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Drug Discovery

Heart Regeneration: Opportunities and Challenges for Drug Discovery with Novel Chemical and Therapeutic Methods or Agents

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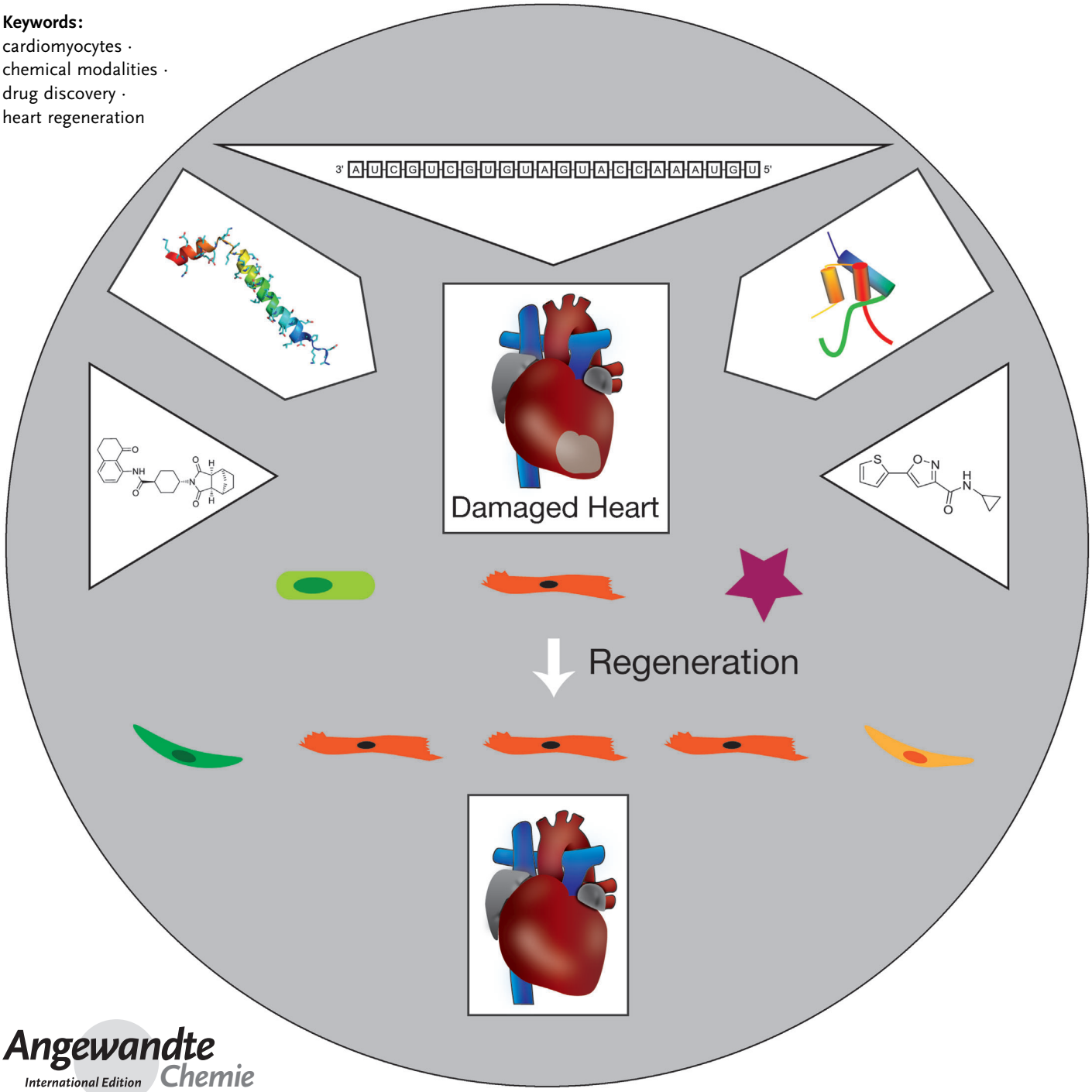

Following a heart attack, more than a billion cardiac muscle cells (cardiomyocytes) can be killed, leading to heart failure and sudden death. Much research in this area is now focused on the regeneration of heart tissue through differentiation of stem cells, proliferation of existing cardiomyocytes and cardiac progenitor cells, and reprogramming of fibroblasts into cardiomyocytes. Different chemical modalities (i.e. methods or agents), ranging from small molecules and RNA approaches (including both microRNA and anti-microRNA) to modified peptides and proteins, are showing potential to meet this medical need. In this Review, we outline the recent advances in these areas and describe both the modality and progress, including novel screening strategies to identify hits, and the upcoming challenges and opportunities to develop these hits into pharmaceuticals, at which chemistry plays a key role.

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1. Introduction

Heart disease often leads to cardiomyocyte death and pathological remodeling of the heart, culminating in heart failure and sudden death. Thus, heart disease is one of the leading causes of human mortality worldwide.[1] Unlike in lower vertebrates, the adult mammalian heart, including the human heart, has very limited regenerative capacity. Following a myocardial infarction (MI), the injured heart undergoes fibrotic repair, which replaces damaged tissue with fibroblasts and extracellular matrix. This generates a robust scar that can withstand elevated filling pressures, but does not conduct electricity or actively contract. One of the greatest challenges of regenerative medicine is the redirection of the heart's innate fibrosis-repair program to generate new contractile muscle instead of scar tissue. The only currently available curative treatment of end-stage heart failure is heart transplantation. However, limited donor availability and immune rejection upon transplantation remain serious clinical limitations. Stem-cell-based approaches for regenerative therapies have also encountered several major obstacles, including the low efficiency of homing and retention of delivered cells, the potential for immune rejection by the host, the risk of teratoma formation, and the translatability of preclinical animal models for assessing cell engraftment.[2]

With the revelation that the adult mammalian heart is not a terminally differentiated organ,[3] and that new cardiomyocytes are generated at measurable rates throughout life, regeneration of heart tissue in situ without the need of exogenous cells could provide a therapeutic solution.[4] Different avenues of research to create new cardiomyocytes are pursued, including the induction of mitosis in pre-existing cardiomyocytes,[5] proliferation of dedifferentiated cardiomyocytes,[6] proliferation and differentiation of cardiac stem and progenitor cells,[7] and reprogramming of cardiac fibroblasts into cardiomyocytes (Figure 1).[8,9] Research in these areas is conducted following different strategies, including the use of a variety of chemical modalities (i.e., chemical methods or agents). These approaches could avoid or at least minimize issues of mutation, immune rejection, and ethical controversies associated with stem-cell-based therapies. In addition,

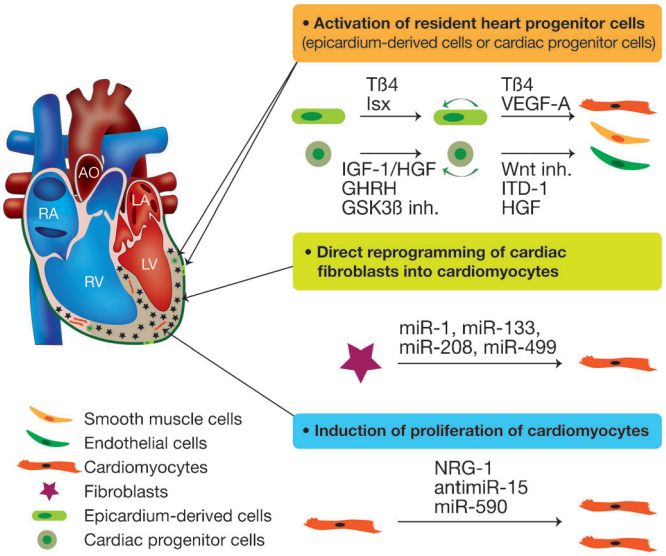

Figure 1. Avenues of research to create new cardiomyocytes. Abbreviations not defined in the text: AO = aorta, inh. = inhibitor, LA = left atrium, LV = left ventricle, RA = right atrium, RV = right ventricle.

