Tailoring Cardiac Synthetic Transcriptional Modulation Towards Precision Medicine

Eric Schoger¹,²,³, Sara Lelek⁴,⁵, Daniela Panáková⁴,⁵* and Laura Cecilia Zelarayán¹,²,³*

¹ Institute of Pharmacology and Toxicology, University Medical Center Goettingen, Goettingen, Germany, ² DZHK (German Center for Cardiovascular Research), Partner Site Goettingen, Goettingen, Germany, ³ Cluster of Excellence “Multiscale Bioimaging: From Molecular Machines to Networks of Excitable Cells”, University of Goettingen, Goettingen, Germany, ⁴ Max Delbrück Center for Molecular Medicine in the Helmholtz Association, Berlin, Germany, ⁵ DZHK (German Center for Cardiovascular Research), Partner Site Berlin, Berlin, Germany

Molecular and genetic differences between individual cells within tissues underlie cellular heterogeneities defining organ physiology and function in homeostasis as well as in disease states. Transcriptional control of endogenous gene expression has been intensively studied for decades. Thanks to a fast-developing field of single cell genomics, we are facing an unprecedented leap in information available pertaining organ biology offering a comprehensive overview. The single-cell technologies that arose aided in resolving the precise cellular composition of many organ systems in the past years. Importantly, when applied to diseased tissues, the novel approaches have been immensely improving our understanding of the underlying pathophysiology of common human diseases. With this information, precise prediction of regulatory elements controlling gene expression upon perturbations in a given cell type or a specific context will be realistic. Simultaneously, the technological advances in CRISPR-mediated regulation of gene transcription as well as their application in the context of epigenome modulation, have opened up novel avenues for targeted therapy and personalized medicine. Here, we discuss the fast-paced advancements during the recent years and the applications thereof in the context of cardiac biology and common cardiac disease. The combination of single cell technologies and the deep knowledge of fundamental biology of the diseased heart together with the CRISPR-mediated modulation of gene regulatory networks will be instrumental in tailoring the right strategies for personalized and precision medicine in the near future. In this review, we provide a brief overview of how single cell transcriptomics has advanced our knowledge and paved the way for emerging CRISPR/Cas9-technologies in clinical applications in cardiac biomedicine.

Keywords: single cell sequencing, CRISPR/Cas9 system, endogenous gene activation, cardiomyocytes, gene regulation

INTRODUCTION

Cardiovascular disease, a leading global cause of death, is accounting for 17.3 million deaths per year; a number that is expected to grow to more than 23.6 million per year by 2030 (1). Common cardiovascular disease conditions leading to heart failure include myocardial ischemia or cardiomyopathies such as ventricular hypertrophy that can be inherited or stress-induced. These
conditions result among others in deficient oxygen supply, eventually affecting cardiomyocytes’ transcriptional and differentiation states that precede substantial cardiomyocyte loss due to the lack of regenerative capacity. Simultaneously, non-cardiomyocytes also undergo significant phenotypic and transcriptional changes leading to imbalanced intercellular communication. Comorbidities, including hypertension, dyslipidemia, and glucose imbalance, produce a hostile environment that modifies disease progression increasing heart failure risk (2). Collectively, these factors determine the extent of myocardial damage, which has a direct impact on patient specific disease progression while also depending on time of injury (3). Hence, a heterogeneous tissue remodeling characterizes heart failure progression. Current therapeutic guidelines for cardiovascular diseases involve reperfusion-based treatments and drug therapies mostly alleviating the symptoms generated by cardiac functional deterioration (1, 4–6). Therapeutic concepts targeting favorable reparative myocardial remodeling remain challenging. A big problem lies in the inefficient regenerative capacity of the heart. Based on the studies of various animal models as well as human data on cardiac regeneration, the attention has recently shifted toward the possibility of repairing diseased hearts by reawakening the intrinsic regenerative potential (7). The analysis of the integration of 14C generated by nuclear bomb tests during the Cold War allowed to estimate that fewer than 50% of cardiomyocytes are physiologically exchanged during the course of life in the human heart (8), indicating the intrinsic potential of cardiomyocytes renewal in the human myocardium. Indeed, in response to heart injury, the rate of cardiomyocyte cell cycling increases in the peri-infarct region; however, this is far too limited to effectively replace the lost cardiomyocytes (9). Thus, efforts have been made toward stimulating cardiomyocytes proliferation based on factors responsible for the transient neonatal heart regeneration in animal models. To promote endogenous cardiomyocyte proliferation, initial approaches targeted universal cell cycle regulators such as cyclins, cyclin-dependent kinases (CDKs), tumor suppressor genes, and cell-intrinsic signaling pathways that regulate cardiomyocyte proliferation during development (7, 10). These include mainly developmental transcription factors comprising the Hippo, Hedgehog (HH), Wnt pathway, HIF1α, SMADs, TBX20, p53, Jarid2, GATA4, MEIS1/2, Retinoblastoma, PITX2, E2F family members, KLF1, REST (11–25) as well as chromatin remodeling proteins (26), and microRNAs (miR-590, miR-199a, miR-548c, miR-509, miR-23b, miR-17-92 cluster, miR302-367, miR-143) (27–30). Recently, induced expression of the pluripotency factors OCT4, SOX2, KLF4, AND C-MYC (OSKM) was shown to trigger cardiomyocyte dedifferentiation; which implies both structural remodeling and dramatic metabolic alterations driven by different mechanisms (38). In order to tackle this problem, the fundamentals of complex regulatory networks that govern cardiomyocyte regeneration and repair embedded in their pathophysiological environment, need to be understood in the disease context (39, 40). This will help to tailor therapeutic strategies correcting specific cellular defects.

