Diplomarbeit

Gene therapy in sudden cardiac death:

Current status and future therapeutic perspectives

eingereicht von

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zur Erlangung des akademischen Grades

Doktor(in) der gesamten Heilkunde

(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt am

Diagnostik & Forschungsinstitut für Humangenetik

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Graz, 28.06.2019

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Danksagung

Mein besonderer Dank gilt all jenen, die mir diese Arbeit ermöglicht haben, insbesondere dem Ideengeber dieses Projektes, Univ.-Ass. Priv.-Doz. Mag. Dr.rer.nat. Dr.scient.med. Thomas Schwarzbraun. Er hat nicht nur dieses interessante und komplexe Thema bereitgestellt, sondern mich ebenfalls mit zahlreicher Fachliteratur bei der Ausführung dieser Arbeit unterstützt.

Des Weiteren möchte ich mich bei meinem Betreuer Assoz. Prof. Priv.-Doz. Mag. Dr.rer.nat. Christian Windpassinger für die immer freundliche und geduldige Betreuung sowie die Begutachtung bedanken.

Ein ganz besonderer Dank gebührt ebenfalls meiner Zweitbetreuerin PhD. Verena Rupp, die mir eine große Hilfe bei der Planung, der Verfassung und schließlich der Begutachtung der Diplomarbeit war.

Zu guter Letzt möchte ich mich bei meiner Familie und meinen Freunden bedanken, die mir das Studium durch ihre Unterstützung ermöglichten, mich motivierten und stets ein offenes Ohr für meine Sorgen hatten.

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INDEX OF ABBREVIATIONS

AAV	Adeno-Associated Virus
AMR	Antibody-mediated allograft rejection
Arch	Archaerhodopsin
ARVC	Arrhthmogenic right ventricular Cardiomyopathy
AV	Adeno-Virus
bp	base pair
BR	Bacteriorhodopsin
BrS	Brugada Syndrome
CDK	Cyclin Dependent Kinases
ChR2	Channelrhodopsin2
CM	Cardiomyocytes
CPVT	Catecholaminergic Polymorphic Ventricular Tachycardia
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DBD	Donation after brain death
DCD	Donation after cardiac death
DCM	Dilated Cardiomyopathy
DD	Deceased Donor
dECM	decellularized Extracellular Matrix
DNA	Deoxyribonucleic Acid
DSB	Double-strand-break
ECG	Electro Cardio Gram
ECM	Extracellular Matrix
FP	Fluorescent Proteins
FRET	Förster Resonance Energy Transfer
FRT	Flippase recognition target
GF	Growth Factor
GPCR	G-protein-coupled receptor
HCM	Hypertrophy Cardiomyopathy
HDR	Homology directed repair
hiPSC	human induced Pluripotent Stem Cells

HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HR	Halorhodopsin
ICD	Implantable Cardioverter Defibrillator
iCM	induced Cardiomyocytes
iKO	induced Knock Out
iPSC	induced Pluripotent Stem Cells
ITR	Inverted Terminal Repeat
LPL	Lipoprotein Lipase
LQTS	Long QT Syndrome
LV	Lenti-Virus
LVEF	Left Ventricular Ejection Fraction
MI	Myocardial Infarction
MMP	Matrix Metallopeptidase
NAB	Neutralizing Antibodies
NHEJ	Non-homologous end joining
NICM	Non-ischemic Cardiomyopathy
NYHA	New York Heart Association
PAM	Protospacer adjacent motif
PGD	Preimplantation genetic diagnosis
PSC	Pluripotent Stem Cells
PVT	Pulseless Ventricular Tachycardia
RCM	Restrictive Cardioymyopathy
RNA	Ribonucleic Acid
RV	Retro-Virus
RVD	Repeat-variable di-residue
SCD	Sudden Cardiac Death
SIS	Small Intestinal Submucosa
SQTS	Short QT Syndrome
TALEN	Transcription activator-like effector nucleases
TG	Triglyceride
VA	Ventricular Arrhythmia
VF	Ventricular Fibrillation
VT	Ventricular Tachycardia

WPW	Wolff-Parkinson-White Syndrome
YAP	Yes-associated protein
ZFN	Zinc Finger Nucleases

ZUSAMMENFASSUNG

Einführung: Der plötzliche Herztod wird definiert als unerwarteter, plötzlicher Tod aufgrund kardialer Ursachen und stellt die häufigste zum Tode führende Manifestation einer Herzerkrankung dar. Bei jungen Patienten und Patientinnen unter 35 Jahren ist eine häufige Ursache hierfür eine angeborene Herzerkrankung. Die derzeitigen Therapiemöglichkeiten, wie beispielsweise Organtransplantation oder implantierbarer Defibrillator, stellen aufgrund einiger Nebenwirkungen beziehungsweise Kontraindikationen ungenügende Behandlungsoptionen dar.
Zukünftige Therapiekonzepte des plötzlichen Herztodes sind gegenwärtig Inhalt der Forschung und zeigen vielversprechende Ansätze.

Methode: Zur Eruierung dieser Thematik wurde eine umfassende Literaturrecherche mit Gegenüberstellung der Aussagen aus unterschiedlichen Quellen und kritischer Analyse betrieben.

Ergebnis: Eine Möglichkeit terminales Herzversagen kurativ zu therapieren, stellt das Bioengineering von menschlichem Myokardium auf nativer extrazellulärer Matrix als Alternative zu Spenderorganen dar. Ziel ist die Kreierung eines artifiziell hergestellten, funktionstüchtigen Herzens, welches mittels dezellularisiertem Gerüst und Rezellularisierung durch Milliarden von kardialen Zellen hergestellt wird. Des Weiteren ist die Korrektur mutierter Gene, beziehungsweise die Normalisierung der Expression von über-/unteraktiven Genen mittels Genome-Editing, eine zukünftige Behandlungsmöglichkeit. Besonderes Augenmerk liegt auf der Einführung spezifischer genetischer Mutationen in humane pluripotente Stammzellen mittels CRISPR/Cas9 Technologie und Übermittlung dieser via Adeno-assoziierten-Viren in die Zielzellen.
Zudem stellen kardiale Regenerierung mittels Proliferation differenzierter Kardiomyozyten und kardiale Optogenetik aussichtsreiche Therapiemethoden dar.

Schlussfolgerung: Die Anwendung dieser Therapieformen hat derzeit noch nicht den Weg in die Klinik gefunden, hält jedoch dank intensiver Forschung vielversprechende Zukunftsaussichten bereit.

ABSTRACT

Introduction: Sudden cardiac death is defined as a sudden, unexpected death due to cardiac causes and represents the most common fatal manifestation of heart disease. In young patients under 35 years, a common cause of this is congenital heart disease. The current treatment options, such as organ transplantation or implantable defibrillator, are due to some side effects or contraindications insufficient treatment options.

Future therapy concepts of sudden cardiac death are currently the subject of research and show promising approaches.

Methods: To investigate this topic, a comprehensive literature research was conducted with comparison of the statements from different sources and critical analysis.

Results: One way to curatively treat terminal heart failure is the bioengineering of human myocardium on native extracellular matrix as an alternative to donor organs. The goal is to create a whole bioengineered heart that is built by using a decellularized scaffold which then gets recellularized by billions of multiple types of cardiac cells.
Furthermore, treating diseases by fixing the mutant gene or normalizing the expression of overactive/underactive genes by means of genome editing is a future treatment option. Particular attention is placed on the introduction of specific genetic mutations into human pluripotent stem cells by means of CRISPR/Cas9 technology and delivery of these via adeno-associated-viruses into the target cells.
In addition, cardiac regeneration by proliferation of differentiated cardiomyocytes and cardiac optogenetics are promising therapeutic methods.

Conclusion: The application of these forms of therapy has not yet found its way into the clinic, but thanks to intensive research, it holds promising prospect.

1.INTRODUCTION

Sudden cardiac death is defined as a sudden, unexpected and natural death due to cardiac causes which occurs within one hour of symptom onset in individuals who are otherwise healthy or in the absence of any other potentially lethal condition (1). It is accountable for around 20 percent of mortality in the general population and leads to significant morbidity amid survivors (2).

In many cases SCD occurs in patients older than 40 years due to coronary heart disease or an ischemic event, but it also emerges in patients under 35 years were it`s more common to result from inherited disorders (3).

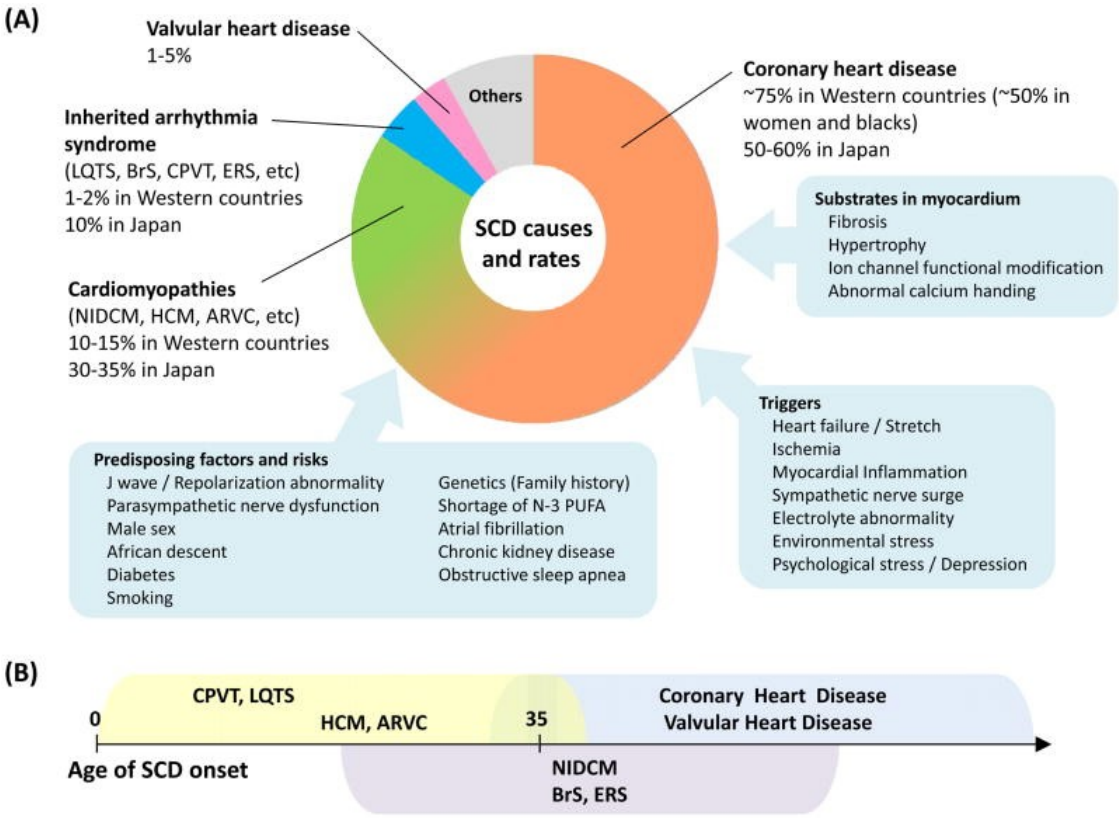

Fig.1: Causes of SCD and rates (A), and age of SCD onset in each disease (B) [Picture re-used without changes with permission from Circulation Research] (4)

1.1 Genetics of sudden cardiac death

Inherited cardiac disorders can be categorized in two main groups:

1. Cardiomyopathies, where mutations result from genes encoding for different categories of proteins, such as desmosomes, sarcomeres and the cytoskeleton, leading to an abnormal structure of the myocardium.
2. Cardiac channelopathies, which are primary electric disorders in a structurally normal heart and result from mutations in genes encoding cardiac ion channels or associated proteins (2).

Studies in Ireland and Sydney showed that sudden cardiac death in individuals younger than 35 years are in 27% to 29% of cases autopsy negative, in a Danish study of SCD this rate was even higher (43%). However, after detailed histological examination, the proportion of biopsy negative findings was significantly lower. Around 50% of the patients with autopsy negative SCD will have inherited arrhythmic syndromes (4).

The cardiomyopathies as well as the primary electric disorders have been considered as monogenic Mendelian disorders. However, these disorders are genetically heterogeneous, meaning that mutations in different genes can lead to the same clinical disease manifestation. This includes also allelic heterogeneity where different mutations within a gene are causative for the disease. Most mutations are unique or have been found in only a few families (2).

