worsening of symptoms | [69, 70] |
| SCA25 | *2p21-p13* | AD | – | Childhood–adulthood | Slow worsening of symptoms | [71] |
| SCA26 | *EEF2* | AD | Non-repeat mutations | Adulthood | | [72, 73] |
| SCA27 | *FGF14* | AD | Non-repeat mutations | Early childhood | Childhood onset with tremor (review Brusse, 2007) | [74] |
| SCA28 | *AFG3L2* | AD | Non-repeat mutations | Adulthood | Nystagmus | [75, 76] |
| SCA29 | *ITPR1* | AD | Non-repeat mutations | Early childhood | Learning deficits | [77, 78] |
| SCA30 | *4q34.3-q35.1* | AD | – | Adulthood | | [79] |
| SCA31 | *BEAN / TK2* | AD | (TGGAA)n repeat expansion | Adulthood | | [80] |
| SCA32 | *7q32-q33* | AD | – | Adulthood | Azoospermia | [81] |
| SCA35 | *TGM6* | AD | Non-repeat mutations | Adulthood | | [82, 83] |
| SCA36 | *NOP56* | AD | (GGCCTG)n repeat expansion | Adulthood | Muscle fasciculations, tongue atrophy, hyperreflexia | [84, 85] |
| DRPLA | *ATN1* | AD | (CAG)n repeat expansion | Adulthood | Chorea, seizures, dementia, myoclonus | [86] |

(continued)

**Table 4.1** (continued)

| SCA type | *Gene or locus* | Inheritance | Type of mutation | Average age of onset (years) | Other distinguishing features (all types show ataxia) | Reference |
|---|---|---|---|---|---|---|
| MTDPS7 | *C10ORF2* | AR | Non-repeat mutations | Childhood–adulthood | Amyotrophy deafness, mitochondrial DNA depletion syndrome | [87] |
| SCAR1/AOA2 | *SETX* | AR | Non-repeat mutations | Adolescence–adulthood | Oculomotor apraxia | [88] |
| SCAR3 | *6p23-p21* | AR | – | Early adulthood | Optic and cochlear degeneration leading to blindness and deafness | [89] |
| SCAR4 | *1p36* | AR | – | Adulthood | Saccadic intrusions | [90] |
| SCAR5/CAMOS | *ZNF592* | AR | Non-repeat mutations | Childhood | Mental retardation, optic atrophy, and skin abnormalities | [91] |
| SCAR6 | *20q11-q13* | AR | – | Early adulthood | | [92] |
| SCAN1 | *TDP1* | AR | Non-repeat mutations | | Axonal neuropathy | [93] |
| SCAX1 | *ATP2B3* | X-linked | – | Early childhood | Hypotonia at birth | [94] |
| SCAX2 | – | X-linked | – | Early childhood | Extrapyramidal involvement | [95] |
| SCAX3 | – | X-linked | – | Early childhood | Optic atrophy, deafness | [96] |
| SCAX4 | *xq26-qter* | X-linked | – | Early childhood | Tremor, pyramidal signs, dementia | [97] |

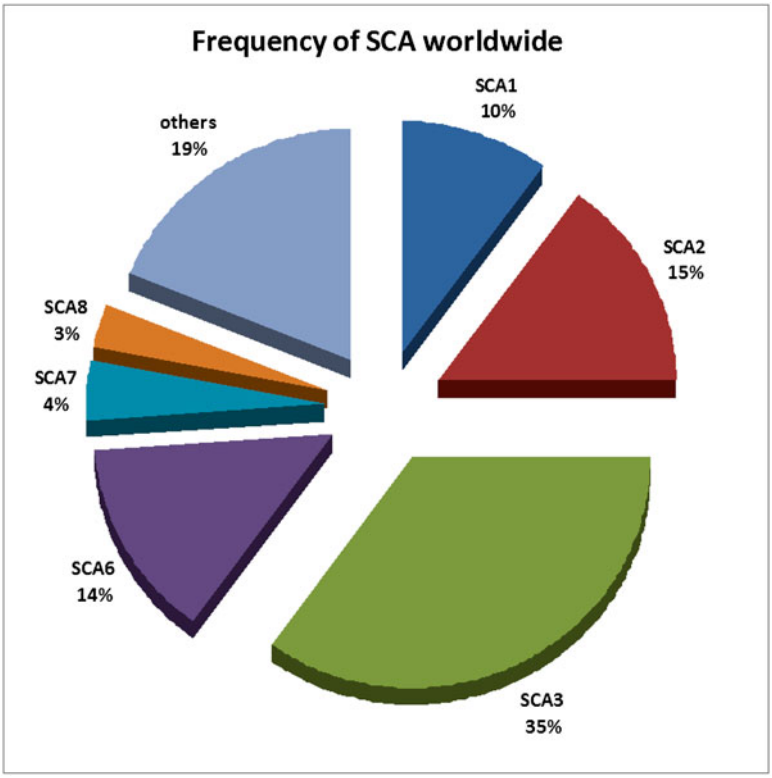


**Fig. 4.1** Frequency of SCA cases worldwide. *Source*: http://neuromuscular.wustl.edu/over/resource.htm

(SCA31); and GGCCTG repeats (SCA36). These nucleotide repeat disorders can be translated into peptides if localized in the exon (e.g., polyglutamine diseases) or the mRNA is transcribed, but is not translated if localized in the intron or untranslated regions (5′UTR or 3′UTR). SCA8 is an exception, in which the expansion is localized in the 3′UTR and peptide are bidirectionally expressed through a non-ATG start codon producing peptides in three coding frames [4]. Non-repeat mutations are also observed in SCAs including point mutations (SCA13, SCA27); frameshift mutations (SCA11); deletions (SCA15); and duplications (SCA20) (Fig. 4.2).

Polyglutamine diseases are a group of disorders characterized by a massive number of CAG repeats located in the coding region of the gene. It includes SCAs 1, 2, 3, 6, 7, 17, and DRPLA; and other diseases such as Huntington's disease and spinal bulbar muscular atrophy (Figs. 4.2 and 4.3). These CAG repeats are translated into multiple copies of glutamine, represented by the letter "Q" and are also known as polyQ tract. PolyQ repeat diseases are aggregation-prone and several other proteins

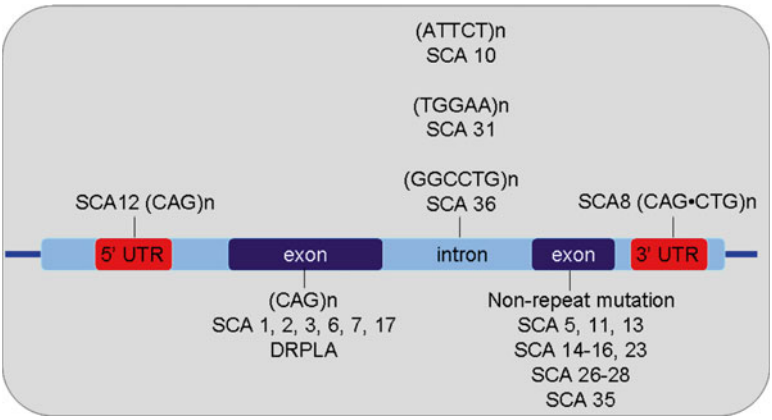


**Fig. 4.2** Schematic figure representing the localization of the nucleotide expansions or mutations in SCA genes

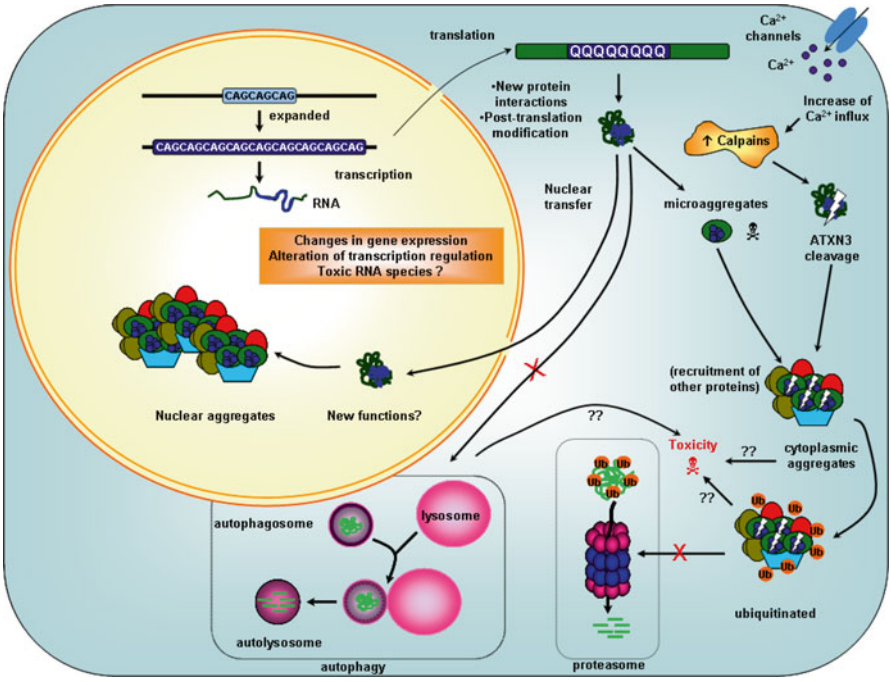


**Fig. 4.3** Schematic figure of SCA pathogenesis caused by polyglutamine expansions showing a cascade of events affecting neurodegeneration including transcriptional dysregulation, new protein interactions, cleavage of polyQ proteins probably by calpain/caspases, formation of micro and macroaggregates with recruitment of other proteins and transcription factors (nuclear aggregates) and alterations of proteasome and autophagy degradation pathways leading to toxicity and cell death

are recruited in these aggregates, including p62 and ubiquitin. Despite the efforts to understand the disease mechanism of polyQ repeat diseases, it is still unclear how and why these proteins are recruited into aggregates, how these repeats trigger the cell death pathway, and if the aggregates are the cause of consequence of neurodegeneration (Fig. 4.3).

## Modelling Spinocerebellar Ataxia Using Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) are an invaluable source of material to study a genetic disease in a petri dish. The breakthrough came in 2006 when Takahashi and Yamanaka isolated four transcriptional factors important for reprogramming skin cells into embryonic stem cells-like cells [5]. Since the development of this technique, alternative protocols have been developed to avoid the integration of the pluripotency factors in the host genome, such as the use of episomal vectors (Sendai vectors) [6], non-integrating viral vectors [7], the use of small molecules [8–10], direct transduction of proteins [11], and the use of microRNA to enhance iPSC derivation [12, 13]. In addition, different primary cellular sources have been tested successfully and it is possible to derive iPSCs from fibroblasts [14], peripheral blood [15], hair follicles [16], amniotic cells [17, 18], urine [19], and others [20–29].

Several genetic disorders have been modelled in vitro using iPSC technology and in this chapter we will focus on spinocerebellar ataxia diseases 2, 3, and 7.

### *SCA2*

Spinocerebellar ataxia type 2 is one of the most frequent SCAs worldwide, coming second after SCA3 (Fig. 4.1). It is caused by a trinucleotide repeat (CAG) in the coding region of *ataxin 2* (*ATXN2*) gene, located on chromosome 12 [30]. The normal CAG repeat length on healthy individuals ranges from 14 to 31 repeats. In general, 32–34 repeats lead to incomplete penetrance and more than 34 repeats cause full penetrance. Often, a CAA interruption is found between CAG repeats. In SCA2, the CAG repeat ranges from 33 to 77 and is unstable within families. Curiously, intermediate repeat lengths of 27–39 repeats, which are below the threshold required for formation of ataxin 2 inclusions are a risk factor for sporadic as well as familial forms of amyotrophic lateral sclerosis (ALS) [31].

*Ataxin 2* codifies for a protein of 150 kDa and is ubiquitously expressed in the brain (cerebellar Purkinje cells and substantia nigra) and systemic tissue. The ATXN2 is a cytoplasmic protein with unknown function; however, studies using mammalian cells, revealed that expanded polyQ repeats formed cytoplasmic microaggregates in the brain of SCA2 patients [32]. ATXN2 interacts with DDX6,

a component of P-bodies and regulates the expression of the poly (A)-binding protein (PABP), a stress granule component. Together, ATXN2 interferes with the assembly of stress granules and P-bodies formation, indicating a putative function on regulating and controlling mRNA degradation, stability, and translation [33].

The clinical symptoms of ATXN2 comprise a progressive cerebellar ataxia, including nystagmus, oculomotor dysfunction, ophthalmoparesis, or parkinsonism in some individuals [1]. Somatosensory deficits and late cognitive decline are also observed. MRI scans reveal atrophy of cerebellum, pons, medulla oblongata and spinal cord. Atrophy of cerebral frontal lobes, brainstem and cranial nerves is also observed. Neuronal loss is observed in the cerebral cortex, basal forebrain, basal ganglia, thalamus, midbrain, pons, medulla oblongata and cerebellum [1].

Ashizawa and colleagues in a brief report described the derivation of iPSC lines from one male patient with 20/44 CAG repeats and one control [34]. They showed that fibroblasts derived from a SCA2 patient showed a similar expression of ATXN2 when compared to controls. However, the levels were decreased in neural stem cells (NSCs) derived from the same patient, suggesting a specific downregulation of ATXN2 in neuronal lines. The expanded ATXN2 lines have a shorter life span compared to control neurons, although no ATXN2 inclusions were detected in the cells.

## *SCA3*

Spinocerebellar ataxia 3 is an autosomal dominant disease, also known as Machado–Joseph disease [35]. It is the most common form of ataxia worldwide and is caused by an expansion of a CAG repeat in the coding region of *ataxin 3* (*ATXN3*) gene, located on chromosome 14q21 [36] (Fig. 4.1). Repeats from 12 to 40 are normally found in healthy individuals, however in disease the number of repeats increase from 51 to 86. Anticipation is often observed in SCA3, especially when transmitted from father to daughter. Male parents transmit the mutant allele 73 % of the time.

SCA3 phenotype is heterogeneous and the symptoms include dysphagia, pyramidal and extrapyramidal signs, sensory deficits, peripheral neuropathy, amyotrophy, and in some cases parkinsonism. The brain pathology displays atrophy of the cerebellum and brainstem. Neuronal loss in the cerebral cortex, basal ganglia, thalamus, midbrain, pons, medulla oblongata, and cerebellum is also observed [1].

Ataxin 3 is also an aggregate-prone protein and the key pathological hallmark for SCA3 is the formation of insoluble aggregates in the neurons [37]. ATXN3 is expressed in the cytoplasm of neuronal and non-neuronal cells and regulates the stability of several proteins. In addition, it is involved in the ubiquitin-proteasome system, recycling ubiquitin that is bound to proteins targeted for degradation [37]. In disease, the long polyQ tract changes the conformation of the protein, recruiting ubiquitin and ATXN3, forming inclusions within the nucleus of the neuronal cells.

Brüstle and colleagues in an elegant study generated iPSCs from four SCA3 patients (two males and two females) with 73–74 CAG repeats and two healthy controls from the same family [38].

It is known that excitation-mediated depolarization of neurons results in increase of intracellular $Ca^{2+}$ influx from the extracellular space or from the reticulum endoplasmic compartment. For this reason, the authors hypothesized that temporary activation of $Ca^{2+}$-dependent proteases might trigger the formation of microaggregates as a seed for macroaggregate formation (Fig. 4.3). Neurons derived from iPSCs were stimulated with L-glutamate, an excitatory neurotransmitter that increases intracellular $Ca^{2+}$ levels, for 30 min inducing cleavage of ATXN3 in SCA3 and normal neurons. These cleaved products from SCA3 neurons were insoluble in SDS, suggesting they contain aggregates. In contrast, control neurons did not contain insoluble material. The SDS-insoluble aggregates are dose-dependent and repetitive excitation of L-glutamate (two stimuli of 30 min) and a waiting period of 24 h dramatically increased the formation of 25–45 kDa fragments and high molecular weight smear in SCA3 derived neurons, but not in controls. In addition, they observed that these SDS-insoluble products recruited other polyQ proteins, including the TATA binding protein (TBP), which has been previously found in ATXN3 aggregates in the brain of SCA patients. This phenotype is rescued after treatment with the calpain inhibitors ALLN and calpeptin. Inhibitors of other proteases had no influence on aggregate formation, suggesting that calpain-mediated cleavage of ATXN3 seems to be important for aggregate formation. The authors show that neurons generated from SCA3 patients recapitulate aspects of the disease, and inclusions were exclusively observed in neuronal cells and were absent in iPSCs, fibroblasts and glia. At this stage of neuronal maturity, no large inclusion bodies or macroaggregates associated with cytotoxicity were observed. The authors believe that these key features of disease progression are normally associated to late stages of SCA3 disease.

This study supports the approach to study disease mechanisms using iPSCs derived from patients with a genetic disorder and potentially the identification of drugs for future treatments for SCA3.

## *SCA7*

Spinocerebellar ataxia 7, also known as olivopontocerebellar atrophy, is characterized by neuronal degeneration in the cerebellar cortex, basal ganglia, thalamus, midbrain, pons, and inferior olives and is associated with visual loss caused by pigmentary macular degeneration. It was first classified as autosomal dominant cerebellar ataxia (ADCA) type II by [2].

SCA7 is caused by an expansion of a trinucleotide CAG repeat in the *ataxin 7* (*ATXN7*) gene, located on chromosome 3 [39]. The expansion is translated into glutamine and as SCA2 and SCA3 is a polyQ disease. Healthy individuals have less than 28 CAG repeats, whereas in disease it is found between 37 and >200. Intermediate range (28–36 repeats) is unstable and it is associated with incomplete penetrance. These intermediate repeats may expand in the successive generations, especially if transmitted by the father, due to a high gonadal instability and mosaicism.

ATXN7 is essential for retinal function and development and when expanded causes retinal degeneration and blindness. The disease onset is frequently observed in adulthood, but due to meiotic instability and anticipation it is also observed in young adults. The lifespan in these patients is shortened by aspiration pneumonia due to dysphagia [1].

Sun and collaborators described the generation of iPSCs from fibroblasts collected from a 56-year-old female patient diagnosed SCA7 with 45 CAG repeats. The authors describe that the iPSCs are capable of differentiating into neurons [40].

## Concluding Remarks

iPSCs hold promises for the understanding of mechanisms that underlie genetic disorders. It also opens a new field for regenerative medicine, including tissue engineering and therapeutic applications for genetic disorders. Modelling a disease in a "petri dish" allows the screening of hundreds of drugs and small molecules in a high-throughput fashion in order to identify a treatment and potentially a cure for such diseases.

However, further considerations are needed before embarking on the iPSC therapeutic crusade, such as: the generation of several lines from different patients with the same mutation to understand the mechanisms of diseases; the use of appropriate controls, similarities, and differences of gene expression of iPSCs generated from different primary cells, teratoma formation as a result of iPSCs transplantation, a consistent protocol to generate specialized differentiated cells and the most important: the mechanism of the disease in question.

Currently, a treatment using iPSCs is far from reality, especially for late-onset expansion diseases such as Huntington's disease and spinocerebellar ataxias. The initial symptoms of such disorders occur later in life, often characterized by a progressive lack of coordination of walking, and progressively impairment of hand movements and speech. These disorders develop slowly and gradually worsen over the years. It is known that misfolded proteins aggregate in SCAs and these proteins recruit other proteins essential for neuronal survival. Such events must occur early in life and build-up over the years and by the time the first symptoms are identifiable a treatment is virtually impossible. At the moment there is no cure for SCA.