A powerful tool capable of deciphering individual cellular responses within tissues is single cell sequencing (SCS). Using the SC transcriptomic data, the spatiotemporal interplay of different cell types within tissues enables to delineate dynamics during disease progression (41, 42). Further bioinformatic assessment of ligand–receptor interactions allows us not only to measure the expression of ligands and receptors in multiple cell types, but also to systematically decode intercellular communication networks that function in homeostasis and are altered in disease states (43). Having a better understanding of how exactly transcriptomic changes in both, healthy and pathological conditions mediate phenotypic effects at the SC resolution, will allow the use of synthetic transcription for correcting disease conditions. This can be achieved by using programmable nucleases such as DNA targeting class II clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 systems (44). These rapidly advancing technologies have expanded the applications of genetic research across the world by mediating transcriptional control of endogenous genes to model and, moreover, to treat common and multifactorial diseases in one-in-a-lifetime approaches in the near future. This review aims to summarize the current status toward deciphering the cardiac disease transcriptome and to describe the novel approaches in which molecular tools including CRISPR/Cas9 can be used to modulate and revert disease conditions.

REAL-TIME SINGLE CELL TRANSCRIPTOME PROFILE OF THE HEART, THE BASIS OF PRECISE THERAPEUTIC TARGET IDENTIFICATION

The heart is composed of four morphologically as well as functionally distinct chambers, which requires an exact orchestration of all heterogeneous cell populations to guarantee its proper function (45). This is governed by a spatiotemporal pattern of gene regulation and cell-cell communication which, when altered upon disease condition, results in
significant phenotypic changes and imbalanced intercellular communication. This leads to organ tissue remodeling triggering a vicious circle driving disease progression. Knowing crucial factors, which perturbation results in alteration of gene regulatory networks, maladapted cellular behaviors, and ultimately to a disease condition, will help to develop more efficient and tailored therapies. Despite decades of research, the medical interventions for treating cardiac abnormalities have not dramatically changed from the classical symptomatic treatment, remaining the clinical need unmet worldwide.

One of the critical advancements to understand disease states in the past years was the adaptation of several SCS platforms for profiling all heart cells’ transcriptome. Even though application of SCS for many tissues was rapidly and successfully adapted, the use of this method in the heart remained quite challenging for some time. While isolation and sequencing of several non-cardiomyocyte cell populations, including immune and endothelial cells, were relatively straightforward, singularization and dispensing of large, elongated cardiomyocytes for sequencing were challenging. To circumvent this issue, single nuclei sequencing (SNS) has been adapted for cardiomyocytes as well as recent advances have been made toward whole cell sequencing (46–48). These technological innovations have allowed accurate use of SCS for all heart cells embedded in a tissue including cardiomyocytes. The different platforms used for SCS of the mammalian heart including human are well-described elsewhere (46, 47), and are not the focus of this review; only few examples of their application for heart tissue and their potential therapeutical implications will be discussed. Several studies provided comprehensive transcriptomic data that can be used to extract valuable information of diseased vs. healthy heart. Litvinuková et al. (41) provided comprehensive transcriptomic data on six distinct cardiac regions of the healthy adult heart deploying SNS of cardiomyocytes and SCS of enriched fibroblast, stromal, vascular and immune cell populations. An important observation in this study was the identification of cardiomyocyte population heterogeneity among the atrial and ventricular compartments (41). This study highlighted not only chamber-specific and lineage-specific profiles, but also sex differences of the healthy heart.

Other heart-disease oriented studies have focused on the cellular composition upon ischemic injury in the murine heart (42, 49, 50). SCS of the interstitial cell population showed comprehensive dynamics of cardiac stromal, vascular and immune cells of healthy and ischemic hearts. A novel activated fibroblast population characterized by an anti-WNT signaling transcriptome signature was identified (49). This particular observation is advancing our understanding of the role of transcriptional activation of WNT effectors and inhibitors, previously observed in the whole heart tissue upon stress (51). WNT signaling plays a complex role in cardiac biology and disease, affecting different cell types including cardiomyocytes, fibroblasts, and endothelial cells (51). Many drugs inhibiting WNT signaling are currently under investigation for their potential impact in heart repair (49, 51, 52). It is therefore pivotal to identify the cell-specific transcriptional profiles in order to target the correct cell population. A transient phenotypic change has been identified upon ischemic injury in a murine model. In this model, endothelial cells undergo a transient mesenchymal activation within the first days after myocardial damage, but do not acquire a long-term mesenchymal fate (42). The authors concluded that the transient mesenchymal fate of endothelial cells may facilitate cell migration and clonal expansion to promote regeneration of vascular networks (42). These data indicate the intrinsic regenerative potential of the heart, which is rendered inefficient in long-term remodeling and can be used as regenerative therapeutic targets. Using a different protocol, SCS was also performed using infarct and border zone regions and was compared to control hearts (50). Similar to other studies, they could detect cell type-specific upregulation of various genes between healthy and diseased subpopulations of various cell types. In this study, Ckap4 was reported as a novel marker specifically upregulated in activated fibroblasts. The authors further identified a subset of epicardially located cardiomyocytes expressing MyoD2, a protein that tethers α-actinin to the hypertrophy inducer calcineurin, thereby inhibiting hypertrophic response (53). This indicates that subpopulations of cardiomyocytes respond differently to known hypertrophic factors, and would limit therapies that assume homogenous hypertrophic response among cardiomyocytes populations.