1.1.1 Cardiomyopathies

Many cardiomyopathies are monogenetic disorders, often leading to heart failure with a steady number of systemic manifestations.

The different types of inherited cardiomyopathies are (1):

Hypertrophy cardiomyopathy (HCM):

In young individuals HCM is the main reason for sudden cardiac death.
The majority of HCM affected individuals presents disease-causing mutations in genes encoding cardiac sarcomeric proteins, most common in *MYH7* and *MYBPC3* (2).
Other mutations leading to HCM occur in *TPM, TNNT2, MYL2, MYL3, TNNI3, ACTC1, TNNC1, MYH6* and *PRKAG2* (1).
The pattern of inheritance is autosomal dominant with variable expression and age-related, incomplete penetrance, therefore it has consequences for biologically related family members. The main pathology is hypertrophy of the left ventricle which can result in outflow tract obstruction, causing symptoms like dyspnea, palpitations or syncope. In many cases sudden cardiac death is the only symptom (5).

Dilated cardiomyopathy (DCM):

DCM is called to be the most severe form of cardiomyopathy, frequently leading to heart failure and requiring heart transplantation (6).
Its pathological hallmarks are dilatation of the left ventricle and systolic dysfunction (2). Frequently found are ventricular and supraventricular arrhythmias, conduction system abnormalities and thromboembolism. Eventually SCD might occur (1).
DCM is associated with mutations in more than 40 genes with those involving the *TTN* gene being the most common ones (6).
In around 20% of adults with DCM potential pathogenic genetic mutations are found (7).
The pattern of inheritance is mostly autosomal dominant and the mutations occur in genes encoding a wide range of proteins, such as sarcomere proteins, structural proteins, ion channels and nuclear envelope proteins (1).
Furthermore, many conditions such as inflammatory, infective and systemic diseases, various drugs and toxins can cause DCM (7).
DCM can get classified by cause in three subgroups: familial form, primary without a family history or secondary (1).

Restrictive cardiomyopathy (RCM):

RCM is a rather rare form of cardiomyopathy characterized by myocardial stiffness that is leading to reduced right or left ventricular filling causing right or left heart failure. It can have inherited or acquired causes, or be a combination of those two. The three main causes of RCM are amyloidosis, cardiac sarcoidosis and cardiac hemochromatosis (8).
Clinical manifestation of RCM are signs and symptoms of biventricular heart failure and the disease is associated with bad long-term prognosis, being the treatment mostly palliative (7).
Mutations have been identified in four different genes encoding for sarcomeric proteins: *MYH7, TNNT2, TNNI3* and *ACTC*. The main pattern of inheritance is autosomal dominant; however autosomal and X-linked patterns have been described (1).

Arrhythmogenic right ventricular cardiomyopathy (ARVC):

ARVC is distinguished by replacement of normal cardiac myocytes with fibrous and adipose tissue due to an inherited disease that concerns cardiac desmosomes. It leads to right ventricular systolic dysfunction and right ventricular deformation with a risk of malignant ventricular tachycardia and sudden cardiac death (9).
It is a major cause of sudden cardiac death among young athletes, having an incidence of sudden death at approximately 2,5% per year (1).
The estimated prevalence of ARVC in the general population is 1 in 1000 to 1 in 5000 (7).
Many causative genes have been identified to leading to ARVC, most importantly mutations found in the desmosomal genes like *JUP, DSP, PKP2, DSG2* and *DSC2*. Its inheritance is autosomal dominant; however, two recessive modes have been identified. In more than 60% of patients familial evidence has been found (1).

1.1.2 Cardiac channelopathies

In the myocardial cellular membrane ion channels for sodium (Na+), potassium (K+) and calcium (Ca2+) can be found whose interplay leads to the heartbeat. Mutations can lead to dysfunction of these channels, leading to life-threatening arrhythmias (10).

Brugada Syndrome (BrS):

Brugada Syndrome is a congenital cardiac channelopathy that comes with a predisposition for SCD in patients where no structural heart disease can be found. It usually leads to symptoms in the fourth or fifth decade of life; however, there are also cases concerning children. The clinical presentation of BrS is right ventricular conduction delay and ST segment elevation in leads V1-V3 in the ECG. One of the main clinical manifestations is syncope (1,11).
The pattern of inheritance is autosomal-dominant with incomplete penetrance which is age- as well as sex-related. More than 450 pathogenic variants in 24 genes encoding sodium (e.g. *SCN5A, SCN1B, SCN2B, SCN3B*) potassium and calcium channels (e.g. *CACNA1C, CANB2b, CACNA2D1*) or linked proteins (e.g. *RANGRF, GPD1L*) are associated with Brugada Syndrome. Around 20%-25% of genetic alterations associated with BrS were identified in the *SCN5A* gene. However, still 65%-70% of BrS patients remain genetically unsolved since BrS-susceptibility genes can only in part explain the clinical diagnosed cases (3).

Long QT Syndrome (LQTS):

The clinical presentation of LQTS is a prolonged QT interval and large T waves on the ECG, which can either be asymptomatic or lead to SCD, syncope, malignant ventricular arrhythmias, VF or torsade de pointes ventricular tachycardia. The clinical diagnosis is made by ECG and an extensive evaluation due to personal/family history. Currently 16 types of LQTS have been specified, the most frequent forms being LQTS1, LQTS2 and LQTS3 (1,12).
LQTS1 is characterized by syncope or sudden death during physical exercise and the ECG shows a broad-based T-wave.

The clinical presentation of LQTS2 is syncope or SCD with strong emotions or sudden auditory stimuli, showing a notched or low-amplitude T-wave in the ECG. Patients with LQTS3 tend to have late-peaked T-waves and long, flat ST segments in the ECG and usually have a tendency to bradycardia and a higher risk for sudden death during sleep (1).

Congenital LQTS, which is associated with mutations in ion channels and/or associated proteins, is distinguished from acquired LQTS, which is linked to drugs and electrolyte imbalance. So far 19 disease-causing genes have been identified in congenital LQTS. In 15 out of these the pattern of inheritance is autosomal-dominant. Approximately 75% of patients with a LQTS diagnosis have mutations in the following three genes: *KCNQ1, KCNH2* and *SCN5A* (3).

Short QT Syndrome (SQTS):

Characterization of SQTS is a reduced QT interval (<340ms) on the ECG, as well as a predisposition to supraventricular arrhythmias and cardiac arrest without an existing structural heart disease. A distinction is drawn between congenital or acquired SQTS, with the last being associated with hypercalcemia, hyperkalemia, acidosis and drugs (2).

Common symptoms are cardiac arrest in approximately 30%, followed by palpitations, syncope and atrial fibrillation, which can occur in different situation e.g. during exercise, at rest, during daily activity. The diagnosis is made by pathological findings in the ECG, syncope, episodes of VF or PVT and family history. The pattern of inheritance is autosomal-dominant and it has a high penetrance. At present, six disease causing genes have been identified (*KCNQ1, KCNH2, KCNJ2, CACNA1C, CACNB2* and *CACNA2D1)* (3).

It is a very severe disease with a high risk of lethality in all age groups. E.g. the risk of having a cardiac arrest by the age of 40 years is more than 40% (7).

Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT):

CPVT is a rare inherited cardiac arrhythmia disorder which can lead to emotion- and exercise-induced polymorphic ventricular arrhythmias. The estimated prevalence of the disease is 1 in 10000 (3).

The ECG of patients with CPVT is usually normal, for diagnosis exercise- or medication-ECG is performed, which unmasks the CPVT. The patterns of inheritance are autosomal dominant as well as autosomal-recessive.

The major CPVT gene is *RYR2*, a gene that is encoding for the cardiac ryanodine receptor gene (approximately 60% of patient with clinical diagnosis of CVPT have mutations occurring in *RYR2*), where more than 150 different mutations have been associated with CVPT so far (3,13).

Signs and symptoms usually occur in the first decade of life and are triggered by physical activity or emotional stress (7).

1.2 Sudden cardiac death in athletes

For the vast majority of the population exercise yields beneficial change in many cardiovascular risk factors such as blood pressure, insulin sensitivity, lipids and weight. However, for patients with hereditary or congenital diseases of the electrical cardiac system or the cardiac structure exercise can be associated with risk of SCD. Causes of SCD in young athletes are e.g. hypertrophic cardiomyopathy, arrhythmogenic ventricular cardiomyopathy or congenital coronary artery anomalies. These cardiac conditions might be clinically silent and undiagnosed, therefore leading to sudden cardiac death being the first incidence (14,15).

Even though it is an unusual event in young athletes, SCD occurs in about 1 out of 50.000 and can happen at all times, be it during a competition, in training or even at rest. The cause of death in these patients needs to be identified by postmortem examination. When a genetic heart disease is suspected, genetic testing should follow to identify other family members at risk of SCD (16).

Sudden death of a young person is a disastrous and emotional event that makes prevention in form of cardiovascular screening very important. Basic screening includes family and/or personal history, physical examination and in most countries also 12-lead resting ECG, which shows electrophysiological abnormalities such as long and short QT syndrome, Brugada syndrome, WPW, high-grade AV-block, ARVC and HCM with high sensitivity. If pathologic findings appear further diagnostic work-up and therapy are needed. However, it will never be possible to identify all individuals with risk through cardiac screening (17).

The main part of diseases leading to SCD cannot be cured, but there are reasonable interventions disposable, such as drug therapy, radiofrequency ablation or implantation of an ICD.

Summing up, an identification of patients at risk as early as possible is very important to reduce SCD in young athletes and identify other family members at risk (15).

1.3 Current treatment options

An effective management of underlying diseases and co-morbidities is a significant aspect in the successful management of sudden cardiac death. Depending on the underlying disease, different up-to-date management, including ICD or avoiding competitive sports, must be optimized.

1.3.1 Implantable cardiac defibrillator (ICD)

The most efficient treatment for prevention of sudden cardiac death is yet the ICD. However, it is still challenging to identify individuals who are at risk of SCD and to figure out who might profit from the implantation of an ICD (18).

Due to the findings of clinical trials the recommendation for primary prevention ICD in individuals with ischemic cardiomyopathy rests upon their LVEF and NYHA functional class. The primary prevention ICD in non-ischemic cardiomyopathy according to earlier clinical trials is presumably best in younger patients, individuals with less grave heart failure symptoms and fewer comorbidities. However, the risk for ventricular arrhythmias is lower in NICM, making the implantation of an ICD being not beneficial. Secondary prevention ICD is the first-line treatment for patients who survived sudden cardiac death from ventricular fibrillation/ ventricular tachycardia or from hemodynamically unstable VT not due to reversible causes. Even though the ICD is an essential treatment option, SCD remains a public health threat, because the majority of SCD occurs in individuals without severe cardiomyopathy and unknown to be at risk (18,19).

ICD implantation in the young is associated with higher risk of complications with devices, such as frequency of inappropriate shocks, lead malfunctions and difficulties in ICD placement due to intracardiac anatomy and small size. Therefore, guidelines for implantation of an ICD in children and young people are written with more latitude compared to those used in adults (20).

Nowadays three different types of defibrillator technologies are used: the transvenous implantable cardiac defibrillator (ICD), the subcutaneous ICD (S-ICD) and the wearable cardioverter defibrillator (WCD).

Indications for S-ICD are younger patients, patients with limited vascular access, patients with higher risk of infection, patients with channelopathies and patients with congenital heart diseases. However, a contra-indication for ICD are patients who necessitate cardiac pacing. The wearable cardioverter is indicated for individuals who are at higher risk of SCD for a limited time, until either definitive therapy is available or the SCD risk resolves over time (18).

1.3.2 Pharmacotherapy for ventricular arrhythmia

Clinical trials showed that an exclusive use of ant-arrhythmic drugs (with the exception of beta-blockers) leads to no significant improvement in the treatment of individuals with life-threatening VAs or in the prevention of SCD under specific circumstances. However, they might be effective when used as additional therapy.
Each anti-arrhythmic drug has the potential to trigger life-threatening ventricular tachyarrhythmias. Using drugs for inherited primary arrhythmia syndromes and cardiomyopathies is an off-label indication (7).

Beta-blockers are effective and safe anti-arrhythmic drugs, being the first-line therapy in the treatment of VA and the prevention of SCD. They lead to a competitive beta-adrenoreceptor blockade and slow of the sinus rate. Furthermore they inhibit excess calcium release (21).