Brain scans of SCA patients reveal degeneration and atrophy of neurons in the cerebellum and replacement of compromised neurons in the affected area is desirable. However, little is known how these disorders are triggered and how to prevent the neurons from dying. Differentiation of specialized neurons is difficult since "in vitro" cellular culture is composed of a mixed population of neurons. Identifying the correct type of functional cells and the number of neurons for transplantation is another challenge to overcome.

At the moment, genetic testing early in life would help to monitor the disease progression and potentially help to identify the moment in which the protein aggregates. However, such DNA testing is not recommended for children and young

adults, and the consequences of a positive result may bring important implications later in life, such as, career decisions, family planning, professional support, and even insurance coverage. Genetic counselling is an important aspect of any DNA testing and psychological support is essential for the individual to cope with a positive result.

Nevertheless, the use of iPSCs to model a disease is essential for the understanding of a disease mechanism and could bring essential cues for a potential drug therapy to slow down the disease progression.

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# Chapter 5
# Induced Pluripotent Stem Cells and Vascular Disease

**Sophia Kelaini, Amy Cochrane, and Andriana Margariti**

## What Are iPS Cells?

Induced pluripotent stem cells (iPS cells) are adult cells which have been reprogrammed to an embryonic-like state by forced over-expression of genetic factors important in the maintenance of Embryonic Stem Cells (ESCs). They are similar to ESCs in both morphology and phenotype, expressing stem cell markers and having the ability to generate all three germ layers [1]. iPS cells are very useful multi-purpose tools offering the potential for exciting possibilities in the field of regenerative medicine.

## Role of iPS Cells in Regenerative Medicine

Pluripotent stem cells like iPS cells could be customised to be patient-specific, avoiding, thus, problems arising due to tissue rejection. It would also circumvent the need for immunosuppressive drugs and their adverse side-effects in patients.

A particularly appealing aspect of using iPS cells is that these cells can be directed to differentiate into any cell lineage, paving the way for treatment of many types of diseases. The potential medical applications are numerous and range from treating many diseases, such as Alzheimer's or Parkinson's disease, cardiovascular disease and diabetes to cellular tissue regeneration [2, 3].

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In fact, iPS cell technology has already revolutionised the fields of regenerative medicine; for example, it has successfully treated sickle-cell anemia in a mouse model [4] and also provided scientists with powerful laboratory models for studying the manifestation of particular diseases. These include hepatic [5], neurological [6], endothelial [7] and cardiovascular [7].

In addition, iPS cells can be useful tools in drug development and also assist researchers in intervening and correcting the genetic defect at its root, before the onset of the disease.

## Advantages and Disadvantages of iPS Cells

### *Advantages*

iPS cells are remarkable research tools. They are similar to ESCs and can serve as models towards the understanding of the complex series of events during embryonic development or a certain disease by allowing researchers the detailed study of their mechanisms. One of the biggest advantages with using these cells is the avoidance of immune rejection, as they can be derived from a patient's own cells. Using iPS cells also avoids the ethical issues linked to the use of human embryos in medical research.

### *Disadvantages*

One of the main disadvantages in using iPS cells in cellular reprogramming and regenerative medicine is the fact that the process is generally slow and with low levels of efficiency [8]. The other major disadvantage is the tumourigenic potential of iPS cells [9]. Indeed, several in vitro studies have shown that the reprogramming process has the ability to produce genetic and epigenetic changes in iPS cells [10, 11]. Another problem with using iPS cells in the study of disease models stems from the fact that iPS cell lines are highly heterogeneous leading to intrinsic variability, and, thus, different observed phenotypes. It is, therefore, important to evaluate several cell lines from both the same patient and different patients.

## iPS Cells Generation

One of the most common methods of iPS cell generation is transduction of the reprogramming genes to the cells of interest using integrating retroviral or lentiviral vectors. The most widely accepted method of iPS cell generation involves the genetic transduction of a combination of reprogramming factors, namely Oct4, Sox2, Klf4 and c-Myc, (OSKM) using retroviral or lentiviral vectors (Fig. 5.1). It was first discovered by Takahashi and Yamanaka after screening of pre-selected

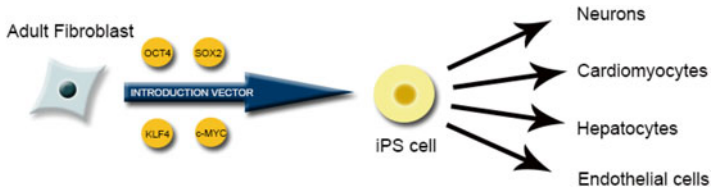


**Fig. 5.1** Classic example of iPS cell generation using the OCT4, SOX2, KLF4, and c-MYC transcription factors through an introduction vector, and subsequent differentiation to the desired cell lineage

factors in mouse embryonic fibroblasts (MEFs) [12]. This combination has been shown to work in other somatic cell types and different species too, including monkey [13] and human [14].

Later studies used different combinations of reprogramming factors such as Oct4, Sox2, Nanog and Lin28 [15] while more recent studies have used even fewer factors; in neural stem cells, expression of only one factor (Oct4) was shown to be sufficient to induce pluripotency [16].

The above approaches, however, present an obstacle towards the clinical translation of iPS cells due to their potential tumorigenicity. Studies have tried to address this problem using either minimal genetic modifications [17] or the use of non-integrating vectors [18]. Non-integrating vectors, either viral or non-viral, that have been used successfully to generate iPS cells include adenoviruses, the Sendai virus, expression plasmids, minicircle vectors, and liposomal magnetofection [19].

Protein transduction of OSKM is another promising alternative approach [20]. In addition, reprogramming of iPS cells and differentiation to the desired cell type has also been made possible with the use of microRNAs (miRNAs), which are small non-coding RNAs that can regulate gene transcription [21, 22].

The chemical approach of using small molecules to enhance reprogramming efficiencies or even replace certain reprogramming factors is also among the methods that may offer an alternative solution. Some DNA methyltransferase inhibitors, histone deacetylase inhibitors (valproic acid) which modulate chromatin modifications have been reported to enhance the reprogramming process [23–26].

Other methods involve the generation of iPS cells using episomal vectors from a variety of cells such as fibroblasts or bone marrow mononuclear cells. They are introduced into the system by electroporation, providing a transgene-free, virus-free iPS cell generation [27].

## Regenerative Medicine and Vascular Disease

Regenerative medicine methodologies that aim to recover cardiac and vascular function are being increasingly explored as management approaches for vascular and cardiovascular diseases. However, one of the biggest obstacles towards applying such therapeutic approaches is the reduced availability of suitable cells needed for clinical

purposes. For example, in the field of cardiac disease, which is a leading cause of mortality and morbidity worldwide [28, 29], a very large number of healthy cells would be required for use in a clinical setting. Since cell regeneration is quite limited in the adult heart [30], urgent development of fast and robust new therapies that will produce clinical-grade cells, suitable for disease modelling, tissue engineering, and cell replacement treatments is imperative. Endothelial cells (ECs) play a very crucial role during the development of vascular and cardiovascular disease. Although just a thin cellular monolayer lining the inner walls of blood and lymphatic vessels, the healthy endothelium is implicated in a wide range of factors. It is central to cardiovascular homeostasis [31], as well as in regulating vascular tone and, thus, blood pressure. It also plays a crucial role in cell adhesion, fluid filtration, smooth muscle cell proliferation and vessel wall inflammation. ECs also play an important role acting as barrier between a vessel's lumen and surrounding tissues, as well as in blood clotting (thrombosis/fibrinolysis) and repair of damaged vascular cells [32, 33]. It is, thus, important to note their key role while researching potential solutions in vascular diseases.

## What Is Vascular Disease?

Vascular disease is an abnormality of the blood vessels and involves a narrowing of a vessel's diameter leading to obstruction of normal blood flow. It is caused by atherosclerosis, which can block arteries in critical parts of the body. Atherosclerosis is a disease, which causes plaque build-up in the inner walls of an artery (Fig. 5.2).

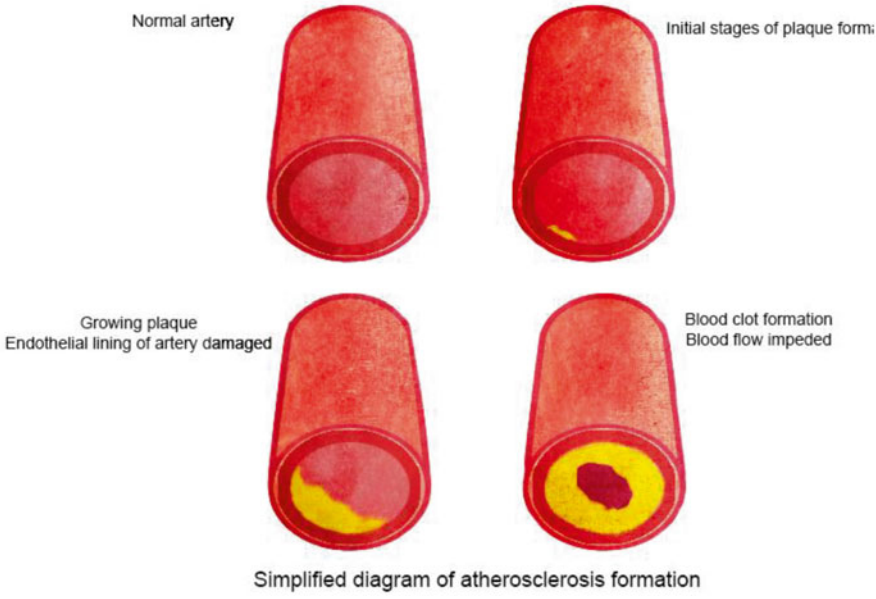


**Fig. 5.2** Simplified diagram of atherosclerosis formation

Plaque is a mixture of fat deposits, cholesterol, calcium and other cellular debris and it can lead to serious problems that include heat attack, stroke and, on certain occasions, even death. It is particularly dangerous as, many times, there are no symptoms preluding the onset of a critical episode.

It is now commonly accepted that at the heart of vascular disease lies endothelial cell dysfunction. The vascular endothelium is not just a simple barrier between intravascular and interstitial compartments, it is also responsible for the regulation of hemodynamics, the angiogenic remodelling of vessels, as well as a plethora of metabolic, anti-inflammatory, and antithrombogenic processes [34].

## Types of Vascular Diseases

There are various types of diseases involving the blood vessels. These include the following:

### *Peripheral Arterial Disease*

Peripheral arterial disease (PAD), also called peripheral vascular disease (PVD) or peripheral artery occlusive disease (PAOD), is a condition in which the fatty deposits build up (plaque) in the outer arteries of the body (arms, legs) cause a narrowing of the artery wall, decreasing blood flow and supply.

*Symptoms*: The symptoms of PAD include pain, numbness fatigue, and muscle discomfort in the lower limbs. They may appear slowly but can increase in frequency over time. In severe cases, PAD symptoms may lead to night cramps, feet and toes tingling, dark and blue skin appearance (cyanosis), non-healing sores and hair loss in the affected area.

*Risk Factors*: The disease most commonly affects men over 50 years of age but it can also affect women. Smoking is the main risk factor, while other factors such as age, abnormal cholesterol, increased blood pressure and having a history of certain diseases such as diabetes, heart and kidney or cerebrovascular disease (strokes) also increase the risk. PAD also increases the risk of coronary heart disease, strokes, and heart attacks.

*Detection*: In an examination, indicative findings of PAD may include decreased blood pressure or weak/absent pulse in the affected limb and calf muscle atrophy. Blood tests may show diabetes or high cholesterol. Other tests include arteriography on the legs, ankle/brachial index (ABI), Doppler ultrasound and Magnetic resonance angiography (MRA).

*Treatment and Management*: Giving up smoking and balancing exercise with rest can help alleviate the symptoms by improving blood circulation. Weight loss in cases of obese patients and lowering of cholesterol may also prove beneficial. Keeping blood sugar under control is also essential in cases of diabetes. Medicines

prescribed by the doctor for controlling the disorder include aspirin, and other anti-coagulants such as clopidogrel for preventing blood clot formation. Artery-dilating drugs such as cilostazol may also be prescribed in more severe cases when surgery is not an option. Anti-cholesterol drugs may also be prescribed. As a last option, surgery may also be performed and may involve artery angioplasty and stent placement or peripheral artery bypass.

*Outlook*: Very rarely the limb may need to be amputated, especially in cases involving gangrene development. In most cases, however, PAD can be sufficiently controlled without the need for surgery [35].

## *Aneurysm*

An aneurysm is a swelling that resembles a balloon-like structure in a vessel. It can grow large and eventually rupture or dissect. If any of these occur, the outcome is usually fatal. Aortic aneurysmal disease was previously believed to be a form of atherosclerosis, but is now recognised as degenerative process involving all layers in a vessel wall. Its pathophysiology mainly involves four events: lymphocytes and macrophages infiltration of the vessel wall; collagen and elastin degradation in the media and adventitia; loss of smooth muscle cells; and neovascularization [34].

There are three main types of aneurysms: aortic, cerebral and peripheral aneurysms.

Aortic aneurysm is sub-classified into thoracic aortic aneurysm (TAA) and abdominal aortic aneurysm (AAA):

TAA occurs in the aorta running through the chest (Thorax), in which the arterial walls close to the heart weaken leading to improper heart valve closure and subsequent blood leakage back into the heart.

AAA occurs in the aorta that runs through the abdominal area and is located between the diaphragm and the aortic bifurcation. It is a full-thickness dilatation on a part of the vessel that exceeds the normal vessel diameter by 50 %. Typically though, an aneurysm diameter of 3.0 cm is usually regarded as the threshold. When identified, these aneurysms are typically monitored for expansion. The growth rate can vary depending on the individual. It is usually characterised by progressive expansion, with some remaining stable for years, while others may grow rapidly. The most common predictor of AAA rapture is the aneurysm's size. Most of them are asymptomatic until they rapture and they can be often lethal. Therefore, the main goal is to be able to identify them and treat them before the point of rapture. Aneurysms are classified as suprarenal if they involve at least one visceral artery, pararenal if they involve the origins of renal arteries, and infrarenal if they begin beyond the renal arteries. Key risk factors for AAA include ageing, male gender, and family history. In men aged 50 or over and women 60–70 or over, the incidence of AAA increases significantly with each passing decade [34]. Other risk factors for AAA include smoking, hypertension, increased cholesterol, obesity and atherosclerotic occlusive disease [34]. Aneurysms are commonly discovered during routine abdominal examinations. However, ultrasonography is the principal method of screening with a very high

sensitivity and specificity [34]. Treatment usually involves risk factor modification such as smoking cessation or control of co-existing conditions that contribute to the risk with the use of medication (for example, statins or antihypertensive agents).

Cerebral or intracranial aneurysm is a cerebrovascular disorder which occurs in an artery of the brain. If rapture occurs, blood leaks into the area around the brain (subarachnoid haemorrhage). Aneurysms are classified as saccular, fusiform and microaneurysms.

Saccular (berry) aneurysms are the most common and appear as a round outpouching. They are almost always the result of an inherited blood vessel weakness and usually occur within the arteries of the Circle of Willis. Fusiform ones usually appear in an arterial segment around the entire vessel rather than just one side of the vessel wall. Microaneurysms (or Charcot-Bouchard aneurysms) occur in small blood vessels. The vessels most commonly affected in this type of aneurysm are the lenticulostriate vessels in the basal ganglia. Small aneurysms are relatively symptomless but if they rapture, they may cause an intracerebral haemorrhage. Larger aneurysms also produce no symptoms, but on occasions a person may experience sudden and severe headaches, nausea, sight impairment and unconsciousness prior to the rapture. If rapture occurs, blood leaks into the area around the brain (subarachnoid haemorrhage). Risk factors include lifestyle-originating diseases such as smoking, excess alcohol consumption, obesity and hypertension. Trauma to the head or infections may also contribute to the development of an aneurysm. Genetic conditions have also been linked to increased risk. They include autosomal dominant polycystic kidney disease, neurofibromatosis type I, Marfan syndrome, pseudoxanthoma elasticum, hereditary hemorrhagic telangiectasia, Ehlers–Danlos syndrome type II and IV and multiple endocrine neoplasia type I [36]. Once suspected, brain aneurysms can be diagnosed with medical tests such as angiography, magnetic resonance imaging and CT scans. Emergency treatment after rapture generally involves improving respiration and reducing intracranial pressure. This is achieved through surgical clipping or endovascular coiling [37, 38].

Peripheral aneurysms occur in areas other than the chest and brain. They most commonly develop in the popliteal artery in the lower part of the thigh and knee but they can also occur in the femoral and carotid arteries or arteries in the arm. As with other types of aneurysms, peripheral ones have common risk factors such as obesity, smoking, high cholesterol and high blood pressure, as well as family history of heart disease. Some of the symptoms include a throbbing lump in the affected limb, claudication (cramping), numbness and pain. Diagnostic tools include CT scans, MRI and ultrasound while treatment may require thrombolytic therapy or surgical repair [39].

## *Renal Failure*

Renal failure (kidney failure or renal insufficiency) is a medical condition that affects the function of the kidneys, which receive their blood supply from the aorta through the renal arteries. Kidneys are particularly sensitive to any decrease in blood flow, and, thus, a narrowing of the renal arteries due to plaque build-up, can

lead to serious complications. One of the main functions of the kidneys is eliminating waste products generated as a result of the body's metabolism, extracting them from the blood and sending them to the bladder through the ureter. Urea is one of the major waste products. In renal failure occurring as a result of vascular disease, the kidneys fail to adequately filter these waste products. Renal failure, which has five stages (number 5 being the most severe), is determined by the decrease in glomerular filtration rate, the rate of blood filtration in the renal glomeruli. It is usually detected by a decrease or non-passage of urine or accumulation of waste products, like creatinine or urea, in the blood [40].

There are two types: acute kidney injury, which is usually reversible and chronic kidney disease, which is usually not reversible and there may be an underlying cause. In acute failure, there is a rapid loss of renal function, which is accompanied by oliguria (decreased urine production) as well as an electrolyte imbalance. It can be the result of a number of causes, which are generally classified as prerenal, intrinsic and postrenal. Chronic renal disease may have numerous causes, the most common being diabetes mellitus and long-term hypertension. Overuse of common drugs such as aspirin and paracetamol may also lead to chronic renal disease [41].

Renal disease symptoms may include nausea and vomiting, weight loss, blood in the urine (uremia), and changes in the frequency of urination (more or less frequent) due to the high urea levels in the blood. Other symptoms caused by build-up of inadequately filtered phosphates in the blood may include bone damage and muscle cramps [42]. Build-up of potassium blood levels may lead to hyperkalaemia and abnormal heart rhythm or muscle [43] paralysis. Other symptoms include pain, swelling, polycystic kidney disease or anaemia with resulting fatigue and dizziness.

Treatment options for renal failure mainly involve dialysis to remove waste products and excess fluid from the blood. Transplantation is also another option; however sometimes health issues may prevent taking this route.

## *Diabetic Vascular Disease*

Diabetic Vascular Disease refers to artery blockages throughout the body because of diabetes. In diabetes, blood sugar levels are elevated due to the body's inability to either produce insulin or to use it effectively. The majority of patients with diabetes exhibit abnormalities of endothelial function and vascular regulation. The factors involved in diabetic endothelial dysfunction are numerous but a key final common pathway is the deregulation of nitric oxide (NO) bioavailability. NO is a key stimulus for vasodilation and also inhibits vascular smooth muscle proliferation migration or proliferation. It also limits activation of platelets. Hyperglycemia inhibits the function of eNOS in endothelial cells and increases reactive oxygen species (ROS) production. In addition, insulin resistance may also contribute to loss of normal NO homeostasis [44]. The sum effect of the deregulation of these mechanisms and of endothelial cell dysfunction increases the inflammatory state of the vessel wall.