Yekelchyk et al. specifically investigated the transcriptional profile of mono and multi-nucleated adult cardiomyocytes under baseline conditions and in pressure-induced cardiac hypertrophy in the murine heart (48). Using an image-based quality control system and strict exclusion criteria, they concentrated on rod-shaped adult cardiomyocytes. This differs from other studies using the same system (3, 41). A noteworthy observation of this study is the elucidation of cardiomyocytes clusters correlating with the expression of basic helix-loop-helix transcription factor HIF1α, a master regulator of hypoxic stress response which was the main driver of heterogeneity in this pathological condition. This is in line with CreERT2-based lineage-tracing studies revealing a population of hypoxic cycling cardiomyocytes resembling neonatal proliferative cardiomyocytes that contribute to the slow cardiomyocyte turnover occurring in the adult mammalian heart (54). Interestingly, overexpression of a downstream target of HIF1α, the Zinc finger E-box-binding homebox 2 (ZEB2), improves cardiomyocyte survival and cardiac function as well as angiogenesis following cardiac damage (55). Activation of HIF1α expression is well described in pathological conditions (56) now the prospective analyses of its activation in distinct cell types will offer a new perspective to interfere with pathological phenotypes in a cell-dependent manner, which may be applicable to other transcription factors. Indeed, HIF1α regulates cardiac fibroblasts activation upon ischemic injury by limiting their proliferative capacity (57), highlighting even more the necessity for cell-targeted therapies.

Furthermore, Wang et al. (3) have analyzed heart cells and their interconnection on normal healthy and patients with heart failure as well as those with functional recovery after treatment with a left ventricular assist device (LVAD) at single-cell resolution. Applying bioinformatic tools, the authors studied transcription-factor-centered regulatory networks and evaluated...
regulon activities in cardiomyocytes. They demonstrated regulation of transcription factor-depending regulons such as JUN, CEBPD and TCF7L2, which were previously described in disease conditions (58–60). However, the data exhibited distinct profiles of regulation among the different conditions, strongly indicating the presence of specific target disease-induced pathways and networks, warranting more disease-specific treatment of cardiovascular diseases. Wang et al. also provided evidence supporting a model, in which non-cardiomyocytes undergo substantial changes during the loss of normal heart function that may direct disease progression and prognosis. The comparison of cell types of diseased and healthy hearts led them to identify non-cardiomyocyte cell types necessary for maintaining myocardial homeostasis and for protecting the heart tissue from failing. As an example, based on the data they obtained, they performed transplantation of ACKR1+ endothelial cells into the ischemic heart, which significantly enhanced cardiac function (3). More importantly, they showed that transcriptome profiles of all cell populations from the patients with improved heart function shifted considerably toward normal physiological state. Thus, this observation implies the plasticity and substantial recovery potential of cardiac cells in the adult human heart, even in end-stage heart failure (3). This data holds promise for the development of strategies that can reverse disease cell-states by exploiting endogenous recovery programs of the heart. Using SCS analysis in cardiac biopsy samples from patients with heart failure before treatment, the presence of failing cardiomyocytes characterized by the activation of DNA damage response genes only in patients showing poor prognosis was validated (61, 62). Hence, these methods present a realistic approach to determine clinical prognosis and treatment response.

Another study analyzed human left ventricular samples including control non-failing, hypertrophic and end-stage cardiomyopathy as well as heart failure samples along with mouse hearts at different stages after experimentally-induced pressure overload to investigate the pathological progression of cardiac hypertrophy (63). Specifically, their findings suggested a pivotal role of macrophage subtype switching toward an inflammatory state upon reduction of cardiac function during pathological cardiac hypertrophy. Therefore, they tested the effectiveness of the anti-inflammatory treatment on the stage-specific macrophages between 2 and 5 weeks after induced pressure overload. This resulted in ameliorated cardiac hypertrophy. However, an earlier anti-inflammatory treatment before 2 weeks failed to avoid decline in cardiac function (63). This suggests that stage-specific targeting of macrophages may serve to suppress pathological cardiac hypertrophy and influence the course of disease progression. This study also revealed conserved cellular and molecular basis of cardiac hypertrophy between mouse and human, providing an excellent platform to investigate mechanisms that can be translated toward improved therapies. The extraction of (sub-)cell types and their respective transcriptome profiles allows for analyses of differential gene and gene cluster expression as well as gene regulatory networks in health and disease states. This will help to exactly specify their role for targeted functional phenotyping (64). For instance, ischemic and non-ischemic human heart samples subjected to SNS yielded a catalog of cardiomyocyte and non-cardiomyocyte (vascular endothelial cells, endocardial endothelial cells, fibroblasts, mesothelial cell, smooth muscle cells, adipocytes, immune cells, and neurons) and their individual, disease-specific gene expression profile (65). The authors identified gene regulatory networks and disease driver candidates by intersection of the SNS data sets with the disease risk GWAS data (65). This data extended descriptive cellular characterization of the human heart (3, 41) toward understanding the precise underlying disease progression mechanisms.

