Chapter 6
Patient-Derived Induced Pluripotent Stem Cells Provide a Regenerative Medicine Platform for Duchenne Muscular Dystrophy Heart Failure

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6.1 Introduction

Mutations in the dystrophin gene cause dystrophinopathy, a hereditary disorder with variable allelic clinical presentations including Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), and X-linked dilated cardiomyopathy (XLDC). Though the disease is well known for the skeletal muscle involvement, most patients develop cardiomyopathy and eventually succumb to congestive heart failure. With ventilatory support preventing respiratory-related mortality, the greater cardiac workload associated with longer life expectancy is believed to increase the incidence of heart failure. Currently there are no effective therapies to contain the decline of heart function in these patients. Thus, it is urgent to devise new strategies to prevent, halt, or reverse the cardiomyopathy. To address this unmet medical need, we modeled dystrophin-deficient cardiomyopathy using cellular reprogramming technology, which involves converting adult somatic cells into pluripotent stem

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cells, termed induced pluripotent stem cells (iPSCs). Upon further induction, iPSCs can give rise to vast number of heart cells that can be used to study disease etiology and screen therapeutic compounds.

6.2 Dystrophin-Deficient Cardiomyopathy

6.2.1 Background

Dystrophinopathy refers to a group of genetic disorders, encompassing DMD, its milder variant BMD, and XLDC [1]. Though varied in clinical presentation, these diseases share common gene defects in dystrophin resulting in varying levels of dystrophin deficiency. Cardiac symptoms are invariably associated with all dystrophinopathy patients. Rare mutations cause localized dystrophin protein defects restricted to the heart, making the heart the only affected organ in XLDC. More commonly, mutations cause devastating skeletal muscle weakness for muscular dystrophy patients that overshadows any underlying cardiac abnormality. 98 % of DMD patients develop cardiac abnormalities, while congestive heart failure (CHF) and sudden cardiac death account for 10–20 % of the mortality in DMD patients. In contrast to their relatively mild skeletal muscle involvement, many BMD patients develop evident cardiac symptoms likely due to the cardiac workload imposed by the longer life span and vigorous physical activities. Cardiac complications are estimated to account for up to 50 % of the mortality in patients with DMD [1]. The mortality associated with cardiac failure is expected to rise even further, due to improved respiratory management that decreases fatal respiratory failure and extends the patients' life span.

Other reports have highlighted the linkage of dystrophin with several forms of acquired cardiomyopathy [2–4], suggesting that dystrophin protein remodeling may represent a common pathway underlying contractile dysfunction in failing hearts [5]. Thus, restoring normal function of the dystrophin-associated glycoprotein complex (DGC) could serve as a potential therapeutic target for heart failure patients.

6.2.1.1 Dystrophin Gene and the Mutations

The gene encoding dystrophin locates to the X chromosome, spanning 79 exons and covering 2.4 Mbp [6]. While the shortest isoform, DP71, is ubiquitously expressed in multiple tissues, the full-length transcript variant Dp427m is mainly expressed in muscle tissue, including the heart [7]. By forming a dystrophin-associated glycoprotein complex (DGC) together with the sarcolemma, dystrophin mainly functions as the hub to connect the intracellular actin filament with the extracellular matrix (ECM), providing mechanical support to reinforce the sarcolemma. On the other hand, dystrophin also serves as a scaffold protein to organize molecules in proper position for function, such as membrane receptors and signaling proteins like neuronal nitric oxide synthase (nNOS) [8]. Hence, dystrophin plays a critical role in both mechanical membrane support and in proper function of certain cell signaling pathways.

Numerous genetic mutations have been identified across the whole length of the dystrophin gene, but mainly enriched within two "hotspots." The most common region, 3' end hotspot lies within exon 45–55 with genomic breakpoints at intron 44, while the 5' end hotspot covers exons 2–19 with breakpoints in intron 2 and 7 [7]. Exon deletions and duplications are the most common forms of mutations. The severity of symptoms heavily depends on the maintenance of the open reading frame, rather than the size of mutated genomic regions. The frame shift hypothesis suggests that mutations maintaining the original open reading frame lead to the production of a truncated but partially functional protein, which usually leads to a milder clinical presentation in BMD. On the other hand, mutations shifting the reading frame completely cease protein production [7] with prominent disease manifestations.

6.2.1.2   Clinical Symptoms and Management

Cardiomyopathy in dystrophic patients is largely underdiagnosed and poorly managed [9], partly due to the fact that symptoms dynamically progress over time. To monitor disease progression, electrocardiogram, echocardiogram, and magnetic resonance imaging (MRI) can be used to determine the appropriate time and course of intervention. Cardiac manifestations associated with dystrophin deficiency include rhythmic disturbance, organ structural alteration, and hemodynamic abnormalities. A typical disease course includes three distinct but continuous stages [10]. The preclinical stage usually presents with an abnormal electrocardiogram, demonstrating a variety of findings such as sinus tachycardia, premature contractions, and conduction delays [1, 11]. As the disease progresses, imaging finds evidence of cardiac hypertrophy such as increase of ventricular septal thickness and left ventricular free wall/septum ratio in the hypertrophic stage. In the advanced dilated cardiomyopathy stage, echocardiogram usually reveals ventricular dilation coupled with hemodynamic disturbances that eventually progresses to congestive heart failure.

Available therapies are limited and palliative. Conventional anti-heart failure regimens, including ACE inhibitors (ACEI), angiotensin II receptor blockers (ARBs), beta-adrenergic receptor blockers, and aldosterone antagonists, are typically prescribed in an attempt to delay heart function decline [12–14]. Corticosteroids also demonstrated benefit in several reports, although most of these studies are retrospective observations with limited sample size. More recently a cohort of 86 patients was retrospectively analyzed, and the investigators concluded on top of ACEI therapy that the use of steroids was associated with a 76 % decrease of mortality, largely driven by the reduction of heart failure-associated death. However, the corticosteroid-treated group received ACEI treatment 3 years earlier, which may be the alternative explanation for the observed effect [15]. Other therapeutic modalities, such as pacemaker [16–18], ventricular assist device (VAD) [19–22], and cardiac resynchronization therapy (CRT) [23–25], may be beneficial in decreasing fatal arrhythmias and temporarily boost heart function.