This process is accompanied by increased leukocyte chemotaxis, adhesion and transformation into foam cells, which is an early precursor of atheroma formation [45]. Apart from changes in pathways involving endothelial cells, diabetes also stimulates pro-atherogenic mechanisms in vascular smooth muscle cells in a similar fashion [46].

Symptoms include blurred vision, limb swelling, foot sores, pain and high blood pressure. Initial assessment in patients with diabetes begins with a thorough medical history and examination [47]. Other than the standard glucose tests, the physician, depending on the affected organ, may order tests to determine and monitor the function of, for instance, blood vessels, eyes and kidneys. The final diagnosis and treatment will usually require the collaboration of physicians from different fields.

## *The Need for Novel Therapies*

Everyone is at risk of developing vascular disease, with millions of people around the world suffering from adverse complications related to it, which are, in many cases, lethal. Vascular disease ranges from diseases affecting the arteries, veins and lymphatic vessels to disorders that affect blood circulation causing ischemia.

Vascular disease is one of the leading causes of death in the western world and results from the monolayer of cells lining the vessels, endothelial cells, becoming dysfunctional. This results in the downstream effects of disease such as atherosclerosis. The repair and regeneration of these cells has therefore been the focus of research for many years however to date still faces many barriers. In recent years, there has been great advancement in the generation of iPS cells and their ability to differentiate towards a specific lineage. In terms of vascular disease, the research is aimed towards the generation of functional vascular cells with the goal of regeneration of the vascular tissue as well as personalised medicine via the use of autologous tissue. However, the underlying mechanisms and signalling pathways that are involved in the differentiation process to produce optimal endothelial cells are generally unknown.

## *Potential for iPS Cells to Differentiate Towards Vascular Cells*

Recent ability to derive vascular cells through reprogramming from iPS cells holds huge therapeutic potential for personalised medicine and vascular cell therapy. Stem cells are intricately coupled with their extracellular surroundings; therefore any range of extrinsic signals that causes change to their environment have a direct effect on their subsequent response, such as remaining in the same state or inducing differentiation towards a specific cell within the three germ layers. For example, cells can remain in a pluripotent state by being cultured in conditions that block reprogramming such as leukaemia inhibitory factor (LIF) [48].

Similarly, adding a dynamic array of factors and signals that mimic the elements seen during organogenesis in development can induce the pluripotent cell to differentiate into the desired specific cell line [49, 50].

One of the many advantages of using iPS cells is the exciting idea of personalised medicine through the use of autologous tissue. For example, the generation of vascular cells from the patient's own cells overcomes the limitations, such as tissue rejection [51], seen in embryonic stem cells.

Cardiovascular disease is one of the leading causes of death in the western world and current therapy is limited. The generation of vascular cells from iPS cells offers a new window for this research that will overcome these limitations. The ability to generate vascular cells from iPS cells allows close study and better understanding of the generally unknown underlying mechanisms in vascular differentiation. Elucidating these mechanisms will result in the generation of efficient protocols for the development of functional vascular cells for therapy.

## *Mechanisms Involved in iPS-Derived Differentiated Vascular Cells*

It is widely known and practiced that vascular cells can be generated from iPS cells; however the underlying mechanisms involved are poorly understood. Signalling pathways involved in vasculogenesis/angiogenesis (Fig. 5.3) and defects in vascular remodelling are seen in pathways such as Notch, Wnt, VEGF, TGFβ [52–56] and mutations in these pathways respectively. These pathways work independently and also simultaneously with each other. Vasculogenesis occurs almost exclusively during embryogenesis as it is the generation of vessels with no pre-existing vessel. The mesodermal differentiates into hemangioblasts which aggregate and form blood islands consisting of endothelial precursor cells (EPCs) and hematopoietic stem cells (HSCs).

## Notch Signalling

These blood islands fuse and become primitive capillary plexus which sends signals to recruit more cells and also for progenitor mural cells to differentiate in order to remodel and develop a mature blood vessel [52, 53, 57] (Fig. 5.3). Angiogenesis will occur after a stimulus such as tissue wounds, inflammation or pathogenic responses such as vascular supply to tumors. These stimuli create a hypoxic environment which in turn results in the production of growth factors such as vascular endothelial growth factor (VEGF). This causes the basement membrane to become disrupted and upregulation of a member of the NOTCH pathway,

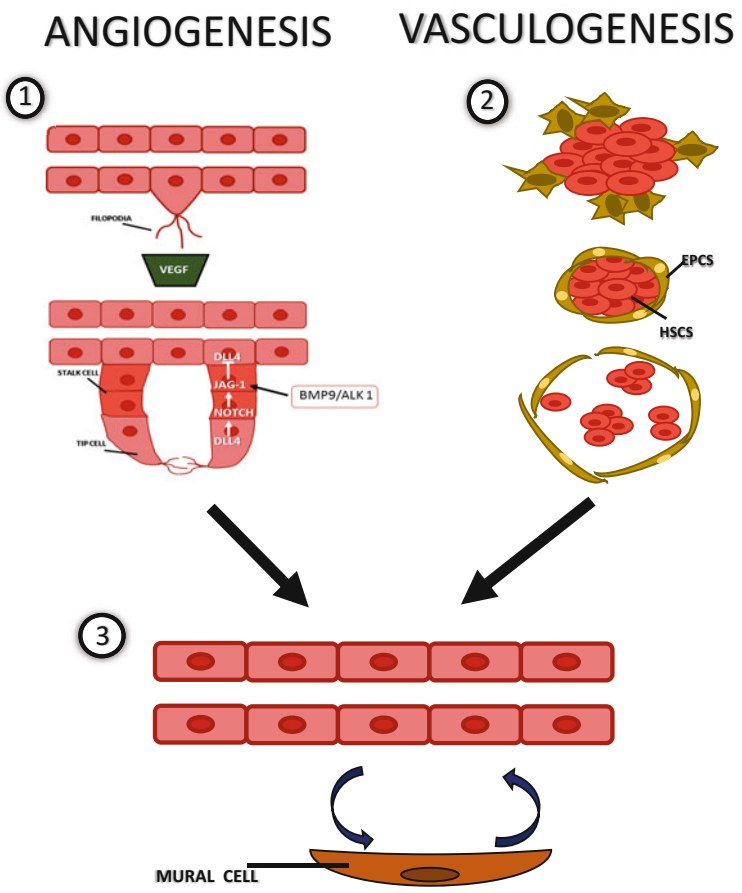


**Fig. 5.3** Diagram showing the stages of angiogenesis [1] and vasculogenesis [2] and the recruitment of mural cells to nascent vessel [3]

delta like ligand—1 (DLL-4) in ECs and causing it to adapt the morphology of a "tip cell." VEGF receptor A (VEGFRA) becomes upregulated and drives the tip cell towards the VEGF stimulus using membrane extensions called filopodia. However, these tip cells do not divide, it is the preceding cells known as "stalk cells" that proliferate and form the new vessel wall. In these stalk cells, DLL4 upregulates NOTCH signalling which aids in proliferation (working alongside Wnt signalling). At the same time, DLL4 upregulates NOTCH receptor jagged-1 which inhibits activation of DLL4 in adjacent cells therefore stopping these cells becoming tip cells which regulates and controls angiogenesis.

### *MicroRNA 199b*

Other factors can also influence the outcome of these pathways, for example, Micro RNA 199b has been shown to modulate vascular cell fate through targeting and suppressing the expression of Jag-1 which then in turn activates STAT3 expression which binds to the promoter of VEGF and results in induction of EC differentiation [58].

## TGFB Signalling

TGFβ signalling can work as pro- or anti-angiogenic in many ways. Here Bone morphogenic protein 9 (BMP9) and activin-like receptor-like kinase 1 (ALK1), members of the TGFβ pathway are seen here to help regulate angiogenesis by regulating jagged-1 expression [52, 53, 59, 60] (Fig. 5.4). TGFβ/BMP work in a sensitive dose-dependant manner which regulate their effect on angiogenesis (Fig. 5.4). TGFβ binds to TGFBR2 recruiting TGFBR1 (ALK-5) and ACVRL1 (ALK-1). TGFB is pro-angiogenic at low doses (parallel to a high concentration of BMP9) causing high binding to ALK-1 receptor and the downstream effects of this results in EC proliferation and migration needed for angiogenesis to occur. The opposite dose of these factors causes this to be inhibited through binding of ALK-5 and the associated downstream responses [52, 59, 60]. Micro RNA 27 has been shown to

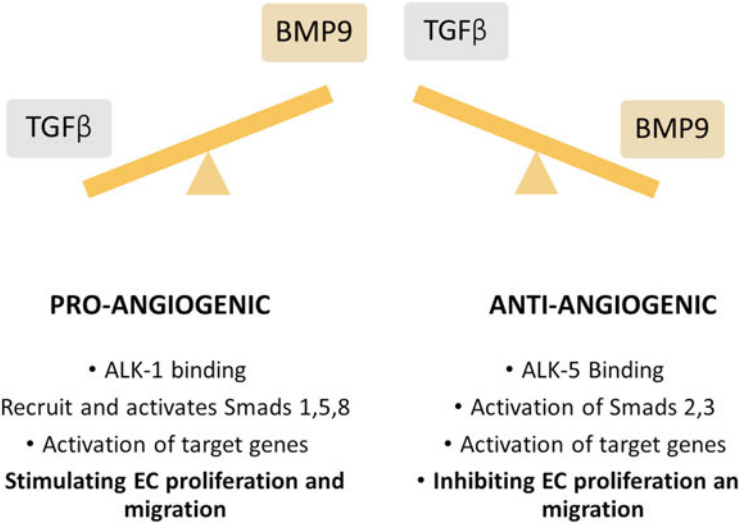


**Fig. 5.4** Diagram illustrating how TGFB family affects angiogenesis in a dose-dependant manner

effect the expression of EC markers as when it is overexpressed there is an increase in the EC marker presence and this is due to an increase in the TGFBR2 [61].

Eventually sprouting tip cells will anastamose forming a new vessel. These ECs require support and structure from mural cells (pericytes and vascular smooth muscle cells, vSMCs) which will also help the vessel with vasoconstriction and dilation. ECs secret platelet-derived growth factor (PDGF) recruits the mural cells and in a reciprocal signalling mechanism, these mural cells secret VEGF [52, 53, 62–65]. Contrastingly, mural cells will then maintain vessel stability through Angiotensin—Tie2 signalling [59, 60].

## Wnt Signalling

VEGF, NOTCH and TGFβ signalling are some of the most prevalent signalling pathways involved in vascular cell development and maintenance. There are however many other signalling pathways that interact and also work independently to obtain the same outcome. Wnt signalling is involved in many cellular processes such as proliferation and maintenance of stem cells in the undifferentiated state. Canonical Wnt signalling has been shown to regulate VEGFA expression through β-catenin expression [52, 66, 67]. β-catenin expression has been seen to increase during proliferating vessels stimulating the VEGFA promoter and therefore angiogenesis [67]. Tight regulation of β-catenin is therefore required to regulate angiogenesis. Histone deacetylase 7 (HDAC7) has been shown to interact with β-catenin reducing the expression and therefore keeping ECs in a low proliferative state [68].

Studying these pathways in detail allows us to elucidate the key factors necessary for differentiating successfully and efficiently iPS cells to functional vascular cells for future developments in therapy.

## RNA Binding Proteins

There are also many correlating genes in vascular generation that can be studied in detail to understand the mechanisms. For example, the quaking gene (QKI) has been shown to have a major role in vascular development. QKI belongs to the family of highly conserved RNA binding proteins called STAR (Signal Transduction and Activation of RNA). It is a pre-transcription regulator, meaning it controls aspects such as pre-mRNA splicing, mRNA stability and protein translation [7, 56, 69–73]. QKI was originally associated and defined for its involvement in myelinisation and oligodendrocyte differentiation [7, 70, 74–76]; however, more recently it has been discovered for its involvement in vascular development [7, 56, 71, 72, 76] prior to the start of myelination, this is seen clearly in vivo where qki null mice were embryonic lethal between E9.5 and E10.5 due to a failure of blood circulation in the yolk sac [7, 56, 69, 72].

QKI has been shown to have a key involvement in embryonic blood vessel formation and remodelling. It is the preliminary defects in the provascular endothelium that cause the lethal vascular defects. During normal vasculogenesis development the endoderm and mesoderm interact producing signals causing the cells to differentiate to endothelium and some erythrocytes which form blood islands. These blood islands fuse and become primitive capillary plexus which sends signals to recruit more cells and also for progenitor mural cells to differentiate in order to remodel and develop a mature blood vessel. QKI is expressed in the endoderm layer regulating its function and when this is not present it causes these series of vascular differentiation events to become dysfunctional. The cells are unable to differentiate to mature vascular smooth muscle cells and it is this perturbed investment of mural cell to the nascent vessels that causes the yolk sac vasculature to become unstable and inhibit the essential remodelling required for progression of development resulting in embryonic death [7, 69, 71].

## Chromatin Remodelling Mediators

A gene defined as "similar to SET translocation protein" (SETSIP) has been discovered to be expressed in parallel with endothelial markers via microarray analysis. The SET protein is involved in essential cell processes such as chromatin remodelling, differentiation [77], apoptosis and cell cycle progression [78]. Several transcript variants encoding different isoforms have been found for this gene. SET protein is part of a complex localised to the endoplasmic reticulum but is also found in the nucleus [79]. Indeed, depletion of SET by RNA interference (RNAi) delays transcription, suggesting a positive role in transcription [80]. Overexpression of SETSIP resulted in a correlating increase in EC markers and contrastingly a decrease in expression when it is knocked out; therefore there is a strong connection with SETSIP and the regulation of endothelial differentiation from pluripotent cells. In particular, luciferase assays have shown that SETSIP translocates to the nucleus and binds to the promoter of the endothelial structural marker VE-cadherin, which is an essential molecule in maintaining EC structure and integrity. Further studies also showed that SETSIP expression was induced by VEGF [81].

At the moment, the potential of the iPS cells to differentiate towards therapeutic cells is only based on directed empiricism, while they are totally dependent on combinations of growth factors, media, and matrices to favour the desired lineage. In regards to vascular regeneration, it is important to understand the key regulatory pathways such as epigenetic alterations, transcriptional activity and RNA binding patterns associated with the differentiation processes. Only then, fully defined experimental protocols could reproducibly guide iPS cells to a vascular lineage [82, 83] and enable clinical application [84–86].

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# Chapter 6
# iPS Cells and Cardiomyopathies

**Hiroko Nakahama and Elisa Di Pasquale**

## Abbreviations

| | |
|---|---|
| AAVS1 | Adeno-associated virus integration site 1 |
| AFM | Atomic force microscopy |
| ANF | Atrial natriuretic factor |
| AP | Action potential |
| APD | Action potential duration |
| ARVD/C | Arrhythmogenic right ventricular dysplasia/cardiomyopathy |
| bFGF | Basic fibroblast growth factor |
| BMP2 | Bone morphogenic protein 2 |
| bpm | Beats per minute |
| BTHS | Barth syndrome |
| CACNA1C | Calcium Channel, Voltage-Dependent, L Type, Alpha 1C Subunit |
| CASQ2 | Calsequestrin gene |
| CMs | Cardiomyocytes |
| CNX43 | Connexin 43 |
| CPVT | Catecholaminergic polymorphic ventricular tachycardia |
| CRISPR | Clustered regularly inter-spaced short palindromic repeats |
| CsA | Cyclosporin A |
| DADs | Delayed afterdepolarizations |
| DCM | Dilated cardiomyopathy |
| DES | Desmin gene |
| Dkk-1 | Dickkopf-related protein |
| DSBs | Double-strand DNA cleavage |
| EADs | Early-afterdepolarization |

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| | |
|---|---|
| EBs | Embryoid bodies |
| END2 | Visceral endoderm-like cells |
| ESCs | Embryonic stem cells |
| ET-1 | Endothelin-1 |
| FPD | Field potential duration |
| FRDA | Friedreich ataxia |
| FXN | Fataxin gene |
| GAA | Acid-α-glucosidase |
| GATA4 | GATA binding protein 4 |
| HCM | Hypertrophic cardiomyopathy |
| HDAd | Helper-dependent adenoviral vectors |
| HR | Homologous Recombination |
| IFNβ1 | Interferon beta 1 |
| iPSC | Induced pluripotent stem cells |
| iPSC-CMs | iPSC-derived cardiomyocytes |
| KCNH2 | Potassium voltage-gated channel, subfamily H (Eag-related), member 2 |
| KCNJ2 | Potassium inwardly rectifying channel, subfamily J, Member 2 |
| KCNQ1 | Potassium voltage-gated channel, KQT-like subfamily, member 1 |
| LAMP | Lysosome-associated membrane protein |
| LQT | Long QT syndrome |
| MEA | Multi-electrode array |
| MEF2C | Myocyte enhancer factor 2C |
| MHY7 | Beta-myosin heavy chain gene |
| MYL2 | Myosin, light chain 2, regulatory, cardiac, slow |
| NE | Norepinephrine |
| NFAT | Nuclear factor of activated T cells |
| NHEJ | Nonhomologous end joining |
| PDTC | Pyrrolidine dithiocarbamate |
| Pkp2 | Plakophilin-2 gene |
| PPARγ | Peroxisome proliferator-activated receptor-gamma |
| PTPN11 | Protein tyrosine phosphatase, non-receptor type 11 |
| RCM | Restrictive cardiomyopathy |
| RNAi | RNA interference |
| Ros | Roscovitine |
| RYR2 | Ryanodine receptor gene |
| SCN5A | Sodium channel, voltage-gated, type V, alpha subunit |
| Serca2a | Sarcoplasmic reticulum $Ca^{2+}$ ATPase |
| sgRNA | Single chimeric guide RNA |
| ssODNs | Single-strand DNA oligonucleotides |
| TALEN | Transcription activator-like effector nuclease |
| TAZ | Tafazzin |
| TGFβ | Transforming growth factor beta |
| TNNT2 | Cardiac troponin T gene |
| VEGF | Vascular endothelial growth factor |
| ZFN | Zinc finger nucleases |

## Part I: Introduction

Most of the knowledge we have gained thus far on the physiological function of a gene comes from in vivo studies on transgenic or knockout models, in particular mouse models, or from heterologous expression systems in vitro. Although genetically modified mice have been crucial to elucidate genetic mechanisms of cardiac diseases and to test novel therapeutic treatments, numerous human phenotypes fail to be successfully replicated in mice often because of biological differences existing between the two species. Human heart function is extremely different from that of the mouse mainly because of the diverse electrophysiological properties of the CMs among species (i.e., ion channel expression pattern and their gating properties are different). These two species show unique ECG profiles, with a QT interval (measure of the time between ventricular depolarization and repolarization) five times longer in human than in mice (400 ms vs. 50–100 ms, respectively) and different duration and shape of the action potential (AP). These differences reflect distinctive ion channels expression and gating properties in the two species. The major differences are related to the outward $K^+$ currents, which play a critical role in human and mouse AP repolarization. The $I_{Ks}$ and $I_{Kr}$ are the major repolarizing currents in human CMs, whereas in mice, the $I_{to}$ is the predominant outward current. Additionally, $I_K$, slow1, $I_k$, slow2, and $I_{ss}$ contribute to repolarization in mouse CMs, but are absent from human ventricular CMs [1]. Furthermore, the normal heart rate in human is 72 beats per minute (bpm), whereas in mouse, it is around 600 bpm [2].