Amiodarone is blocking the depolarizing sodium currents and potassium channels, eventually leading to an inhibition or termination of VAs by influencing automaticity and re-entry. However, in the therapy of Sudden Cardiac Death in individuals with a LVEF under 35% Amiodarone shows a lack of benefit (7).
A combinational therapy of beta-blocker and Amiodarone reduces the number of ICD shocks, but side effects of the therapy might lead to drug discontinuation (22).

Another anti-arrhythmic drug is Sotalol, which leads to potassium current inhibition and is effective in suppressing VA. It is used for patients with Coronary Artery disease without heart failure and requires careful ECG monitoring. Non-antiarrhythmic drugs like angiotensin-converting enzyme (ACE) inhibitors,

angiotensin II receptor blockers (ARBs) and mineralocorticoid receptor antagonists (MRAs) can have an impact on the prevention of Sudden Cardiac Death as well by reversing the remodeling in the ventricle and reducing the rates of SCD (7).

1.3.3 Interventional therapy

ICDs decrease mortality and have therefore become the main therapy option for individuals with VT or VF. However, they do not cure ventricular arrhythmias and recurrent ICD shocks may result in anxiety and depression in more than half of the patients. One option to reduce the incidence of VT or VF in a scar-related heart disease is catheter ablation (23).

Ablation strategies often rely on identification of a reentrant circuit and targeting a critical isthmus (24).

For planning and guiding the ablation procedure non-invasive imaging of cardiac structure including magnetic resonance imaging can be utilized. Different ablation techniques such as point-by-point ablation at the exit site of the re-entry circuit, deployment of linear lesion sets or ablation of local abnormal ventricular activity can be utilized. Even though catheter ablation is a treatment possibility for a great variety of VT substrates, the theory whether it reduces mortality or not has not been well enough studied yet. If patients with VT are refractory to anti-arrhythmic drug therapy or have a failed catheter ablation, surgical ablation is an option (7).

1.3.4 Organ donation

Organ transplantation is in certain cases of end-stage organ failure the only treatment option for patients and can help to save their lives. However, the shortage of donors leads to many patients dying on the wait-list (e.g. in the US the number of individuals on the wait-list in 2006 was over 95000, whereas the number of deaths of people on the wait-list was over 6300).

Living donation, where an alive human is donating an organ, has to be distinguished from postmortem donation, where the donor has had either a brain- or a cardiac death (25).

Brain dead donors:

The main part of organs from deceased donors comes from patients who are declared brain death by neurologic criteria (26).

Brain death is defined as an irreversible cessation of all clinical brain functions, meanwhile the respiratory, as well as the circulatory functions, remain obtained by mechanical ventilation. Irreversible implies here that no available technology can rebuild the brain function once death has been confirmed by diverse tests.

Cardiac death donors:

Approximately a quarter of the organs from deceased donors in the US comes from patients who are declared death by circulatory criteria. Confirmation of cessation of circulation and respiration are the criteria of cardiac death (27).

1.3.4.1 Problems of organ transplantation

Acute and chronic rejection

After transplantation donor-specific antibodies are produced by long-lived plasma cells and bind directly to graft endothelium. They activate the complement system and recruit natural killer cells, macrophages and neutrophils, leading to acute antibody-mediated allograft rejection (AMR). In chronic AMR, a repetitive pattern of thrombotic events and inflammatory changes induces cell- and repair damage, which leads to late glomerulopathy and reduction of renal function. These humoral responses have to be controlled to sustain the allograft for the recipient (28).

Lifelong immunosuppressive multidrug combination therapy is used to prevent rejection; however, it can also lead to unwanted side effects, which also influence long-term success after transplantation. Among these are infections and malignancies, which count to the leading cause of death with a functioning graft. Furthermore, side effects of current immunosuppressive regimens are e.g. nephrotoxicity, metabolic, and cardiovascular side effects and nonadherence (29).

Organ shortage

Each year the number of individuals on the waitlist increases due to increased incidence of vital organ failure and the improvement in post-transplant outcome,

leading to a bigger interval between the need and the supply of organs. This shortage of organs cannot be fully covered by DBD and/or DCD alone and other approaches are needed to overcome the unbalanced distribution between organ availability and need. One strategy is the use of organs that are less ideal (e.g. elderly donors, pediatric donors, diabetic donors, split-liver DD donors) (25).

Another strategy is the potential pool of donors, which is significantly larger than the actual number of donors (e.g. estimated number of donor-eligible deaths: 10500-16800/year in the US, actual DD: 8500-9000/year) (26).

2. METHODS

An essential part of this work consists of a summary of current knowledge from various sources in the sense of a meta-analyses. For this purpose, the primary aim was to gain as many reviews as possible on this topic from medical and biological databases such as PubMed or bioRxiv. Due to the numerous publications, the focus was clearly on literature from the year 2010 and onwards.
The search in such databases is carried out with the help of keywords or looking for high ranked journals through Clarivate Analytics by comparing the impact factor of different journals concerning the categories “Genetics and Heredity”, “Engineering, Biomedical”, “Biochemistry and Molecular Biology” and “Cardiac and Cardiovascular System”.
Furthermore, the reviews were searched for references concerning work that provided important insights or gave new impulses to research.

Following search yielded the best results: In PubMed, the search for reviews and papers after 2010 has been restricted under “Advanced Search”.
Moreover, by using the commands “AND” and “OR” as well as brackets papers that contained words including “Sudden Cardiac Death”, “Future gene therapies”, “CRISPR/Cas9”, “Genome Editing”, “Bioengineering Human Myocardium”, “Induced Cardiomyocytes”, “Cardiac Regeneration”, “Cardiac Optogenetics”, “Ethics in Genome Editing”, were searched for.

This search was compared with many other combinations that subsequently proved to be less optimal, but served to confirm the validity of this search result. In addition, the databases were searched for all the reviews of authors who were intensively involved with the material, without restricting the search to keywords.

The literature was summarized depending on the focus of the topic and arranged categorically according to different perspectives. The results and contents of similar studies were compared to address differences and objections and were then combined to a uniform work.

3. RESULTS

3.1 Bioengineering human myocardium on native extracellular matrix as an alternative to donor organs

The final therapy option for end-stage heart failure is heart transplantation. However, the problem of organ shortage, the adverse effects from life-long immunosuppression after heart transplantation, and chronic rejection, make it necessary to pursue new alternatives for donor organs. Bioengineering human myocardium as well as bio-artificial hearts could be a new pathway in this field (30).

The heart is composed of muscles, nerves and blood vessels which are integrated into a scaffold that supplies both form and function for the cells. It contains growth factors and sugars, which are important for the cell behavior, structural features such as valves as well as a complex vascular network to conduct nutrients to the cells. The ECM framework is made up of basement membrane and interstitium, containing collagens, laminins, proteoglycans and polysaccharides that bind growth factors and other chemokines. The goal for heart transplantation is to create a whole bioengineered heart that is built by using a decellularized scaffold which then gets recellularized by billions of multiple types of cardiac cells (31).

Heart failure comes along with myocardial necrosis, fibrosis and extracellular matrix remodeling, leading to a progressive loss of architecture and contractile function. In order to accomplish successful clinical application of myocardial regeneration it is important to establish physiological tissue architecture and immediate graft perfusion. For creating the scaffold to engineer bio artificial myocardium, native human heart matrix provides the physiological micro- and macro architecture, extracellular matrix composition and the perfusable vascular bed. To replace or augment lost myocardial function cardiomyocytes are derived from nontransgenic adult-derived induced pluripotent stem cells and the native human heart matrix gets repopulated (30).

3.1.1 Induced Cardiomyocytes (iCM)

In the majority of heart diseases the contractile cardiomyocyte force, which is an important parameter of the cardiomyocyte function, is reduced either in a direct way, concerning the sarcomeric proteins, or in an indirect way with effect on ion channels (32).

Producing patient-specific, functional cardiomyocytes still remains challenging due to the restricted in vitro expansion potential of CMs (33).
There are three differentiation protocols which have been established to extract highly enriched CM cultures:

1) CMs derived from induced PSCs
2) Direct reprogramming of adult non-CM cells into induced CMs
3) Reprogramming somatic cells into CM via temporary pluripotency and consecutive differentiation

1) As a source for patient-specific CMs, induced pluripotent stem cells are a promising approach. Compared to embryonic stem cells iPSCs avoid the ethical issues since no cells from blastocysts are used. Furthermore, they show great potential to simulate myocardium for the function of drug screening. They are capable of differentiating into cells of several lineages, are easy to expand and readily available (34).
   The maturation of human iPSC-derived CMs results from long-term culture and electrical, hydrodynamic and mechanical stimulation. This in-vitro maturation benefits from high stimulation, compared to the native heart which beats more slowly following birth (35).

   Yamanaka and Takahashi (36) started with the production of iPSCs from somatic cells in 2007, using a gammaretrovirus to express four transcription factors responsible for pluripotency. Since then several methods to produce iPSCs more efficiently have been established, including viral and lentiviral integration, non-integrating viral vectors and protein-and small molecule-based reprogramming.

To convert somatic cells into iPSCs, cells from multiple different tissue sources can be used, e.g. skin fibroblasts or mononuclear cells from peripheral blood (34).

Methods for differentiating iPSCs into human cardiomyocytes include the matrix sandwich technique where iPSCs are cultured as monolayers on Matrigel and are subsequently overlaid with Matrigel. Furthermore, the matrix sandwich gets combined with sequential application of growth factors, leading to CMs with high purity and yield from multiple PSC lines. However, studies show that this method only works well for certain cell lines (34,37).
Another method for deriving CMs is temporal regulation of Wnt/β-catenin signaling, either using shRNAs or small molecules, resulting in an efficient production of human cardiomyocytes with a high degree of purity (38).

However, compared to the native CMs, one challenge is the immaturity of the iPSC-derived CMs that do survive after injection in the myocardium.
Changes in the fundamental parameters like the cylindrical shape, length-to-width ratio, sarcomeric length or mitochondria volume are found in iCM, implying their immaturity. One approach to improve this situation is the patterning of culture substrates by using continuous surface grooves to align sarcomeric structures and increase anisotropic contractility (33).

In vivo cells exist in 3D, hence creating a 3D pattern mimics the natural cell-surface and favors a more physiologically mature phenotype (39).
One method to produce scaffolds with 3D features is photolithography and cell imprinting with the goal of achieving better cell reprogramming from iPSCs to iCMs, to induce the physiological 3D shape and orientation of iCMs and to produce highly functional and mature CMs (33).

Another challenge is the heterogeneity of CM subtypes which are generated by CM differentiation. Those mixed populations consist of cells with different action potential conducts and can lead to life-threatening arrhythmogenicity when inaugurated in the heart. The approach is to produce pure populations of each CM subtype (39).

Finally, the risk of teratoma formation after transplantation of differentiated cardiac cells that might still contain undifferentiated hiPSCs is given and therefore it is critical to determine the purity of the cardiomyocyte cell population prior to implantation into the heart (40).

2) Direct reprogramming with CM-transcription factors, such as Gata4, Mef2c and Tbx5, translates adult non-CMs into iCMs. According to recently published data this method is highly preferable for in vivo heart regeneration therapies (39).
   Studies showed that a greater number of CMs can be induced if cells do not have to go through an iPSC intermediate state, leading to this method having an important impact on personalized medicine strategies in the future (34).

3) The indirect reprogramming of somatic cells into CM via temporary pluripotency and consecutive differentiation fuses two methods: Temporary induction of pluripotency as well as subsequent cardiac differentiation. This method offers a simpler and faster way than derivation of CMs via iPSCs and it is more efficient than direct reprogramming. Furthermore, the risk of teratoma formation is reduced (39).

3.1.2 Bioengineering functional myocardial tissue

At the moment, there are several different techniques available to generate extracellular human tissue. Those techniques differ in the size of the construct, the choice of extracellular matrix, the mode of tissue anchoring and the techniques which are used to analyze the tissue. One big advantage of human engineered tissues is their lack of species difference, making it easier to develop a surrogate for the human heart (32).
Regeneration of functional myocardial tissue is based on the creation of biocompatible acellular whole-organ scaffolds from human donor hearts by perfusion-decellularization; the differentiation of iPSCs into mature cardiomyocytes; the adhesion of iCMs on native ECM; and the up-scaling to biomimetic organ culture to create functional myocardial tissue. Studies show that after decellularization, a network of macro- and microvascular channels is preserved, which supplies the basis for the generation of metabolically active myocardium. Furthermore, the native

fiber framework within the myocardium is preserved, as the anisotropic behavior of myocardium on passive mechanical testing is not affected (30).