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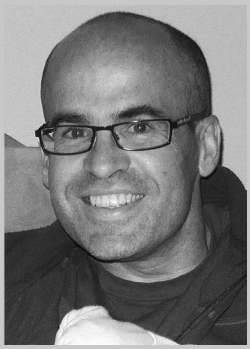
improving the understanding of the myriad of signals that regulate replication and differentiation of various cardiac cells may well reveal mechanisms that underlie the potential of the adult heart to replace muscle cells after injury and ultimately lead to strategies to discover regenerative therapies.[10] A variety of chemical modalities, ranging from RNA-based approaches, such as anti-microRNA (antimiR) oligonucleotides to peptides, proteins, and small molecules, are showing potential to meet this medical need. This emerging area of science will provide many drug discovery and development challenges that need to be overcome; thus, novel innovations will be required.

The role of chemistry to synthesize, modify, and optimize a wide variety of therapeutic modalities (i.e., therapeutic methods or agents) has broadened over recent years with the development and application of new chemical approaches to tackle difficult and novel target classes. Medicinal chemistry to discover small molecules to treat human disease is well established, and the use of small molecules in regenerative medicine has attracted much interest during recent years.[11] In addition, larger molecules, including peptides, antimiRs, and proteins, are all under active investigation as potential treatments for degenerative disorders and damaged organs (including regeneration of the heart).[12] Each modality has its strengths and weaknesses and will encounter unique challenges. However, novel opportunities will be created to utilize chemistry in different contexts that span across the traditional borders of small and large molecules. Chemistry is nowadays playing a key role in the modification of larger molecules to provide compounds with the optimal absorption, distribution, metabolism, and excretion (ADME) properties, disease efficacy, and safety profiles to meet the requirements for a novel treatment. Furthermore it helps to understand mechanisms of action. In addition, the role of chemistry within biotechnology cannot be understated with the future application of devices and methods to target a compound to the organ of interest.



Alleyn Plowright obtained his PhD in organic chemistry with Prof. G. Pattenden at the University of Nottingham in 1999, and continued with postdoctoral studies with Prof. A. Myers at Harvard University in 2000–2001. In 2002, he joined AstraZeneca UK as a medicinal chemist. He moved to AstraZeneca, Sweden, in 2006 and in 2012 became Senior Principal Scientist, Medicinal Chemistry in the Cardiovascular and Metabolic Diseases Innovative Medicines unit. His research interests include drug design and drug discovery for cardiovascular and metabolic diseases.

Ola Engkvist obtained his PhD in Computational Chemistry at the University of Lund in 1997, and continued with postdoctoral studies at the University of Cambridge and the Czech Academy of Sciences. Between 2000 and 2004, he worked for two Biotech companies before joining AstraZeneca Sweden in late 2004. He is currently team leader in computational chemistry within the Discovery Sciences, Chemistry Innovation Centre. His research interests include cheminformatics, molecular modeling, phenotypic screening, target deconvolution, and open innovation.

Adrian Gill obtained his PhD in Organic Chemistry with Prof. P. J. Parsons at the University of Sussex in 1997. He has worked for a number of Biotech and Pharmaceutical companies as a medicinal chemist, including Roche Discovery UK as team leader, Astex Pharmaceuticals as associate director, and currently AstraZeneca, Sweden, where he is Head of the Medicinal Chemistry Department in the Cardiovascular and Metabolic Diseases Innovative Medicines unit.

Laurent Knerr obtained his PhD in Organic Chemistry in the group of Prof. G. Ourisson under the direction of Dr. B. Luu at the University Louis Pasteur, Strasbourg in 1998. Following a postdoctoral stay with Prof. R. R. Schmidt at Konstanz University, he joined UCB Pharma in Braine l'Alleud, Belgium, in 2000 as a team leader in the Parallel Synthesis and Automation group. He joined the Medicinal Chemistry Department of AstraZeneca, Sweden, in 2004, where he is now a principal scientist in the Cardiovascular and Metabolic Diseases Innovative Medicines unit.

Qing-Dong Wang studied medicine and started his career in cardiology at Shandong Medical University. He then obtained his PhD in cardiology with Prof. J. Pernow at Karolinska Institute in 1996. In 1997, he joined AstraZeneca Sweden as a cardiovascular pharmacologist. His research interests include pharmacology, stem cells, and drug discovery. He initiated the Cardiac Regeneration Program at AstraZeneca and is currently leading the Bioscience Regenerative Medicine Innovation team to discover novel molecules for heart failure therapy.

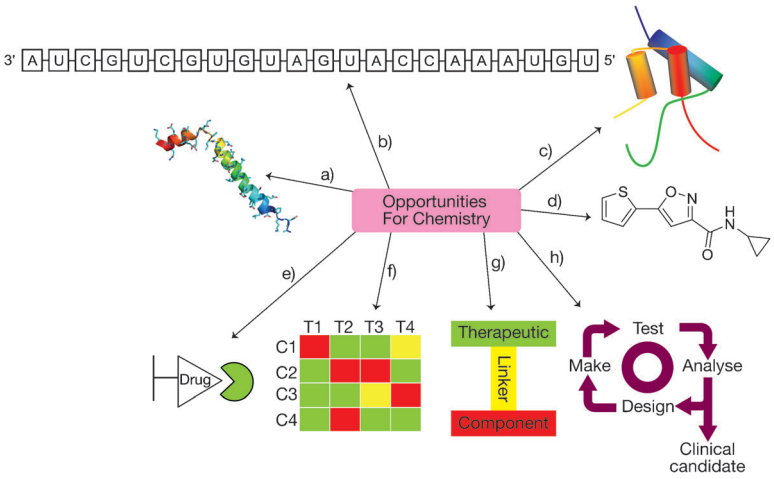


Figure 2. Chemical approaches that impact cardiac regeneration. Range of therapeutic modalities: a) peptides, b) antimiRs, c) proteins, d) small molecules.[13] Techniques required: e) target deconvolution, f) polypharmacology, g) linker and conjugate chemistry with "component" being a therapeutic, targeting, or stabilizing agent, h) developing hits to clinical candidates.