These diverse, distinctive functional properties also determine a diverse physiological response to exercise and stress. Thus, therapeutic strategies that are effective in animal models do not always translate into therapies in humans. Furthermore, considerable differences exist between mouse and human genomes, with many genetic modulators being human specific.

Therefore, the availability of in vitro human models that accurately reflects human disease phenotypes is of utmost importance for understanding the pathological mechanisms and developing efficient therapies.

Primary CMs isolated from human heart tissue are difficult to obtain in quantities that are sufficiently large for experimental studies and challenging to maintain in culture for a long period of time. On the other hand, functional studies on heterologous cell lines or tissues may reveal to be irrelevant. [3] The discovery of a strategy that induces human adult somatic cells back to a pluripotent state by Yamanaka's group marked the beginning of a new era and revolutionized the approach to study human cardiac diseases [4]. These iPSC have the dual ability to proliferate indefinitely and to spontaneously differentiate into any cell type of the human body, including functional CMs (Fig. 6.1). These features make iPSC a valuable source of cardiac cells not only for modeling diseases, but also for studying early development and for drug discovery/testing applications.

A limiting step for use of iPSC in cardiovascular biology has been the low efficiency of differentiation toward the cardiac lineage. In the last few years, new developments of differentiation strategies have become more efficient and reliable.

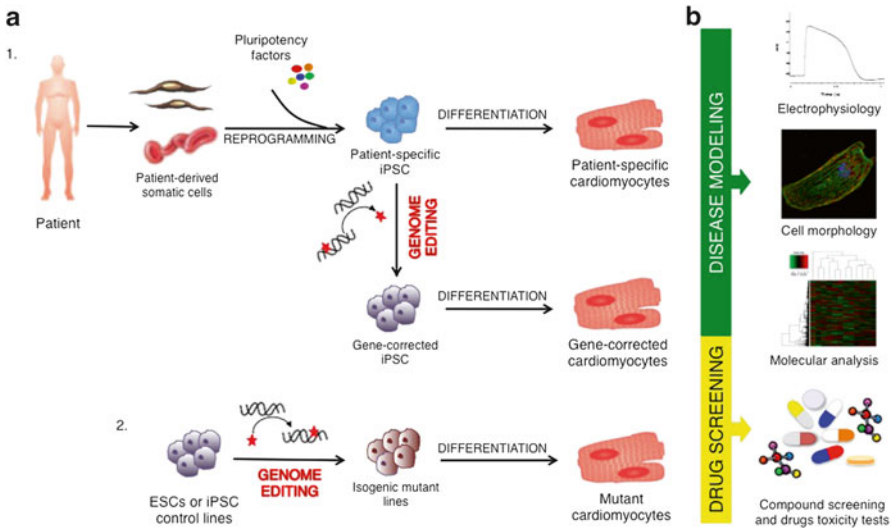


**Fig. 6.1** iPSC technology to study cardiac diseases. (**a**) Generation of patient-specific and reverted "genetically matched" iPSC for studying cardiac diseases. (1) iPSC can be derived by patients' somatic cells by several reprogramming strategies that usually rely on the forced expression of pluripotency factors; induction of cardiomyocytes follows. Gene-correction of patient-specific iPSC lines by homologous recombination enables the generation of CMs in which the specific mutation is reverted to wild type, in the same genetic background as of the patient. This approach allows to prove the causal link between the mutation and the phenotype observed in vitro and facilitate the study of the involvement of putative modifier gene/variants in determining it. (2) Alternatively, isogenic cell lines for mutations of interest can be generated by site-specific genome editing; CMs differentiated from those isogenic mutant lines will carry the genetic mutations of the gene of interest within the same genetic background; this approach is important to establish the function of a defined defect, avoiding confounding effect due to the different genetic background of each individual. Also this strategy won't need to start from specimens of the patients, often difficult to obtain. (**b**) Major applications of iPSC and assays to study iPSC-CMs phenotype. Disease modeling is so far the main application for which iPSC have been employed. iPSC-CMs are a valuable and reliable platform for investigating electrophysiological (action potential properties and intracellular calcium handling), morphological, and molecular properties of disease-CMs. Drug discovery and testing is also a promising area of use of iPSC: many studies have now proved the reliability of iPSC-based cardiac models to test efficacy of chemical compound and toxicity of drugs

These developments have made the generation of large pure population of CMs feasible and have contributed to the burst of employment of iPSC-based disease models in cardiovascular research. Part II of this chapter gives an overview of the available methods for obtaining CMs from iPSC. The limitations related to differentiation protocols will be addressed in the final remarks.

The ability of iPSC to generate a large amount of patient-specific cardiac progenitor cells and CMs makes these cells a useful tool in regenerative medicine. Generating autologous cells should overcome the immunological limitations that currently hamper the use of donor cells and derivatives of embryonic stem cells (ESCs). In this regard, use of iPSC-derived cells/tissue is free from ethical issues linked to cells derived from human embryos. The therapeutic efficacy of iPSC has

recently been proven in several animal models of disease: dopaminergic neurons differentiated from iPSC have been demonstrated to engraft into the brain of a rat model of Parkinson's disease and improve symptoms [5]; in another series of experiments, Hanna et al. used gene-corrected iPSC-derived hematopoietic cells to revert sickle cell anemia in a mouse model of the disease back to normal physiology [6]. As demonstrated, iPSC technology is an ideal platform for corrective gene targeting and gene therapy approaches. Gene-targeting strategies and available models will be discussed in Part V of this chapter.

Similarly, small animal studies have shown that CMs derived from pluripotent stem cells, either ESC or iPSC, form stable cell grafts in the infarcted heart and attenuate ventricular remodeling and heart failure progression and improve ventricular function [7–9]. Importantly, Chong et al. recently made a step forward in the translational application of these cells by showing that intramyocardial delivery of ESC-derived CMs can lead to extensive heart remuscularization in a non-human primate model of myocardial ischemia [10, 11].

Altogether, these studies provide the proof of principle for using iPSC-based therapies for treatment of degenerative and genetic disorders. However, safety issues still remain a limitation, and addressing this issue is a long-term goal for the scientific community.

On the other hand, their application for drug testing is promising. There is accumulating evidence from disease models generated so far that have strongly demonstrated the reliability of models to recapitulate phenotypes associated with different cardiac diseases, spanning from inherited arrhythmias to familial or acquired cardiomyopathies. These studies indicate the ability of such models to respond to conventional drugs in most cases. Part III and IV will provide an overview of the existing models and examples of how these models have been used to test efficacy of therapeutic drugs.

To conclude, in this chapter we will give an overview on the impact of recent advances of iPSC-based strategies in the cardiac field, with a special emphasis on diseases modeling, and address current challenges and promises for their use in modeling and treating cardiovascular disorders.

## Part II: Generation of Cardiomyocytes from Human iPSC

iPSC have become an essential part of investigating patient-specific disease behavior; yet, the differentiation of iPSC into CMs remains the crucial bottleneck for investigating cardiac diseases in vitro and for regenerative medicine approaches due to variable differentiation efficiency, impure cell populations, and fetal-like immature cell morphology and phenotype [3, 12]. However, significant advances have been made in effectively differentiating iPSC into functional, spontaneously beating cardiomyocytes. Methods of differentiation can be broken down into three major categories: co-culture with visceral endoderm-like cells (END2), the embryoid bodies method, and the monolayer method. These methods all have shown great potential in differentiating iPSC into CMs.

### *Co-culture with END2*

ESCs were first used to demonstrate induction of CMs by co-culturing END2 cells activated by mitomycin C [13] or with the addition of bone morphogenic protein 2 (BMP2) [14]. Addition of molecules such as P38-MAPK inhibitor [15] and cyclosporin-A [16] also increased the induction of CMs. Since then, this method has successfully been employed to differentiate more than ten different human iPSC lines into CMs, although the efficiency of differentiation varied between cell lines, ranging from 5 to 25 % efficiency [17–20].

### *Embryoid Bodies Method*

One of the most commonly employed methods of differentiation involves the generation of 3D cell aggregate clusters called embryoid bodies (EBs), which is less expensive and simple to execute compared to other methods described. Many derivatives of this method have been developed to improve the efficiency of cardiac induction. The "hanging-drop" method, suspending cells from a petri dish [21], and the "Spin EBs" method, generating CMs via forced aggregation with the supplement of growth factors or in the presence of END2 medium [22] has been demonstrated. Addition of BMP2 in suspension [23] or 5-aza-cytidine 1–3 days after EBs attachment has also been shown to enhance the differentiation of iPSC into CMs [24]. Moreover, temporal addition of growth factors and signaling molecules that mimic known steps of cardiac development have improved efficiency of differentiation of spontaneously beating CMs. Examples include the Wnt signaling pathway with Wnt3a, Dickkopf-related protein (Dkk-1) and IWP2/4; transforming growth factor beta (TGFβ) family including activin A and BMP2/4; basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) [15, 18, 25–30]. Growth factor supplementation induced approximately 70–90 % of contracting EBs [18, 25, 31].

Most of these earlier techniques incorporated the use of fetal bovine serum in culture, but recent advancements have successfully developed methods for serum-free derivation of CMs [32, 33]. Addition of Activin A and BMP4 supplements to serum-free medium have also demonstrated differentiation of beating CMs. CMs derived from this method observed beating at around 11 days of differentiation with 20–80 % beating efficiency [33].

### *Monolayer Method*

The monolayer method also has several derivations for differentiation into CMs. The "matrigel sandwich" method cultures a monolayer of cells sandwiched between extracellular matrix with addition of BMP 4, Activin A and bFGF [34]. Similar to the EBs method, differentiation into CMs can also be directed with sequential addition of

Activin A and BMP4 in RPMI containing B27 medium [9], or temporal activation and subsequent inhibition of the Wnt signaling pathway using CHIR99021 and IWP2 to activate and inhibit the pathway, respectively [35, 36]. Although this method depends highly on the correct timing of the addition of these small molecules to regulate cardiogenesis, these methods can generate up to 95–98 % of highly purified CMs [34, 36].

A recent work from Tohyama S et al. further improved these protocols and, taking advantage of the differences in glucose and lactate metabolism between CMs and non-CMs, demonstrated that lactate supplementation (in glucose-depleted media) was sufficient to obtain 99 % pure populations of iPSC-derived CMs (iPSC-CMs) that did not form tumors after transplantation [37]. This technical advancement expands the areas of application of iPSC-CMs and will definitely have a positive impact toward their application in regenerative medicine approaches.

## Part III: Overview of the Currently Established Models of Inherited Cardiac Diseases

iPSC have the unique ability to regenerate an unlimited supply of cells that have identical genetic properties as that of the patient [38]. As such, they have been indispensable in studying patient-specific diseases and they have pioneered the way to understanding various mechanisms and pathophysiologies of a number of cardiac diseases. There has been an exponential increase in the number of disease models that have been demonstrated since the advent of iPSC and the impact on healthcare and future potential treatment options are insurmountable. The following section contains an overview of the cardiac disease models that have been established so far using iPSC-CMs to elucidate the disease mechanisms and to test therapeutic drugs to investigate future treatment potentials.

Methods of investigations include morphological evaluation of the sarcomere and cytoskeletal structure by immunofluorescence and electron microscopy, analysis of the electrophysiological properties of the iPSC-CMs (AP properties and intracellular calcium handling) and definition of the contractile properties.

Overall, the data accumulated so far indicate iPSC-CMs recapitulate the main characteristics of any investigated disease, strongly indicating their reliability to be used as a heart-like model to study disease mechanisms and efficacy of drugs.

A list of the presented diseases, complete with phenotype description, involved genes and references is provided in the Table 6.1.

### *Long QT Syndrome*

Long QT syndrome (LQT) is one of the most extensively investigated diseases utilizing human iPSC-CMs and is the first cardiac disease for which an iPSC-based model has been created [39]. Characterized by prolonged QT-intervals,

**Table 6.1** Abbreviated summary of the major previous studies modeling cardiac diseases utilizing iPSC-CMs

| Disease | Gene mutation | Detected in vitro phenotype | Pharmacological intervention/ treatment | First author (year) |
|---|---|---|---|---|
| *Long QT type 1* | KCNQ1 (R190Q) | $K^+$ channel subunit enrichment on endoplasmic reticulum, prolonged APD and FPD | Propranolol pretreatment: eliminated EADs induced by isoproterenol | Moretti et al. (2010) [39] |
| | KCNQ1 (P631fs/33) | | | Egashira et al. (2012) [41] |
| *Long QT type 2* | KCNH2 (M4109R) | Prolonged APD, reduced $I_{kr}$, EAD and arrhythmic activity | Nifedipine, nadolol, propranolol: reduced APD, FPD; eliminated EADs and arrhythmogenicity | Itzhaki et al. (2011) [42] |
| | KCNH2 (G1681A) | | Homologous recombination, RNAi: Rescued electrophysiological phenotype | Matsa et al. (2011, 2014) [44, 46] |
| | KCNH2 (R176W) | | | |
| | KCNH2 (N996I) | | | Lahti et al. (2012) [43] |
| | | | | Bellin et al. (2013) [45] |
| *Long QT type 3* | SCN5A (F1473C) with KCNH2 Polymorphism | Increased late $Na^+$ current, shifted steady state, prolonged APD | Mexiletine: rescued electrophysiological disease phenotype | Terrenoire et al. (2013) [47] |
| | SCN5A (V1763M) | | | Ma et al. (2013) [48] |
| *Long QT type 8/ Timothy syndrome* | CACNA1C (G406R) | Decreased HR, irregular contractions, DAD | Roscovitine: normalized $Ca^+$ transients, decreased APD and abnormal depolarizing events | Yazawa et al. (2011) [49] |

| | | | | |
|---|---|---|---|---|
| *Catecholaminergic polymorphic ventricular tachycardia (CPVT)* | RYR2 (T7447A) | RYR2: Increased frequency of $Ca^{+2}$ sparks, DAD, EAD | Flecainide, Thapsigargin: eliminated DAD | Fatima et al. (2011) [52] |
| | RYR2 (M4109R) | CASQ: narrow myofibrils, abnormal, wide SR cisternae, reduced caveolae, DAD | Dantrolene: restored $Ca^{+2}$ sparks activity | Itzhaki et al. (2012) [51] |
| | RYR2 (S406L) | | KN-93: normalized $Ca^{+2}$ upstroke, restored single beating initiation site | |
| | RYR2 (P2328S) | | | Jung et al. (2012) [50] |
| | CASQ2 (D307H) | | | Kujala et al. (2012) [54] |
| | RYR2 (E2311D) | | | Novak et al. (2012) [55] |
| | | | | Di Pasquale et al. (2013) [53] |
| *Dilated cardiomyopathy (DCM)* | TNNT2 (R173W) | TNNT2: sarcomeric disorganization, small $Ca^{+2}$ transients, weaker contractile force | Beta-blocker: Improved sarcomeric organization | Sun et al. (2012) [59] |
| | LMNA (R225X) | LMNA: nuclear senescence and apoptosis | Serca2a: Rescued contractile force and global $Ca^{+2}$ expression | Siu et al. (2012) [60] |
| | DES (A285V) | DES: abnormal desmin and sarcomere, decreased $Ca^{+2}$ uptake, beating rate | U0126 and Selumetinib AZD6244: diminished apoptotic effect | Tse et al. (2013) [61] |
| *Hypertrophic cardiomyopathy (HCM)* | MYH7 (R663H) | Upregulation of hypertrophic genetic markers, enlarged cells, multinucleation, increased calcineurin and NFAT, sarcomeric disorganization, irregular $Ca^{+2}$ transients | Cyclosporin A and FK506: Reduced hypertrophy by over 40 % | Lan et al. (2013) [63] |
| | | | Verapamil, beta-blockers, $Ca^{+2}$ channel blockers, antiarrhythmics: Ameliorated hypertrophic events | Uesugi et al. (2014) [64] |
| | | | | Tanaka et al. (2014) [65] |

(continued)

**Table 6.1** (continued)

| Disease | Gene mutation | Detected in vitro phenotype | Pharmacological intervention/ treatment | First author (year) |
|---|---|---|---|---|
| *Arrhythmic right ventricular dysplasia/ cardiomyopathy (ARVD/C)* | PKP2 (2484C>T) | Abnormal translocation of plakoglobulin, adipocytic change, reduced CNX43, increased desmosomal gap, desmosomal dissymmetry, increased lipid droplets | Induction of beta-catenin/ canonical Wnt: reduced lipid droplet accumulation due to adipogenic stimulus | Kim et al. (2013) [68] |
| | PKP2 (L614P) | | | Ma et al. (2013) [69] |
| | PKP2 (A324fs335X) | | | Caspi et al.(2013) [67] |
| *Viral cardiomyopathy* | – | Abnormal cardiac beating 6 h post-infection and cessation of beating 12 h post-infection with coxcackievirus B3, abnormal intracellular calcium handling | IFNβ1, ribavirin, Fluoxetine, and pyrrolidine dithiocarbamate (PDTC) all reduced viral proliferation | Sharma et al. (2014) [70] |
| *Barth syndrome* | TAZ (517delG, 328 T>C) | Smaller, fragmented mitochondria, low basal ATP levels, irregular sarcomere, elevated ROS production, decreased contractility, | MitoTEMPO: improved sarcomere organization and contractility, suppressed excessive ROS | Wang et al. (2014) [74] |
| | | | Arginine plus cysteine: increased ATP levels Linoleic acid: normalized ATP, improved sarcomeric organization | |
| *Friedreich ataxia (FRDA)* | Expanded GAA repeats on FXN | Dark, hypertrophic cristae mitochondria accumulation | – | Hick et al. (2013) [77] |

| | | | | |
|---|---|---|---|---|
| *Diabetic cardiomyopathy* | – | Accumulation of intracellular lipid and lipid peroxidation | Thapsigargin and fluspirilene: improved DCM phenotype | Drawnel et al. (2014) [78] |
| *Pompe disease* | GAA [c.1935C>A (p.D645E)/c.1935C>A (p.D645E); c.1935C>A/c.2040+1G>T; c.1062C>G (p.Y354X)/c.1935C>A (p.D645E)] | Increased glycogen content, large glycogen storing vacuoles, abnormal mitochondria, and autophagosome-like structures | L-carnitine: increased oxygen consumption rate | Huang et al. (2011) [79] |
| | GAA (mutation not indicated) | | rhGAA, 3-methyladenine: reduced glycogen content | |
| | | Reduced GAA activity, increased glycogen storage, and hypoglycosylation of LAMP | – | Raval et al. (2015) [80] |

multiple subtypes have been established based on the gene mutation and clinical presentation. Studies utilizing iPSC-CMs have been done for LQT type 1, 2, 3, and 8.