With existing regimens, the majority of patients still face inevitable cardiac failure. This cruel reality makes it imperative to pursue new strategies. However, this

attempt is largely hindered by the lack of a reliable disease model. Existing animal models such as the *mdx* mouse fail to precisely reproduce human pathophysiology. For example, pharmacotherapy proven effective in *mdx* mice failed to demonstrate the equivalent efficacy in DMD patients and even worsened heart performance [26]. On the other hand, utilization of primary human cardiomyocytes is limited by risky isolation procedures and poor proliferation capacity of cells captured from human biopsy material. Therefore, a disease model system that closely mimics human symptoms and is capable of predicting in vivo efficacy is invaluable.

6.2.2 Pathogenesis

A plethora of evidence suggests that the outer cell membrane of the skeletal or cardiac muscle cell, the sarcolemma, is abnormally susceptible to mechanical stress in the face of dystrophin deficiency. This "vulnerable membrane" is characterized by the decrease of membrane stability when subjected to mechanical stretch during contraction, predisposing muscle cells to rupture. On the other hand, a spectrum of abnormal phenotypes across multiple physiological domains have also been linked to the absence of dystrophin, including but not limited to disturbance of calcium homeostasis, mitochondria dysfunction, and aberrant nNOS-cGMP signaling (Fig. 6.1). The pleiotropic effects of dystrophin deficiency are likely due to the
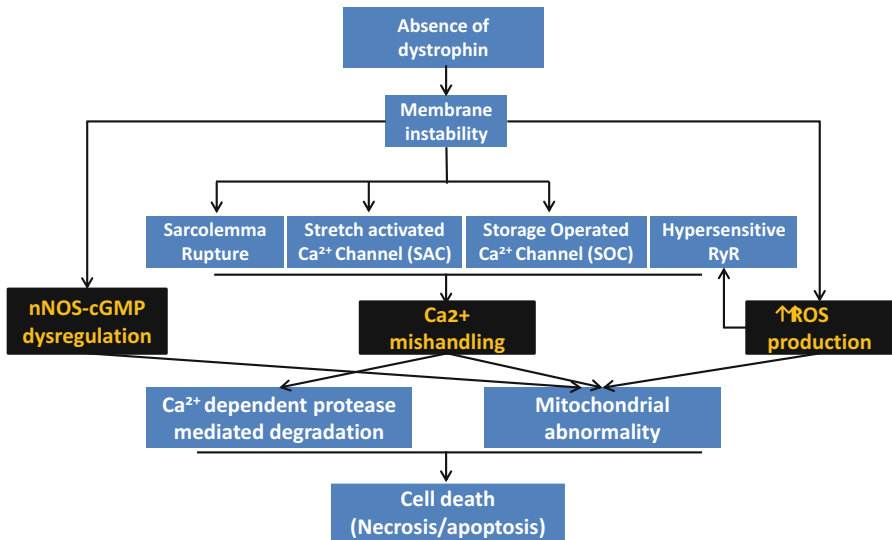
Fig. 6.1 Mechanistic scheme of the dystrophin-deficient cardiomyopathy. The absence of dystrophin causes membrane instability, triggering the abnormal calcium influx through various calcium channels. Improper accumulation of extracellular calcium together with hypersensitive ryanodine receptor leads to calcium mishandling. Coupled with dysregulated nNOS-cGMP pathway and heightened oxidative stress, the disease network triggers cell death through calcium-dependent protein degradation and mitochondria-mediated apoptosis

multifaceted function of dystrophin and to some degree complicate the clear delineation of the pathogenic process. Consequently, it is still elusive how dystrophin deficiency leads to abnormal phenotypes. Earlier work suggests that those aforementioned phenotypes are not merely concomitants of dystrophin deficiency, but actively contributing to disease progression. Corrections of these abnormalities, such as amplifying cGMP signaling or correction of calcium mishandling, are accompanied by ameliorated tissue pathology and improved muscle function. Because successful disease modeling in vitro is determined by faithful reproduction of in vivo disease characteristics, confirmation of the presence of the disease features in dystrophin-deficient cardiac cells renders credibility to further exploration.

6.2.2.1 Impairment of Membrane Barrier Function

Dystrophin is a cell membrane anchoring protein. Based on its sarcolemma localization, others postulated that the absence of dystrophin causes membrane barrier dysfunction. This idea is supported by the findings of "leaky sarcolemma." For instance, measuring serum levels of intracellular proteins, such as creatine kinase (CK), has been widely employed to assess the degree of muscle damage in muscular dystrophy. Experimentally introduced membrane impermeable substances such as albumin [27–31] and Evans Blue dye were found to accumulate within damaged dystrophin-deficient myofibers. Although these early studies confirmed the presence of an altered membrane barrier function, they failed to precisely define the biophysical nature of the membrane lesion. Normally, skeletal and cardiac muscle endures mechanical strain during contraction. In dystrophin-deficient DMD muscle, discontinuation of the normal sarcolemmal membrane structure in non-necrotic muscle fibers was observed by transmission electron microscopy. This observation was coupled with pathological intracellular changes and hyper-contracture of the surrounding myofibers leading to the speculation that physical breakage of the membrane, or "micro-ruptures," induced by mechanical strain during muscle contraction is the underlying membrane defect in dystrophin deficiency [32]. This notion is further supported by the observation that a synthetic polymer, poloxamer 188, seals membrane ruptures and reversed the dystrophic phenotype [33, 34]. In contrast to this prevailing view, Allen et al. argued that in dystrophin-deficient muscle, slow kinetics of trans-sarcolemmal calcium ingress following injury could not be accounted for by abrupt physical breakage. Instead, the investigators proposed that pathological activation of preexisting membrane channels are responsible for heightened membrane permeability [35]. Various calcium-permeable channels have been scrutinized for this purpose and will be discussed in the following section.