This review covers recent advances that span a diverse range of therapeutic modalities and describes recent progress from a drug discovery perspective, including novel screening strategies for hit identification and the upcoming challenges to develop these hits into pharmaceuticals for cardiac regeneration (Figure 2).

2. Opportunities with Different Modalities

2.1. Small Molecules

Disease intervention using small molecules has been central to healthcare for many years. The art of medicinal chemistry to modify a hit structure to improve all compound properties (including potency against the required target, properties to allow suitable dosing and administration, reducing off-target effects, improving in vivo safety profiles, and providing high levels of efficacy against the disease) has provided drugs across multiple indications.[14] In comparison, the development of small-molecule therapeutics for the regeneration or repair of damaged tissue in the heart is at an early stage.[15] To date, only a handful of chemical mediators of cardiogenesis have been reported, and the amenability of manipulating the critical signal-transduction mechanisms that are involved with small molecules will vary enormously (Figure 3).[16]

Small molecules offer opportunities to explore the underlying biology of a disease with specifically developed tool compounds and to develop therapies.[17] They have the advantage of temporally modulating biological systems, either through specific or multiple effects in a cell, thus allowing flexible regulation of complex signaling networks. Furthermore, their concentration can be varied with a variety of administration options (including oral administration) to achieve an optimal effect. This allows the clinician to control the dosage and to balance efficacy with safety concerns. In addition, small molecules are often relatively straightforward and inexpensive to synthesize and store.

It is very difficult, if not impossible, to create tool compounds with absolute selectivity, and prescribed drugs rarely show absolute selectivity.[18] In order to generate large changes in biological systems, such as the induction of cell differentiation or reprogramming cells, it may be advantageous to be less selective and hit multiple targets to modulate different pathways simultaneously. Hence, developing a highly efficacious treatment may require less selectivity for different targets.[19] However, lack of selectivity may hamper the use of a compound as a tool to explore biology, because the effect on a specific pathway may be difficult to deduce.[17]

Several approaches exist to exploit small molecules in the modulation of complex biological systems in the context of cardiac regeneration, ranging from the exploration of an already well-established target to a diversity screen to identify completely novel molecules and/or targets. These include:

1) Exploitation of known targets. Here, the main objectives are to better understand the target in this specific context by using tool compounds or, alternatively, to start a target-based drug-discovery campaign to identify novel modu-

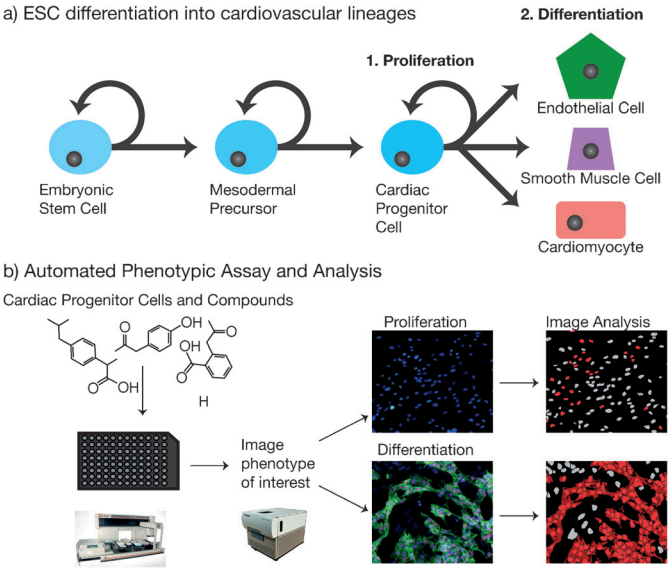


Figure 3. a) Self-renewal and differentiation of embryonic stem cells (ESC) into cardiomyocytes, endothelial cells, and smooth-muscle cells through intermediate mesodermal precursor and progenitor cells. b) Schematic representation of a high-content screen assay. Cells are plated in 384- or 96-well format prior to compound addition. Immunocytochemistry for markers of interest is performed with automation and subsequent imaging, and analysis is completed on plate-based imaging systems. The specific images shown are proliferation (Ki67 staining) and cardiomyocyte differentiation (troponin T staining) of cardiac progenitor cells.

lators. If a complex biological assay is used, it is essential to know the selectivity profile for the compound. There are cases where compounds have been thought to be selective, but in practice have modulated more than one target, for example, telmisartan, which besides being an angiotensin II receptor antagonist also modulates PPARγ.[20]

2) Exploitation of a known pathway. The main objective can be to either identify novel compounds to explore the relevant biology without identifying the specific target, or to deconvolute the target that gives rise to the biological effect.[21] The difference of this approach to a more usual phenotypic screen is that the readout of the assay is coupled to the pathway, with the advantage that an assay with a higher throughput can be developed.
3) Performing an unbiased phenotypic screen.[22] The screen can be performed with a set of tool compounds with known targets or with a diversity set. Assays that accurately mimick the biology and provide a variety of relevant endpoints can be used (Figure 3B).[23] Screening of tool compounds is a cost-efficient method to identify targets, because fewer compounds need to be screened. High-quality tool compounds exist for only a few hundred targets, depending on the criteria that are set, for example, drug on the market.[24] As the potential targets are at least partially known, the results can be followed up by validating the targets with the use of, for example, siRNA knockdown. The drawback of this approach is that the chances of identifying novel drug targets are reduced. To increase the chances, a diversity screen can be performed followed by medicinal-chemistry optimization and target deconvolution. A diversity screen should be large to cover a reasonable portion of chemical space. The hit compounds should then be optimized to reach a certain desired effect in the phenotypic assay. There will be no structural information available to guide the design, and the phenotypic effect may originate from the modulation of multiple targets. Hence, structure–activity relationship (SAR) data may be more difficult to interpret and the optimization more challenging. The optimized compound could be progressed directly or a target deconvolution strategy could be used. Several target deconvolution methods, including chemical proteomics, siRNA, and in vitro panel screens, exist and have recently been reviewed thoroughly.[25] All methodologies have their pros and cons. For instance, chemical proteomics has been much more successful in identifying soluble enzymes than membrane bound receptors.

In this section, an overview of recent publications in screening small molecules will be given.