## *LQT1*

Long QT syndrome Type 1 (LQT1) is caused by a mutation in the gene KCNQ1, which encodes a potassium channel subunit responsible for the slow component of the delayed rectifier potassium current ($I_{Ks}$) [40]. iPSC-CMs derived from patients with this mutation expressed channel subunit enrichment in the endoplasmic reticulum as compared to the control, which were localized on the cell surface [39]. Electrophysiological tests with patch clamp and multi-electrode array (MEA) systems measured prolonged action potentials duration (APD) [39, 41] and field potential duration (FPD) [41], respectively. Treatment with chromanol 293B that blocks $I_{Ks}$ did not affect iPSC-CMs, indicating insensitivity or dysfunctional $I_{ks}$ channels, whereas treatment with the rapid component of the rectifier potassium current ($I_{kr}$) channel blocker E4031 induced early-afterdepolarizations (EADs) and induced arrhythmic events at high doses [41]. Furthermore, treatment with beta-adrenergic stimulant, isoproterenol, increased APD and induced EAD, which were ameliorated with pretreatment with nonselective beta-blocker propranolol [39, 41].

## *LQT2*

Long QT syndrome Type 2 (LQT2) is due to a mutation in the KCNH2, or hERG, which affects the potassium ion channel involved in $I_{kr}$. Electrophysiological tests on patient-derived iPSC-CMs detected prolonged APD [42–44], reduced $I_{kr}$ [42–45], and arrhythmic activity due to the presence of EADs [42, 44]. Treatment with nifedipine, calcium channels blocker, reduced APD and FPD and eliminated EADs and arrhythmic activity [42]. Further drug tests with beta-adrenergic receptor agonist, isoprenaline, shortened APD and FPD, and induced EADs. Treatment with beta-adrenergic antagonist, such as nadolol or propranolol, and potassium channel activators eliminated EADs and rescued arrhythmic activity [44]. Blocking $I_{kr}$ with E4031 resulted in EADs and prolonged APD [44] and increased arrhythmogenicity [43].

Bellin et al. used homologous recombination for targeted gene correction in patient iPSC-CMs to compare genetically matched lines. These corrected CMs showed reduced APD compared to mutated counterparts and rescued $I_{kr}$ density. They also found that the mutation that affects $I_{kr}$ current was isolated to the N9961 hERG mutation [45]. Finally, Matsa et al. in 2013 used RNA interference (RNAi)-based therapy to knockdown the expression of the mutant hERG, which showed to successfully rescue electrophysiological phenotypes, even under adrenergic stimulating conditions [46].

## *LQT3*

Long QT syndrome Type 3 (LQT3) is caused by a mutation in the SCN5A gene, which encodes a sodium ion channel subunit. Terrenoire et al. derived iPSC-CMs from patients with mutations in SCN5A gene with polymorphisms in KCNH2 [47]. These CMs exhibited increased late sodium current, shifted steady-state properties, and faster recovery from inactivation, which explained the arrhythmic risk in these patients. However, they found little contribution of the KCNH2 polymorphism to the disease phenotype [47]. Ma et al. found electrophysiological measurements with prolonged APD and increased sodium current, indicative of clinical ECG measurements [48]. In both studies, treatment with mexiletine, an antiarrhythmic drug, recovered the disease phenotypes [47, 48].

## *LQT8*

Long QT syndrome Type 8 (LQT8) is characterized by a mutation in the L-type calcium channel CaV1.2. Yazawa et al. derived iPSC-CMs from patients with Timothy Syndrome with a mutation in CACNA1C gene that encodes CaV1.2. They observed decreased heart rates with irregular contractions, and electrophysiological tests revealed decreased voltage-dependent inactivation and DAD-like characteristics. To test therapeutic potential to treat electrical defects of this disease, iPSC-CMs were treated with a cycline-dependent kinase inhibitor, Roscovitine (Ros). IPSC-CMs treated with Ros had significant decrease in the amplitude and frequency of calcium transients, duration of action potentials, and frequency of abnormal depolarizing events, successfully demonstrating the effective use of future therapeutic drugs on iPSC-CMs [49].

## *CPVT*

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited disease that leads to stress-induced arrhythmia, syncope, and sudden cardiac death. Two forms have been identified: CPVT1, an autosomal dominant mutation in the ryanodine receptor (RYR2) gene and CPVT2, a more rare recessive mutation in the calsequestrin-2 gene (CASQ2). Many studies utilizing iPSC-CMs have been performed, the majority of which investigated the more prevalent form, CPVT1. Studies utilizing RYR2 mutant iPSC-CMs detected increased frequency of calcium sparks [50] and diastolic calcium concentrations with beta-adrenergic stimulation with isoproterenol [50, 51] and arrhythmogenic activity [52]. Whole cell patch clamp revealed delayed afterdepolarizations (DADs) characteristic to RYR2 mutant patients [51–54] and in some cases, EAD [54].

Drug efficacy on iPSC-CMs was tested with various treatments including an antiarrhythmic drug, flecainide, and calcium pump blocker, thapsigargin, which eliminated afterdepolarizations [51]. Dantrolene, which blocks ryanodine receptors, restored calcium spark activity [50]. Treatment with beta-blocker, propranolol, reversed calcium abnormalities induced by isoproterenol [51]. Finally, treatment with KN-93, a Ca/calmodulin-dependent serine-threonine protein kinase II inhibitor, normalized calcium upstroke and reverted beating cluster of CMs to a single initiation site, similar to that observed in normal cardiac tissue [53].

Novak et al. in 2012 studied two patients with the CASQ2 variant and studied iPSC-CMs [55]. They observed narrower myofibrils, abnormal, wider sarcoplasmic reticulum cisternae, and reduced number of caveolae compared to the control. In addition, adrenergic stimulation via isoproterenol treatment caused arrhythmogenic propensity in mutant iPSC-CMs with DAD equivalents in electrophysiologic experiments. They conjectured that the increase in diastolic cytosolic calcium levels caused the DADs and arrythmogenicity, similar to those to clinical symptoms.

## Part IV: Focus on Primary Cardiomyopathies

Primary cardiomyopathies are diseases of the myocardium and comprise a heterogeneous group of disorders associated with mechanical and/or electrical dysfunction of the heart that usually exhibits inappropriate ventricular hypertrophy or dilatation. A significant percentage of cardiomyopathies underlie genetic mutations of genes acting via various pathways: these encode components of a wide variety of cellular compartments and pathways, primarily involving the nucleo-cytoskeletal apparatus, including the sarcomere (α-cardiac actin; α-tropomyosin; cardiac troponin; α/β-myosin heavy chain, titin) and the force transduction apparatus (Z-disk and costamere) [56].

Based on the clinical phenotype, cardiomyopathies are classified into four major groups: dilated (DCM), hypertrophic (HCM), restrictive (RCM), and arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) [57]. As a general rule, HCM is defined as "disease of the sarcomere," since mutations in genes encoding sarcomeric proteins are responsible for two thirds of the cases, while DCM is characterized by diverse causes that lead to a final common phenotype (dilation); ARVC is instead referred to as a disease of the desmosome, due to the high percentage of mutation involving desmosomal proteins [57].

Despite this classification, all the inherited cardiomyopathies are genetically heterogeneous; within each category there are multiple disease genes, and many different mutations. It is not uncommon that different mutations within the same gene can underlie different disorders; mutations that affect adjacent regions in the β-myosin heavy chain, for example, can cause either HCM or DCM [57]. This phenomenon is probably due to other genetic variations or epigenetic and environmental determinants (i.e., modifier genes, variable expressivity and penetrance, post-translational modifications, epigenetic modifications, exercise, stress, other diseases).

From a clinical point of view, this heterogeneity renders very difficult when establishing genotype/phenotype correlations and making decisions for therapeutic intervention based on genetic information.

The combination of patient-specific cells and genome-editing strategies opens a new perspective for investigating this group of diseases. This strategy allows us to determine the contribution of genetic background and other potentially modifying factors as the main causative mutation in the onset and progression of the disease and in the determination of the cellular phenotype.

In the last 3 years, studies on iPSC-based cardiac models highly contributed to our knowledge on the underlying mechanisms of different forms of cardiomyopathies, either genetically derived or acquired, and provided the "proof of principle" that such models can be used for pharmacological screening to test the efficacy of "personalized" therapies and to discover new drugs.

Below we will examine in depth the models of cardiomyopathies that have been generated so far using iPSC-based cardiac model systems.

## *DCM*

DCM is characterized by ventricular dilation and systolic dysfunction that can progressively lead to heart failure. DCM has most commonly been associated with defects or mutations in genes encoding components of the cytoskeleton, sarcomere, or nuclear lamina [58]. Several studies have investigated various mutations from DCM patients via iPSC-derived patient CMs. First in 2012, Sun et al. investigated a three-generation family with point mutations in cardiac troponin T gene (TNNT2) [59]. At day 30 of differentiation, they observed heterogeneous sarcomere alpha actin distribution. Furthermore, treatment with beta-adrenergic agonist, norepinephrine (NE), for one week in culture exhibited a marked increase in sarcomeric alpha-actin disorganization and a more scattered distribution of Z-bodies. On the other hand, treatment with beta-adrenergic blocker for one week in culture improved sarcomeric alpha actin organization. $Ca^{+2}$ handling activities also measured significantly smaller calcium transients and atomic force microscopy (AFM) measurements found weaker contractile forces of mutant cells compared to control CMs. Overexpression of Serca2a, a treatment for heart failure, had rescued the contractile force and restored global $Ca^{+2}$ concentrations. Thus, they were able to identify key phenotypes in their in vitro models and test potential therapeutic interventional strategies [59].

Also in 2012, Siu et al. performed a similar study except with patient cells with mutations in the lamin A/C gene, which accounts for about 6 % of overall familial DCM [60]. They showed that electronic stimulation of iPSC-CMs resulted in increased nuclear senescence and apoptosis. Blockage of the MEK1 pathway with anticancer drugs, U0126 and Selumetinib AZD6244, diminished the apoptotic effect [60].

In a recent work, Tse et al. identified mutations in the desmin gene (DES), which are associated with cytoskeletal structure and maintenance. iPSC-CMs derived from

patients with this mutation exhibited diffuse, disorganized desmin network and abnormal sarcomeric architecture. Functional tests revealed decreased calcium uptake, beating rate, and response to stress-induced environment via isoproterenol injection [61]. These studies prove that iPSC-CMs can recapitulate in vitro the phenotype of a late-onset disease such as DCM and strengthen their usefulness as a tool for further investigating DCM mechanisms.

## *HCM*

Familial HCM, an autosomal dominant disease due to mutations in cardiac sarcomere genes, causes abnormal thickening of the left ventricular myocardium, eventually progressing to arrhythmia, heart failure, and sudden cardiac death [62]. Patient iPSC were derived from fibroblasts with missense mutations in the beta-myosin heavy chain gene (MYH7) and differentiated into CMs [63]. Key genetic players of hypertrophy such as GATA4, MEF2C, TNNT2, MYL2, and MYH7 were upregulated in diseased human iPSC-CMs at day 40 of differentiation. The phenotypic characteristics of beating CMs showed cellular enlargement, higher frequency of multinucleation, and expression of HCM markers such as atrial natriuretic factor (ANF), increased beta-myosin/alpha myosin ratio, sarcomeric disorganization, and elevated calcineurin and nuclear factor of activated T cells (NFAT) than that of the control. The latter two signaling factors, calcineurin and NFAT, are key transcriptional regulators of hypertrophy. Lan et al. demonstrated that blocking this pathway with cyclosporin A (CsA) and FK506 reduced hypertrophy by over 40 %, thus demonstrating a therapeutic potential of this disease model in vitro [63].

$Ca^{+2}$ handling plays a critical role in the clinical presentation of arrhythmia in HCM. The $Ca^{+2}$ handling data showed irregular $Ca^{+2}$ transients, occurring even before the onset of cellular hypertrophy, suggesting that irregular $Ca^{+2}$ transients may contribute to the pathogenesis of HCM. Several pharmaceutical drugs were tested, including L-type calcium channel blocker verapamil, beta-blockers, $Ca^{+2}$ channel blockers, and antiarrhythmic drugs, most of which demonstrated to have potentially therapeutic effects in ameliorating hypertrophic events [63].

Interestingly, a couple environmental factors have shown induction of hypertrophy of iPSC-CMs. Uesugi et al. demonstrated that low density cell culture conditions revealed a significant enlargement of CMs, expression of hypertrophic markers, desensitization to $I_{ks}$ blockers chromanol 293B and HMR1556, and reduced expression of KCNQ1 and KCNJ2 genes responsible for potassium ion channels, thus recapitulating hypertrophy-like phenotypes [64]. Tanaka et al. treated HCM patient-derived iPSC-CMs with endothelin-1 (ET-1), which promoted further HCM phenotype including CMs enlargement, myofibril disarray, and unequal CMs contractile patterns [65]. These in vitro models of HCM could help further investigation into HCM disease mechanism and treatment.

## *ARVD/C*

ARVD/C is an inherited heart disorder frequently caused by a mutation in cardiac desmosomes [66]. This mutation results in fibrous or fibro-fatty replacement of CMs, resulting in ventricular arrhythmia and cardiac death. Three studies pertaining to this disease have been published in 2013. The studies involved generation of iPSC-CMs of cells from patients with a frameshift mutation of the plakophilin-2 (Pkp2) gene, a component of desmosomes [67–69]. Kim et al. found abnormal nuclear translocation of Pkp2 anchoring protein plakoglobulin protein, which decreased beta-catenin activity. They also treated iPSC-CMs with activators of peroxisome proliferator-activated receptor-gamma (PPARgamma), which may be upregulated in ARVD/C patients; this test resulted in high levels of lipogenesis and apoptosis. Finally, they found a metabolic shift from fatty acid oxidation for major energy source to glycolysis, efficiently recapitulating metabolic occurrences of the disease to that in the clinical setting [68].

In addition to decreased plakoglobulin protein expression, Ma et al. observed darker, abnormal lipid droplet morphology in mutant iPSC-CMs and induced adipogenic stimulatory conditions exhibited an increase is potential for adipocytic change compared to that of control [69]. Similarly, Caspi et al. found a reduction in plakoglobulin expression but also detected a significant reduction in the gap-junction protein connexin 43 (CNX43). They also observed morphologic differences in iPSC-CMs including increased desmosomal internal gap, dissymmetric, pale desmosome appearance, and increased clusters of lipid droplets. Finally, treatment with adipogenic stimulus significantly increased lipid droplet accumulation, which was reversed by inducing beta-catenin/canonical Wnt signaling pathway, a pathway previously shown to decrease lipid accumulation in a mouse model [67].

## *Viral Cardiomyopathy*

Infections by some strains of viruses can cause myocarditis, which can lead to cardiac arrhythmias, cardiomyopathy, and heart failure. Sharma et al. differentiated iPSCs into CMs from healthy patients and infected them with the B3 strain of coxsackievirus, the most common known cause of viral myocarditis [70]. Cytopathic effects of coxsackievirus-infected iPSC-CMs could be observed at around 6 h after infection, with decreased, erratic beating. Beating stopped at 12 h after infection and most cells were detached after 24 h. Calcium assays detected abnormal intracellular calcium handling with elongated calcium transient time, time to transient peak, and standard deviation between transient intervals, a possible explanation for arrhythmic activity seen in some virally infected patients.

Drug therapeutic potential was tested on these iPSC-CMs with IFNβ1, ribavirin, Fluoxetine, and pyrrolidine dithiocarbamate (PDTC). IFNβ1, previously demonstrated to eliminate cardiotropic viruses in in vitro CMs, reduced viral activity in

infected iPSC-CMs by greater than 50 % after 12 h of pretreatment. Treatment with ribavirin, an inhibitor of RNA synthesis, reduced around 50 % of viral activity. Fluoxetine, which inhibits coxsackievirus in HeLa cells, decreased viral activity in moderate levels but showed cardiotoxicity at high levels. Finally, treatment with PDTC, an antioxidant, showed concentration-dependent reduction in viral proliferation both with pretreatment and with concurrent addition of the drug with the viral vector into the iPSC-CMs [70]. Since no antiviral treatment is currently available for viral myocarditis, these results indicate that virally infected iPSC-CMs can successfully test therapeutic potential for drug treatment of viral myocarditis [71].

### *Multisystem Disorders*

An increasing number of studies have now been published that address the cardiac counterpart of multi-organ diseases using iPSC-based systems. These studies comprise LEOPARD syndrome, Barth syndrome, Friedreich ataxia, diabetic cardiomyopathy, and Pompe disease.

### *LEOPARD Syndrome*

LEOPARD syndrome (lentigines; electrocardiographic abnormalities; ocular hypertelorism; pulmonary valve stenosis; abnormal genitalia; retardation of growth; deafness) is a multisystem disorder that causes a multitude of symptoms, one of which includes HCM. iPSC-CMs were derived from patients with a missense mutation in the PTPN11 gene that encodes tyrosine phosphatase SHP2. They observed gene expression profiles consistent with HCM along with a significant increase in the median surface area and sarcomeric assembly compared to that of wild type. These genetic and morphological changes were consistent with that of HCM, thus providing evidence that a part of this disease could be recapitulated in in vitro studies [72].

### *Barth Syndrome*

Barth syndrome is a rare X-linked disorder associated with mutations in the Tafazzin (TAZ) gene. These mutations cause abnormal phospholipids of the mitochondrial inner membrane, which leads to mitochondrial cardiomyopathy [73]. Two unrelated disease lines, one with a missense mutation and another with a frameshift mutation in the TAZ gene were induced into iPSC and subsequently differentiated into CMs. Diseased iPSC-CMs presented with smaller, fragmented mitochondria than that of the control. Basal ATP levels and electron transport activities were low, while the oxygen consumption rate was elevated, demonstrating defective ATP production

but functioning ATP synthase oxygen consumption. Sarcomeres were irregularly aligned and sparse compared to the control and patient iPSC-CMs had elevated mitochondrial ROS production. Treatment with mitoTEMPO, a mitochondrial ROS suppressor, improved sarcomere organization and contractility of patient-derived CMs [74].

Myocardial constructs were engineered from patient iPSC-CMs and contractile properties were observed. Patient cells had markedly decreased contractile properties but were reversed upon TAZ replacement. Furthermore, three proposed treatment options targeting the mutant phospholipid of the inner membrane of BTHS patients were tested on these constructs. Although the degree of effectiveness varied by treatment, they rescued contractile properties and partially corrected the metabolic presentation of the BTHS engineered constructs [56].

## *Friedreich Ataxia*

Friedreich Ataxia (FRDA) is an autosomal recessive disorder that affects multiple systems but predominantly comprises neurological and cardiac clinical presentations such as ataxia and cardiomyopathy, respectively [75]. Patients with FRDA usually have expanded triplet acid-α-glucosidase (GAA) repeats in the fataxin (FXN) gene, which plays a role in mitochondrial development [76]. Researchers derived iPSC from two patients with the mutation and differentiated them into CMs. Ultrastructural observations of the diseased iPSC-CMs mitochondria showed several types of mitochondrial abnormalities including accumulation, darkened matrix, and hypertrophic cristae mitochondria, consistent with findings from other studies. Time-lapse measurements detected variable arrhythmic activities depending on the clone, however, not necessarily significantly different from that of the control. Thus, although iPSC-CMs were variable in the in vitro measurements, they have the potential for future investigation of the disease and therapeutic application [77].