6.2.2.2 Dysregulated Calcium Handling

Calcium is a critical ion with diversified biological functions in both physiological and pathological processes of muscle. As a result, muscle has evolved highly regulated machinery to keep calcium flow in check. Thus, malfunction of critical
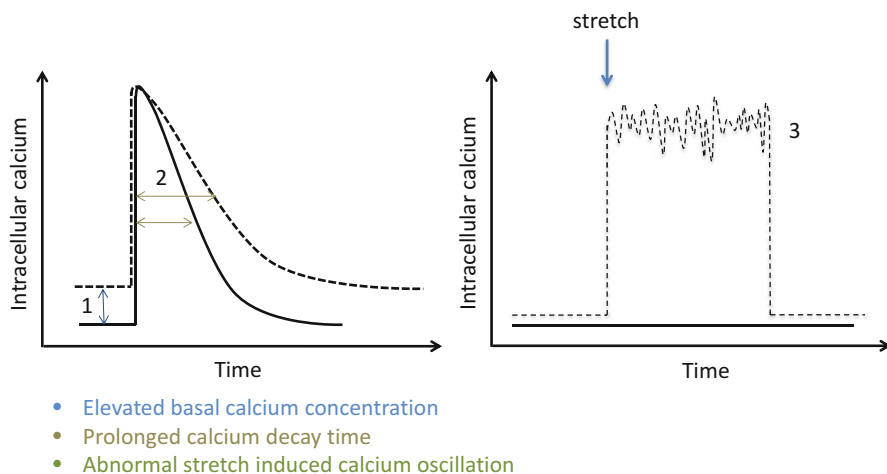
proteins in this system may jeopardize the delicate balance with a detrimental effect. Increase of intracellular calcium concentration triggers muscle contraction and attenuates cellular compliance [36]. Excessive activation of calcium signaling networks could lead to cell death [11], partly through the mitochondrial death pathway [5, 37]. It is also worth noting that skeletal muscle differs from cardiac muscle in calcium-handling processes. Opening of the membrane-bound L-type calcium channel in skeletal myocytes physically interacts and activates the ryanodine receptor (RyR), the gatekeeper of sarcolemma reticulum (SR) calcium storage. While in cardiomyocytes, it is the local elevation of subsarcolemmal calcium concentration, following calcium influx through the dihydropyridine receptor (DHPR), that activates the SR RyR. This difference in calcium handling could potentially contribute to different pathophysiologies and account for organ-specific phenotypes such as heart rhythm disturbances.

1. [Ca2+]$_i$ handling in dystrophin-deficient cardiomyocytes

   Aged *mdx* cardiomyocytes demonstrate elevated resting [Ca2+]$_i$ [38] and attenuated SR calcium storage [39] (Fig. 6.2), not present in young *mdx* mice. When young *mdx* cells were stressed by mechanical stretch, they responded with a profound calcium transient that largely surpassed the WT controls [34, 39, 40] (Fig. 6.2). Both adult and young *mdx* cardiomyocytes demonstrated prolonged calcium reuptake [38, 41, 42]. On the other hand, overexpression of sarco-/endoplasmic reticulum Ca^{2+}-ATPase 2 (SERCA2), the pump responsible for sequestering calcium in SR during repolarization, normalized intracellular calcium load and corrected the abnormal EKG [43].

2. Extracellular calcium entry

   It is generally accepted that abnormal extracellular calcium entry through a disrupted membrane triggers downstream disease networks. In support of this

- Elevated basal calcium concentration
- Prolonged calcium decay time
- Abnormal stretch induced calcium oscillation

Fig. 6.2 Schematic representation of the abnormal calcium handling detected on cellular level

idea, removal of extracellular calcium completely abrogates pathological $[Ca^{2+}]_i$ accumulation [40]. However, the biophysical identity of the entry route is still obscure. Though membrane tears resulting from mechanical injury has long been suspected to be the culprit instigator, recently Whitehead et al. argued that the delayed [Ca2+]i uprise following mechanical stretch contradicts membrane micro-rupture to be the leading path of calcium entry [44]. Alternatively, they suggested a group of calcium-permeable cation channels. The so-called stretch-activated channels (SACs) were first described by Franco and Lansman to be abnormally active in *mdx* mice myotubes [45]. Squire et al. later confirmed that the occurrence and opening probability of this channel were greater in *mdx* myofibers [46]. Blocking this channel by gadolinium, streptomycin, and GsMT-4 significantly ameliorated intracellular calcium overload induced by mechanical stretch [38, 47, 48]. Unfortunately, though the functionality of SAC has been confirmed by electrophysiological exams, little is known about its protein identity. Some suggested involvement of a transient receptor potential channel (TRPC). For example, a recent study revealed in *mdx* heart a twofold increase of TRPC vanilloid channels type 2 (TRPV2) expression and a mislocalization from the cytoplasm to the sarcolemma. Knocking down TRPV2 by siRNA or antibody blocking TRPV2 channel abrogated mechanically induced intracellular calcium accumulation [49].

Other membrane-localized Ca^{2+} channels have also been investigated in the context of dystrophin deficiency. The sarcolemma L-type calcium channel, DHPR, is the main calcium channel that triggers excitation-contraction coupling and directly interacts with dystrophin in the t-tubule system [50] [51]. DHPR channel inactivation was delayed in cardiomyocytes from neonatal [50] and adult *mdx* mice [52, 53]. Moreover, on *mdx* cardiomyocytes, the L-type calcium channel mediates an enhanced calcium influx that contributes to prolonged action potential duration [52]. These findings inspired studies to test the effect of calcium inhibitors in DMD patients. However, neither animal experiments nor clinical trials have demonstrated significant benefit [54, 55].

3. Intracellular calcium release through hyperactive RyR

   Trans-sarcolemmal calcium influx is augmented in DMD cardiomyocytes. However, this change alone is insufficient to account for the cyclic abnormal calcium oscillations observed in DMD cardiomyocytes, as shown in Fig. 6.2 [36, 40]. The self-sustaining nature of the calcium oscillation suggests the involvement of intracellular calcium storage of the SR. Several groups have reported that the function of RyR, the molecule gating SR calcium release, is altered in DMD cardiomyocytes [36, 56]. A "leaky" RyR loses control over intracellular SR calcium release, contributing to calcium dysregulation. Ullrich et al. reported that the sensitivity of RyR is increased in *mdx* cardiomyocytes, responding to low concentration of extracellular calcium that could not activate wild-type RyR [37]. Prosser et al. further demonstrated that sensitized RyR responds to stimuli by a profound SR calcium liberation, which potentially accounts for DMD cardiac rhythm disturbances [36].