The canonical Wnt signaling pathway is a key regulator of cardiomyocyte differentiation and proliferation (Figure 4).[26] A number of studies have highlighted small-molecule Wnt pathway modulators,[27] which drive cardiomyocyte differentiation from embryonic stem cells (ESC). In these studies, the timing of treatment with the compound was extremely important to achieve the desired effect.[28] Activation of the canonical Wnt pathway is required in the early stages of both human ESC (hESC) and human induced pluripotent stem cell

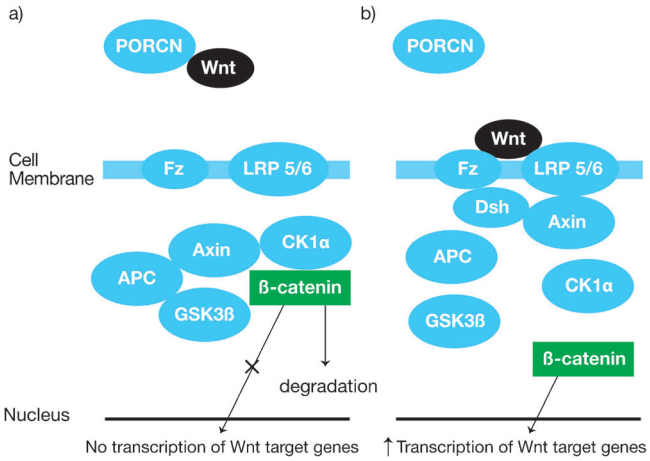


Figure 4. Simplified overview of the canonical Wnt signaling pathway. a) In the absence of Wnt ligand, β-catenin in the cytoplasm interacts with a complex made up of APC and axin scaffold proteins and the kinases CK1 and GSK3β, leading to β-catenin phosphorylation and degradation. b) Binding of Wnt ligand to a Frizzled receptor and a LRP-5/6 co-receptor inhibits the APC/Axin/CK1/GSK3β complex, and β-catenin enters the nucleus and regulates target genes.

(hiPSC) differentiation to early mesoderm cells, whereas suppression of the pathway is needed in the later stages of differentiation to cardiomyocytes.[28,29] While these compounds are unlikely to aid regeneration of the injured human heart, as they target early phases of differentiation, they are useful tools for generating cardiomyocytes, understanding the underlying biology, and timing the signaling pathway intervention. Key recent studies are summarized below.

Willems et al. developed a high-content screen (HCS) using hESC that carried a cardiac-specific α-myosin heavy chain (MYH6) mCherry reporter gene, with which hits that promote the generation of cardiomyocytes triggered the production of red-fluorescent protein.[30] The HCS was applied to a library of 244 kinase inhibitors and 305 pathway modulators and a potent hit IWR-1 (**1**, Figure 5), an inhibitor of canonical Wnt signaling by stabilizing the axin complex through direct interaction with axin, was identified.[31] IWR-1 produced maximal cardiac induction at day 4–5 with an EC_{50} in the low micromolar range. Flow cytometry analysis demonstrated that IWR-1 yielded up to 30% cardiomyocytes and did not increase the formation of other mesoderm lineages (Figure 3A). The effect of different inhibition of the Wnt pathway was studied using three structurally diverse Wnt inhibitors; the more potent IWR derivative 53AH (**2**),[30] the porcupine (PORCN) inhibitor IWP-3 (**3**),[31a] and the tankyrase (TNKS) 1 and 2 inhibitor XAV939 (**4**).[32] All three inhibitors promoted cardiomyocyte induction, with 53AH possessing an EC_{50} in the submicromolar range. A SAR study was performed through modification of the three main regions of IWR-1, leading to **5**, which possesses a 30-fold improved potency and greater cardiomyocyte induction compared with IWR-1.[33]

To search for inducers of cardiomyocyte differentiation, Ni et al. developed an in vivo phenotypic screen based on the

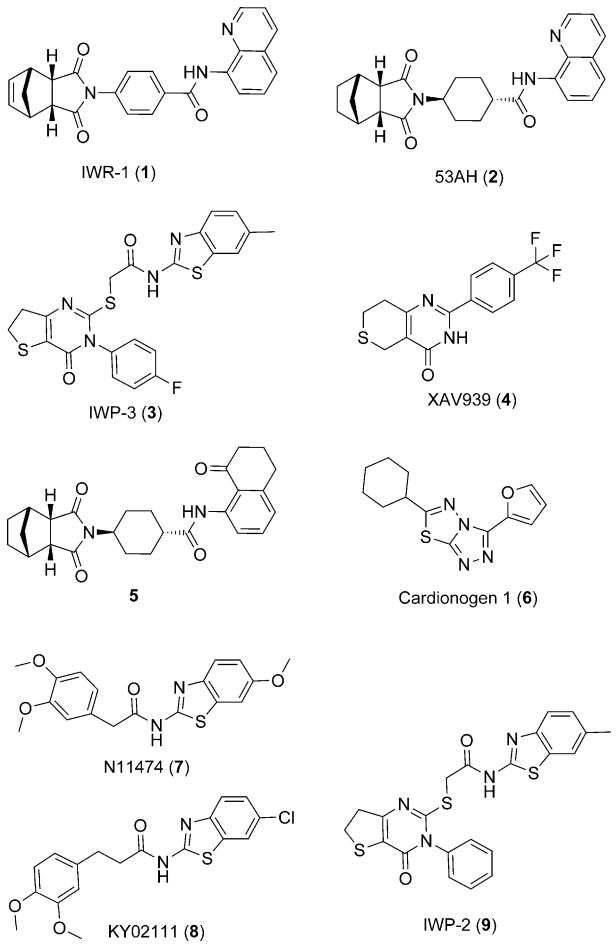


Figure 5. Inhibitors of the canonical Wnt signaling pathway.

use of transgenic zebrafish embryos.[34] The endpoint of the screen was the expression of enhanced green-fluorescent protein (EGFP), which is under the control of the late-stage cardiomyocyte differentiation marker, cardiac myosin light chain 2 (cmlc2) promoter. Compounds were dosed (5 μM) and heart size, cardiac morphology, and contractility were monitored, as well as observing organs for indications of compound selectivity and potential toxicity. A set of 4000 compounds was screened and provided 17 compounds that induced an enlarged heart phenotype. Among these, cardionogen 1 (**6**), 2, and 3, which possess a common [1,2,4]triazolo[3,4-b]-[1,3,4]thiadiazole scaffold, were characterized further. Cardionogen 1 showed a biphasic pattern of activity, inhibiting the formation of cardiomyocytes when treatment took place before gastrulation, while inducing it when administered during or after gastrulation. Cardionogens were shown to induce heart hyperplasia not through cardiomyocyte proliferation, but by increasing the progenitor cell population. The results were translated to murine ESC by promoting ESC differentiation to cardiomyocytes. While the exact target was not identified, mechanistic studies indicate that cardionogen 1 antagonizes the canonical Wnt signaling pathway. Interestingly, IWR-1 was shown to antagonize Wnt signaling in different cell lines (ESC and HEK), while cardionogen 1 proved to be selective for ESC. The results suggest the possibility of a cell-type/tissue-specific inhibition by cardionogen 1.