## *Diabetic Cardiomyopathy*

The potential use of patient-specific iPSC in the iPSC-based disease-modeling platform has now extended beyond genetically related diseases. Drawnel et al. recently published a study using iPSC-CMs to characterize type II diabetes mellitus [78]. First, non-patient-specific iPSC-CMs were tested after maturing with maturation media and culturing in pro-diabetic conditions (glucose, ET-1, and cortisol). This conditioning caused cellular hypertrophy, loss of sarcomeric integrity, lipid accumulation in the cytosol, and protein production dysfunction, all characteristic to that of the clinical manifestation of diabetic cardiomyopathy. Next, they produced iPSC-CMs from patients with type II diabetes and found characteristics of diabetic cardiomyopathy such as lipid accumulation and lipid peroxidation that paralleled the

degree of severity to that of the clinical condition of the patient. Molecules to normalize cardiomyopathy phenotype were screened and tested and they were able to identify compounds, the most potent of which were thapsigargin and fluspirilene, that effectively improved the cardiac phenotype of the diseased iPSC-CMs. Taken together, this study developed an in vitro simulation of diabetic cardiomyopathy and established a screening platform for patients with type II diabetes mellitus using patient-specific iPSC-CMs, demonstrating great potential in iPSC technology for patient-specific clinical use [60].

### *Pompe Disease*

Pompe disease is an autosomal recessive lysosomal storage disease caused by a mutation in the gene encoding GAA, which can cause cardiomyopathy and heart failure particularly in the infantile-onset type. A couple studies have utilized iPSC-CMs in modeling this disease. Huang et al. produced iPSC by inducing the overexpression of GAA in patient dermal fibroblasts and then differentiating them into CMs [79]. They found increased glycogen content, large glycogen storing vacuoles, abnormal mitochondria, and autophagosome-like structures in these iPSC-CMs. Treatment with L-carnitine increased oxygen consumption rate, partially rescuing mitochondrial function. In addition, co-treatment of rhGAA, a human recombinant GAA often used to treat Pompe disease, and 3-methyladenine, an autophagy inhibitor, significantly reduced glycogen content, showing successful disease modeling and treatment [79].

Another group, Raval et al. used iPSC-CMs and found reduced GAA activity, increased glycogen storage, and hypoglycosylation of lysosome-associated membrane proteins due to deficient Golgi glycosylation [80]. Using this model, further detailed investigation can be achieved to further elucidate the disease mechanism.

## Part V: Genome Editing in iPSC-Based Models

iPSC allow the investigation of a gene/genetic mutation of interest in human CMs that are genetically identical to the patient. Although this possibility has greatly impacted the discovery of new mechanisms of disease, the demonstration of the causal contribution of a genetic mutation in the disease phenotype requires the study of cells where the genetic alteration has been corrected. Also, as mentioned in the previous section, creation of isogenic lines is fundamental to determine the role of modifiers factors (genetic and nongenetic) on the phenotypic traits of the diseases. Insertion of a specific genetic modification in isogenic human model systems, rather than the correction of patient-specific lines, is an alternative approach to prove its direct involvement in the pathogenesis of the disease (Fig. 6.1).

A well-designed study by Bellin et al. employed both patient-specific iPSC cells carrying the N996I KCNH2 mutation and corrected lines together with isogenic ESCs lines with the specific mutation introduced into the gene by homologous recombination (HR) to study LQT2 syndrome [45].

In another study, using a zinc finger nucleases (ZNF) based-targeting approach (see below), Wang Y et al. re-created in vitro isogenic iPSC models of LQT1 and LQT2 syndromes by stably integrating the dominant negative mutants of the ion channel genes KCNQ1 and KCNH2, respectively, into a safe harbor AAVS1 locus and demonstrated that CMs derived from these lines recapitulate the main feature (QT elongation) of the diseases and are able to respond to specific drug treatments [81]. Such findings indicate the feasibility of using isogenic iPSC lines for future drug testing.

Gene targeting has been routinely achieved by electroporation in mouse ESCs; however, this methodology is extremely inefficient in human iPSC and as such its application is limited in these models [82]. Viral vectors have been utilized as an alternative strategy to overcome this issue and improve the efficiency of HR events in human pluripotent stem cells (both embryonic and induced pluripotent). In particular, use of high capacity helper-dependent adenoviral (HDAd) vectors have been demonstrated to be highly efficient and accurate in driving homologous recombination in human iPSC, with no detectable adverse effects on the undifferentiated state and pluripotency [83, 84].

More recently, further progress in gene editing and gene delivery technology have been made, which fix or insert defected mutations without inserting or adding unnecessary viral gene or non-human genomes.

Such methods are based on use of nucleases that are able to target and cut specific DNA sequences within chromosomes. These genome engineering strategies comprise three different types of nucleases: (1) ZNF, transcription activator-like effector nuclease (TALEN); and (2) clustered regularly inter-spaced short palindromic repeats (CRISPR). All these, with their unique features, have the ability to target specific DNA sequences and, as such, constitute very useful tools for manipulating pluripotent cells [85].

Among all, CRISPR/Cas9 system is emerging as the most promising approach, mainly because of its ease to handle, the facile design and the wide range of different applications besides the gene editing itself.

CRISPR/Cas9 has been demonstrated as a powerful and versatile site-specific tool for genome modification in several model systems and has significantly improved our ability to generate genetically modified human iPSC [86, 87].

The main components of this are the DNA endonuclease Cas9 and a single chimeric guide RNA (sgRNA), which recognizes a specific DNA sequence (only 20 nucleotides long) on the genome and directs Cas9 for double-strand DNA cleavage (DSBs). This induces nonhomologous end joining (NHEJ)-mediated disruption of the target gene and may also allow its HR-mediated editing. In the latter case, use of a mutated form of Cas9 (Cas9D10A) allows an induction of a DNA "nick" rather than DSBs, resulting in preferential repair through HR, a minimized risk of indel mutations from off-target DSBs, and improved specificity.

Furthermore, single-strand DNA oligonucleotides (ssODNs) have been successfully used in combination with sgRNAs to achieve HR for short modifications within a defined locus in place of the targeting plasmids [88].

Using a CRISPR/Cas9-based strategy Wang G et al. achieved gene replacement and genome editing in Barth Syndrome iPSC and provided the causal demonstration that TAZ gene mutations are necessary and sufficient to lead to the impaired CMs function typical of the diseases [74] (see Table 6.1 and Part IV).

Importantly, CRISPR/Cas9 system has been demonstrated to be effective also in vivo: Long C et al. recently showed that correction of dystrophin gene mutation through CRISPR/Cas9-mediated genome editing in the germ line of a mouse model of Duchenne muscular dystrophy prevents muscle fibers degeneration and attenuates the associated dystrophic phenotype, suggesting a potential of CRISPR/Cas9 system in future gene therapy applications [89].

## Part VI: Challenges and Future Perspectives (How Close We Are to Use Them in Clinic?)

The value of iPSC technology to the study of human diseases is unquestionable and evidence from the studies reported in this chapter clearly indicate the growing importance of iPSC-based models also in the cardiovascular field for understanding molecular and cellular mechanisms of cardiac diseases and for developing alternative and "personalized" therapeutic interventions.

However, although structural and functional maturation of CMs derived from iPSC improves over time, these cells remain immature and equate more to fetal cells than to adult CMs. These cells do not possess a well-developed T-tubule system, show spontaneously beating activity and display AP parameter similar to the embryonic human heart, probably because of the retention of a fetal transcriptional program.

As a consequence, these fetal-like features may determine a lack in the disease phenotype in iPSC-CMs, especially for those diseases that have a late onset or that are caused by alteration of genes and pathways expressed later in development.

Research is moving toward the development of robust and large-scale protocols to differentiate human CMs that better reproduce the physiology of an adult cardiomyocyte. Accomplishment of this goal is expected to require the integration of treatments that mimic more accurately heart development process with 3D-systems, electrical pacing, mechanical strain, and fiber alignment.

Further refinements of the methodology are also needed to specifically generate the three major subtypes of CMs, atrial, ventricular and nodal cells that are currently induced as a mixed population of cells by most of the available protocols.

Such advancements in the differentiation methods together with the improvement of high-throughput techniques are fundamental for the setting of iPSC-based cardiac platforms that are recognized for "clinical-grade" testing of efficacy and cardiotoxicity of new drugs and personalized intervention.

Even if "the road to the clinic" is still long for iPSC, the ability to generate high-yield, pure CMs with definite phenotypes from these cells is a prerequisite for their potential application to heart replacement therapies. One of the main issues to the clinical application of iPSC-based treatments is the potential tumorigenicity associated with iPSC-CMs due to residual undifferentiated cells. Aside from the efforts in searching for cell-specific surface markers that allow for antibody-based purification of cardiovascular progenitors and CMs subtypes, no unequivocal candidate have been identified. Moreover, potential arrhythmic effect, long-term engraftment ability and efficacy of iPSC-based therapies are still a grey area and need further investigations.

Less than a decade has passed since the advent of iPSC but progress in this field have been exponential and many studies have provided proof of the reliability of this technology in the study of cardiovascular diseases. A step forward is now required to overcome the current challenges and limitation and to realize the full potential of iPSC.

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# Chapter 7
# Cancer Stem Cells and Chemoresistance

**Toshio Kubo, Nagio Takigawa, Katsuyuki Kiura, and Mitsune Tanimoto**

## Abbreviations

| | |
|---|---|
| ABC | Adenosine triphosphate-binding cassette |
| BCRP | Breast cancer resistance protein |
| BMI1 | B-cell–specific Moloney murine leukemia virus integration site 1 |
| CSC | Cancer stem cell |
| EMT | Epithelial to mesenchymal transition |
| ERCC1 | Excision repair cross-complementation group 1 |
| iPSC | Induced pluripotent stem cell |
| MDR1 | Multidrug resistance transporter 1 |
| ROS | Reactive oxygen species |
| TS | Thymidylate synthase |

## Introduction

Cancer stem cells (CSCs) are a rare population of undifferentiated cells that are responsible for tumor initiation, maintenance, and spreading. They are resistant to anticancer agents and can self-renew and generate progeny in the form of

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differentiated cells that constitute most of the cells in tumors [1–3]. Because a surviving population of CSCs after conventional treatment might be responsible for tumor regrowth, identifying and eradicating the CSC population are very important. The mechanisms causing drug resistance in CSCs are still poorly understood; CSCs may be intrinsically resistant to chemotherapeutic agents due to their low proliferation rate and may have different expressions of adenosine triphosphate (ATP)-binding cassette (ABC) transporters, molecular metabolic mediators, cell-cycle kinetics, and the antioxidant system from non-CSCs. In this section, we describe the resistant mechanisms of CSCs to conventional chemotherapy and radiotherapy, and advance some strategies to eliminate CSCs.

## Multidrug Efflux Mechanism

Both normal stem cells and CSCs commonly express drug pumps such as ABC transporters that include multidrug resistance transporter 1 (MDR1)/P-glycoprotein/ABCB1 and breast cancer resistance protein (BCRP)/ABCG2 [4]. Leukemic side population (SP) cells, which are enriched for CSCs, have an amplified ability to pump chemotherapeutic drugs such as daunorubicin and mitoxantrone out of the cell, suggesting that increased drug removal ability contributes to chemotherapy resistance of CSCs [5]. The SP fraction expressed both BCRP and MDR1 in lung cancer cell lines [6]. In addition, stemlike neuroblastoma cells displayed a similar ability to pump mitoxantrone, resulting in increased cell survival [7]. MDR1 has been shown to remove vinblastine [8] and paclitaxel [9], and BCRP prevented accumulation of mitoxantrone, doxorubicin [10], imatinib mesylate [11], topotecan [12], and methotrexate [13]. Therefore, the overexpression of ABC transporters is implicated in expelling chemotherapeutic agents from cells and mediating chemotherapy resistance in CSCs.

## Molecular Metabolic Mechanism

CSCs also express molecular metabolic mediators such as aldehyde dehydrogenase 1 (ALDH1), which have been shown to confer resistance to cyclophosphamide in hematopoietic cell lines [14]. ALDH1 activity was revealed to be amplified in leukemic CSCs and contributed to their chemoresistivity including cyclophosphamide [15]. ALDH1 was also expressed in breast CSCs and its high expression was associated with a poor prognosis [16], suggesting that the molecular metabolic mechanism expressed in CSCs may directly affect patient outcome. Moreover, ALDH1 positive lung cancer cells displayed significant resistance to epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor (gefitinib) and anticancer chemotherapeutic drugs (cisplatin, etoposide, and fluorouracil), compared to ALDH1 negative

lung cancer cells [17]. The finding shows that ALDH1 contributes to the resistance of both cytotoxic agents and molecular targeting therapeutics.

Similarly, there are some enzymes associated with chemoresistivity, such as excision repair cross-complementation group 1 (ERCC1) and thymidylate synthase (TS). ERCC1 is one of the DNA repair proteins and is associated with cisplatin resistance. Non-small lung cancer patients who received cisplatin-based adjuvant chemotherapy showed a significantly longer survival when their tumors were ERCC1 negative [18]. Also in advanced disease, lower *ERCC1* mRNA levels were highly predictive for longer survival after cisplatin-based treatment [19, 20]. Ota et al. demonstrated a correlation between protein expression of ERCC1 and BCRP which is one of the ABC transporters [21]. In addition, TS, which is one of the key enzymes in the folate metabolism, was also overexpressed in CSCs. The overexpression of TS showed strong association with response to folate antagonists such as pemetrexed [22]. Salnikov et al. demonstrated a correlation between protein expressions of TS and the stem cell antigen CD133 [23]. These findings indicated that tumor cells with predominant expression of these enzymes might bear stem cell characteristics.

## Cell-Cycle Kinetics

Cellular sensitivity to chemotherapeutic agents also relies on cell-cycle kinetics. Highly proliferative cells undergo lethal cellular damage by anticancer agents, but quiescent cells are believed to escape the process. Because the turnover of normal stem cells and CSCs are less frequent than differentiated amplifying cells, cytotoxic agents are considered to have little effect on CSCs. For example, CSCs in acute and chronic myelogenous leukemia were relatively quiescent, contributing to therapeutic resistance [24, 25]. In lung cancer cell lines, the SP cells showed lower mRNA levels of *MCM7*, a member of the minichromosome maintenance family and proliferation marker, suggesting that a majority of the SP fraction was in the G0 quiescent state [6].

## Epithelial to Mesenchymal Transition

Epithelial to mesenchymal transition (EMT) has been broadly shown to convert mature cancer cells into CSCs [26, 27], which is mediated by EMT-associated genes and microRNAs [28–30]. CSCs as well as cells undergoing EMT are considered to be more resistant to chemotherapy and radiotherapy than differentiated daughter cells. Cells undergoing EMT become invasive and develop resistance to anticancer agents. In fact, EMT can be induced by stresses such as exposure to cytotoxic drugs, radiation, and hypoxic conditions [31, 32]. Mani et al. initially disclosed that

immortalized human mammary epithelial cells (HMLEs) undergoing EMT were CSC-like as characterized by their CD44-high/CD24-low phenotype [27]. They revealed that the cells undergoing EMT had the properties of CSCs, including self-renewal and the capacity to form mammospheres. These findings suggest that EMT may play a role in the development of CSCs and properties of invasiveness, metastasis, recurrence, and chemoresistivity. In addition, upregulation of *TWIS*T was also associated with cellular resistance to paclitaxel in human nasopharyngeal, bladder, ovarian, and prostate cancers [33].

## Activation of BMI1

Emerging evidence shows that the chemoresistivity of CSCs is in part due to the activation of B-cell–specific Moloney murine leukemia virus integration site 1 (BMI1), a stem cell factor and polycomb group family member. BMI1 is reported to regulate the proliferation activity of normal, stem, and progenitor cells. BMI1 plays a role in cell cycle, cell immortalization, and senescence. Numerous studies demonstrate that BMI1, which is upregulated in various cancers, has a positive correlation with clinical grade/stage and poor prognosis. Although much evidence supports the role of BMI as a factor in chemoresistance, its mechanism of action is not fully understood [34]. BMI has been reported to be associated with the protection of tumor cells from apoptosis. Ovarian CSCs exhibiting high BMI1 levels have increased resistance to cisplatin and paclitaxel [35]. In addition, silencing of BMI1 significantly enhanced the antitumor efficiency of docetaxel against prostate cancer cells [36]. Modulation of reduced glutathione and CHK2 and H2AX molecules by BMI1 was reported as the underlying mechanism for chemoresistant behavior of ovarian tumor cells [37].

## Cell Surface Markers

Several cell surface markers, such as CD24, CD34, CD44, and CD133 are used for the identification and prospective isolation of CSCs. Here, we focus on CD133 and CD44.

CD133 is one of the most appropriate markers for human CSC [38, 39]. In small-cell lung cancer cell lines, CD133 and CD87 positive cells showed higher chemoresistivity than negative cells [40, 41]. And CD133+ lung cancer cells showed coexpression of BCRP and embryonic stem cell transcription factors *Oct 3/4* and *Nanog*, and were highly resistant to various chemotherapeutic drugs [42]. The stemness of CD133+ cells was lost when the expression of *Oct 4* was knocked down by siRNA [43]. These data suggested that CD133 might be just a marker of CSCs and not directly affect the chemoresistant mechanism. Moreover, CD133+ brain tumor stem cells were selectively resistant to radiation, both in vitro and in vivo, and the

mechanism for this resistance was considered to be preferential activation of the DNA damage checkpoint response and an increase in DNA-repair capacity [44].

CD44 is also an extensively researched marker for CSCs in many epithelial tumors [45] and is associated with tumor growth, invasion, and metastasis. After alternative mRNA splicing, numerous variant (v) isoforms of CD44 are generated. Interaction of CD44v with cystine transporter subunit xCT enhances the ability of CSCs to defend against reactive oxygen species (ROS) and thereby promotes tumor growth [46]. Sulfasalazine, a specific inhibitor of xCT-mediated cystine transport, selectively kills the stemlike tumor cells [47]. Based on these results, a phase 1 study of sulfasalazine in patients with gastric cancer was conducted. Patients with high CD44v expression achieved reduced expression of CD44v after the administration of sulfasalazine for 2 weeks as well as a decreased level of glutathione [48].

## CSC Targeted Therapy

Combination therapy targeted for CSCs and differentiating cells is a key strategy to eliminate cancer cells. Therefore, we have to screen potential new drugs that are effective for CSCs for more efficient cancer treatment. As described above, sulfasalazine for CD44v positive cells is based on this concept. Moreover, some signal pathways are considered to be important in CSCs, such as Hedgehog, Notch, and Wnt/β-catenin pathways [49].

Activation of Notch signaling occurs when the Notch receptor undergoes a conformational change that allows proteolytic cleavage by γ-secretase, releasing an intracellular domain that undergoes nuclear translocation and modulates Notch-specific gene expression. The inhibition of Notch signaling promotes cell differentiation, increases sensitivity to chemotherapy and reduces metastasis [50]. Some clinical trials with inhibitor of γ-secretase–mediated Notch cleavage were undergone [51]. Other clinical trials targeting Hedgehog or Wnt/β-catenin pathways have also been conducted, although there have been no conclusive results that support the efficacy.

## Investigating Human Cancer Using Stem Cell Models

Induced pluripotent stem cells (iPSCs) from primary cells in patients with specific diseases have been used as valuable human disease models [52]. However, iPSC cell lines from human cancers had not been reported until 2013. Kim et al. could reprogram a cell from a recurrent, late-stage human pancreatic cancer to a near-pluripotent state [53]. Although it remains unclear whether the approach using iPSC will work with other solid tumors, their model will be very useful to identify real CSCs and to screen the drugs overcoming treatment resistance.