Altogether, these recent discoveries serve as a blueprint for how knowledge about gene expression dynamics, collected from single cell responses under physiological and pathological conditions, provides unprecedented information. Invaluable insight that supports context-specific therapeutic approaches based on transcriptional modulation is the observation that altered SC transcriptomic profiles in the human diseased hearts seem to reverse toward normal state with improved organ function (3). This indicates that transcriptional profile and function are coupled. Furthermore, as abovementioned, the information collected from current studies, such as HIF1α and ZEB2 expression promoting cardiomyocytes proliferation endogenously, can be used to transiently boost clusters of cardiomyocytes toward a more regenerative state in ischemic conditions. Based on the data that ACKR1+ endothelial cell transplantation preserves cardiac function upon ischemia, an approach using suitable adeno-associated virus (AAV) serotype or other non-viral vector delivery approach can be designed for enhancing Akr1 expression in this cell population. This is particularly motivating toward developing more specific strategies that can restore homeostatic cell states for a wide number of human cardiovascular pathologies ranging from adaptive cardiac remodeling to heart failure. The next logical step is to actively interfere with the identified aberrant gene expression and to rewire gene programs for the prevention of heart failure progression. In this context, synthetic control of transcription to restore endogenous homeostatic transcriptional programs of the heart specifically in the desired cell-types offers a suitable platform. In terms of budget, current advances of protocols as well as standardization of bioinformatics pipelines warrants the SCS approach as a realistic option for clinical applications in the near future. Importantly, conventional single-cell RNA-seq analysis may not be sufficient for obtaining the information necessary for a deeper understanding of molecular behavior and therefore combined bulk sequencing analysis will be mandatory for a more precise analysis (66).

ADAPTING SYNTHETIC CONTROL OF TRANSCRIPTION TO THE HEART

Regulation of gene expression relies on transcription factors availability and their activity, as well as on chromatin state and nucleosome positioning that determines RNA polymerases recruitment to a specific gene locus (67). This complex
process has turned transcriptional control “undruggable” for many years (67). Recent approaches tackling this issue deploy epigenetic modifiers and synthetic transcription factors driven by DNA binding element systems such as engineered zinc finger, transcriptional activator-like elements (TALEs) or aim at repurposing programmable CRISPR/Cas9 systems (68–70). Beyond genome editing activity of the CRISPR system, epigenomic modifications can now be achieved at a specific genomic locus by using mutated catalytically inactive dead (d) Cas9 protein fused to effector domains (68). This works by carefully choosing where guide (g) RNA molecules bind relative to transcriptional start sites (TSS) in the genome. This allows for recruitment of dCas9 with activator or inhibitor domains to a specific locus of interest. Consequently, chromatin landscape modification or further recruitment of factors that lead to tailored transcriptional modulation is possible. This is extensively reviewed elsewhere (67, 71, 72). Hence, the limitation of strict control over endogenous gene expression in vivo that has long been a tedious work for researchers is now alleviated by the use of the RNA-guided programmable endonuclease systems associated with transcriptional modifiers (67). This offers the ability to precisely modify endogenous gene expression to program cell and tissue behavior (67). Thus, all the efforts that have been conducted for decades in order to understand how exactly transcriptomic changes cause disease conditions can now be exploited to develop therapeutic concepts by applying rapidly evolving CRISPR/Cas9 technologies.

CRISPR/Cas9 technology is rapidly advancing in the medical world with the development of therapies for blood disorders, Duchenne muscular dystrophy, cystic fibrosis, and cancer (73–75). Yet, the CRISPR-mediated transient transcriptional activation or repression of genes, desirable when considering changing the course of complex diseases such as metabolic diseases or tissue regeneration (76), is still in its infancy. Endogenous regulatory mechanisms of genome function as well as issues concerning CRISPR-mediated transcriptional engineering need to be addressed before this technology reaches use in the clinic, and is discussed elsewhere (67). Initial generation of dCas9-based transcriptional modulation platforms consisted of transcriptional activators derived from herpes simplex virus, VP16. Second-generation systems resulted from combination of bi/or tripartite activators such as VP64, VPR, SAM, the peptide scaffold-based activator SunTag-VP64 and RNA containing aptamers with increased activation efficiency (67, 72). The same is true for synthetic repression, where the initial KRAB repressor domain has been improved by several bipartite repressors consisting of KRAB and a secondary repressor domain (ZIM3, KOX1, MeCP2, DNMT3A, DNMT3L) (72, 77, 78). All these systems allow to fine-tune the intensity of gene modulation according to the biological needs and represent promising tools to modulate the cellular epigenome. Importantly, titration of gene activity is possible with the development of advanced gene activator platforms by expanding homomeric (79) or by using heteromeric transactivation domains (80). Additionally, the selection of gRNA target sites upstream of the TSS was sufficient to modulate drug resistance phenotypes according to expression levels indicating fine-tuning of gene activity to biologically relevant levels (79). Furthermore, tiling of gRNAs in the TSS upstream region was consistently reported as an option to adjust gene activation strength (81–83). Altogether, Cas9-transcription factor characteristics and careful gRNA selection are therefore suitable for unprecedented control of endogenous gene activity, an advantage over classical cDNA delivery via AAV which will be further discussed below.