6.2.3 Excessive ROS Production

Cumulative evidence suggests a key role of elevated oxidative stress in the degeneration of dystrophin-deficient skeletal muscle [44, 57, 58]. Exposing *mdx* cardiomyocytes to hypoosmotic or stretch stress triggers higher-than-normal reactive oxygen species (ROS) production [36, 40], likely through upregulating key enzymes in ROS production such as NAPDH oxidase 2 (NOX2) [36], NOX4, and Lysyl oxidase (LOX) [59]. Antagonizing ROS by supplementing the ROS scavenger, N-acetylcysteine (NAC), attenuates calcium-handling abnormalities, preserves heart function, and mitigates inflammation and fibrosis [60].

The role of oxidative stress and its downstream molecular consequences has been well documented in other forms of cardiomyopathy [61]. Though direct evidence is lacking, it is reasonable to argue that a similar pathway may also be activated in dystrophin-deficient cardiomyocytes. Excessive oxidation of functional proteins inflicts cell damage and deteriorates heart function. Those susceptible targets include membrane channels, contractile proteins [62], signaling proteins [63], and EC coupling components [64]. For example, Prosser et al. revealed that the ROS generated via NOX2 oxidize RyR, which is the molecular basis of its hypersensitivity [36].

6.2.4 Mitochondria Dysfunction

Functional imaging studies through positron emission tomography (PET) identified regional abnormalities in the DMD heart, characterized by energy substrate shift from fatty acid to glucose [65–67]. Lately, alterations of metabolic substrate were experimentally confirmed in young *mdx* heart, prior to overt cardiomyopathy, coupled with increased oxygen consumption, glycolysis, and ATP production rate [68]. The same group also discovered that the mitochondria permeability transition pore (mPTP), an important mediator of mitochondrial apoptosis pathway, is more susceptible to open when challenged by stressors [5], suggesting that mitochondria are actively involved in disease progression and may represent an intervention target.

6.2.5 Abnormal nNOS-cGMP Signaling

In skeletal muscle, the neuronal nitric oxide synthase (nNOS) is anchored to the c-terminus of dystrophin to control nitric oxide (NO) production. While active NO modulates target proteins by binding to a thiol residue via s-nitrosylation, NO can also function through activating soluble guanylyl cyclase (sGC) to produce cGMP as the secondary messenger. In DMD patients, the absence of dystrophin causes nNOS mislocalization, which greatly dampens NOS function by up to 80 %. The benefits of

nNOS overexpression in skeletal muscle include anti-inflammation, anti-fibrosis, and additional protection of myofibers against mechanical stress [69]. nNOS-mediated NO production also exerts a protective effect in the heart [8]. Differing from its sarcolemmal localization in skeletal myofiber, nNOS mainly associates with SR [70] and mitochondria [71] in the heart, without direct interaction with dystrophin. Surprisingly, Bia et al. discovered in dystrophin/utrophin double knockout mice an 80 % decrease of cardiac nNOS activity. Encouraged by their previous success in skeletal muscle [69], overexpressing nNOS in *mdx* heart demonstrated salutary effect to decrease inflammation and fibrosis [72]. Another group overexpressed the sGC and administration of the PDE5 inhibitor sildenafil with the goal to activate the sGC pathway. Remarkably, both these strategies improved mitochondrial function, attenuated stress-induced mPTP opening, and exerted sarcolemma protection against workload-induced damage [73, 74]. Sildenafil has also been shown capable to reverse cardiomyopathy in aged *mdx* mice, boosting depressed cardiac function to comparable levels to age-matched controls [75]. Taken together, evidence suggests that cGMP-mediated effects account for benefits associated with modulating the nNOS pathway. Commercially available PDE5 inhibitors may hold promise in treating heart disease in DMD.

6.3 Regenerative Cellular Technologies

Advances in genetics, epigenetics, and stem cell biology have empowered scientists with new tools to control cell fate. A variety of human tissues, including cardiomyocytes, neurons, hepatocytes, and endothelial cells can now be produced from stem cells in large quantities. This new technology paves the way for a myriad of downstream applications, such as studying disease mechanisms, screening therapeutic compounds, and enabling cellular replacement therapy.

6.3.1 The Germination of Nuclear Reprogramming

Mammalian development is accompanied by diversification of progeny cell identity, realized by a gradual loss of cell plasticity. It was once believed that development is an irreversible process. Unidirectionality was ensured by a gradual loss of genetic material [76]. Consequently, cell fate was considered to be static and inter-lineage switch of cellular identity impossible. In the middle twentieth century, this dogma of a "one-way street" was challenged. John Gurdon discovered that differentiated tadpole muscle and intestinal nuclei could generate mature fertile adults after being transplanted into enucleated Xenopus eggs. This discovery unequivocally proved that the genome is relatively stable across the life span of individual organisms. The development process is not coupled with attrition of genetic material. When provided with the correct cue, an adult nucleus is capable to initiate and maintain

normal development in a similar fashion as zygotes [77]. Miller and Ruddle had also shown that differentiated thymus cells could be reset to a primitive stage equivalent to pluripotent embryonic stem cells by fusing with embryonic carcinoma cells [78]. These findings broadened the "one-way street" to "two-way traffic" but were still constrained within a longitudinal developmental path. Later, Helen Blau utilized a similar cell fusion technique, termed heterokaryon, to successfully convert human non-muscle cell nuclei to acquire a skeletal muscle gene expression profile. This was the first demonstration of an inter-lineage switch [79]. Together, these findings revealed the surprising plasticity of nuclei and foreshadowed an era of nuclear reprogramming to manipulate cellular fate by altering gene expression.