More recently Minami et al. developed a HCS using monkey-ESC-expressing EGFP under the control of the promoter for human MYH6.[35] Treatment with a library of 9600 compounds (1–5 μM) for eight days (days 6–14) identified the molecule N11474 (**7**), which produced cardiac differentiation. As observed previously, the timing of N11474 addition was important with optimal results by treatment on days 4–8. A synthesis campaign led to the discovery of KY02111 (**8**) with a 6-chloro substituent on the benzothiazole ring and an ethylene linker on the acyl side chain. KY02111 promoted cardiac differentiation about seven times more efficiently than N11474. Extensive characterization suggested that the majority of cardiomyocytes were functional mature ventricular cardiomyocytes with expression of cardiac markers and ion channels at levels similar to adult heart tissue. Mechanism-of-action studies with KY02111 using gene-expression profiling showed profiles similar to known Wnt inhibitors, XAV939 and IWP-2 (**9**).[31a] Further studies showed that KY02111 acts differently to IWP-2 and XAV939 (probably downstream of APC and glycogen synthase kinase-3β (GSK3β)) and is more potent.

In a 2011 report, Wang et al. focused on the TNKS 1 and 2 inhibitor, XAV939, and established a robust protocol of cardiac differentiation of mouse ESC.[32] The timing of XAV939 treatment from days 3–5 of differentiation resulted in over 95% of embryonic bodies that beat spontaneously at day ten. Mouse ESC expressing the nuclear red-fluorescent protein under the control of the α-myosin heavy chain (MHC) promoter were exposed to XAV939 and indicated a 30-fold increase in the relative abundance of cardiomyocytes as well as significantly decreased mRNA expression levels of diverse mesoderm-derived hematopoietic, endothelial, and smooth-muscle cell markers. In addition, XAV939 had no effect on ectoderm and endoderm cellular markers. These results indicated that XAV939 induces cardiac differentiation at the expense of cells of other mesoderm lineages.

In contrast to inhibition of canonical Wnt signaling, activation of the pathway is required in the early stages of both hESC and hiPSC differentiation to early mesoderm cells.[28,29,36] This has been achieved, for example, using CHIR99021 (**10**, Figure 6),[36b] which inhibits GSK3β, leading to nuclear accumulation of β-catenin and activation of gene transcription. However, Tseng et al. showed that 6-bromoindirubin-3-oxime (BIO, **11**), another inhibitor of GSK3β, also stimulated proliferation of both neonatal and adult rat cardiomyocytes.[37] Exposure of neonatal cardiomyocytes to BIO (5 μM) for 48 hours increased the incorporation of 5-bromo-2′-deoxyuridine (BrdU), a uridine analogue that is incorporated into newly synthesized DNA, by a factor of 10. BIO was active at a concentration of 1 μM, but not at 0.1 μM. A close analogue, 1-Me-BIO (**12**), which is inactive against GSK3β, was unable to promote BrdU incorporation, suggesting a role for GSK3β inhibition in the entry of neonatal cardiomyocytes into the cell cycle. Subsequent studies showed that BIO treatment increased levels of positive cell cycle regulators (cyclin D1, cyclin A) and decreased the level of a negative effector (CDK inhibitor p27), evidence for cells

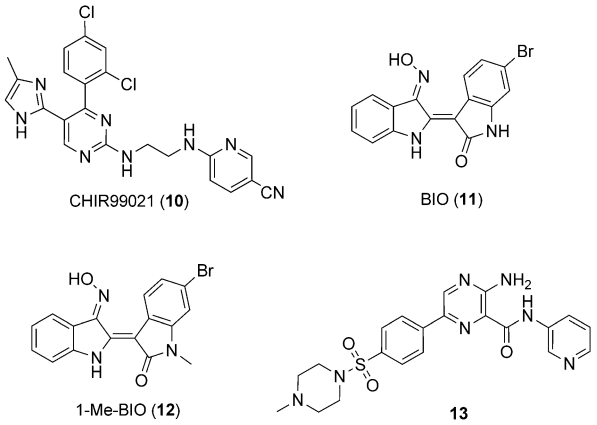


Figure 6. GSK3β inhibitors and the inactive analogue 1-Me-BIO.

entering mitosis and undergoing cytokinesis. The capacity of BIO to induce cell-cycle progression in adult cardiomyocytes was subsequently confirmed by the incorporation of BrdU and cells entering mitosis. Using hiPSC-derived cardiomyocytes, we recently demonstrated that BIO and a further GSK3β inhibitor (**13**, Figure 6)[38] increased the incorporation of 5-ethynyl-2-deoxyuridine (EdU), another marker of DNA synthesis (Figure 7), thus suggesting that GSK3β inhibition also promotes cell-cycle entry in human cardiomyocytes (unpublished work).

As described, canonical Wnt signaling is a key regulator of cardiomyocyte differentiation and proliferation, and compounds have been discovered that can either antagonize or stimulate the pathway (Figure 8). However, other signaling pathways also play an important role. Willems et al. used a mouse ESC HCS to identify compounds capable of converting uncommitted mesoderm into cardiomyocytes.[39] Screening a 17000 member unbiased diversity library between days 2–6 of differentiation identified the hit 1,4-dihydropyridine, ITD-1 (**14**, Figure 9). ITD-1 promoted cardiogenesis when added from days 3–5, while completely abolishing it when added earlier. Study of the mechanism of action of ITD-1 utilized a panel of tyrosine kinase inhibitors and showed that blocking of transforming growth factor β (TGF-β) signaling upregulated cardiogenesis significantly. In

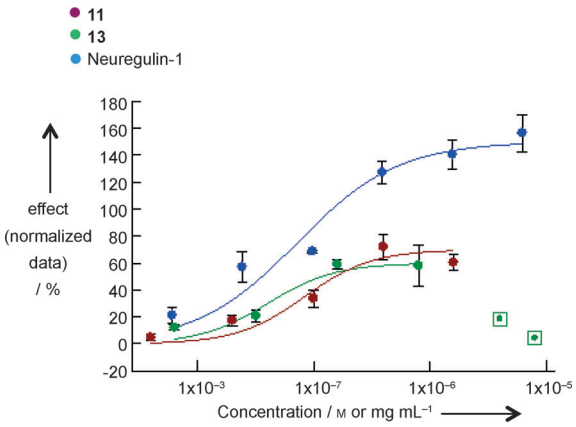


Figure 7. Effect of GSK3β inhibitors, BIO (**11**) and **13**, and neuregulin-1 on proliferation of human induced pluripotent stem cell derived cardiomyocytes. Concentration unit is M for **11** and **13**, and mg mL^{-1} for neuregulin-1. The y axis is the effect (%) relative to positive control medium. At the two highest concentrations **13** showed toxicity.

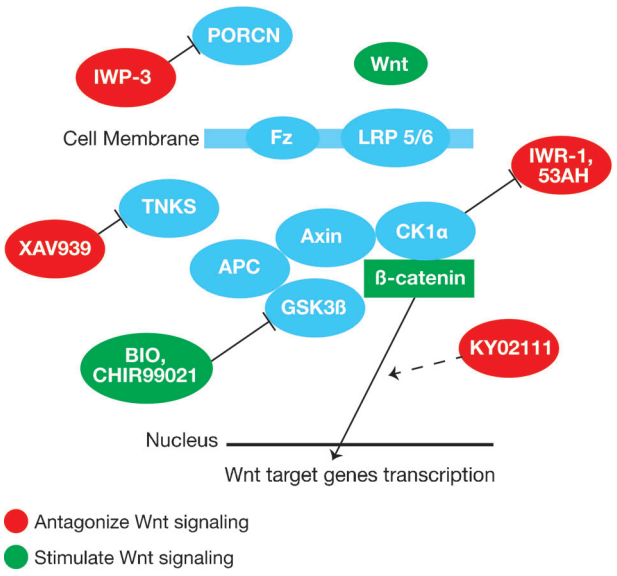


Figure 8. Differential modulation of the canonical Wnt signaling pathway by small molecules.