Decellularized extracellular matrix catches the complex array of proteins, glycosaminoglycans, proteoglycans and several other matrix components which are found in native tissue. Therefore, dECM is a promising biomaterial for repairing cardiovascular tissue, providing cues for regeneration, repair and remodeling of damaged myocardium. dECMs from primary cardiac or secondary tissue sources have been used for heart-based applications, whereat primary cardiac-derived dECMs have a more tissue-specific biochemical profile and structure composition that leads to a more effective cardiac repair compared to secondary dECMs.
dECMs have been used for cardiac therapy as either solid scaffolds, that have been decellularized and maintain native matrix structure, or as soluble materials, which, after decellularization, break down the ECM structure and solubilize the ECM into a liquid form (41).

3.1.2.1 Solid dECM

Solid dECM scaffolds are used directly after decellularization and preserve the native tissue structure and vasculature. The material gets used either as a solid patch or therapies attempt to recellularize the dECM toward functional tissue development.

<u>Decellularization Methods:</u>
The most common method is perfusion decellularization since it minimizes ECM damage and leaves ECM structure and vasculature intact. To remove cells via perfusion, either chemical treatment, enzymatic reaction, or mechanical degradation can be used. However, no method has turned out to be optimal due to variability in tissue composition. Chemical decellularization uses acidic or basic solutions, alcohols or detergents to lyse and remove cells. In contrast, enzymatic decellularization involves using biological enzymes such as MMPs, proteases, or nucleases to interrupt cell-cell and cell-matrix attachments and cells get lysed by membrane cleavage. Mechanical degradation uses freeze-thaw cycles to disrupt

the cellular components, agitation during immersion in chemical solution, or application of pressure to damage cells and lead to cell removal (42).
For the clinical value of organ matrix, the complete removal of cell surface xeno- or alloantigens as well as significant reduction of remaining double-strand DNA is important. Moreover, perfusion-decellularized human heart should be free from HLAs and antibody induction (30).
Quantifying cellular remains after decellularization can get done by using fluorescent immunostaining for cardiomyocyte marker or by histology. Furthermore, ECM composition gets observed to see if no damage to ECM components and macrostructure was caused. Finally, compressive or tensile strain measurements and transmural pressure analysis are made to ensure preservation of ECM mechanics (41).

Tissue engineered construct:
To completely repopulate an acellular human heart, several billion cardiomyocytes would be required (30).
However, for repairing damaged myocardium, cardiac patches descended from sections of decellularized heart tissue might be just as effective as whole hearts.
Studies which focus on cardiac patches follow methods, where 3D scaffolds are built from decellularized cECM and are then seeded with stem cells or differentiated cells generated from stem cells (41).
Overall, the use of solid dECM scaffolds with cells may be a potent method in the future, however, at the moment improved techniques are necessary to gain full recellularization of the myocardial matrix and the valve structures, in order to bring this technology to clinical scale and applications (30).

3.1.2.2 Soluble dECM

Soluble dECM scaffolds are derived from myocardium, pericardium or secondary sources such as SIS, placenta and omentum, who are decellularized and subsequently the ECM structure is broken down and solubilized into a liquid form (41).

It can form either 2D or 3D hydrogels in vitro or in vivo after injection into the myocardium (43).

Studies focus on building and modifying pure dECM for direct injection into damaged myocardium, or on dECM combined with cells and/or additional biomaterials to generate bioactive gels or cardiac patches. Soluble ECM keeps up ECM composition but misses structural and mechanical similarity to solid dECM scaffolds. It contains the ECM profile of native tissue, but can be injected into the myocardium without the requirement of surgical implantation. However, it is much more multisided than solid dECM scaffolds (41).

Synthesis methods:

To decellularize organ tissue, similar methods are used as for solid dECM. Specific areas of the heart get isolated and sectioned into small pieces, thereafter they get decellularized in solution rather than through perfusion (44).

Then the dECM gets lyophilized, is grinded into a fine powder and pepsin digestion leads to the breakdown of the dECM to make it more soluble. dECM materials are derived from specific tissue areas and sources and are therefore homogenized tissues which have similar qualities if batch-to-batch variability is reduced through combination of multiple animals. This is performed with porcine or murine dECM, cECM or pECM, but gets problematic when isolating human dECM. However, human cardiac ECM needs rougher decellularization protocols as well as additional steps of lipid removal after formation (41).

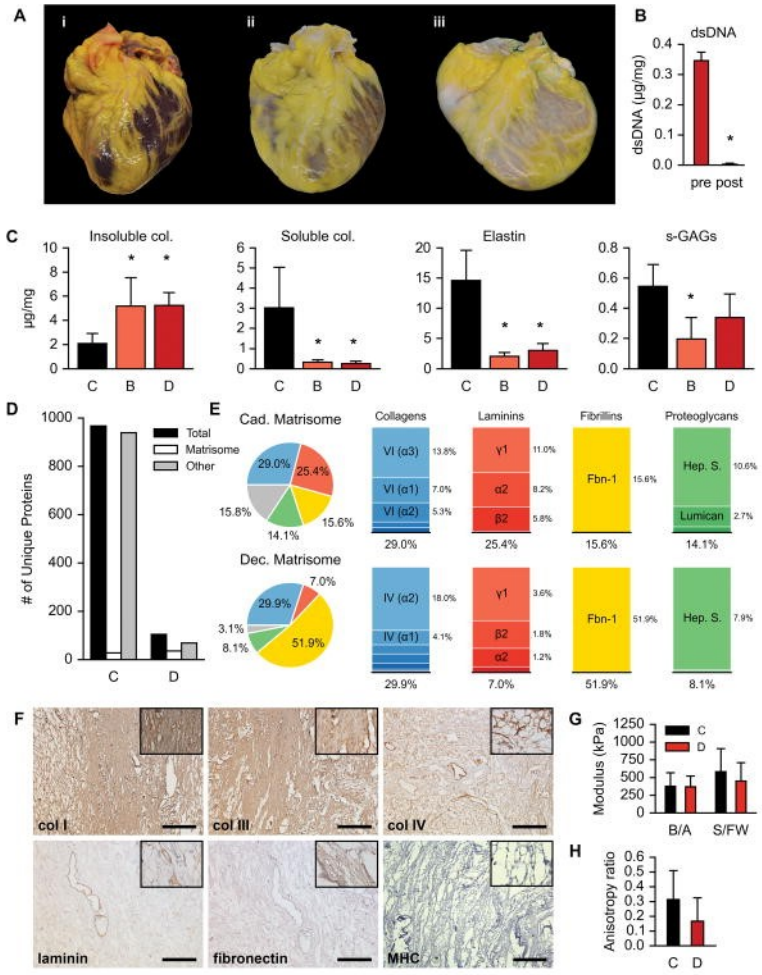

Fig.2: Biological and mechanical characterization of decellularized human myocardium:

A. Human heart before (i), during (ii) and after (iii) decellularization
B. DNA content of decellularized myocardium
C. Biochemical analysis for insoluble collagen, soluble collagen, α-elastin and sulfated glycosaminoglycans on normal cadaveric human myocardium, decellularized human cardiac matrix from brain dead donors and decellularized human cardiac matrix from cardiac death donors
D. Number of unique proteins identified through analysis in cadaveric and decellularized human myocardium
E. Left: Major components in the cadaveric and decellularized matrisome; Right: Breakdown of the major components into specific identified proteins
F. Immunohistochemical staining of decellularized human myocardium
G. Mechanical testing of cadaveric and decellularized human myocardium
H. Anisotropy of cadaveric and decellularized human myocardium

[Picture re-used without changes with permission from Circulation Research] (30)

In Vivo Evaluation of dECM Therapies:

The effectiveness of dECM as therapy for cardiovascular diseases is depended on in vivo implementation and evaluation of functional outcomes.
Injectable cECM (VentriGel) and SIS cardiac patches (CorMatrix) are the two main dECM technologies that have been evaluated in clinical testing (41).
The first studies about injectable myocardial matrix gel as a myocardial ECM mimic was performed in-vitro in rat models by Syngelin et al (45). They demonstrated the clinical potential of this material for minimal invasive delivery as well as the ability to form a nanofibrous gel in vitro and in situ. Furthermore, they showed the migration of vascular cells towards the myocardial matrix in vitro and the ability of the matrix to promote vascular cell infiltration (45).
The VentriGel is now the only soluble dECM therapy that is used for clinical testing for treating MI so far (41).
The solid dECM patch CorMatrix is widely used in clinical applications as a scaffold for tissue repair as well as for carotid repair and pericardial reconstruction, being cardiac closure the most common use (46).

3.1.3 Challenges and Future Directions

For scoring a higher effectiveness of dECM therapies improvements, including the development of technologies to reduce problems like sterilization, potential arrhythmia from dECM injection, requirement of surgical implantation of patches, and immune reactions from xenogeneic scaffolds, must be made (41).
Decellularization methods lead to nearly sterile materials (42).
However, to further sterilize, the only method in most studies is to maintain sterile conditions throughout the whole process of fabrication and implantation/injection.
Injectable dECM materials may induce arrhythmia in islands of non-contractile material within the myocardium, leading to a worsening of cardiac function.
Cardiac patches, in contrast, require surgical treatment which might not be performable in all patients according to age and cardiac condition.
Using human tissue for dECM is much rarer, more expensive and there is a higher patient-to-patient variability than with xenograft dECM. Nonhuman dECM materials, on the other hand, may cause increased inflammation and rejection after treatment,

due to an activation of the immune cascades. Studies have shown that combinatorial therapies with pure dECM materials joined with cells, additional biomaterials or paracrine factors lead to improvement in cardiac function compared to the use of pure dECM materials (41).

Furthermore, growth factor addition to biomaterials can improve regenerative outcomes in vivo, which is seen in dECM therapies for other tissues (47).

The addition of GFs leads to tissue remodeling, reduces inflammation, enhances angiogenesis in vivo and modulates stem cell response. More and more studies include GFs within scaffolds, which might be leading to an increase of functional improvement in treating damaged cardiac tissue. Another approach is exosome loaded dECM material that may show improvements in cardiac repair over other methods, due to their powerful effects in leading to cardiac repair with direct injection. In conclusion, dECM materials, which can be generated and modified through many methods, are a powerful tool in the future treatment of cardiovascular diseases. Two products of dECM materials to treat human cardiovascular diseases are in clinical trials at the moment. Whereas the results of these are still unknown, it is hoped that these therapies move forward in the clinic together with a variety of additional therapies (41).

3.2 Gene Editing

After deciphering the genetic code and developing strong genome editing tools, the idea of a direct correction of genetic mutations in the affected tissues arose.
Diseases, especially monogenic diseases, could be treated at the molecular level by fixing the mutant gene or normalizing the expression of overactive/underactive genes (48).

Human pluripotent stem cell lines are beneficial tools to understand regulatory processes during early development and diseases pathogenesis under the human genetic background.
Genetic modification of hPSCs, like gene knockout, enables more detailed studies on gene function in human embryogenesis or human genetic diseases (49).
Using targeted gene modification tools, disease-causing genetic variations could get corrected in patient-derived cells by eliminating these mutations. For the development of future therapies, the correction or silencing of a pathological genetic variant could be a promising approach.
There are three different methods in gene targeting based on sequence-specific nucleases to generate double-strand breaks in targeted DNA which have been developed recently (50):

1) Zinc finger nucleases

2) Transcription activator-like effector nucleases

3) CRISPR- Cas system

These double-strand breaks are then repaired by either fault-prone nonhomologous end-joining or the more accurate template-based homology-direct repair.
However, the generation of inducible gene knockout hPSC lines still remains challenging (49).