6.3.2   Nuclear Reprogramming 2.0

6.3.2.1   Induced Pluripotency

A lesson learned from the somatic nuclear transfer and heterokaryon experiments is that certain molecules within the recipient's cytoplasm redirect a terminally differentiated cell type to acquire a different identity. Consequently, identifying these limited molecules and overexpressing them in donor cells might simplify the process. The cosmologically vast number of molecules contained in the cytoplasm made this a formidable task. After decades of exploration, a seminal breakthrough occurred in 2006. Yamanaka and Takahashi identified ectopic expression of four transcription factors, Oct-4, Sox-2, c-Myc, and Klf4 [80], that converted both mouse [80] and human [81] fibroblasts to pluripotent stem cells. Another report around the same time demonstrated that nanog and Lin28 could replace c-Myc and Klf4 [82]. Converted somatic cells, termed, induced pluripotent stem cells (iPSCs), possess indefinite self-renewal capacity and the potential to generate virtually any cell types within the body. To date, although small differences have been reported between iPSC and hESC [83–87], these two behave largely the same. Not only do iPSC and ES cells share similar morphology and gene expression profiles, more importantly, iPSC cells acquire bona fide pluripotency, forming teratoma tumors after engrafting into immune-compromised animals. Mouse iPSCs could even pass the most stringent tetraploid complementation assay to generate progenies solely composed of cells differentiated from iPSCs [88].

Recent technique evolution has overcome some major hurdles for clinical translation of iPSC technology. The poor reprogramming efficiency and slow kinetics once the bottlenecks of iPSC derivation were overcome by optimizing the reprogramming factor combination and starting cell population [89–90]. Lately, a remarkable 100 % efficiency was achieved by simultaneously knocking down the MDB3 gene [91] together with reprogramming factor delivery. On the other hand, the concern of insertional mutagenesis, elicited by random viral integration, is addressed by "footprint-free" reprogramming technology. Non-integration delivery vectors, such as sendai virus, episome, or mRNA, can achieve efficient reprogramming without any genome perturbation.

6.3.2.2   Inter-Lineage Cell Fate Conversion

Derivation of iPSCs by transient, ectopic expression of transgenes demarcates the new era of nuclear reprogramming. Compared to previous SCNT experiments, the major improvement of this new version of nuclear reprogramming is that cell fate conversion is achieved through manipulating a small set of predefined factors. This breakthrough considerably lowered the technical barrier and encouraged further exploration. Following experiments demonstrated that other cell fates could also be rerouted via ectopic expression of a handful transgenes (Table 6.1). Inter-lineage cell fate conversion bypasses the pluripotent stem cell stage. This process, termed "trans-differentiation," usually occurred with a donor cell developmentally related to the target cells, although trans-germ layer conversions have also been documented.

Table 6.1 Summary of human cells inter-lineage conversions

Donor cells	Target cells	Reprogramming factors
Fibroblasts	Melanocyte	Mitf, Sox10, and Pax3 [92]
Pancreatic exocrine cells	Pancreatic beta cells	Activated MAPK and STAT3 [93]
Fibroblasts	Hematopoietic progenitor with macrophage potential	Sox2 [94]
Fibroblasts	Multipotent neural crest progenitor	Sox10, Wnt activation [95]
Endothelial cells	Hematopoietic progenitors	Fosb, Gfi1, Runx1, and Spi1 with vascular niche monolayers [96]
Fibroblasts	Hepatocytes	FoxA3, Hnf1a, and Hnf4a [97]
Fibroblasts	Hepatocytes	Hnf1a, Hnf4a, Hnf6, Atf5, Prox1, and CEBPA [98]
Fibroblasts	Retinal pigment epithelium like cells	c-Myc, Mitf, Otx2, Rax, and Crx [99]
Fetal lung fibroblasts	Cholinergic neurons	Neurogenin2 supplemented with forskolin and dorsomorphin [100]
Cardiac fibroblasts	Cardiomyocytes	Gata4, Mef2c, and Tbx5 Mesp1 and Myocd [101]
Proximal tubule cells	Nephron progenitors	Six1, Six2, Osr1, Eya1, HoxA11, and Snai2 [102]
Fibroblasts	Cardiomyocytes	GATA4, Hand2, Tbx5, myocardin, miR1, and miR133 [103]
Fibroblasts	Cardiomyocytes	Ets2 and Mesp1 [104]
Fibroblasts	Multipotent neural stem cells	Sox2 [105]
Fibroblasts	Dopaminergic neurons	Mash1, Ngn2, Sox2, Nurr1, and Pitx3 [106]
Fibroblasts	Motor neurons	Brn2, Ascl1, Myt1l, Lhx3, HB9, Isl1, Ngn2 [107]
Fibroblasts	Neurons	Brn2, Myt1l, miR124 [108]
Fibroblasts	Neurons	Ascl1, Myt1ll, NeuroD2, miR9/9*, miR124 [109]

The majority of these trans-differentiation transgenes are transcriptional factors, chromatin modifiers, and miRNAs critical to normal development of certain lineages. Surprisingly only a handful of factors are required to achieve these transformations, which may emphasize that cell identities are maintained through certain "master regulators," such as MyoD for skeletal muscle [110]. Several studies have demonstrated that similar cell fate conversion could be achieved in vivo, such as cardiomyocytes [111], pancreatic beta cells [112, 113], and neurons [114, 115], showcasing the versatility of the reprogramming technology.

6.3.3 Guided Differentiation

An alternative strategy to achieve in vitro cell fate control is to guide pluripotent stem cells to differentiate into desired cell types. Insights gained from embryonic development have enabled scientists to harness the physiological cues to hijack the intrinsic developmental program. The key is to recapitulate those early events governing lineage commitment and germ layers' specification, by temporally modulating critical signaling pathways [116]. A broad spectrum of cell types including cardiomyocytes, hepatocytes, pancreatic beta cells, endothelium, and dopaminergic neurons have been generated in this fashion. The advantages of this method are twofold. First, large quantity, highly purified target cells can be manufactured through an optimized induction protocol. For example, cardiomyocyte production can be over 90 % in purity with 3 output cardiomyocytes for every input stem cell. This scale is compatible with an industrial setting to produce clinically relevant cell numbers, usually in the billions [117]. Secondly, starting from a pluripotent stem cell offers a unique opportunity for genetic engineering to generate homogenous cell line with desired genetic modifications. The following section will focus on cardiac induction. The specification of other germ layers is well reviewed by Murry et al. [118].