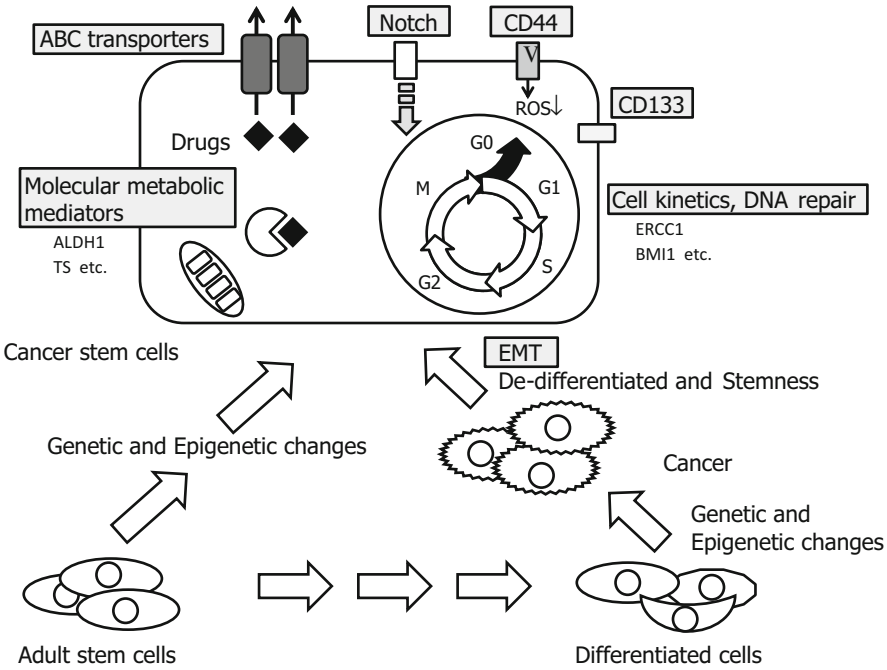


**Fig. 7.1** The diverse chemoresistant mechanisms of cancer stem cells

## Conclusion

The chemoresistance of CSCs seems to be caused by diverse mechanisms such as increased drug efflux, metabolic alterations, cell-cycle kinetics, and EMT with different circumstances involved (Figure 7.1). The discovery of compounds targeting CSCs will allow these resistant populations to be eradicated and will prevent recurrence of the disease.

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# Chapter 8
# Stem Cells to Understand the Pathophysiology of Autism Spectrum Disorders

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## Introduction

Autism spectrum disorders (ASD) are a heterogeneous group of neurodevelopmental disorders characterized by severe developmental defects in social response and communication accompanied by inappropriate repetitive behavior [1]. ASD has an overall prevalence of ~1 % [2]. There is a strong male bias, with a ratio of about 4 males to 1 female, particularly among those with milder ASD forms [3]. ASD can be a clinical manifestation of several well-characterized monogenic disorders. The ASD penetrance in these disorders varies, and may reach values of ~60 % in Fragile X syndrome (FXS), tuberous sclerosis, and Timothy syndrome [4–6]. ASD is also a relevant phenotype in Rett, Rett-like or *CDKL5*-associated syndrome, Angelman and Phelan–McDermid syndromes (PMDS) [7–9]. The molecular genetic

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mutational mechanism is very well characterized in all of these conditions. For didactic purposes, we will refer to the group of monogenic disorders frequently associated with ASD as syndromic ASD forms, in contrast to ASD or nonsyndromic ASD, in which the major clinical manifestation is autism, associated or not with other clinical features, such as epilepsy and hyperactivity, and in which clinical features are not sufficient to define a syndrome.

A high heritability component in ASD has been estimated, varying from 50 to 90 % [3, 10]. The recurrence risk in families with only one ASD-affected individual is about 10 %, and male siblings of ASD individuals have a ~3-fold increase of the risk of being affected by ASD in comparison to female siblings [3, 10]. Recurrence risk also increases according to the number of ASD-affected individuals in the family, thus reinforcing the genetic basis of ASD. The disease model seems to be very complex, with a high level of heterogeneity. Based on several worldwide genome-wide association studies (GWAS), there is a consensus that common variants, each with a low predictive risk, play a role in ASD etiology; however, the actual contribution of these variants to ASD heritability is still unclear [3, 10, 11]. Genomic studies, in contrast, have revealed that rare CNVs or rare sequencing variants with moderate-to-high penetrance are associated with the etiology of ASD in at least 20 % of the cases [3, 10, 12, 13]. These studies have revealed a remarkable genetic heterogeneity, as less than 20 of the 100 candidate genes present recurrent putative pathogenic variants. Despite the high heterogeneity of ASD, a growing number of evidence has shown that candidate genes belong to convergent pathways, nowadays represented by regulation of transcription (chromatin remodeling genes and transcription factors), regulation of protein abundance (splicing, translation, and ubiquitination genes), synapse (cell adhesion, sodium and calcium channels, *N*-methyl-D-aspartate (NMDA) receptors, and synaptic scaffold genes), and intracellular signaling factors that regulate cell growth and proliferation (mTOR/PI3K and RAS genes) [3, 12, 13].

The majority of these candidate pathogenic variants have been classified as loss-of-function variants, which occur with a population frequency of about 5 % [14]. Therefore, one of the current challenges in the genomic analysis of ASD is pinpointing causative variants; moreover, it is necessary to define the penetrance associated with each one of them and how many pathogenic variants are sufficient for a complete ASD penetrance per individual. Finally, the functional effects of the identified variants must be demonstrated and be translated to the phenotypic effect. One approach to address the above-mentioned questions is to conduct functional studies in order to verify which of these candidate variants are sufficient to cause morphological and functional changes in neurons.

Stem cells have recently emerged as a promising alternative to conduct functional cellular studies, particularly in disorders where the tissue of interest is of limited or nearly no access. This is exactly the case for ASD and several other

neurodevelopmental disorders, in which the ideal tissue of interest to conduct cellular and molecular studies is the developing brain. Another drawback is the impossibility to get an accurate diagnosis for these disorders before 1 year of age. Murine model systems provide genetic homogeneity and allow the study of behavioral phenotypes, but show limited applicability to understand how diseases affect human neocortical regions, and often do not recapitulate the complex human neurodevelopment [15, 16].

Stem cells from human exfoliated deciduous teeth (SHED) are an easily accessible cell type comprised by populations of mesodermal and neuroectodermal origin [17–19]. The identification of cellular pathways associated with diseases can be successfully achieved by studying SHED, particularly when they share the same embryological origin with cells of the main disease-affected tissue, as in the case of ASD [20–23]. However, the use of SHED is restricted by their limited potential to be differentiated into functional neurons. These difficulties have been overcome, thanks to the possibility of reprogramming somatic cells to a pluripotent state by overexpressing specific transcription factors [24, 25]. These cells, known as induced pluripotent stem cells (iPSCs), have opened a new world of possibilities, as they can be differentiated toward multiple cell lines, including neurons; therefore, the generation of disease-specific neurons by reprogramming somatic cells from ASD patients have empowered researchers to functionally characterize genetic alterations and determine how they lead to ASD neuronal phenotypes. In this regard, both glutamatergic and GABAergic neurons as well as astrocytes, which are of interest to study ASD-related phenotypes, can be obtained [26]. Further, cells from different sources, such as SHED and erythroblasts, in addition to fibroblasts, which were the first type of cells used to obtain iPSCs, can be reprogrammed by these transcription factors with similar efficiency [23, 27]. Even though human embryonic stem cells (hESCs) and neural stem cells (hNSCs) can also be employed to model diseases in a dish, they are in practice, more difficult to be obtained, and their use still represents an ethical issue. Also, the use of these cells is hampered by the impossibility to establish the diagnosis in the early developmental period, particularly for nonsyndromic forms of ASD (Fig. 8.1).

We are currently at the dawn of human stem cell modeling of ASD and ASD-associated monogenic disorders. Although much effort still needs to be gathered to fully understand the etiology of these disorders, the results obtained so far have been encouraging and exciting, as they have unraveled new biological pathways and provided a causal relationship between pathogenic mutations and morphological and functional neuronal alterations. Importantly, in several situations, such findings recapitulate relevant cellular and/or molecular phenotypes previously reported in murine models or other approaches.

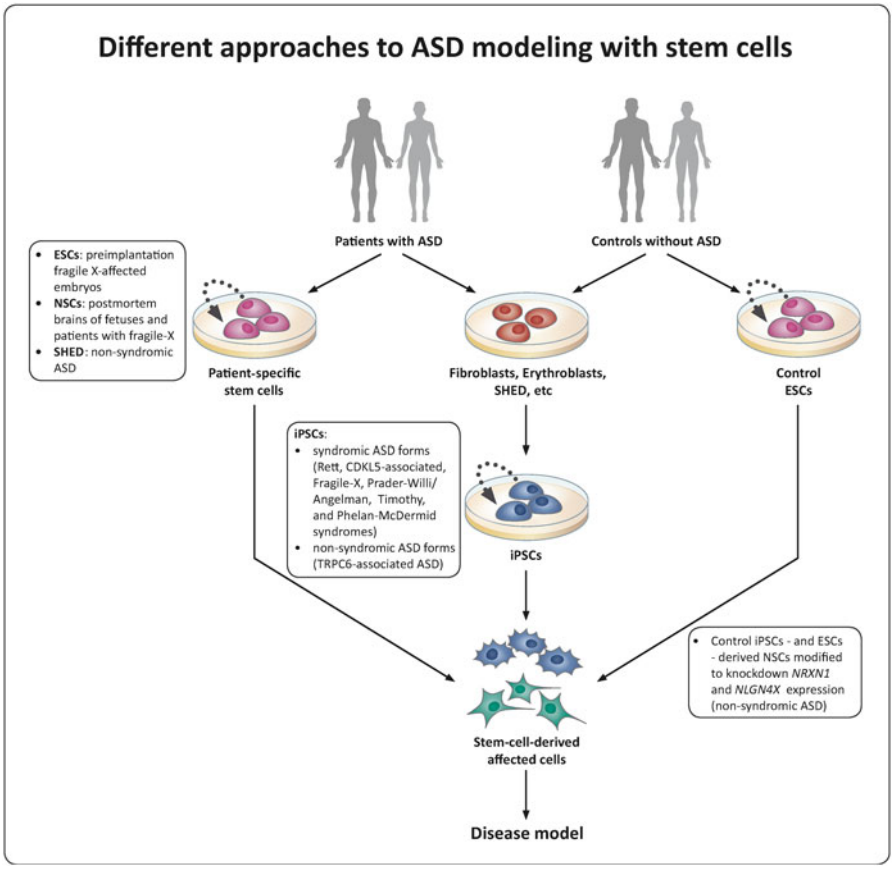


**Fig. 8.1** Schematic summary of different approaches to ASD modeling using human stem cells. Patient-derived embryonic stem cells (ESCs) and neural stem cells (NSCs) were used to model the syndromic ASD fragile-X syndrome. Although these patient-derived stem cells have the advantage of not requiring any genetic or epigenetic manipulations to be relevant for disease modeling, they are, in practice, difficult to be obtained. Stem cells from human exfoliated deciduous teeth (SHED) represent an accessible source of patient material and have recently been used to model nonsyndromic forms of ASD. Induced pluripotent stem cells (iPSCs) can be generated from adult cells from ASD patients and can be differentiated into the disease-affected cells. At least six monogenic disorders that include ASD features as part of the phenotype have been modeled by the use of iPSC methodology: Rett syndrome, *CDKL5*-associated ASD, Fragile-X syndrome, Prader–Willi/ Angelman syndrome, Timothy syndrome, and Phelan–McDermid syndrome. iPSCs have also been used to model nonsyndromic forms of ASD, such as TRPC6-associated ASD. Finally, control-derived stem cells engineered to reduce (knockdown) a particular gene's expression have been used to model the neurodevelopmental impact of Neurexin 1 (*NRXN1*) and Neuroligin 4X (*NLGN4X*) deletions, associated with nonsyndromic ASD and other neurodevelopmental disorders. Figure modified from Sterneckert et al. (2014)

## iPSCs: Modeling Monogenic Disorders Featuring ASD

To date, six genetically well-characterized monogenic disorders with ASD features have been modeled with the use of iPSC technology. In the following segment, we provide a short background and summarize the most significant findings and current state of the art for each disorder.

### *Fragile X Syndrome*

FXS is the most commonly inherited form of intellectual disability, which may be accompanied by a characteristic appearance in affected males (large head, long face, prominent forehead and chin, protruding ears), connective tissue defects, and postpubescent macroorchidism [28, 29]. Autistic features may be present in up to 60 % of all cases, depending on the clinical criteria adopted [30–33]. FXS is caused by expansions of CGG trinucleotide repeats in the 5′ untranslated region of the *FMR1* (fragile X mental retardation 1) gene in the X chromosome. The expansions lead to hypermethylation of the *FMR1* promoter and consequent silencing of FMRP (fragile X mental retardation protein), an RNA-binding protein involved in mRNA localization and protein synthesis during synaptic plasticity [34, 35]. In hESCs derived from FXS-affected blastocyst-stage embryos, *FMR1* expression is active and gene silencing takes place upon differentiation [36]. However, this is not observed in iPSCs derived from FXS patients, in which reprogramming adult cells to a pluripotent state does not reset the epigenetic marks associated with *FMR1* silencing [37]. These findings illustrate how iPSCs and ESCs, albeit similar in many aspects, still exhibit differences that might be relevant to the disease under investigation and that should be considered when selecting cell types for disease modeling. In the case of FXS, since *FMR1* is kept silenced during neuronal differentiation, FXS-derived iPSCs remain a suitable model to study neuronal changes caused by the expansion mutations. Additionally, one advantage of FXS-derived iPSCs is the ability to generate cells from clinically well-characterized patients and the possibility of investigating different-sized mutations present in the same individual (a consequence inherent to the dynamic nature of the expansions; [38, 39]). Such approach has shed light on the genotype–phenotype relationship in FXS, showing defective neurite formation and outgrowth occurring prior to synaptogenesis, during neuronal differentiation [38, 40]. Importantly, a previous study using an *Fmr1*-knockout mouse model and in vitro neural stem cells (NSCs) from postmortem brain of a fragile-X fetus also described reduced neurite outgrowth and branching and altered neuronal differentiation in FMRP-deficient NSCs [41].

## *Timothy Syndrome*

Timothy syndrome (TS) is a rare, severe neurodevelopmental disorder accompanied by cardiac defects/arrhythmia and facial dysmorphisms, and one of the most penetrant monogenic forms of ASD. This disorder is caused by mutations in *CACNA1C* (calcium channel, voltage-dependent, L type, alpha 1C subunit), a gene encoding an alpha subunit of a voltage-dependent calcium channel ($Ca_v1.2$) [5]. Neurons derived from TS iPSCs showed action potentials and increased intracellular $Ca^{++}$ concentration indicative of loss of calcium channel inactivation. TS neurons also showed abnormal expression of tyrosine hydroxylase, which could be rescued with the drug roscovitine, a compound that increases the inactivation of L-type calcium channels. Moreover, gene expression studies revealed that iPSC-derived neural progenitor cells (NPCs) and iPSC-derived neurons from TS patients show alterations in expression of genes previously associated with ASD, as well as similarities with gene expression patterns observed in postmortem idiopathic ASD brains [42, 43]. Also in TS-derived iPSCs, Krey et al. [44] observed that depolarization causes dendritic retraction in TS neurons, which occurs independently of the excessive $Ca^{++}$ influx seen in these cells. In fact, their results suggested that the dendritic phenotype was caused by decreased binding between TS $Ca_v1.2$ and the GTPase Gem, leading to ectopic activation of RhoA. These findings directly link $Ca_v1.2$ channels to RhoA signaling in the brain and provide new grounds for studying ASD neuronal phenotypes in vitro.

## *Rett Syndrome*

Rett Syndrome (RTT) is a severe progressive neurodevelopmental disorder mainly caused by mutations in the X-linked gene *MECP2* (methyl CpG-binding protein 2) [45, 46]. RTT individuals undergo apparently normal development until 6–18 months of age, followed by impaired motor function, stagnation and regression of developmental skills, hypotonia, seizures, and autistic behavior [45, 47]. MeCP2, the encoded protein, is involved in transcriptional regulation by binding to methylated CpG dinucleotides, and recruiting proteins involved in chromatin remodeling [48]. Marchetto et al. [47] were the first group to model RTT in human cells. By generating iPSCs from RTT patient-derived fibroblasts, they found that deficiency of *MECP2* in RTT neurons resulted in smaller soma, fewer dendritic spines and synapses, and impairment in calcium signaling and in excitatory synaptic transmission, by comparison to control, unaffected neurons. Most of these neuronal phenotypes, such as reduced soma size and dendritic arborization, have been subsequently corroborated by groups employing different techniques to generate *MECP2*-deficient neurons from pluripotent stem cells [49, 50]. Li et al. [50] used gene-editing techniques to generate *MECP2*-deficient hESCs, and showed that mutant neurons exhibit global reduction in translation and protein synthesis, and reduced AKT/mTOR activity. Studies in mouse models have suggested a role for astrocytes in RTT pathogenesis, which has

been recently confirmed in human cells. Through differentiation of RTT iPSCs to astrocytes and employment of a series of co-culture experiments, Williams et al. [51] showed that *MECP2*-mutated astrocytes lead to reduction in neurite length and in the number of terminal ends of wild-type neurons, and this non-cell-autonomous influence was partially mediated by factors secreted by mutant astrocytes.

Modeling RTT cells in a dish has shown that neuronal disease-related phenotypes can be rescued in vitro. Treatment with insulin-like growth factor-1 (IGF-1) or gentamicin has been shown to improve synaptic density in RTT neurons [47], and addition of IGF-1 or BDNF (brain-derived neurotrophic factor) improved protein synthesis through activation of AKT/mTOR pathway. Importantly, these findings provide proof-of-principle evidence for the application of ASD iPSC-derived neurons in drug discovery.

## *CDKL5-Associated Syndrome*

Dominant-negative mutations in *CDKL5* (cyclin-dependent kinase-like 5), also located in the X chromosome, are responsible for a RTT-like phenotype [52, 53], herein referred to as *CDKL5*-associated syndrome. Patients mainly exhibit early-onset intractable seizures before 6 months of age, severe developmental delay, and autistic features [52–55]. *CDKL5* encodes a serine/threonine kinase whose role in brain development is not fully understood. *Cdkl5* silencing in a mouse model has shown the importance of this gene for dendritic spine morphogenesis and maintenance of synaptic contact, which occurs via interaction between the postsynaptic proteins NGL-1 and PSD-95, stabilized through phosphorylation of NGL-1 by *CDKL5* [56]. The use of patient-derived iPSCs harboring loss-of-function mutations in *CDKL5* further confirmed those findings, as patients' iPSC-derived neurons exhibited a significantly reduced number of synaptic contacts and lacked presynaptic terminals [56].

## *Angelman Syndrome and Prader–Willi Syndrome*

Angelman syndrome (AS) and Prader–Willi syndrome (PWS) were the first imprinting disorders described in humans, in which alterations in the chromosomal region 15q11-q13 lead to different phenotypes depending on which chromosome (paternal or maternal) is affected [57]. AS is characterized by significant intellectual disability, absent speech, frequent seizures, motor impairment, and a typical happy demeanor [58]. PWS is characterized by small stature, neonatal hypotonia, hypogonadism, mild-to-moderate intellectual disability, and compulsive hyperphagia [59]. AS is caused by loss of function of the maternally inherited allele of *UBE3A* (ubiquitin-protein ligase E3A), which undergoes tissue-specific genomic imprinting with silencing of the paternally inherited allele in brain tissues [60, 61].