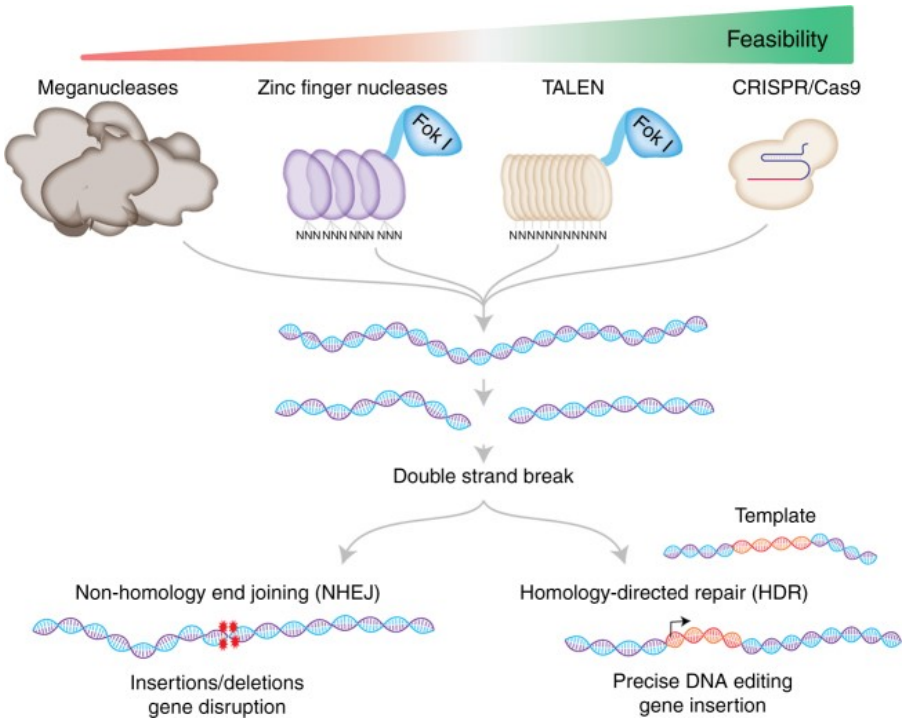

Fig.3: Working principle of Genome-Editing tools [Picture re-used without changes with permission from Creative Commons License: https://creativecommons.org/licenses/by/4.0/] (51)

3.2.1 Zinc finger nucleases (ZFN)

ZFNs are artificial produced restriction enzymes, created by fusion of a zinc finger DNA-binding domain to the endonuclease domain of the bacterial FokI (Flavobacterium okeanokoites) restriction enzyme. The zinc finger proteins have short sequences of amino acids that bind a zinc atom. Given that they are viewed as fingers sensing the DNA molecules, they are called “zinc-finger” (52).

The DNA binding domain can recognize a 3-to 4bp DNA sequence. Furthermore, tandem domains could also bind to a, for a cell’s genome unique, extended nucleotide sequence. For binding on a specific site in the genome, ZFNs recognize two sequences, one on the forward strand and one on the reverse strand (53). Meanwhile the FokI domains dimerize and create a DNA double-strand break at the site (54).

The double-strand break gets repaired either by using error-prone non-homologous end-join repair to generate small alterations at targeted genomic loci, which can occur during any phase of the cell cycle, or by homology-directed repair, which

occurs during late S-phase or G_2-phase and uses a sister chromatid or another closely matching DNA sequence as a repair template. One of the disadvantages of ZFNs is the difficulty to engineer them. Furthermore, the target site selection is limited, which may present challenges if a particular site is required such as to knock-in a specific mutation. Ultimately, concerns exist that ZFNs do not only introduce a double strand break at the desired location but also at off-target sites, leading to unwanted off-target events (53).

3.2.2 Transcription activator-like effector nucleases (TALENs)

It has been demonstrated that transcription activator-like effector proteins (TALE) from the bacterial genus *Xanthomonas* contain a unique DNA-binding domain.
They are made up of an N-terminal translocation signal as well as a central DNA binding domain. Furthermore they include a C-terminal region which contains nuclear localization signals and a transcriptional activation domain (55).
The TALE repeats cover tandem arrays with 10 to 30 repeats that links and detects extended DNA sequences. The length of each repeat varies from 33 to 35 amino acids. Two adjoining amino acids are referred to as repeat-variable di-residues (RVDs) which guarantee the specificity for one of the four base pairs.
Hence, there is a direct concordance between the repeats and the base pairs in the target DNA sequences. With this RVD code a new way of nuclease got created, where a domain of TALE repeats is fused to the FokI endonuclease domain (TALEN). TALENs can be used to knock-out genes or knock-in mutations, making double-strand breaks at wanted target sites in the genome (53).
TALENs can bind a wider range of DNA sequences than ZFNs and can be programmed in a short time and large number. To transfer TALEN into primary cells, viral vectors, e.g. adenoviral vectors, are proven to be the most effective so far (56).
As with ZFNs, one drawback is the TALEN-mediated DNA cleavage at off-target sites that can result in mutations in genomic loci, highlighting the importance of improving the specificity of this tool to minimize unwanted effects (57).
Another disadvantage of TALENs are their high repetitive sequences, which impair their use with viral vector gene delivery vehicles, most likely by causing sequence

instabilities. However, this can be managed by modifying the coding sequences of the TALE repeats (58).

Finally, the significantly larger size of TALENs compared to ZFNs makes it more difficult to supply and express them in cells. Furthermore, TALENs have a rather huge size, which makes them less appealing for therapeutic use, because viral vectors, used for delivering them, have restricted load size (53).

3.2.3 Clustered regularly interspaced short palindromic repeats (CRISPR)/ Cas9

CRISPR/Cas9 has become the genome editing tool of choice, since it does not require the production of a target-specific protein. The Cas9 simply interacts with a short single-guide RNA to identify the target region and induce a cut. It is sought to be less cytotoxic but as efficient as other gene editing methods. The CRISPR system was originally found in bacteria and archaea where it functions as a RNA-based bacterial adaptive immune system. The protein Cas9 can ligate 2 short RNA sequences serving as a site-specific endonuclease. Small guide RNAs, termed crRNA, are applied for sequence-specific interference with invading nucleic acids. A transactivating crRNA (tracrRNA) is partly complementary to the crRNA. By overlapping they form a RNA structure that attracts Cas9 to the cleaving complex, provided that there is also a protospacer adjacent motif (PAM) sequence in the target DNA. PAMs are very short, conserved sequences that are specific for Cas9s of different species, with “NGG” from SpCas9 being the most common one. Cas9 then binds to the PAM in the DNA as well as to the non-protospacer portion of the guide RNA, whereon the protospacer of the guide RNA hybridizes with one strand of the genomic DNA. Cas9 then induces a DSB in the DNA three bp’s before the PAM (59).

The cell can repair the damage after a DSB by two mechanisms: either by error-prone NHEJ, which introduces insertions or deletions in the cleavage site useful for generating loss-of-function mutations, or by HDR using a simultaneously introduced DNA repair template that corrects existing mutations, knocks-in desired mutations or reporters (60).

To deliver CRISPR/Cas9 reagents into postnatal organisms and human cells, non-integrating adeno-associated virus (AAV) and integrase-deficient lentivirus vectors are most widely used (59).

AAVs have two advantages: First of all, they are not recognized by the human immune system. Secondly, they do not integrate DNA randomly into the genome, whereas lentiviruses do (61).

With CRISPR/Cas9 gene knock-downs as well as gene-knock-outs can be accomplished. By introducing mutations in the nuclease motifs of Cas9, “dead” variants are created, which are unable to cleave DNA and can be used to repress expression of multiples genes simultaneously without altering the genome. In contrast, activation of transcription can be accomplished by fusing dCas9 to a transcription activator, which recruits the RNA polymerase and induces gene expression.With this application of CRISPR, gene editing with no double-stranded breaks is possible, making this method one of the most powerful gene editing tools (62).

Another approach of the CRISPR method is epigenome editing by introducing modifications such as DNA methylation or histone acetylation. This could lead to new therapeutical strategies due to helping to explore the regulation of gene expression (63).

In spite of the capability of CRISPR/Cas9 to become a safe and simple genome editing tool, some challenges, like off-target mutations, PAM dependence, gRNA production and delivery methods, still remain.

Compared to ZFN and TALEN, CRISPR/Cas9 holds a relative high risk of off-target mutations. That is because in large genomes you often find DNA sequences being identical or very highly homologous to target DNA sequences. Those unwanted sites then also get cut by CRISPR/Cas9 resulting in mutations that can lead to cell death or transformation (64).

Finally, one of the greatest obstacles with the CRISPR/Cas9 system is to find an all-purpose delivery method which is both safe and specific.

As many researches are done concerning CRISPR, it becomes even more precise and can be delivered with increasing precision (63).

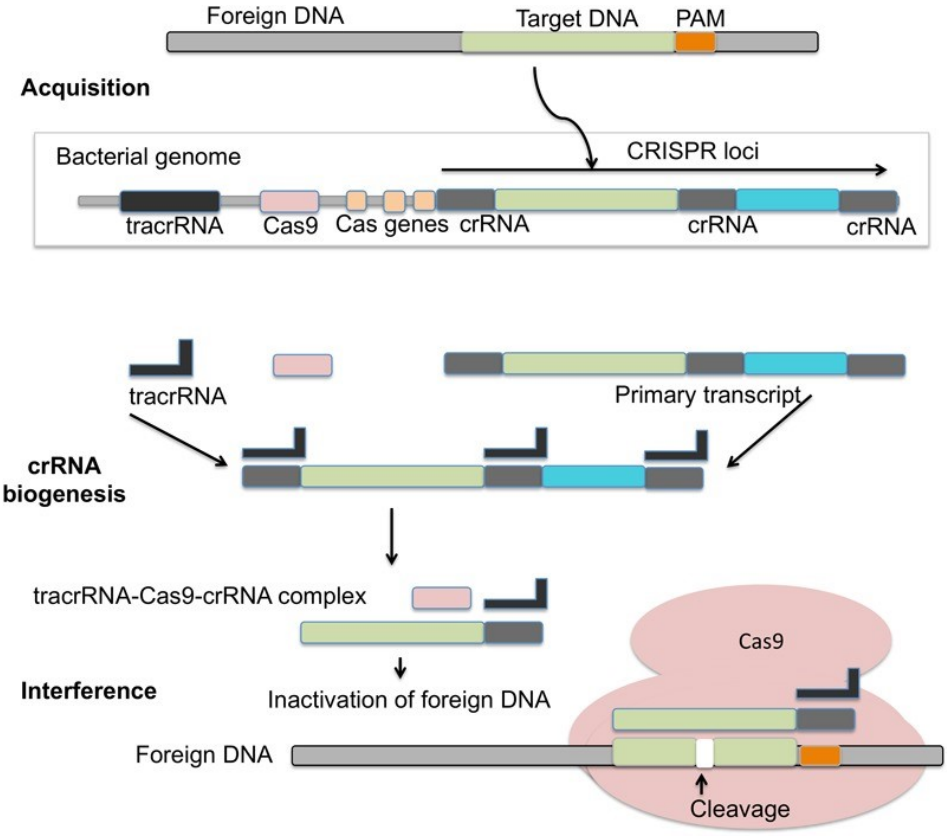

Fig. 4: Mechanism of CRISPR/Cas9 [Picture re-used without changes with permission from Frontiers] (65)

3.2.4 Genome editing in human pluripotent stem cells

Human pluripotent stem cells, inclusive of human embryonic stem cells and human induced pluripotent stem cells, are useful tools to model human organ development as well as to analyze disease mechanism and develope potential therapies.

To accomplish that, developing methods for adequate genetic manipulation in hPSCs is important (60).

With the new gene-editing tools, TALENs and CRISPR/Cas9, specific genetic mutations can get introduced into the hPSCs (66).

They act as “DNA-scissors” by introducing DSBs at desired genomic loci, which are then repaired by either NHEJ, leading to small insertions and deletions useful for generating loss-of-function mutations; or HDR which allows targeted integration of exogenously provided DNA sequences for introducing precise nucleotide alterations or knocking-in reporter genes (60).

The first efficient method to generate iKO hPSC lines was created by González et al. (66) in 2014 with a master hPSC line containing a doxycycline (DOX)-inducible Cas9 expression cassette inserted in the AAVS1 site, which has been shown to support robust and sustained transgene expression. Then two rounds of single guide RNA transfections were performed at specific times. This introduced random indels into targeted gene sites via NHEJ which eventually led to a potential gene KO. The resulting cells were a mixed population of KO and non-KO cells with unpredictable genotypes, making this method less practical for inducing gene KO in most of the cells during stem cell differentiation or at a particular stage of the pathological process (66).

Another approach is the dual-sgRNA mediated gene targeting method by Chen et al. which allows predictable KO of genes with different structural organization and/or expression patterns. First, an hPSC line gets generated by using the dual sgRNA targeting strategy, resulting in exons flanked by flippase recognition target (FRT)-sites. Subsequently, the drug-resistance expression cassette in the FRT-flanked gene locus gets removed and the activity-controllable enhanced-flippase (Flpe-ER) gets inserted into the AAVS1 locus to establish the iKO hPSC line. By administration of 4-OHT, the Flpe-ER will translocate into the nucleus and recombine the FRT-flanked exons, thereby resulting in frame-shift of a protein-coding sequence and KO of the targeted gene. One major concern with using CRISPR/Cas9 are off-target effects. However, this study showed relatively specific genome targeting in hPSCs, after sequencing 114 potential off-target sites (49).