It was noticed that beating cardiomyocytes could be generated from embryoid bodies (EB), stem cell aggregates mimicking early developing embryo structure. The efficiency of spontaneous cardiogenesis is low (less than 10 %) with large batch-to-batch variation [118, 119]. Not until insights gained from development were applied have investigators started to consistently generate adequate cardiomyocytes for downstream studies. In the embryo, cardiac progenitors are derived from *brachyury T-* and *Emos*-positive mesoderm cells, which further give rise to *KDR*- and *PDGFRα*-positive cardiac mesoderm cells and gradually turn on the cardiac master regulator *MESP1*. Further specification of cardiac mesoderm generates cardiac progenitors, characterized by the expression of a panel of cardiac-specific transcriptional factors such as *Nkx2.5, GATA4, Tbx5,* and *Isl1*. Temporally and spatially orchestrated Nodal, BMP4, and Wnt3 signaling ensure this organized sequential progression. Artificially supplementing these ligands in culture medium, mimicking the physiological strength and timing, can also differentiate pluripotent stem cells into cardiomyocytes. With some technical variation, this scheme consistently generates

cardiomyocytes in around 2 weeks, with an efficiency ranging from 30 to 90 % [120]. It is important to note that the Wnt signaling has a unique biphasic role in cardiac development. Lately the Palecek group had shown that by fine-tuning Wnt pathway alone by two molecules, GSK-3β inhibitor CHIRO-99021 and Wnt inhibitor IWP-4 or IWR-2, cardiomyocytes can be generated from multiple cell lines with an efficiency over 90 % [121].

Various strategies have demonstrated that in vitro cardiac induction follows the same gene expression pattern that would occur during embryonic development. Downregulation of pluripotent genes is accompanied by upregulation of mesoderm markers Brachyury T and MESP1, which peak around day 2. Cardiac progenitor markers KDR, ISL1, and PDGFR-α peak at day 5, followed by a steady increase of mature myocyte markers such as Nkx2.5, myosin heavy chain (MHC), and troponin [122–125]. Differentiated cells manifest sarcomere striation, typical action potentials and associated ion channels [126], potent gap junctions for synchronized contraction, as well as positive humeral regulation response following isoproterenol stimulation [119, 127, 128]. Klug et al. demonstrated that stem cell-derived cardiomyocytes express dystrophin [129]. While this evidence suggests that these are bona fide cardiomyocytes, stem cell-derived cardiomyocytes largely mimic a developmentally immature cardiac phenotype. Transcriptional profiling suggested that the cardiac gene expression pattern was similar to 20-gestational-week fetal cardiomyocytes [130]. Electrophysiological assays indicated that stem cell-derived cardiomyocytes possess fetal-type ion channels [122], leading to a fetal-like negative force frequency relationship, blunted post-rest potentiation [131], higher resting potential, and slow action potential upstroke [130, 132]. Ultrastructure analysis revealed that the characteristic components, such as contraction machinery, sarcomeric reticulum, transverse tubules (t-tubule), and mitochondria, were all present, but their abundance, distribution, and organization failed to reach the level of adult cardiomyocytes [133].

6.3.4 From Urine to Dystrophin-Deficient Cardiomyocytes

Our group demonstrated that urine harbors a unique cell population capable to adhere to plastic and undergo extensive proliferation. These urine-derived cells manifest spindle-shape morphology and express classic MSC surface markers including CD44, CD73, CD90, CD105, and CD146 [134, 135]. Functional assays reveal that urine-derived cells possess progenitor properties and give rise to several somatic cell types [136]. When transduced with lentiviral vector containing classic OKSM reprogramming factors, these cells demonstrate faster reprogramming kinetics compared to mesenchymal stem cells, generating iPSC colonies within 2 weeks [134]. Urine-derived iPSC colonies, from both normal individual and DMD patient urine, can give rise to embryonic tumors containing characteristic tissue structures of all three germ layers, demonstrating bona fide pluripotency.

Pluripotent stem cells
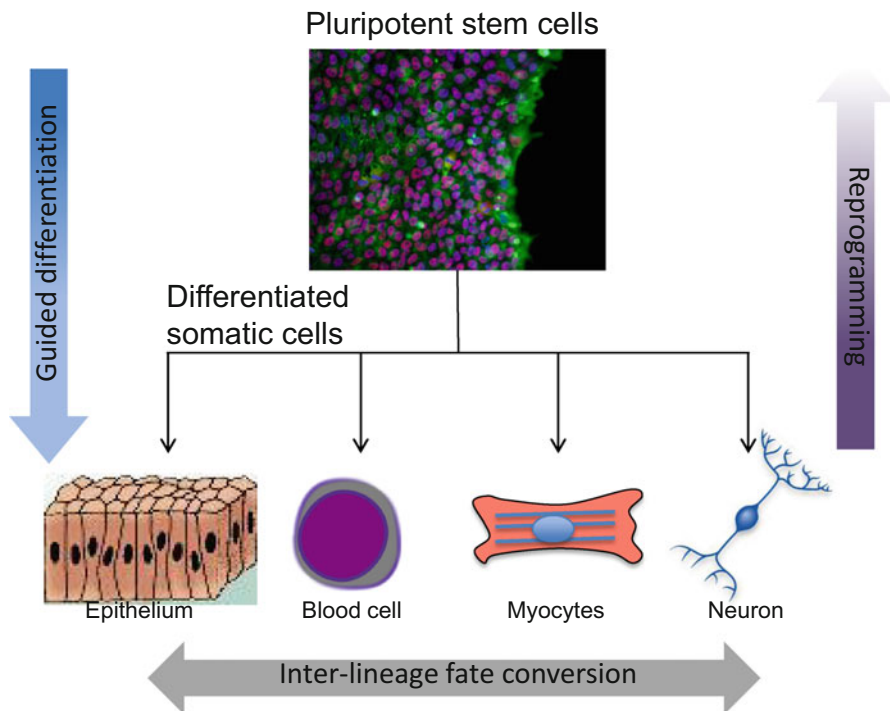
Fig. 6.3 Cell fate controlling. Strategies to ex vivo manipulate cellular identities, including guided differentiation following developmental cues, reprogramming to pluripotency by forced overexpression of reprogramming factors, and inter-lineages by various techniques

Exposing the urine-derived iPSC cells to a combination of growth factors, including Activin A, bone morphogenetic protein 4, and dickkopf-1, in a strict sequence and defined duration [137] forms beating cardiomyocytes with an efficiency from 40 to 90 %. These differentiated cardiomyocytes show typical sarcomere structure and are positive for sarcomeric α-actinin, cardiac myosin heavy chain, as well as membrane-localized connexin43. They also exhibit functional cardiac properties, spontaneous action potentials characteristic of nodal, ventricular, and atrial subtypes. On the other hand, dystrophin expression was absent from DMD cardiomyocytes, recapitulating the essential aspect of the DMD disease [134] (Figs. 6.3 and 6.4).