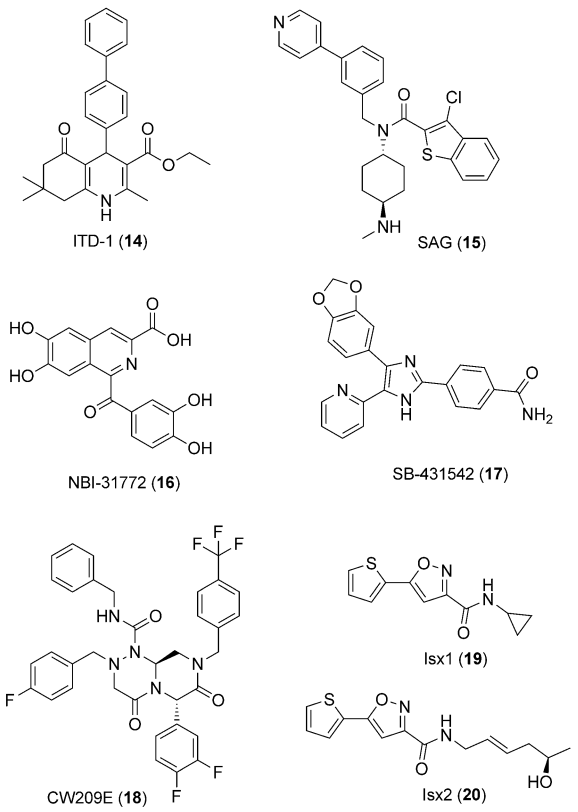


Figure 9. Small molecules that impact differentiation and proliferation of cardiomyocytes.

addition, the inhibitory activity of ITD-1 was independent of toxicity, calcium channel blockage, and other developmental pathways such as Wnt and BMP. Synthesis of 200 analogues of ITD-1 allowed the identification of compounds from the same series with greater selectivity.[40] Extensive mode-of-action studies of ITD-1 on the TGF-β pathway showed induction of proteasome-mediated degradation of the cell surface TGF-β type II receptor through a ubiquitin-independent mechanism. This study highlighted an interesting and unexpected dual role of the TGF-β pathway in cardiac differentiation in that it first promotes mesoderm formation and subsequently inhibits cardiomyocyte formation.

Choi et al. recently described the use of a transgenic system to visualize cardiomyocyte proliferation in zebrafish embryos.[41] The fluorescent ubiquitylation-based cell-cycle-indicator (FUCCI) system was adapted to employ a fluorescent protein (venus) fused to a regulator of the cell cycle (hGeminin) expressed behind the cmlc2 promoter.[42] This system allowed the sensitive identification of cardiomyocytes at different cell-cycle stages. The transgenic embryos were screened against a panel of modulators of developmental signaling pathways three days after fertilization (dpf), as cardiac growth at this stage would occur through cardiomyocyte proliferation. Inspection of the embryos four days after fertilization showed compounds targeting the Hedgehog (Hh), insulin-like growth factor (IGF), and TGF-β pathways all had an effect on cardiomyocyte proliferation. Implication of the Hh signaling pathway was demonstrated with smoothened agonist (SAG, **15**, 5 μM),[43] which triggered a 60% increase in the number of proliferating cardiomyocytes. Treatment with the IGF agonist NBI-31772 (**16**, 2.5 μM) increased proliferation by 41%.[44] The TGF-β pathway was studied using the TGF-β receptor antagonist SB-431542 (**17**, 5 μM), an inhibitor of the TGF-β/activin pathway,[45] which reduced cardiomyocyte proliferation (30%). To assess the effects on the regenerative capacity of the heart after ventricle resection or genetic ablation of cardiomyocytes, adult zebrafish were treated six days after injury with SAG (2.5 μM) and NBI-31772 (10 μM). This stimulated cardiomyocyte proliferation (65%), thus suggesting that agonizing the Hh and IGF pathway could promote cardiomyocyte proliferation in the injured adult heart. These results showed that the described embryonic chemical screen was useful for the discovery of inducers of cardiomyocyte proliferation in injured adult zebrafish.

A novel series of β-turn peptidomimetics with cardiogenic properties were identified by Oh et al.[46] A reporter-gene assay in mouse ESC based on the expression of EGFP-1 under the control of the promoter region of α-MHC was used. 200 compounds were screened (10 μM) for five days and eight of them gave a greater than two-fold increase in the number of fluorescent cells. Three active compounds were then confirmed in dose–response experiments. The triazinopyrazone derivative CW209E (**18**), a 6,6-bicyclic β-turn mimetic, represents a distinct type of cardiomyogenesis-inducing molecule. Screening of structural near neighbors confirmed that this scaffold formed the basis of a hit series and the activity of CW209E was translated to human cell lines. This study presents the discovery of an original chemotype for inducing cardiomyogenesis, which may be involved in modulation of protein–protein interactions.

In an early report, Sadek et al. designed a high-throughput screen (HTS) assay using a reporter luciferase gene inserted into the Nkx2.5 locus in genetically engineered P19CL6 PSC.[47] The choice of Nkx2.5 as a marker is in contrast to the later stage differentiation markers that are often employed (e.g. α-MHC) and constitutes an alternative screening strategy. A compound library with 147000 members was screened, leading to the discovery of a number of interesting series, including a series of 3,5-disubstituted isoxazoles (Isx).[13d]

Isx1 (**19**), a cyclopropyl amide derivative, was selected for in vivo characterization and assessed using Nkx2.5-luc-BAC transgenic reporter mice, which are animal counterparts to the cells used in the original HTS. Once daily dosing (16 mg kg^{-1} intraperitoneally) during one week resulted in increased steady-state luciferase activity in two tissues expressing Nkx2.5, the heart and the stomach. Significantly increased cell-cycle activity was demonstrated in the heart, and in particular in the cardiomyocyte population, as shown by increased DNA synthesis assessed by BrdU incorporation and mitotic activity using phosphohistone H3 as a marker. Isx1 also dramatically altered the gene-expression profile of Notch-activated epicardium-derived cells (NEC), a population of cardiac-resident progenitor cells, toward cardiomyocyte-like precursors. Isx1 was subsequently administered immediately after induction of MI, but effects on cardiac muscle genes were overridden by MI-triggered activation of fibrosis genes. Administration of Isx1 three days after MI resulted in significant improvement in cardiac ventricular function. However, this beneficial effect was not durable and disappeared completely 21 days after MI. Moreover, Isx1 treatment had no effect on early scar histology. Although Isx1 lacked in vivo efficacy, this study constituted an encouraging example of inducing proliferation of cardiac-resident progenitor cells and differentiation toward cardiomyocytes using in vitro discovered small molecules.