A study of Li et al. (67) used episomal vectors based on oriP-EBNA1 to derive vector-free cells. The CRISPR/Cas9 system can effectively generate vector-free mutations in mammalian cells by delivering Cas9 and gRNA as episomes into the cells. This new system shows great functionality by inactivating visible reporter gene, by specifically deleting a single genomic region and editing multiplex genes in a transgene-free manner (67).

Another study by Xie et al. (60) also used an episomal CRISPR/Cas9 system for efficient genome editing in hPSCs with a vector containing oriP-EBNA1 components termed epiCRISPR. This system supports efficient double-gene knockout, large

genomic deletions and can be removed after genome editing, so that edited cells are free of exogenous gene expression. Genome editing with this system is done in two ways: Transfected cells are continuously selected due to the reporter genes on the episomal vector, reducing the need of a high editing frequency. Furthermore, the episomal vector allows long-term genome editing, meaning that the editing efficiency increases over time. Altogether, the epiCRISPR system is a great platform for genome editing in a variety of cells (60).

3.2.5 Vector systems

The delivery of genome engineering tools can be conducted by either viral- or non-viral vector systems with the major goal of developing safe and efficient delivery platforms. The advantages of viral vectors are efficient transduction and high transgene expression levels, but keeping in mind that they can lead to immunologic reactions and insertional mutagenesis. In contrast, non-viral vectors have diverse available chemistry, capacity for functionalization and targeting and are easy to manufacture. However, they are lower in efficiency than viral vectors. The most common non-viral delivery vectors are carrier-free delivery, physical membrane disruption, liposomes, polymers, nanoparticles and cell-penetrating peptides (68).
The choice of vector should be carefully considered, taking in mind genotoxic events such as inflammation, random insertion disrupting normal genes, activation of proto-oncogenes and insertional mutagenesis. Genotoxic events can be influenced either by virus type, integration target site or target cell type, or by non-viral factors such as patient age, disease and dose. The most frequently used viruses for gene therapy are adenovirus (AV), adeno-associated virus (AAV), retrovirus (γ-RV) and lentivirus (LV), with AAV being the most widely used viral vector for genome engineering to date. Retroviruses and lentiviruses have the ability to permanently integrate DNA into the genome, resulting in a long-term expression of the gene. However, at the same time other genes are most likely disrupted by the random integration into the genome and become therefor inactive (69).

3.2.5.1 Adeno-associated Virus Vector (AAV)

The treatment of genetic disorders by in vivo gene replacement is one of most stupendous concepts in modern medicine, with AAV vectors serving as the most suitable tool for the gene delivery. They have superior efficiency of transduction of several tissues such as the liver, muscle or nervous tissue. In humans, studies have shown promising results when AAV was used to transfer therapeutic genes to the retina for the treatment of congenital blindness, to the liver to treat Haemophilia B and to focal areas of the brain to improve the course of Parkinson´s disease (70).
In November 2012, the first gene-therapy medicine, Glybera, has been granted permission in Europe. Glybera is a medication for the treatment of individuals with familial lipoprotein lipase deficiency and is applied as local *in vivo* gene therapy. It is an AAV vector serotype 1 encoding a naturally occurring gain-of-function variant of LPL and leads to lower plasma TG levels and a lower frequency of heart diseases (71).

The AAV belongs to the *Parvoviridae* family and was identified in 1965. AAV needs to be co-infected with a helper virus such as the adenovirus or the herpesvirus for an active infection to occur. The AAV is not associated with any human illness, even though the seroprevalence in the general population is very high with approximately 80% (72).
The virus is non-enveloped and three capsid proteins (VP1, VP2, and VP3) generate an icosahedral capsid structure. It has a single stranded DNA genome of approximately 4.7-kilobases (kb) which carries two different genes: rep (replication) and cap (capsid). Both of them have multiple open reading frames which express proteins required for genome replication and packaging (73).
These proteins can either positively or negatively regulate AAV gene expression by response to environmental cues such as presence or absence of a helper virus (48).
The AAV2 DNA termini has a 145 nucleotide-long inverted terminal repeat (ITR) which can fold itself, form a T-shaped hairpin structure and is required for viral genome replication and packaging (72).
Recombinant AAVs have capsids that are made up of capsid proteins of the serotypes 1 to 9, but additional serotypes, isolates and designer variants exist as well and play more and more a role in AAV gene therapy (74).

The AAV vectors are used for gene therapy treatments of inherited disorders and therefore the native genome of AAV needs to have the rep and cap genes removed and replaced by the genetic elements necessary for gene therapy.
The recombinant AAV then has no viral DNA and can cross the cell membrane, where it can deliver its DNA load into the nucleus of a cell (73).
The added genetic elements get flanked by the so-called transgenes, which are the inverted terminal repeat sequences (ITRs). They generate circular concatemers that subsist as episomes in the nucleus of transduced cells. Recombinant episomal DNA does not get integrated into the host genome. After some time, the episomal DNA gets eliminated as the cell continues to repeat his rounds of replication. This leads to a loss of transgene and transgene expression depending on the conversion rate of the cell. Because of that rAAV is ideal for particular gene therapy applications. Each AAV vector can provide a ssDNA version of the transgene to the target cell which can express the desired therapeutic protein only if it is converted into a double-strand (48).
The packaging capacity of recombinant AAV vectors is limited to therapeutic transgenes up to the size of the wild-type AAV genome (4.7 kb). As the length of the transgene increases, the packaging efficiency into the capsids decreases.
The structure of a therapeutic transgene requires inclusion of a promoter, the gene of interest and a poly-adenylation signal flanked by ITRs, meaning that the treatment of disorders caused by defective genes over 3.5kb is right now not realizable, because the transgene would not fit into a AAV capsid. Therefore, if large coding sequences need to be packaged, the use of dual, overlapping vector strategies should be considered (73).
Cell- or tissue-specific expression is often wanted, because systemic delivery can lead to transduction and expression of the gene of interest in undesired cells or tissues. Currently, technologies exist which have the capability to produce novel, tissue-specific promoters, predicated on DNA regulatory element libraries.
Which particular AAV gets chosen for gene therapy is depending on which cell or tissue types are being targeted. Furthermore, the safety profile linked with the delivered gene and the option between systemic or local delivery is important. Finally, also the use of tissue-specific versus acrtive promoters gets considered (48).

Another challenge the AAV vector system is facing, is the immune recognition of the viral capsid by neutralizing antibodies (NABs), due to prior exposure to natural virus as well as following antibody responses against an administered vector. Further challenges are cytotoxic T lymphocyte recognition of infected cells and pattern recognition receptors. Whereas CD8+ T cell responses to the capsid can be blocked by transient immunosuppression, pre-existing NABs can lead to an immune response toward an administered vector causing rapid seroconversion. However, increasing knowledge of AAV biology is allowing the rational design or chemical alteration of capsids to protect them from detection.
Furthermore, aligned evolution can create variants that are resistant to neutralizing antibodies and can be joined with evolution for targeted delivery to specific tissues and cells in vivo (75).

In conclusion, AAV is the most popular vector for most gene therapy applications due to its unique biology, structure and no-known disease associations. In the future, the knowledge of AAV biology and the growing toolbox for applied AAV technology will continue to increase (48,76).

3.2.6 Cardiac delivery

Cardiac gene therapy is an up-and-coming method in treatment for inherited cardiac disorders and acquired cardiac diseases, such as heart failure. Currently, the only curative treatment for those diseases is heart transplantation. With cardiac gene therapy the underlying cause of cardiac diseases could be treated as well as cured (74).
Intra-myocardial, intra-vascular and pericardial injection are the three primary approaches for cardiac specific vector delivery. In clinical studies, the most common one is the antegrade intracoronary injection, because it is thought to be less invasive and can be performed in any catheterization facilities (77).
Intra-myocardial injection can be done direct via invasive thoracotomy, offering the greatest flexibility and precision of the injection or percutaneous via catheter based injection which is minimal invasive and therefor more desirable. The advantages of this injection method are that the virus can be delivered at a very high local

concentration, it bypasses the endothelial barrier which is usually an obstacle for gene transfer, off-target organ transduction is kept very low and the neutralizing effect of pre-existing antibodies is inhibited. However, it is a suboptimal delivery method for diseases such as heart failure because the expression of the transgene is narrowed to a very small area surrounding the injection site (74).

AAVs vectors are the most promising cardiac delivery systems due to their efficient transduction into cardiomyocytes, their moderate cellular immune response and their persistent expression of therapeutic gene of interest (78).

The most promising AAV serotypes for cardiac gene transfer are AAV1, AAV6 and AAV9, with AAV9 being the most powerful one to transduce efficiently cardiomyocytes in mice and rats if injected systemically (74).

One of the largest studies of gene transfer done in a cardiac insufficiency population was CUPID2, a study that was designed to assess whether AAV1/SERCA2a administration improves the clinical outcome of patients with moderate to severe heart failure and reduced ejection fraction or not. Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) activity is insufficient in the failing heart and CUPID2 thought to improve cardiac function by gene transfer. However, the results showed that AAV1/SERCA2a at the dose did neither reduce heart failure nor terminal events in the overall study population (79).

Currently, two studies using different AAV capdsids are being designed, one trial being sponsored by UniQure where S100A is delivered directly to the heart with an AAV9 vector, using retroinfusion and left anterior descending coronary occlusion as delivering method, is currently in preclinical development (48).

The other trial aims to deliver a constitutively active form of protein phosphatase 1 inhibitors, I1-c, with a chimeric capsid between AAV2 and AAV8 (74).

3.3 Cardiac Regeneration

In fetal hearts the major determinant of heart growth is cardiomyocyte proliferation. Then, after birth, physiological hypertrophy of the heart due to increase of the cardiomyocyte size appears. In contrast, adult cardiomyocytes loose most of their mitotic potential, being this the biggest hurdle of cardiac regeneration after heart injury (80).

Proliferation of differentiated cardiomyocytes is one approach to gain cardiac regeneration. Even though it seems to be a promising method and quite some research is done on cardiac cell replacement/bioengineering of human myocardium, it seems to be difficult to apply this method to the clinic, because long-term engraftment is difficult to accomplish. The perfect cell type has not been discovered yet and after using PSC CMs ventricular arrhythmias can appear more frequently (81).

Therefore, regulation of the cardiac cell cycle could be a promising approach for improving cardiac regeneration by inducing adult cardiomyocyte proliferation. In lower organisms such as Zebrafish, CMs maintain the ability for cardiac regeneration into adulthood, mainly through cardiomyocyte proliferation. In mammals, the cell-cycle exit is one of the most stable (82).

Their regeneration of myocardium is depending on the development stage, according as embryonic, neonatal or adult. The mammalian cell-cycle is regulated by complexes of cyclins and cyclin-dependent kinases which decrease simultaneously with their exit from the cell cycle. In comparison to neonatal and fetal hearts in adults, cell-cycle genes are downregulated. After birth, the cardiomyocyte cell cycle activity gets decreased due to a downregulation of pro-mitotic factors like cyclins and cyclin-dependent kinases. In contrast, cyclin inhibitors like p21, p27 and p57, get upregulated and favor cell cycle exit of cardiomyocytes. When the heart gets hypertrophic, cell cycle variants in CM appear, notably endoreduplication and acytokinetic mitosis. Endoreduplication leads to polyploid CM, whereas acytokinetic leads to multinuclear and binuclear cells (81).

Adult, differentiated CMs generally get postmitotic and permanently withdraw from the cell-cycle. This results in a reduced regenerative capacity.

Studies by Mohamed et al. (82) demonstrated that cardiomyocyte proliferation and subsequent cell survival can get achieved by a combination of four cell-cycle regulators. Two of them forming the CDK1:CCNB complex which gets inactivated by phosphorylation of CDK1 by Wee1. Therefor a Wee1 inhibitor is used to allow an efficient cell-cycle entry of CMs. P27 inhibits the function of G1 phase cyclins and TGFβ directly activates the p27 expression, hence using a TGFβ inhibitor leads to an enhancement of cell-cycle progression.
Cyclins and CDKs decrease dramatically after a few days of cells not dividing any further, thus being beneficial since ongoing cellular division is an undesired outcome of a regenerative approach (82).

Furthermore, prolonged, severe hypoxia induces metabolic reprogramming of adult cardiomyocytes which results in cell-cycle re-entry and myocardial regeneration. Studies with mice put in hypoxic environment have shown CM proliferation. However, further findings by other study groups need to confirm this method (83).