CRISPR-MEDIATED CONTROL OF TRANSCRIPTION IN PRECLINICAL MODELS

CRISPR-based synthetic transcriptional control may lay the basis for personalized and precision medicine. Efficient transcription regulation mediated by CRISPR-mediated gene activation (CRISPRa) systems was demonstrated in vivo in the brain, liver, kidney and skeletal muscle as well as mouse model of human diseases including muscle dystrophy, diabetes, kidney and brain diseases using different delivery methods (84–87). Preclinical models using dCas9-targeted transcription factor regulation have shown great promise for treating disorders such as Duchenne’s muscular dystrophy, type 1 diabetes, acute kidney disease and retinitis pigmentosa (84, 85, 88). Liao at al. developed a mouse model, in which transcriptional activators were separated from constitutively expressed Cas9 (active or inactive, Cas9a and Cas9i, respectively) (85). This consisted of a combination including gRNAs engineered to contain two MS2 domains for recruiting the MS2-P65:HSF1 (MPH) transcriptional activation complex to the target locus, which was introduced with an AAV serotype 9. MS2 binds a specific stem-loop structure allowing assembly of RNA-protein complexes and it is used as tagging technique (89). Using this system, they showed amelioration of acute kidney injury by induced expression of the protective protein Klotho or the anti-inflammatory IL-10. Next, they triggered trans-differentiation of liver cells into insulin-producing cells by induction of pancreatic and duodenal homeobox gene 1 (Pdx1) in liver cells; which improved hyperglycemia in streptozotocin (STZ)-induced diabetes model via tail vein injection of AAV-gRNAs. Moreover, they showed that transcriptional induction of utrophin, a protein product which is very similar to dystrophin, improved muscle strength in a mouse model of Duchenne muscular dystrophy (DMD) by local application of the AAV-gRNAs (85). Additionally, a kidney-specific epigenetic modifier with dCas9TET3 fusion proteins to induce gene activity was shown to be efficient by Xu et al. (90). A double transgenic mouse was generated in a Sim1 heterozygous background, which normally develops obesity. The second transgene consists of a dCas9 fused to a transcriptional activator VP64 as well as a sgRNA targeting the Sim1 promoter or enhancer (88). By using this system to activate Sim1 expression from the healthy intact allele, the obesity phenotype was rescued upon targeting of both, Sim1 promoter or enhancer. The same rescue was observed upon direct delivery of three different AAV particles carrying the dCas9VP16, the gRNA-promoter Sim1 and the gRNA-enhancer Sim1 into the hypothalamus. These examples elegantly demonstrate that
CRISPR-based transcriptional modulation can also be applied for epigenetically-mediated correction of a genetic disease without the need of modifying the mutated gene-coding DNA sequence. Meng et al. engineered bone marrow derived mesenchymal stem cell (MSC) to overexpress IL-10 using CRISPRa based on dCas9-VP64-MS2 system (91).

IL-10 improved myocardial infarction; which is hampered in patients with diabetes due to MSC dysfunction. Engineered MSCs overexpressing IL-10 were transplanted in a diabetic mouse model with myocardial infarction, which substantially suppressed inflammation, improved cardiac functional recovery, alleviated cardiac injury, decreased apoptosis of cardiac cells, and increased angiogenesis (91). Other studies showed that CRISPRa approaches can decrease seizures and rescue cognitive deficits in a rodent model of epilepsy as well as demonstrated the utility of CRISPRa system for in vivo screening, e.g., in liver or brain (86, 87). Common efforts from E. Olson's and L. Zelarayán's Labs resulted in the establishment of a mouse model for cardiomyocyte-specific, CRISPR-mediated transcriptional modulation. The system is based on the constitutive expression of dCas9VPR combined with systemic administration of gRNA driving dCas9 to specific loci via AAV serotype 9, which showed robust, safe and specific single or multiplex activation of targeted genes (92). This model represents a rapid and powerful technical platform for gene activation in postnatal cardiomyocytes in preclinical proof-of-concepts. All of these studies demonstrate the feasibility of CRISPR-based methods for transcriptome modulation and set the stage for future optimization in both, basic and clinical research.

CRISPR-MEDIATED CONTROL OF TRANSCRIPTION TO ENHANCE CARDIAC REGENERATION

Although modulating individual factors in adult cardiomyocytes did enable some proliferative activity (93, 94), overexpression of a combination of cell cycle regulators increased the effect on cardiomyocyte proliferation and improved cardiac function after ischemic injury (95). Moreover, as aforementioned, effective cardiomyocytes' regeneration requires coordinated structural and metabolic alteration, which demands the targeting of multiple mechanisms. A highly attractive feature of the CRISPR-based technology is the possibility of simultaneous manipulation of multiple genes that can be exploited to efficiently induce cardiomyocytes renewal. The notion that a disease phenotype is triggered by dysregulation of several factors affecting one or more networks supports the use of multiple genes manipulation targeting dynamic gene networks, which perturbation results in a disease phenotype. In this context, CRISPR-associated RNA scaffolds were shown to provide a powerful way to construct not only multiple, but also orthogonal synthetic gene expression programs (96, 97). Such system was applied to modulate a branched metabolic pathway in yeast, in which multiplexed transcription activation and repression is carried out using distinct single gRNAs modified with RNA aptamers, termed scaffold RNAs. These aptamers can recruit either binding protein fused to a Krüppel-associated box (KRAB) domain for transcription repression or the MS2 coat protein (MCP) fused to VP64 for transcription activation (97). Another strategy made use of different dCas9 orthologs in a dual inducible and repressible systems for precise and dynamic control of CRISPR-dCas9- and 12a-mediated epigenetic editing tested in HEK293T cells (98). These studies provide promising evidence of the ability to use CRISPR-mediated gene modulation for modeling complex gene networks and reversing a disease condition using orthogonal systems for parallel activation and repression in the same cell. The combination of single cell transcriptomics and the bioinformatic assessment of network activities will provide the information for tailored CRISPR-based synthetic control of transcription. This will allow steering gene expression profiles in order to detour a cell toward a physiological state and prevent organ deterioration in a multifactorial and disease-specific manner (Figure 1).