The mode of action and molecular target of Isx1 was subsequently identified.[48] Around 100 analogues of the Isx starting points were synthesized, enabling the discovery of more potent analogues, such as Isx2 (**20**). The authors showed that Isx were involved in the regulation of intracellular Ca^{2+} flux in NEC, suggesting modulation of G(q)PCR signaling. A GPCR panel screen (GPCR = G protein-coupled receptor) resulted in one hit, GPR68, an extracellular proton/pH-sensing GPCR that was not known to have cardiac function up to that point, for which Isx act as direct agonists. GPR68 was subsequently shown to be upregulated in MI-spared cardiomyocytes in mice, and GPR68-expressing cells form a proton-sensing buffer zone around the infarction. Isx1 targeted this buffer zone in vivo. This study provides a good example of the application of in vitro generated compounds to the discovery and validation of a novel target for cardiac regeneration.

As shown, small molecules led to success in elucidating underlying biology in heart regeneration, including the role of the Wnt pathway. The investigations have included the use of single tool compounds, the screening of small compound sets, and HTS with greater than 100000 compounds (Table 1). Hits

Table 1: Examples of small molecules, the screening strategy, and target deconvolution. n.a. = not applicable.

Strategic goal	Library size	Assay type	Identified hits	Key molecules	Deconvolution	Chemistry follow-up	References
Cardiomyogenesis	550 tool compounds	cell reporter gene	yes	IWR-1, **5**	known mechanism	yes	[30,33]
Cardiomyocyte differentiation	4000 diversity set	zebrafish	yes	cardionogen 1	pathway level	no	[34]
Cardiomyocyte differentiation	9600 diversity library	cell reporter gene	yes	N11474, KY02111	pathway level	yes	[35]
Cardiomyocyte differentiation	one compound	mouse ESC	n.a.	XAV939	known mechanism	no	[32]
Cardiomyogenesis	17000 diversity library	cell reporter gene	yes	ITD-1	pathway level	yes	[39]
Cardiomyogenesis	200 diversity set	cell reporter gene	yes	CW209E	unsuccessful	near neighbor screening	[46]
Cardiomyogenesis	147000 diversity library	cell reporter gene	yes	Isx1, Isx2	yes, GPCR panel screening	yes	[13d,47,48]
Cardiomyocyte proliferation	tool compounds	zebrafish	yes	SAG, NBI-31772	pathway level	no	[41]
Cardiomyocyte proliferation	tool compounds	mammalian cardiomyocytes	yes	BIO	known mechanism	no	[37]

have been identified and potency can be improved through medicinal chemistry optimization. While many of these compounds may be good tools for use in vitro, in many cases they suffer from poor physicochemical and ADMET properties, thus making them unsuitable for in vivo characterization. A key challenge will be the development of a hit into a compound that can elicit an effect in vivo, requiring both engagement and activity at the target as well as suitable properties to achieve sufficient exposure in the required tissue for a sufficiently long time.[49] For example, poor solubility, low permeability, and compound instability in the blood, often driven by high molecular weight and lipophilicity, as well as metabolic soft spots in the molecule can all lead to low concentrations of compound after in vivo dosing, and thus minimize the chances of observing efficacy.[50] The optimization of multiple compound parameters to develop hit molecules into in vivo tools and ultimately clinical candidates is an important aspect to develop treatments based on small molecules.[51]

A recent example was described by Shultz et al. for the optimization of XAV939,[52] a potent inhibitor of TNKS 1 and 2 with good ligand lipophilicity efficiency (a measure of the differential between potency and lipophilicity, LLE = pIC_{50}−logD).[53] However, XAV939 shows a number of liabilities, including low solubility and low stability in rat liver microsomes, thus hindering its use for in vivo studies. The initial optimization strategy focused on less potent molecules, but with higher LLE. This approach led to compounds with improved stability and solubility as well as increased potency and reduced lipophilicity, ultimately showing good exposure and in vivo activity in tumor-bearing mice.

While it has been possible to deduce which pathway is involved in the biological effect of small molecules, it has often been difficult to deconvolute the exact target. The only successful target deconvolution published in the context of heart regeneration has been through panel screening of a set of GPCRs. This highlights the challenge with deconvoluting targets and is an area that requires significant investment going forward.

These recent disclosures have provided techniques to discover and utilize small molecules for generating cardiomyocytes, and the article series by Schneider et al. is a nice example of the steps from an HTS to a new target and effects in vivo.[13d,47,48] Much work is needed to build on these studies to discover compounds that target later stages of cardiomyocyte differentiation and to generate therapeutically relevant cardiomyocyte populations (and ultimately improved cardiac function) that can progress successfully into the clinic.[54]

2.2. MicroRNA and Anti-microRNA Oligonucleotides

MicroRNAs (miRs) are short (around 22 nucleotides in length), single-stranded noncoding RNAs that regulate gene expression by targeting complementary oligonucleotide sequences usually located at the 3′-untranslated region in messenger RNAs (mRNAs). These interactions result in the inhibition of the mRNA translation and hence suppression of protein expression, or degradation of the mRNA. Individual miRs can bind to many mRNA targets, often encoding multiple components of complex intracellular networks. Hence, by manipulating miR expression or function, a significant impact on cellular phenotypes can be achieved.

Methods are available to either effectively upregulate miR expression or inhibit their activity. Synthetic miRs can be administered to enhance function or replace miRs that are lost or downregulated in disease.[55] This is challenging, and more effective gain of function can be achieved using adeno-associated viruses (AAV), resulting in robust expression of the miR of interest.[56] In contrast, antimiR oligonucleotide molecules (**21**) can be synthesized that bind to the target miRs through complementary base pairing, thus leading to inhibition of the miR and hence increased availability of mRNA for translation (Figure 10).[57] Application of antisense oligo-