Another important tool in cardiac regeneration is the Hippo pathway. It inhibits cell proliferation, promotes apoptosis and regulates fates of stem/progenitor cells to control heart size. By either inactivating the Hippo pathway or activating the Yes-associated protein (YAP) cardiac regeneration gets enhanced.
YAP can control the fate of cardiomyocytes through several transcriptional mechanisms. The YAP activity as well as the TAZ (=coactivator with PDZ-binding motif) gets regulated depending on strength and duration of inhibiting mechanisms. YAP as well as TAZ do not have DNA-binding domains and hence have to use transcription factors to bind exact DNA elements and stimulate gene transcription. Another approach in cardiac physiology are upstream signals of the Hippo pathway like mechanical stress, oxidative stress or the G-protein-coupled receptor (GPCR). In mammals, the core components of the Hippo pathway are highly conserved. However, the Hippo pathway and its mechanisms are still not entirely understood and further investigations are needed (80).

In addition, microRNAs (miRNAs) regulate the CM biology, ranging from contractility to hypertrophy during development as well as in pathological conditions of CM. A single injection of synthetic micro RNA imitates the action of miR-199a-3p and miR-

590-3p and leads to significant cardiac functional improvement in mice. Furthermore, it stimulates endogenous cardiomyocyte proliferation after myocardial infarction. Studies by Lesizza et al. (84) showed that by delivering transient miRNA into injured, adult hearts, cardiac regeneration can be accomplished. With these results, miRNA therapeutics are one step closer to clinical administration (84).

Cardiac regeneration by induction of proliferation in differentiated CMs raises hope to be a future therapeutic method for clinical use. However, some hurdles still remain and further researches need to be done until it finds its way into clinical everyday life (81).

3.4 Optogenetics

Mostly found in archaea and bacteria, microbial opsin genes are encoding light-sensitive proteins functioning as ion channels, ion pumps or signaling receptors (85).
Channelrhodopsin-2 is a seven-transmembrane domain light-gated cation channel and is currently the most used opsin. It belongs to class I microbial opsins and conducts cations along the electrochemical gradient during opening (86).
The light-sensing element used by channelrhodopsin is retinal, a chromophore, which is covalently bound to the ion channel and undergoes isomerization upon interaction with a photon, in this way triggering the ion channel to open. In the dark, the channel closes rapidly again. With the discovery and understanding of channelrhodopsins, a new tool in science was found: the optogenetics, a method of genetic modification of mammalian cells and tissues by microbial opsins. One of the simplest and best studied light-gated active ion pumps is Bacteriorhodopsin (BR), another class I microbial opsin. The chromophore is likewise covalently bound to the ion pump and captures photon energy, leading to move protons against the electrochemical gradient from the cytoplasm across the membrane (87).
Optogenetics is used to accomplish precise temporal control over the activity of selected cells and especially has led to a revolution in neuroscience by probing brain function on health and disease (88).

According to Miesenböck (89) the word optogenetic gets defined as “the branch of biotechnology which combines genetic engineering with optics to observe and control the function of genetically targeted groups of cells with light, often in the intact animal.”
The crucial factor of optogenetics is the effects of light on the protein product of genes. The structure and function of biomolecules is getting represented in real-time by using fluorescent proteins (FPs). FPs have an intrinsic chromophore and therefor can reveal a visible wavelength fluorescence. Introducing a gene encoding a FP into a living cell enables that cell to emit a light upon excitation. Optogenetics combines light and genetic manipulation to disturb, control or analyze the function of cells (90).

Two types of light-sensitive proteins are used in optogenetics: Sensors, which make cellular function visible through translating cell physiological signals into optical signals, and actuators, which make cellular function controllable due to transducing optical signals into physiological signals. Those two devices together form an experimental unit with actuators delivering controlled interferences and sensors reporting system responses back. Optogenetic sensors respond to a change in their environment by an alteration in molecular conformation that leads to a modification in fluorescence intensity, fluorescence color or other spectral characteristics (89).
The optogenetic sensors consist of a sensing domain connected with one or more FPs. They can be activated by the parameter of interest which leads to a conformational change. The FPs then either change the brightness of a single FP or have alterations in Förster resonance energy transfer (FRET) efficacy between two FPs. Recently, the number of FPs and the colors which can be used have increased (91).

The so-called optogenetic toolbox includes different microbial opsins which can generate depolarizing/excitatory currents as well as inhibitory/hyperpolarizing currents. Therefore, they truly open the possibility for optical control of membrane potential. Through genetic engineering currently available opsins can get optimized respective light sensitivity, speed and spectral response (87).

3.4.1 Cardiac Optogenetics

The present knowledge of cardiac physiology is mostly predicated on studies using fluorescent organic dyes that enable the study of intracellular ions, transmembrane potential or pH using light microscopy. Due to that, many regions of an example and subcellular mechanisms such as three-dimensional and temporal resolution of calcium sparks can be studied. However, organic dyes have some obstacles, including that it can be used only once. For further studies of the fluorescent signal, the coloring will have to be repeated on another example. Furthermore, organic dyes cannot be used for in *vivo* studies, but only on isolated hearts or cardiomyocytes. By using optogenetic sensors *in vivo* studies of cardiac parameters are possible (91).

Microbial opsins get expressed in mammalian cells and modulate membrane voltage by either depolarizing or hyperpolarizing. Channelrhodopsin2 (ChR2), an excitatory opsin, produces depolarizing transmembrane current in answer to illumination with blue light. In contrast, Archaerhodopsin (Arch), an inhibitory opsin, can repress activity. Thereby, molecules are either flowing passively with the electrochemical gradient with ChR2-cations or are actively pumped against it with Arch-H^+, affecting the transmembrane potential in very short time (88).

ChR2 photocurrent can trigger action potentials in ChR2-expressing mammalian cardiomyocytes and therefore several studies have pursued to show that optogenetics-based stimulation is a possible replacement for cardiac tissue excitation (92).

Early publication in cardiac optogenetics appeared in 2010 by Arrenberg et al. (93), who combined ChR2, an excitatory opsin, and Halorhodopsin (HR), an inhibitory opsin, in zebrafish to locate and control cardiac pacemaker cells. Furthermore, through photostimulation-methods optically controlling of the heart rate, reversing of cardiac conduction and inducing disease-like states was possible (93).

Another study by Bruegmann et al. (94) in 2010 demonstrated the first application of optogenetics in the heart of a mammalian. They showed that optogenetics could be applied to control cardiac rhythm, generate ectopic sources of heart activity or cause heart slowing or block by interference with the cardiac conduction system.
This study demonstrated the significance of optogenetics in the heart, showing that light can modulate the heart rate (87,94).

One study by Abilez et al. (95) in 2011 showed that ChR2 can be expressed in human embryonic stem cells, which then can be differentiated into CM. By photostimulation, ChR2 opens and sodium ions can penetrate the cell which triggers the action potential. Like this the cardiac system can get controlled by photostimulation (95).

Heart function is not only regulated by intrinsic factors, but is also dependent on the extrinsic parameters, such as neurogenic mechanisms. Control of the heart rate and

contractility gets accomplished by innervation of the myocardium through autonomic neurons. Central nuclei and the brainstem are the origin of the cardiac efferent neuronal network, which then joins the heart walls where postganglionic neurons get in contact with their effector cells. By activating central nuclei or selected neuronal circuits of the cardiac autonomic network via light optogenetic stimulation can get accomplished (86).

A study by Tsubota et al. (96) in 2012 used optogenetic stimulation to demonstrate the role of cerebellar Purkinje cells of the uvula in blood pressure regulation during postural alterations (96).

The most aspired therapeutic potential of optogenetics in cardiology is the treatment of arrhythmias to improve heart activation sequence and prevent sudden cardiac death. Now the therapy of arrhythmias consists on the use of electronic devices such as pacemakers or ICDs. However, those devices have some cutbacks like tissue damage because of electrical currents or physical contact with the pacemaker leads. In contrast, the use of light is contact-less and almost damage free.
Moreover, cardiac optogenetics are sought to be used as optical heart defibrillation. Studies concentrate on how opsin channel kinetics, delivery mode and cardiac distribution can be altered to achieve optical defibrillation (86).

Another study in mice showed that optogenetics in the heart can efficiently interrupt arrhythmias and that defibrillation energies can get reduced by applying re-entry based interventions. They demonstrated that by light-induced depolarizations, Ventricular Tachycardias can be interrupted by reducing the irradiated area as well as the total irradiation energy (97).

Furthermore, non-specific electrical stimulation of relatively large regions of the myocardium can get achieved by using the cell type selectivity of optogenetics. Thus, heart activation at particular levels of the conduction system, like the His-bundle or the Purkinje fibers, could get accomplished. Stimulating the His-bundle can lead to ventricular resynchronization, meaning the correction of the differences in timing of activation of right and left ventricles. To achieve an efficient and

controlled way of ventricular pacing, using optical stimulation of the Purkinje fibers is an approach (86).

The major challenge of optogenetics is introducing light sensitivity in cardiac tissue. Highly efficient transgene delivery vehicles are needed for transduction. Currently, three different main gene delivery ways are existing: direct plasmid transfection, virally mediated or cell mediated transduction. The most common method is lentivirus or adeno-associated virus delivery (87).
As already mentioned, the advantages of AAVs contain long-term expression, tissue tropism from 13 serotypes, the ability to transduce dividing and non-dividing cells (98).

One concern regarding to viral-mediated delivery are the pre-existing neutralizing antibodies to the ChR2-carrying virus. Furthermore, arrhythmias could occur due to the non-uniform expression of ChR2. Finally, another limitation of this delivery method is the ectopic expression of the transgene in other cell types which are infected by the virus. Currently, cardiac optogenetics has not yet found its way into the clinic due to already mentioned limitations. Nevertheless, the field holds promising prospects as it is moving forward at fast pace (86).

4. DISCUSSION

The present work was designed to investigate the future genetic therapeutic perspectives in sudden cardiac death. Current knowledge and future relevance concerning this topic are the main questions discussed.
What concrete conclusions can we draw for the clinical practice?

To begin with, we need to understand that our knowledge about future gene therapies in SCD is currently still quite insufficient. Most of the literature concerning this subject is based on animal research with just few studies on humans. Nevertheless, intensive research in this field is conducted and high-quality, up-to-date publications are released frequent, showing promising future prospects.

4.1 Summary

As showed above, current therapy options concerning sudden cardiac death, such as organ transplantation, are inadequate due to undesired side effects e.g. acute/chronic rejection (28).

One important option for treating patients with end-stage heart failure could be bioengineering human myocardium on native extracellular matrix as an alternative to donor organs (30).
Extracellular human matrix (ECM) can get generated by several different techniques (32).
First, ECM gets decellularized and used either as solid scaffold which maintains native matrix structure or as soluble material that gets broken down the ECM structure and is solubilized into liquid form (41).
Later on, the scaffold gets recellularized by billions of multiple types of cardiomyocytes which are produced by either deriving them from induced pluripotent stem cells, by direct reprogramming of adult non-cardiomyocytes into induced cardiomyocytes or by reprogramming somatic cells into cardiomyocytes via temporary pluripotency and consecutive differentiation (31,34).

At the moment, two clinical trials are investigating decellularized ECM materials to treat human cardiovascular diseases and results are expected soon (41).

The second promising therapy to cure genetic diseases that can lead to sudden cardiac death is genome editing. With the introduction of various gene editing tools, disease causing genetic variants could get corrected in patient-derived cells (48).
The most promising tool is CRISPR/Cas9, a system established from various Cas-proteins and a DNA-array that consists of repetitive DNA-sequences. After invasion of mobile genetic elements short sequences of the foreign DNA get integrated in the locus of the CRISPR-array. Then the CRISPR-array gets transcribed to a pre-crRNA and, in a complex with other RNAs, litigated to the mature crRNA. The complete ribonucleoprotein-complex detects immersive foreign-DNA and induces double-strand breaks (59).

For the first time ever, in 2012, the group of Emmanuelle Charpentier and Jennifer Doudna (99) demonstrated that by using a single-guide RNA in combination with Cas9 DNA can be specifically cut. With this technique, genes can get deactivated, changed or newly integrated in a cell. Studies in mice showed that monogenic diseases such as Duchenne Muscular Dystrophy or Hemophilia B could get improved by using CRISPR/Cas9 to edit the affected gene. Another research approach is using genome editing to combat human virus infections such as HIV (100).

To deliver genome editing tools into the target cells either viral-or non-viral vector systems can be used (59).
The most frequently used tool is the Adeno-associated virus vector due to its unique biology, simple structure and no-known disease associations (48).