When the paternal chromosome is deleted in the same chromosome region, individuals develop PWS due to loss of a cluster of several species of small nucleolar RNAs [62]. Although autistic features are reported in AS but not in PWS, the 15q11-q13 region has been systematically associated with ASD [63]. Chamberlain et al. [64] were the first to show that iPSCs derived from AS and PWS fibroblasts maintain the genomic imprinting at 15q11-q13. Moreover, they confirm that expression of the paternally inherited *UBE3A* is repressed upon neuronal differentiation in AS cells, recapitulating the main epigenetic characteristics of AS in vitro. Additionally, in neurons differentiated from PWS-derived iPSCs, Cruvinel et al. [65] showed that the zinc-finger protein ZNF274, in association with the histone methyltransferase SETDB1, might protect against methylation of the small nucleolar RNA cluster in the PWS region. Together these findings suggest that iPSC modeling of alterations at 15q11-q13 is a promising strategy to better understand PWS, AS and ASD.

### *Phelan–McDermid Syndrome*

Another syndrome frequently associated with ASD is Phelan-McDermid syndrome (PMDS). This syndrome is caused by heterozygous deletions of variable sizes in chromosome 22 (region 22q13.3). Besides ASD, PMDS patients may exhibit hypotonia, normal to accelerated growth, and minor dysmorphic features [66]. Studies attempting to establish a critical region for the syndrome, in combination with analysis of rare ASD-related mutations and functional studies have appointed *SHANK3* to be the most likely candidate responsible for the neurological phenotype in PMDS [7, 67]. Produced iPSC-derived neurons from PMDS patients and observed a reduced amplitude and frequency of spontaneous excitatory synaptic events. Such phenotype was caused by impaired AMPA- and NMDA-mediated transmission. Overexpression of *SHANK3* rescued the electrophysiological alterations found, showing that this gene significantly contributes to the neuronal alterations in PMDS. By treating the PMDS neurons with IGF-1, the authors were also able to restore the excitatory synaptic defects. Intriguingly, treatment with IGF-1 decreased the expression of *SHANK3* in control and PMDS neurons. They found that IGF-1 actually increases the number of synapses that lack *SHANK3* but contains PSD-95, which have faster deactivation of excitatory currents, a kinetics that resemble that of neurons appearing later in development.

## Stem Cells to Model Nonsyndromic ASD

Lymphocytes and fibroblasts have been used in several studies in order to dissect the cellular pathways altered in nonsyndromic forms of ASD [68, 69]. However, even though acquiring these cells is relatively simple and with minor ethical implications,

they present limitations to study neurodevelopmental pathways, as they are mature, post-natal cell types, and possess non-neural embryological origins.

SHED are an alternative and interesting source of patient-derived cells to be studied, as they can be noninvasively isolated, they show the same early embryonic origin as neurons, and they express neural progenitor markers [70]. Therefore, SHED may bear genetic regulatory networks that resemble those found in neurons. As detailed below, in three recent studies, we show the applicability of SHED in dissecting the genetic regulatory circuitry in nonsyndromic ASD.

Griesi-Oliveira et al. [22] assessed the gene expression profile of SHED from seven nonsyndromic ASD individuals, with no defined genetic mechanism. By comparing cases and controls, the authors identified 683 differentially expressed genes (DEGs), of which a significant number is expressed in brain and is involved in mechanisms and molecular pathways previously associated with ASD, such as cytoskeleton regulation, axonal guidance, protein synthesis, and cellular adhesion. Among the identified DEGs, one of the upregulated genes was *CHD8*, which has been found to be mutated in about 0.3 % of ASD cases in more than one study [12, 71–74]. Interestingly, *CHD8* is a co-regulator of androgen-responsive transcription [75] and androgen receptor, as well as a significant number of genes regulated by this receptor presented overexpression in the studied set of patients. The authors suggested that this might be a possible mechanism through which *CHD8* can contribute to ASD, especially considering the skewed male-to-female prevalence in such disorders.

Suzuki et al. [76] used SHED to investigate the role of mTOR (mammalian target of rapamycin) signaling pathway in nonsyndromic ASD pathophysiology. mTOR-signaling pathway regulates several essential cellular processes including cell growth, proliferation, autophagy, and protein synthesis [77]. In the central nervous system, mTOR signaling is crucial from the early stages of neural development, controlling self-renewal and differentiation of hNSCs and, in neurons, mTOR signaling is involved in synapse formation and plasticity. Dysfunctional mTOR signaling and dysregulated protein synthesis in neuronal cells have been associated with several monogenic syndromes with high prevalence of autism, such as FXS, tuberous sclerosis, and *PTEN*-related syndromes, which are caused by mutations in, respectively, *FMR1*, *TSC1/2*, and *PTEN*, molecules known to be negative regulators of mTOR pathway [78]. Functional studies addressing mTOR-signaling activity in patients with nonsyndromic ASD were lacking. To examine this important question, Suzuki et al. have made use of cultured SHED derived from 13 patients with nonsyndromic ASD, who were negative for *FRM1*, *TSC1*/*2*, and *PTEN* mutations, and 11 age- and sex-matched controls. They observed that SHED derived from three patients (23 % of the patient sample) showed dysregulation of mTOR-signaling pathway in response to extracellular nutrient availability, enhanced proliferative capacity at higher cell densities, and reduced response to the antiproliferative effect of rapamycin, a specific mTOR inhibitor. Together, the results suggest that dysregulation of mTOR signaling plays an important role in the pathogenesis of a subgroup of nonsyndromic ASD, and that mTOR pathway components might be promising therapeutic targets for these patients. Interestingly, these results were further

corroborated by two studies showing altered mTOR signaling in postmortem brain of patients with nonsyndromic ASD [79, 80].

Griesi-Oliveira et al. [23], by molecularly characterizing the breakpoints of a balanced translocation between chromosomes 3 and 11 in a patient with nonsyndromic ASD, evidenced disruption of *TRPC6*, a gene that encodes a cation channel. They demonstrated that the transcriptome of SHED from this patient with haploinsufficiency of TRPC6 (TRPC6-mut) is dysregulated as compared to control SHED, with enrichment for genes important for cytoskeleton structure and regulation, such as *SEMA3A*, *EPHA4*, *INA*, and *MAP2* [23]. Moreover, a significant number of differentially expressed genes between the TRPC6-mut patient's SHED and control SHED are putative targets of CREB, a transcription factor known to be activated via calcium influx through TRPC6 [81]. Using hyperforin, a specific activator of the channel, the authors also confirmed that part of such genes could in fact be regulated by TRPC6 activation. In order to evaluate how representative these findings would be in neurons, Griesi-Oliveira et al. assessed the phenotype of neuronal cells derived from TRPC6-mut patient iPSC lines obtained from SHED, which represents the first work to evaluate a nonsyndromic case of ASD using such model. Calcium influx upon activation of TRPC6 was reduced in NPCs from the TRPC6-mut patient. Paralleling the results found in SHED, activation of TRPC6 in NPCs leads to expression regulation of some of CREB target genes. TRPC-mut neurons have shorter neurites, with a reduction in arborization complexity and lower density of dendritic spines and glutamatergic vesicles compared to control neurons. These results are consistent with previous and authors' findings in rodent models [81, 82]. Moreover, using gain and loss of function models, authors demonstrated that such alterations could indeed be attributed to TRPC6 function. Interestingly, by taking advantage of a pair of isogenic iPSC line of a RTT patient, one with the mutated copy of MeCP2 inactivated by X-chromosome inactivation, and one with this copy activated, authors showed that MeCP2 is involved in TRPC6 expression regulation, pointing to a shared molecular pathway between a syndromic and nonsyndromic form of ASD. Although using cells in different developmental states, the work points to a mechanism in which haploinsufficiency of TRPC6 leads to reduced calcium influx and consequent dysregulation of the expression of neurodevelopmental genes, at least in part by CREB activity modulation. Such expression dysregulation then would lead to neuronal morphological and functional alterations (Fig. 8.2). Finally, Griesi-Oliveira and colleagues demonstrated that neuronal abnormalities in TRPC6-mut neurons could be rescued with hyperforin or IGF-1 treatment.

The use of control-derived stem cells engineered to reduce (knockdown) a particular gene's expression can also be an approach to in vitro model molecular dysfunction associated with nonsyndromic ASD. In two recent studies, the neurodevelopmental impact of *Neurexin 1* (*NRXN1*) and *Neuroligin 4X* (*NLGN4X*) deletions, known to be associated with nonsyndromic ASD and other neurodevelopmental disorders, were investigated using human iPSCs and hESCs as in vitro models [83, 84]. *NRXN1* is a presynaptic neuronal adhesion molecule that interacts with postsynaptic neuroligins, such as *NLGN4X*, in excitatory and inhibitory synapses in the brain to form an inter-synaptic complex required for synapse formation and

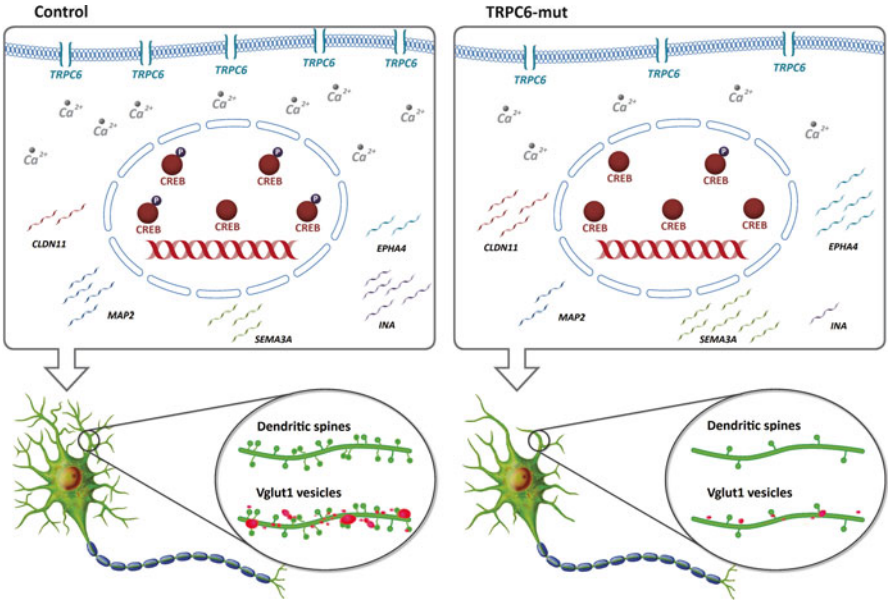


**Fig. 8.2** TRPC6 haploinsufficiency consequences in neuronal phenotype: TRPC6 disruption leads to a lower expression of these channels in cell membrane and consequent lower $Ca^{++}$ influx into cells. Calcium signaling through TRPC6 leads to CREB activation, which is consequently diminished in TRPC6-mut cells. This leads to gene expression abnormalities, probably due to dysregulation of CREB activation. A significant number of such dysregulated genes are important for neuronal development and function, especially genes related to cytoskeleton dynamics. Indeed, TRPC6-mut neurons presented less and shorter neurites and a reduction on spine density and glutamatergic vesicles, when compared to controls

function. Zeng et al. [83] showed that reduction of *NRXN1* expression in both iPSC-derived hNSCs and hESC-derived hNSCs leads to alterations in the expression levels of several genes involved in cell adhesion and neuron development during differentiation of the hNSCs into mature neurons. Additionally, NSCs with *NRXN1* knockdown showed reduced astrocyte differentiation potential. These results suggest that *NRXN1* deletion might impair nervous system development and synaptic adhesion and transmission. Using a similar approach, Shi et al. [84] knocked down *NLGN4X* expression in iPSC-derived NSCs and observed transcriptome alterations as well as morphological changes during differentiation of NSCs into mature neurons over a 6-week period. The authors observed that *NLGN4X* knockdown alters the expression patterns of several biological pathways including nervous system development and neuron differentiation, impairs the differentiation of the NSCs into neurons, and compromises neurite formation and inter-cell connections. In conclusion, these two studies combined in vitro stem cell models and targeted gene silencing to explore molecular, cellular, and neurodevelopmental effects of loss-of function mutations in ASD-associated genes.

## Conclusions and Perspectives

Disease modeling in a dish with the use of stem cells has proven to be, so far, a very promising avenue to study ASD, both in its syndromic and nonsyndromic forms. In general, most of the alterations found in iPSC-derived neurons possessing different ASD pathogenic mutations are comparable to data obtained from animal models or brain-derived tissues or cells (Table 8.1). This remarkable concordance thus validates disease modeling with iPSCs, which, despite being an in vitro biological system, is able to reproduce in vivo observations. Nevertheless, in its current state, the use of reprogrammed cells should always be viewed as a complementary approach, as there are still limitations to translating how the morphological, functional or transcriptional changes observed in neural iPSC-derived cells lead to alterations in the human phenotype. In the near future, an extensive cellular characterization of a wide spectrum of ASD variability and mutational mechanisms is anticipated to unveil these relationships.

The monogenic ASD syndromic forms are caused by mutated genes belonging to different but related cellular pathways. The phenotypes can be caused by a variety of mutational mechanisms, including loss-of-function, gain-of-function, and dominant-negative mutations, associated with different functional effects at the cellular level. In spite of the genetic heterogeneity associated with the monogenic ASD syndromic forms, in general, the major pathophysiological consequences of the mutations in iPSC-derived neurons apparently are altered dendritic arborization and impaired synaptic function. It is also relevant to mention that comparable neuronal changes have been observed in iPSC modeling of nonsyndromic ASD. These observations raise some possibilities that deserve our attention: (a) impaired dendrite formation can be a feature shared by many neurodevelopmental disorders, as previously suggested [44]; (b) if impaired dendrite formation is such a common feature in these disorders, it will be impossible to assign this phenotype to specific neurodevelopmental phenotypes (e.g., ASD or cognitive deficit alone); (c) we still need to search for more specific synaptic changes or molecular markers at the cellular and molecular levels in order to establish precise correlations between neuronal phenotypes and clinical phenotypes, as suggested in *CDKL5*-associated syndrome. In *CDKL5*-mutated neurons, PSD-95, a protein that plays a significant role in learning and memory, is compromised as a consequence of the dominant-negative effect of the *CDKL5* mutations, which would explain the severe mental impairment in these patients.

Mesenchymal stem cells can be easily accessed and manipulated. The few studies conducted on this type of cells have shown promising results. For example, transcriptome analysis conducted on SHED from idiopathic ASD patients and on one patient with haploinsufficiency of TRPC6 revealed cytoskeleton dynamic genes to be one of the most relevant dysregulated pathways [22, 23]. Such dysregulation would predictably result in abnormal dendritic development, which was found in TRPC6-mutated iPSC-derived neurons exhibiting less dendritic arborization and extension [23].

Finally, dysregulation of mTOR signaling has been found in about 25 % of ASD patients through analysis of SHED cultures. This is quite an unexpected proportion, as pathogenic variants in mTOR-related genes have been found in a much smaller

**Table 8.1** Studies using iPSC-derived neuronal cells to model syndromic and non-syndromic ASD cases

| Disease/gene | iPSC-derived neuronal phenotype | Rescue | References | Similar results in animal models? | Similar results in postmortem or embryonic-derived hNSC? |
|---|---|---|---|---|---|
| *Syndromic ASD* | | | | | |
| Rett syndrome | Reduced glutamatergic synapses | IGF-1 | [47, 49, 51] | [85, 87, 92, 94, 95, 96] | [84, 86, 91] |
| | **Smaller soma and nuclear size** | BDNF | Ananiev et al. 2011 | | |
| | Reduced dendritic spine density | | Djuric et al. 2015 | | |
| | Alterations in calcium signaling | | | | |
| | Decreased frequency and amplitude of spontaneous postsynaptic currents | | | | |
| | Decreased transcription | | | | |
| | Defect in AKT/mTOR pathway (protein synthesis) | | | | |
| | Reduced capacity of mitochondrial electron transport chain | | | | |
| | **Reduced neurite complexity** | | | | |
| | **Reduction in action potential rates** | | | | |
| | Non-cell-autonomous glial effect | | | | |
| CDKL5-associated syndrome (CDKL5-dominant-negative mutations) | Reduced glutamatergic synapses | | [55] | [55] | n.r. |
| | Longer dendritic spines | | | | |

(continued)

**Table 8.1** (continued)

| Disease/gene | Disease neuronal-associated phenotype | Rescue | References | Similar results in animal models? | Similar results in postmortem or embryonic-derived hNSC? |
|---|---|---|---|---|---|
| Fragile-X syndrome (FMR1 loss-of-function) | **Fewer and shorter neurites** | | [38, 40] | [89] | [90] |
| | Reduced neurite outgrowth | | | | |
| Timothy syndrome (CACNA1C gain-of-function) | Defects in calcium signaling | Roscovitine | [42, 43, 44] | [42, 44] | n.r. |
| | Abnormalities in lower cortical and callosum neurons differentiation | | | | |
| | Abnormal expression of tyrosine hydroxylase and increased production of norepinephrine and dopamine | | | | |
| | Activity dependent dendritic retraction | | | | |
| Phelan McDermid syndrome (*SHANK3* loss-of-function) | Decreased frequency and amplitude of spontaneous postsynaptic currents | IGF-1 | Shcheglovitov et al. 2013 | [88] | n.r. |
| | Decreased expression of excitatory neurotransmitters receptors | | | | |
| | Reduced glutamatergic synapses | | | | |

| | | | | | |
|---|---|---|---|---|---|
| *Non-syndromic ASD* | | | | | |
| *TRPC6* loss-of-function | Defects in calcium signaling | Hyperforin | [23] | [79, 80] | n.r. |
| | Reduced dendritic spine density | IGF-1 | | | |
| | Reduced neurite complexity | | | | |
| | Shorter neurites | | | | |
| | Reduced glutamatergic synapses | | | | |
| *NRNX1* loss-of-function | Reduced astrocyte differentiation potential | | [81] | n.r. | n.r. |
| *NLGN4X* loss-of-function | Impaired neuron differentiation | | [82] | n.r. | n.r. |
| | Reduced neurite complexity and inter-cell connections | | | | |

In bold: phenotypes observed in more than one different study of the specific disease, *n.r.* not reported

proportion of ASD patients. Thus, the application of stem cells in the investigation of ASD etiology can not only be useful for dissecting the functional consequences of known mutations but also for aiding in the identification of common mechanisms involved in different ASD cases, even in those in which genetic alterations have not been identified.

Language impairment, difficulties in social interaction, and abnormal behavior with repetitive stereotyped movements are the main clinical hallmarks of ASD patients, and pharmacological treatments have yet to be elected to ameliorate these symptoms. Furthermore, due to the high genetic heterogeneity of nonsyndromic forms of ASD, personalized treatment for each patient has been expected to take place. However, the current studies in iPSC-derived neurons, both from syndromic and nonsyndromic patients, together with mouse models with different mutations, have revealed IGF-1 as a candidate molecule to rescue the phenotype in more than one situation. In this regard, in a phase one clinical trial using IGF-1 in PMDS, Kolevzon et al. [98] suggested in this pilot study that this drug was associated with improvement in both social impairment and restrictive behaviors in autistic children. The overall results indicate that IGF-1 might act in neuronal regulation in a very downstream manner, thus compensating any genetic alteration acting upstream. It will be important to investigate this hypothesis further, as understanding how IGF-1 regulates neuronal morphology and function can aid in finding a more universal drug for ASD treatment.

We stand in a very exciting period with great expectation to move toward a better understanding of ASD etiology and pathophysiology, and it seems that the use of stem cells will certainly change our knowledge in this field.

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