Besides targeting cardiomyocytes, which depending on the disease condition and extent of the damage may not be efficient, a promising approach to promote regeneration of heart tissue is to convert resident non-cardiomyocytes cells directly into de novo cardiomyocytes (10). Direct reprogramming in vivo has been reported in mice by using GATA4, MEF2C, and TBX5 (GMT) or GMT factors plus HAND2 (GHMT) reprogramming cocktails with retroviral delivery in order to infect proliferating cells such as activated cardiac fibroblasts after myocardial injury (99, 100). This approach generated new cardiomyocyte-like cells from activated cardiac fibroblasts. However, direct reprogramming showed relatively low reprogramming efficiency (10). Adding of ZNF281 to the reprogramming cocktail repressed genes associated with the inflammatory response as well as regulated cardiac gene expression by interacting with the transcription factor GATA4 (101). Additional factors improving cardiac reprogramming efficiency include the modification of endogenous signaling pathways such as RAC-α serine/threonine-protein kinase (AKT1), transforming growth factor-β (TGFβ), WNT, and Notch signaling (10, 102, 103). Furthermore, enhanced cardiac reprogramming was observed by suppressing the expression of the Polycomb complex protein BMI1 and the splicing factor polypyrimidine tract-binding protein 1 (PTB), while indicating the repressive role of these factors for cardiac reprogramming (104, 105). Trans-differentiation induced by activating endogenous gene expression with the use of the CRISPR-dCas9 system has been reported in different cell lines including neonatal mouse fibroblasts (10, 85, 106–108). However, it was shown that endogenous cardiac transcription factor activation is necessary for expression of maturation genes but not sufficient to induce efficient cardiac fibroblast transdifferentiation (108). It will be interesting to evaluate whether combinatorial activation and/or repression, allowing for more precise control of multiple pathways in orthogonal directions, could enhance cardiomyocytes reprogramming efficiencies by harnessing knowledge about the epigenetic landscape and modulating factors of cardiomyogenic cells (109).

A further application of CRISPR-dCas9 systems will include patient-specific induced pluripotent stem cells (iPSC) for derivation of specific cell types for transcriptomic approaches.
The iPSCs offer an attractive experimental platform, paving the way for the development of personalized medicine in cardiovascular diseases (110). CRISPR/dCas9 activation and interference systems were widely used for genome-scale screening (96, 111–113). Upon identification of transcriptional networks dysregulation in patient specific iPSC-derived cells, the amalgamation of CRISPR-mediated gene modulation with iPSC technology may allow reverting disease condition in a dish as a basis to translate the personalized approach to the patient without affecting the genomic DNA. This will include the delivery of a CRISPR-gene modulation molecular tool that will restore the altered transcriptome in specific cells for precise (patient and disease specific) therapeutic applications. With these improvements, personalized medicine could be a reality for many patients, minimizing side effects (Figure 2).

The concept of using single cell (SC) transcriptome for personalized medicine is already an important research focus for the investigation and treatment of multifactorial diseases. Recently, a pan-European initiative (“Life Time”: Revolutionizing Healthcare by Tracking and Understanding Human Cells during Disease) as well as national initiatives (e.g., Berlin Cell Hospital and Virchow 2.0, Germany) have been initiated. These and similar consortia aim at targeting human cells during the onset and progression of complex diseases as well as at analyzing their response to therapy at single-cell resolution (114). Integration of large molecular and clinical datasets will identify molecular mechanisms and create predictive computational models of disease progression allowing the implementation of gene or pathway-directed targeted therapy (114, 115). SCS technology has already contributed to the identification of novel disease biomarkers helping in the diagnosis and refinement of treatments. High-resolution SC transcriptomics will be vital in dissecting how these new treatments affect cell populations receiving the cell precision therapies (115). The CRISPR toolbox is an emerging opportunity to therapeutically modulate cellular states by the use of gene or base editing and synthetic transcription. CRISPR/Cas9-based gene editing approaches for prevention of cardiovascular disease have been demonstrated (71, 116). CRISPR base editors that are delivered in vivo using lipid nanoparticles were shown to efficiently and precisely modify disease-related genes in living cynomolgus monkeys (117). In this study, PCSK9, a well-established target in atherosclerosis, was mutated in vivo using CRISPR base editors leading to a variant that resulted in lower levels of low-density lipoprotein (LDL) cholesterol in the blood and reduced the risk of atherosclerotic cardiovascular disease (117). In the same manner, identification of specific targets by omics approaches will allow to combine with CRISPR-based modulation approaches in order to target a specific (genomic or epigenomic) perturbation in a disease and patient-specific manner. Logistically, primary patient tissue or iPSC cells, differentiated into a desired cell type, can be used for extracting omics information to be further applied in pathway analysis and network perturbation identification. This would lead to deciphering the set of factors that may need to be modulated for reverting a disease condition, which will be tested experimentally in vitro before ultimately reaching...
the patient. Technical challenges that need to be overcome include standardization of the methods, costs and user-friendly analysis tools.

ADVANTAGES AND CHALLENGES OF CRISPR-MEDIATED TRANSCRIPTIONAL CONTROL

While the CRISPR/Cas9 system has demonstrated a great promise for a variety of applications, there are several factors that influence its efficacy as well as its safety which must be addressed, especially when the goal is in vivo human gene therapy. Last but not least, there are ethical implications that need to be carefully considered, and are discussed extensively elsewhere (118–120). A more worrisome problem includes specific biosafety regulatory challenges and ethical issues concerning applications of CRISPR technology for irreversibly editing the human genome. Nevertheless, ethical concerns need to be addressed properly, which may not be unique when considering other interventions that influence human biology (119).