Another therapy approach is the regulation of the cardiac cell cycle to improve cardiac regeneration by inducing adult cardiomyocyte proliferation (82).
The expression of pro-mitotic factors like cyclins and cyclin-dependent kinases is downregulated after birth, leading to a decrease of the cardiomyocyte cell-cycle activity (81).

Studies from Mohamed et al. (82) in mice have shown that cardiomyocyte proliferation and following cell survival can get achieved by a combination of four cell-cycle regulators, namely CDK1:CCNB and CDK4:CCND, in vitro and in vivo.

The last demonstrated method is cardiac optogenetics, a science that uses the effects of light on the protein product of genes. Light and genetic manipulation are combined to disturb, control or analyze the function of cells (89).
The main field of research in optogenetics is neuroscience, but since 2010 some studies on cardiac optogenetics have been published. These animal studies show that by using optogenetics in the heart, cardiac rhythm can get controlled, ectopic sources of heart activity can get generated or heart slowing can be caused (93).
A promising potential of this therapy is the treatment of arrhythmias to prevent sudden cardiac death (86).

4.2 Ethics

Medicine as we know it is standing before a quantum leap. With introduction of new techniques, especially CRISPR/Cas9 gene editing, new ways in science are paved and we are facing the discovery of the 21st century which will very likely revolutionize medicine.
However, on the one side, hope is raising to cure genetic diseases by editing genes in either the germ-or somatic-cell-line. On the other hand, the intervention in the genetic makeup is coming along with a lot of ethical hurdles.

Germline vs somatic human genome editing:
Somatic gene editing targets genes in specific types of cells and therefore only affects this type of cell and no other. Given that the edited gene is only in the somatic cells, it is not passed on to future generations. Only the patient treated gets affected by the changes due to the gene editing of only some of his/her cells.
In contrast, germline modification changes the genome of a human embryo at its earliest stage. Therefore, every cell contains the edited gene, including sperm or

egg-cells, having an impact not only on the treated patient, but also on the descendants (101).

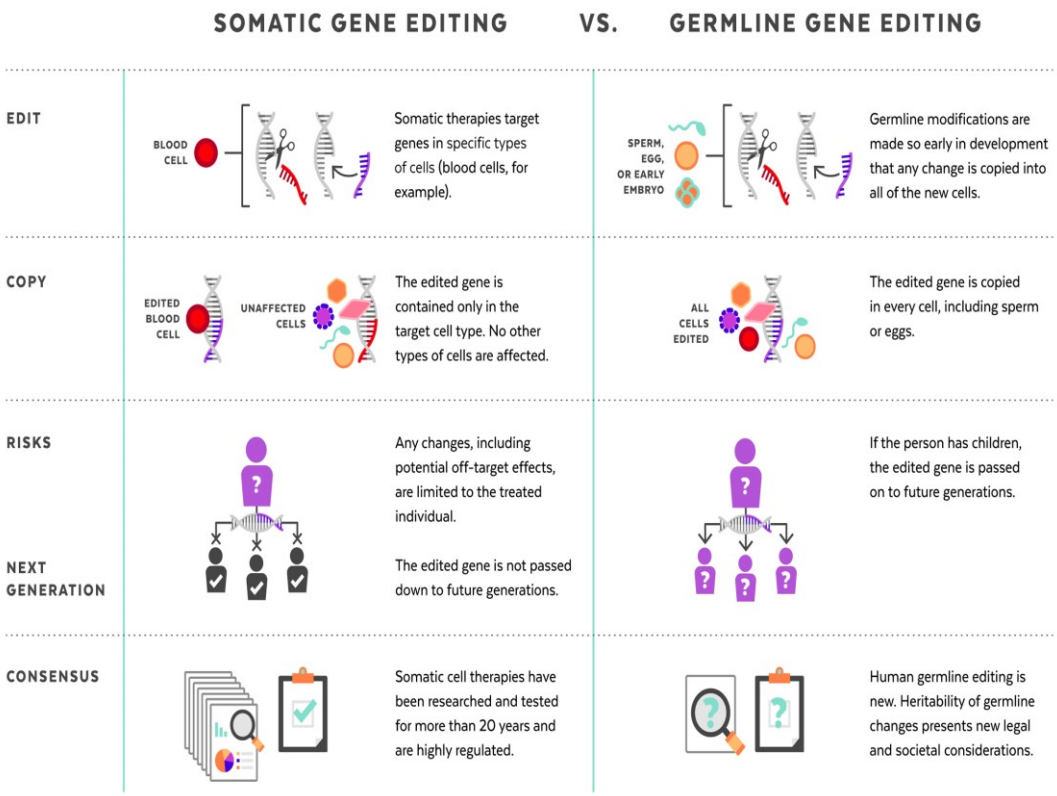

Fig. 5: Somatic gene editing versus germline gene editing by Judy Blomquist [Picture re-used without changes with permission from Harvard Gazette] (101).

By editing the genome of germ cells, disease-causing mutations in all cells of the patient could get corrected. One potential indication for editing the germline would be to alter the genome of an early embryo if the variant predisposed to a clinical recognized disease. A major concern of germline editing is the safety issue regarding possible off-target-effects and mosaicism. At this point, no one can assure that off-target effects created through an editing procedure will not result in a fatal long-term outcome like cancer or in adverse consequences on the development. Despite major advances in the understanding of the human genome, large parts still remain unclear (102).

Somatic gene editing has been used e.g. in trials for the treatment of leukemia with TALEN-mediated editing to cut the T-cell-receptor gene to prevent alloreactivity as

well as in trials for HIV-1 by using ZFN-mediated disruption of a co-receptor of HIV. Again, potential adverse effects cannot be predicted, but changes are limited to the treated individual (103).
Beyond issues of safety and efficacy, somatic gene and cell therapies are mostly seen as acceptable concerning the moral (101).

However, the increasing progress in decivering more efficient ways of genome editing also raises concerns of misusing this technique to enhance or modify the DNA in general, opening up the field of gene doping. The term “enhancement” stands for an improvement of an existing condition, ranging from cosmetic changes such as the eye color to enhancing musculature for individuals to improve efficiency (104).

In the late 19th century the word “eugenics” first appeared, meaning the goal of improving the human species by encouraging the “more suitable races” to have more children than those with less “good” bloodlines. It refers to selection of positive qualities and the removal of diseases or qualities that are seen negatively.
During Nazi Germany, the term eugenics reached an extreme: People with so called genetic limitations were first sterilized and later killed. “Eugenic purity” led to the assassination of millions of people who were distinguished as genetic inferiors.
With the introduction of genome editing in humans, people with genetic diseases will no longer see the light of day and only people with “superior” genes will be born. Parents can design their children by their own will and imagination, creating “the best children possible”. Some people therefor refer to genome editing as the new name for eugenics (105,106).

The natural wish of most parents is that their children are healthy, smart, beautiful, athletic, in general that they have a good life. By modifying their DNA, parents make a definitive decision for their children, taking away their child’s personal autonomy and narrative identity. The child could therefore hold its parents responsible for changing its life and complain that its life could have been different if modification would not have taken place. There are stories of children who do not agree with medical decisions made by their parents during childhood or who wish, if they would have had the choice, that their own medical condition would not have been changed.

On the other side, parents are making all sorts of decisions for their children. Let alone the decision to create a new human is a process children have no bearing on (104).

Some people refer to germline genome editing as “playing God” by making changes which affect the future generation and selecting individuals which are “fitter”, thus marking individuals which have such “unfit” traits or conditions as not valuable and not live worthy (107).
Others say genome editing is less offensive than e.g. prenatal testing because genes get modified instead of selecting against individuals (108).
However, using preimplantation genetic diagnosis (PGD) is sufficient to avoid most disorders, which might be potential candidate applications for gene editing. In this technique, embryos created via in vitro fertilization are screened for specific mutations, avoiding the side-effects of gene editing (109).

Furthermore, if human genome editing in the germline should ever become reality in the daily life, it is very likely to be expensive and limited geographically. Therefore, access to it will be unequal and large differences between populations could arise. Likewise, genetic diseases that now affect both, rich and poor, could become an artifact of class, geographic location and culture (104).

Another ethical concern about genome editing is the research on embryos in order to provide the foundation for human investigation. The embryos used are already existing as leftovers from fertility treatment and are donated to research. Every country has different laws concerning embryo research, but nowhere it is legal to establish a pregnancy with embryos which underwent genome editing (110).

In 2018, the Chinese scientist He Jiankui (111) pronounced the birth of twin girls whose genetic makeup got changed via CRISPR/Cas9 germline editing to disable the gene *CCR5*, which encodes a protein that allows HIV to enter cells. With this, He could have caused undesired mutations/mosaicism somewhere else in the genome, leading to unpredictable consequences for the health of the children. Even though He has been criticized and fired from his university for creating the first

CRISPR-babies, some scientists predict that this event might propel human gene editing forwards (111).

Taking all this in mind, we need to ask ourselves in what kind of society we want to live in. It is beyond question that genome editing is a ground-breaking discovery of the 21st century. Nevertheless, it leaves us with many challenges. Where will we draw the line between changing genes associated with diseases and those controlling our beauty or intelligence?
Gene editing holds the curing of till-now untreatable diseases ready, but before it becomes reality in the everyday clinical life, all the surrounding hurdles must be overcome.

4.3 Prospects and Drawbacks

Being a carrier of an inherited genetic disease can be a significant burden to those who are affected in a physical, psychological, social as well as financial way.
Currently, treatments for many genetic disorders are limited to symptomatic therapy, palliative care and in case of end-stage hear failure organ transplantation. Therefore, great hope lies in future genetic therapies to cure those diseases (102).

As mentioned before, creating bio-artificial hearts could solve the problem of organ shortage patients with heart failure are facing. Indeed, this therapy is facing some challenges as well. Firstly, the number of normal scaffolds available is limited due to the use of healthy donor hearts for transplantation and scaffolds derived from damaged hearts being compromised by scarring or remodeling. One possibility would be the use of xenogeneic scaffolds such as porcine-derived scaffolds (112).

Furthermore, bioengineered human myocardium could lead to arrhythmias from dECM injection, problems with sterilization, requirement of surgical implantation of patches and immune reactions (41).
Another hurdle is creating induced cardiomyocytes for recellularization of the dECM, because difficulties such as immaturity of CMs, heterogeneity of CM subtypes or the risk of teratoma formation could occur (33,40).

As many of the previous discussed studies have shown, gene editing tools, especially CRISPR/Cas9, will ultimately lead to improved infertility treatments and to the use of pluripotent stem cells in regenerative medicine. However, many obstacles have to be overcome before clinical application of those methods becomes reality (113).
Despite the great potential of CRISPR/Cas9 to become a reliable genome editing tool useable for curing diseases, some challenges like off-target mutations, mosaicism, finding an appropriate delivering method and eventual the ethical and moral concerns remain (64).
Regarding to the ethical concerns, restrictions to genome editing in human embryos need to be defined in all countries and regulations concerning the reproductive uses

of any gamete or embryo that has been subjected to scientific research or treatment to produce inheritable genetic changes need to be made (113).
Another major hurdle that comes along with genome editing is using an appropriate delivery method. Currently, the most common one is the AAV vector system, which comes along with the problem of immune recognition of the viral capsid by neutralizing antibodies (48).

Diverse mechanisms like cell cycle regulators, the Hippo pathway, oxygen exposure, energy metabolism, ECM and growth factors play a crucial role in cardiomyocyte proliferation. Uncovering those molecular mechanisms led to potential targets for cardiac regeneration and repair (114).

Even though Mohamed et al. (82) did not find cardiac tumors in the treated mice in his study, there is a potential for ectopic proliferation due to these cell-cycle regulators not only controlling the cardiac cell-cycle but many cell types within organs that have limited regenerative capacity, and there for caution in future clinical development is advised (82).

The last discussed future therapy is cardiac optogenetics. Along with promising prospects, this method also comes with some obstacles. One of the biggest challenges of optogenetics is finding a highly efficient delivery vehicle for transducing light sensitivity in cardiac tissue. Here as well, AAV vehicle systems are the method of choice, facing the challenge of immune recognition of the viral capsid by neutralizing antibodies (75).

All in all, the therapies discussed and listed in detail above lead to the conclusion that the future holds a great variety of methods for treating not only sudden cardiac death, but a various number of genetic diseases ready. However, ongoing studies on animals as well as human researches are necessary to further understand and gain knowledge about the complexity of these therapies and possible undesired effects.

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