6.4 Dystrophin-Deficient Cardiomyocytes Generated Via Cellular Reprogramming Are a Novel Biological Reagent for the Study of DMD Cardiomyopathy

The list of iPSC-based disease models is steadily growing. Impressively, different diseased cells manifest phenotypes analogous to symptoms in patients in culture dishes. A partial list includes models for amyotrophic lateral sclerosis, spinal
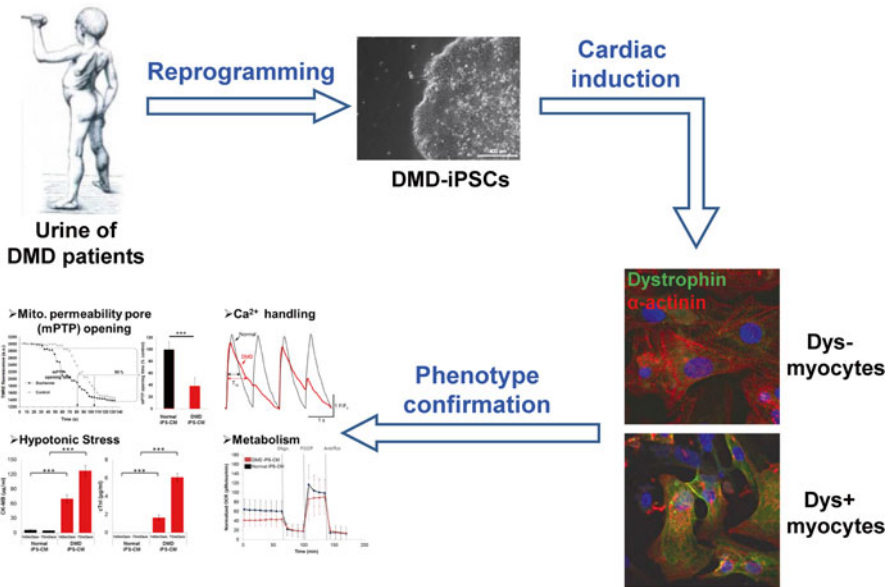
Fig. 6.4 A strategy to model dystrophin-deficient cardiomyopathy via cellular reprogramming

muscular atrophy, familial dysautonomia [138], Rett syndrome [139], schizophrenia [140], Parkinson disease [141–144], Timothy syndrome [145], and long QT syndrome [146]. The explosion of the iPSC application is largely due to the fact that cellular reprogramming is a powerful tool that strongly resonates in the era of personalized medicine, which enables identifying and optimizing solutions for specific mutations. DMD cardiomyopathy is well suited to iPSC-based "disease-in-a-dish" platform. First, there is currently no effective treatment for DMD cardiomyopathy. Secondly, the molecular mechanism of the cardiomyopathy is not well understood. Though the disease root cause is clear, a variety of subdomains, such as membrane stability, calcium handling, and mitochondria abnormalities, have been suggested in the pathogenic process. The complexity of the underlying pathogenic network makes identifying therapeutic targets a daunting task. Thirdly, the most widely used DMD animal model, the *mdx* mouse, does not accurately reproduce the cardiac pathology observed in human patients [148]. Thus it is imperative to study this disease in a human context to render clinical relevance. Furthermore, the risk associated with cardiac biopsy makes acquiring samples from DMD patients nearly impossible. These limiting factors impose significant constraints in developing therapies for human patients. As a result, human cardiomyocytes differentiated from patient iPSCs represent an attractive option to overcome these limitations.

It is technically favorable to model Duchenne cardiomyopathy using the iPSC approach mainly because DMD is a classic Mendelian monogenic disease with complete penetrance in male patients. A large body of literature suggests that the disease phenotype is cell-autonomous, indicating that disease traits can be readily observed independent of the diseased body environment. Compared to polygenic

genetic disorder or complex disorders involving strong environmental attributes, the technical complexity associated with modeling dystrophin-deficient cardiomyopathy ex vivo is considerably lower. Moreover, the existence of a highly efficient cardiac induction regimen bolsters this strategy by lowing the technical hurdle [121, 137].

6.4.1 Personalized Diagnosis: Exploring Disease Etiology

Presently, disease diagnosis has evolved from clinical and functional to molecular diagnosis using biomarkers [148, 149] and genome sequencing [150, 151]. However, genotype-phenotype relationships are not always strongly correlated, and sometimes it is difficult to elucidate a causal relationship. Toward this end, iPSC technology represents a unique opportunity to converge information from multiple levels and directly link them to a change in cellular function. For example, in iPSC-differentiated disease target cells, the mutated gene can be sequenced, biochemical reactions can be measured, and abnormal metabolites can be quantified. Readouts from these measurements can be further linked to cellular responses in a quantifiable fashion. In addition, cell culture systems allow intricate experimental interventions at virtually any level, from genetic to environmental, greatly aiding in determining the disease root cause. Lastly, because clinically relevant cells are differentiated stepwise from stem cells in a fashion that recapitulates the embryonic development, disease initiation and early progression, hard to assess by other modalities, can now be closely monitored. It is possible to exploit these models to uncover early disease markers before overt symptoms, increasing the opportunity for early intervention or even prevention. For instance, Kim et al. identified in patient iPSC-derived cardiomyocytes an abnormal peroxisome proliferator-activated receptor gamma (PPAR-γ) activation that underlies the pathogenesis of arrhythmogenic right ventricular dysplasia (ARVC), a previously unrecognized mechanism [152]. iPSC-based platforms also simplifies the diagnosis procedure since tests only need to be conducted on single diseased lineage in isolation, removed from potential secondary effects of residing within a sick animal.