In respect to tool design, the necessary factors that require careful examination include target DNA site selection, sgRNA design, off-target effects and the method of delivery, the latter representing a major obstacle for use of CRISPR-based editors for in vivo applications (121). A powerful advantage of the CRISPR/Cas9 system is the ability to specifically target any 23-bp sequence that contains a PAM motif on either strand of DNA (121). However, single and multiple-base mismatches can be tolerated specially at greater distances from the PAM resulting in off-target effects (122–129). Importantly, the catalytically inactive Cas9 leaves the genome unaffected, significantly reducing the concerns over off-target effects (92, 130). Therefore, lower risk of side-effects are introduced by using a dCas9; however, this needs to be addressed on individual sgRNAs and in a context-specific manner. In order to reduce off-target events, rational design of the sgRNA has been the subject of a significant body of work resulting in many criteria and no simple rules (121). Comparing predictions from several sgRNA design tools with experimental results published in SpyCas9 off-target studies, showed evidence of algorithmic overfitting (124). They indicated the importance of using a model trained on data from the same gRNA expression system, which are currently few, especially for tissues when in vivo experiments are deployed.

From the clinical perspective, CRISPR-mediated control of gene expression offers several advantages compared to previous methods based on the expression of an open reading frame of the gene of interest, lacking physiologically relevant splice variants with exogenous, and thus uncontrolled expression. CRISPR-mediated regulation has overcome these obstacles and can now generate unprecedented levels of endogenous control while simultaneously offering a multiplexing possibility (131). The major challenge of CRISPR-based therapies is the delivery. Delivery systems for the CRISPR machinery can be classified into three general groups: physical delivery, viral vectors, and non-viral vectors. Viral delivery vectors include specifically engineered AAV, and full-sized adenovirus and lentivirus vehicles. These are the most common CRISPR/Cas9 delivery methods for in vivo approaches (121). AAV is considered a suitable vehicle for gene therapy since it is not known to cause any pathologies in humans, and there is a wide range of serotypes allowing for infection of a multitude of cells with different specificities. Moreover, the virus itself is able to efficiently transduce cells, while provoking little to no innate or adaptive immune response or associated toxicity, at least upon the first treatment with a certain serotype (121, 132). Thus, due to their well-proven safety profiles, AAVs are currently the best choice for nucleic acid-based therapy in clinical trials. AAVs, however, have the disadvantage of a small payload of ~4.7 kb, which can become a limitation considering all the necessary components of the CRISPR activation or repression systems (76, 133). Several approaches are under development to circumvent these hurdles, including the profiling of non-viral nanoparticles for gene delivery and decreasing the size.
of individual Cas9 components (134). Successful packaging of the SpyCas9 and sgRNA into two separate AAV particles and using them for co-transduction has been already reported (135). On one hand, this increases the overall size of the constructs that can be used. On the other hand, this naturally adds more complexities than those existing with a single vector (121). A further approach includes a split Cas9 system, in which the Cas9 C-terminal region is packaged into one AAV vector and the Cas9 N-terminus is packaged into a second AAV vector (136, 137). Reconstitution of the two Cas9 halves results in a functional Cas9 with editing efficiency comparable to the native Cas9, allowing for the use of larger overall Cas9 variants with AAV particles. This has also been proven to be effective in gene editing in pig and human models of Duchenne muscular dystrophy (116, 138). Moreover, the identification of a small Cas9 ortholog from Staphylococcus auricularis (SauriCas9) that can be packaged into an AAV for genome editing, has broadened the possibilities of efficient delivery and can be adapted for gene modulation, further expanding the CRISPR toolbox for epigenetic regulation (139). Non-viral delivery of Cas9 for genome editing have been demonstrated less efficient than viral methods, however, they could allow repeated dosing by using, e.g., lipid-based nanoparticles (140). Other delivery strategies have been applied in vivo, including direct mRNA delivery and ribonucleoprotein (RNP) delivery with lipid nanoparticles (LNP), especially for genome editing (140). Lipofectamine has been used to deliver base editors to the murine ear, however, entailing toxicity, which promoted the development of more biocompatible lipid formulations that can be used to deliver the Cas9 RNP in vivo. These formulations include gold nanowires, gold nanoclusters, black phosphorus nanosheets and nanoscale zeolitic imidazole frameworks (ZIFs). In vivo efficacies of these delivery systems are yet to be determined. Thus, there is a growing need for the next-generation more efficient vectors to be developed (141–144).

Despite these hurdles, CRISPR/Cas9-based therapies have begun their path into the clinic. CRISPR-based gene editing clinical trials for sickle cell disease and beta-thalassemia (CTX001) have paved the way for CRISPR-mediated therapies and further optimizations (145). This has been followed by AAV-based clinical trials and planned non-viral nanoparticle-based delivery of CRISPR to the liver (NTLA-2001) (145). Future studies are necessary to determine pre-existing immunity against candidate Cas9 proteins in humans. Also, the combination of cell- and tissue-specific regulatory components with broad tropism AAV vectors will help to fine-tune the localization of the effector components, while providing increased specificity and safety (76). Many classical targets considered “undruggable” came into play with expression interference strategies such as siRNA (146) and proteolysis-tags technologies (147). With CRISPR/Cas9 gene activity modulation, a powerful approach to precisely target candidate expression mechanisms at the transcriptional level emerges, further expanding our targeting scope. While enzyme activation with classical pharmacological approaches such as small molecules is limited to a small fraction of candidate targets (148), CRISPR gene modulation harbors the potential for gain-of-function mode of actions including transcription factors [i.e., Pdx1 (85) and c-myc (86)], formerly deemed difficult-to-drug (149). Furthermore, CRISPR gene modulation was shown to be efficient for congenital diseases based on haploinsufficiency and diseases caused by loss of a gene product in animal models. An endogenous gene product can be normalized from the healthy allele [as shown for Sim1 haploinsufficiency in obesity (88)] or replaced by a similar transcript [as shown for utrophin, replacing the lack of dystrophin for Duchenne muscular dystrophy (85)]. These therapies present challenges when using pharmacological applications.

Simultaneous activation and repression of multiple genes leading to network modulation rather than unidirectional regulation may be of more therapeutic relevance. With the intensified investigation of endogenous gene regulatory networks in the SC-specific context, the gene network engineering via CRISPR systems is highly attractive for the reestablishment of homoeostatic gene regulatory networks upon disease conditions. Such a high precision tuning of the defined sets of synergistic genes will result in the extraordinary control over cell behavior (131), allowing the induction of tailored reparative responses using the own cell machinery in the mature organ. While they can be exploited to enhance regenerative processes of cells, tissues, and organs, these advances need further technological development along with a better understanding of how exactly epigenomic and transcriptomic changes mediate phenotypic effects at the single cell resolution (67). This will allow for more precisely targeted approaches adjusted to the physiological needs.

### CONTROLLING Cas9 FUNCTION AND SIDE-EFFECTS

To restrict Cas9 activity, and thus reduce the off-target effects, attempts for temporally restricted (d)Cas9 expression were developed including chemical and light controlled gene activity modulation. Detailed reviews regarding inducible Cas9 systems discussed background (leaky) activity, editing effectiveness, and reversibility for gene editing approaches (150, 151). We therefore summarize inducible systems specifically adapted for endogenous gene activity modulation here. Temporal control was harnessed by decoupling Cas9 from transcriptional modulators with conditional chemical or light induced assembly of the synthetic transcription factor. Tested chemical and light inducible elements included: (1) Abscisc acid (ABI-PY11), Gibberellin (GIB1-GAI), and Rapamycin (FKBP-FRB) systems as well as (2) red-light (PHYB-PIF), and blue-light (CRY2PHR-CIBN) inducible systems (98, 152, 153).

Small molecule based Cas9-DNA interference was demonstrated and applicable for CRISPRa approaches reducing gene expression of up to 89% (154). While effectively limiting (d)Cas9 activity, these systems rely on constitutive expression and presence of (d)Cas9 and effector domain proteins harboring potential for unwanted cellular and organismal effects. To overcome this, Trimetoprim or Doxycycline responsive promoter elements driving dCas9 transcription were successfully tested for induced dCas9 expression with concomitant
(multiplexed) gene activation (155–157). Additionally, degron motif-based “suicide-tags” for protease inhibition-dependent Cas9 expression was presented as a promising option for Cas9 activity restriction (158). Taming Cas9 expression or activity using pharmacological approaches might therefore be a prospective route for temporally restricted or pulsed endogenous gene activation, possibly reducing expected immune responses upon constitutive Cas9 expression (159) and deserves a future validation in in vivo models. Bioengineering of Cas9 proteins as well as elaborate cell-type specific and temporally resolved Cas9 expression systems (151) are therefore essential ventures for safe and limited CRISPR/Cas9 based applications (71). Protein-based anti-CRISPRs, which are accessory proteins with fewer than 200 amino acids called “anti-CRISPRs,” can function as antagonists of CRISPR systems and achieve context-specific inhibition of Cas9. This will offer a solution for mitigating the problem of off-target cleavage as well as for limiting Cas9 activity on the genome (160). To what extent these approaches can be transferred to the clinic is still uncertain, nevertheless they warrant further studies.

CONCLUSION

Research efforts during the past decades have broadened our insights into the molecular determinants of cardiac disease. With the recent emergence of the SCS multi-omics profiling, more detailed and comprehensive understanding of the basic molecular profiles of disease-associated perturbations within each cell in the human heart could be achieved, fine-tuning our previous knowledge. Combining this information with the revolutionizing CRISPR technology will enormously advance medical research and open a new chapter of precision and personalized medicine. Within the last few years of research, CRISPR-mediated gene editing has already entered the clinic, making the application of further CRISPR approaches for synthetic transcription a realistic option for more specific treatments of different cardiac disease entities. Furthermore, integration of patient-specific data and human-based in vitro models, will help to identify more personalized therapies for rare diseases. Such an approach will necessarily include collection of biological information from a patient, integration of a suitable iPSC-model to test patient- and/or disease-specific synergistic network modulation and their safety, and ultimately fine-tuning of a precise therapeutic approach. All these tools will broaden our understanding of human- and disease-specific effects as well as provide us with information about safety of proposed modulations. With these advances, a reshaped personalized management of complex human diseases will become a realistic approach. Reduction of costs for development and production of such approaches is mandatory to allow disease-specific and individualized therapies in the future. Joined efforts of clinicians, basic researchers and industry partners is already now facilitating rapid advancements of the discussed technologies that will dramatically impact biomedical research and disease treatments in the future.

AUTHOR CONTRIBUTIONS

ES, SL, DP, and LZ conceptualized, wrote, and revised the manuscript and figures. All authors contributed to the article and approved the submitted version.

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