6.4.2 Personalized Therapy: In Search for the Ideal Treatment

Because of its individualized nature, iPSC technology can also serve as an invaluable tool to identify new therapies. First, it can be employed as a platform to predict the potency and toxicity of existing therapies [153, 154]. It is not uncommon that a spectrum of drugs is available for a single disease, yet not every drug is equally effective for all patients. The potency and toxicity, which largely depend on individual's unique genetic background, are hard to predict based on existing modalities. On the other hand, iPSC cells have the potential to be the stage for such forecasts, since the patients' own cells are tested. Such tests are not necessarily

limited to chemical compounds. As a cellular assay, other therapeutics, such as gene therapy vectors, can also be assayed as a quality control measure to predict its clinical efficacy. For those disorders without effective treatment, like DMD cardiomyopathy, de novo screening against an extensive compound library can potentially identify new treatments with no risk to the patient [155].

The differentiated cells themselves can also serve as therapeutics. One route to cure a genetic disorder may involve replacing the diseased cells with genetically corrected counterparts. The emergence of novel genetic engineering tools, such as CRISPR and TALEN enzymes, has greatly improved the feasibility of complex genome modification of human stem cells [144, 156–159]. Barrier of immunogenicity could be overcome by autologous transplantation of the progeny differentiated from iPSC. This strategy has been applied to improve skeletal muscle function in dystrophin-deficient *mdx* mice [160]. In terms of the heart, though previously it has been shown that genetic heart disease benefited from embryonic stem cell transplantation [161], the delivery method still imposes the biggest challenge for inherited disorders including DMD.

6.4.3 Dystrophin-Deficient Cardiomyocytes Generated Via Cellular Reprogramming Recapitulate Certain Aspects of the Disease Phenotype

Though studies have shown that disease target cells differentiated from patient's iPS cells faithfully reproduce disease-associated abnormalities, it is still questionable whether the cells created in laboratory, at a developmentally naïve stage, will be able to fully demonstrate a phenotype of late-onset pathology observed in DMD. Therefore the first task is to demonstrate progeny differentiated from iPSC cells that retain certain disease hallmarks, which will then lend credibility to novel insights gained on an iPSC platform. For example, ventricular cells differentiated from a long QT syndrome patient demonstrated the characteristic prolongation of the QT interval as well as an abnormal potassium channel [146, 154, 162].

In our hands, to model DMD cardiomyopathy, cardiomyocytes were assessed both molecularly and phenotypically for well-established DMD features. For example, the absence of dystrophin protein is the root cause of DMD. Confirmed both by immunostaining (Fig. 6.4), cardiomyocytes differentiated from DMD iPS cells were negative for dystrophin expression, recapitulating an essential aspect of the disease. It is well established that the absence of dystrophin causes a spectrum of physiologies deviating from normal control. As discussed earlier, membrane fragility is suspected to be a direct consequence of the absence of dystrophin and initiates downstream pathological cascades. One way to assess the membrane barrier function is measuring the release of intracellular content during a hypotonic stress challenge. In vitro, an increase of intracellular hydrostatic pressure passively stretches the cell's sarcolemma. DMD cells are abnormally susceptible to mechanical stress,

and their fragile membrane predisposes cells to rupture, leading to the liberation of intracellular contents. As an example of a hypotonic stress assay, red blood cells are subjected to a series of graded hypotonic solutions, and intracorpuscular hemoglobin release is assayed to diagnose *hereditary spherocytosis* [163]. We employed a similar strategy to mechanically stretch the cardiac sarcolemma by incubating normal and DMD cells in hypotonic solutions ranging from normal tonicity to 1/8 tonicity. The cardiac-specific injury marker CK-MB, a widely used clinical marker, was measured after 30 min. Dystrophin-deficient cardiomyocytes demonstrated an abnormally high release CK-MB profile. Measured as CK-MB concentration, increased levels were demonstrated even at relatively normal tonicity and became marked greater than normal cells as tonicity decreased (Fig. 6.4). This evidence supports the notion that dystrophin deficiency leads to sarcolemma fragility, which predisposes cardiomyocytes to mechanical stretch damage.

Calcium mishandling is another characteristic feature of DMD. Loaded with the calcium indicator Fluo-4, iPSC-derived DMD cardiomyocytes were paced by external field stimulation. Compared to dystrophin replete (normal) control cells, DMD cells demonstrated a prolonged calcium decay time T_{50}, consistent with previous reports in the dystrophin-deficient mouse [38, 41, 42].

Mitochondria dysfunction observed in DMD patients was recently recognized as an important mediator of disease progression. Two prominent features, a lowered opening threshold for mitochondrial permeability transition pore (mPTP) and altered bioenergetics, have been linked to the disease in the *mdx* mouse. To evaluate mPTP pore opening, cardiomyocytes were loaded with mitochondria potential ($\Delta\psi m$) indicator tetramethylrhodamine ethyl ester (TMRE) and then exposed to focused laser to induce oxidative stress-mediated mPTP opening. The opening of mPTP leads to the decrease of $\Delta\psi m$, reflected by lowering of TMRE fluorescence. Dystrophin-deficient cardiomyocytes manifested a 50 % shorter mPTP time compared to normal cells. In other experiments, an extracellular biochemical analyzer (Seahorse) was employed to examine the bioenergetics of cardiomyocytes. Oxygen consumption rate (OCR) was measured as the parameter of metabolism activity. Surprisingly, DMD cells demonstrated augmented basal and maximal oxygen consumption, different from the report on *mdx* skeletal muscle but in agreement with a study using a Langendorff perfused *mdx* adult heart, in which the *mdx* heart exhibited elevated level of glycolysis, carbohydrate utilization, oxygen consumption, and overall ATP production [5].

6.5   Summary

Duchenne muscular dystrophy causes degeneration of both skeletal and cardiac muscle. All DMD patients inevitably develop heart disease, but the mechanism of DMD heart failure is still elusive, partly due to the hard-to-access heart cells from patients. We modeled heart disease in DMD using cellular reprogramming by converting patient urine cells into induced pluripotent stem cells or iPSCs. IPSCs were

further transformed into heart cells for study. This regenerative medicine technology provided an unprecedented opportunity to study DMD heart disease from several physiological perspectives including membrane fragility, calcium handling, and energy metabolism. This regenerative medicine platform will facilitate exploration of DMD disease etiology and provide unlimited patient biologic material to screen new therapeutic compounds.

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