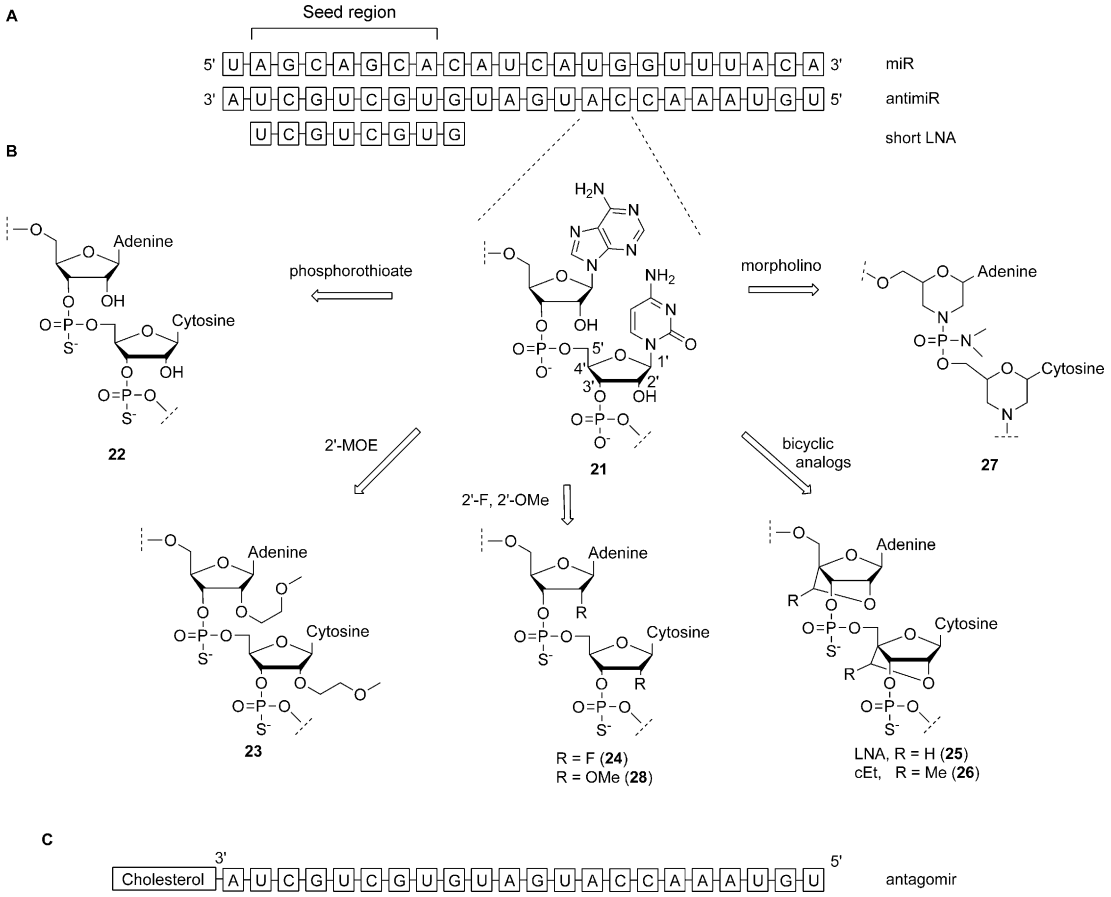


Figure 10. MicroRNA (miR) and antimiR oligonucleotides and chemical modifications. A) Illustration of a miR, the corresponding antimiR, and a tiny locked nucleic acid (LNA) molecule targeting the seed region of the miR. B) More common 2′-hydroxy and ribose chemical backbone modifications are illustrated on the adenine–cytosine residues. An antimiR can contain single or mixed modifications. C) Antagomir structure, in which the 2′-hydroxy groups are replaced with a methoxy group, some of the phosphodiester linkages are changed to phosphorothioates and a cholesterol molecule is conjugated to the 3′ end.

nucleotide chemistry to antimiRs has enabled the rapid discovery of compounds that bind to the miR of interest with high specificity and affinity, are cell permeable and stable, and have long half-lives in vivo.[58] Most antimiR chemistry has involved the introduction of phosphorothioate groups (**22**) into the backbone to increase nuclease resistance as well as plasma protein binding, and hence to reduce clearance of the antimiRs by urinary excretion. In addition, replacement of the hydroxy group in the 2′ position of the ribose sugar moieties has enabled stronger interactions and more stable duplexes with the miR target. These sugar modifications include 2′-O-methoxyethyl (2′-MOE, **23**), 2′-fluoro (2′-F, **24**), and bicyclic systems, such as locked nucleic acid (LNA, **25**) and constrained ethyl (*R*- and *S*-cEt, **26**), in which the oxygen atom in 2′ position is bridged to the 4′ position through a methylene linker to form a rigid bicycle, locked in a C3′-endo sugar conformation.[59] In particular, oligonucleotides that contain bicyclic modified nucleotides possess extremely high binding affinity, as evidenced by thermal denaturation studies,[59b,60] and therefore show efficient inhibition of the miR with a much shorter antimiR. For example, a tiny LNA consisting of eight nucleotides has been used to target the seed region (nucleotides 2–8 at the 5′ end) of the miR-15 family, hence inhibiting the action of multiple miR-15 family members.[61] Nuclease resistance can also be increased by replacing the ribose with a morpholine ring (**27**).[62] The properties of antimiRs can be modified further with antimiR molecules known as antagomirs. Here, the 2′-hydroxy groups are replaced with methoxy groups (**28**), some of the phosphodiester linkages are changed to phosphorothioates, and a cholesterol molecule is conjugated to the 3′ end of the antimiR, thus leading to enhanced cellular uptake and in vivo stability, as well as promoting hepatic uptake of the antimiR while lowering delivery to other tissue types.[63]

As miRs act on multiple mRNAs it is likely that miRs, or miR inhibitors, will moderately regulate several target genes. Hence, the definition of the functional relevance of a miR and the deconvolution of the key targets is challenging. However, understanding the proteins and pathways that are affected by these miRs and that drive the desired phenotype may result in interesting biological targets for intervention with alternative chemical modalities. Because of the often challenging biological readouts, as well as to understand the ADME properties of the antimiR, it is necessary to rapidly test the compounds in animal models relevant to the disease in question. The highly charged nature and large molecular

weight of the antimiRs make them not amenable to oral administration because of poor intestinal absorption, and thus they need to be dosed either through intravenous or subcutaneous injections or infusions. Once an antimiR is taken up into cells, its clearance is significantly reduced and the antimiR can be stable with half-lives of a few weeks, leading to a long duration of biological action. In addition, as multiple genes and pathways can be modulated and the timing to readout the different responses can be variable, fully understanding the pharmacodynamics (PD) of drug treatment is complex. Hence, a significant drug development challenge is to understand the relationships between pharmacokinetic (PK) and PD and treatment regimens to deliver the required therapeutic concentrations in the target tissue to treat disease in humans. It will be important to not only monitor the positive effects of modulating gene expression through the antimiRs, but also the potential adverse effects through modulating genes and pathways. Avoiding the innate immune response has been one of the major challenges for oligonucleotide-based therapeutics. However, chemical modifications of oligonucleotides, particularly those at the 2′ position of the ribose sugar, have led to significant improvements.[59,64]

A number of miRs that regulate cardiac function and regeneration have recently been reported, providing examples of either enhancing or inhibiting function using both miR and antimiR modalities.[65,12a] Eulalio et al. performed an HCS based on fluorescence microscopy in neonatal rat cardiomyocytes using a whole-human-genome miR library consisting of 875 miR mimics.[5c] The screen identified 204 miRs that significantly increased the proliferation of neonatal rat ventricular cardiomyocytes by more than two times, as measured by three markers (sarcomeric α-actinin, Ki-67, and EdU). Forty of the miRs also enhanced EdU incorporation and the Ki-67 marker in mouse cardiomyocytes, and two, miR-590-3p and miR-199a-3p, induced reentry of postnatal rat cardiomyocytes into the cell cycle, leading to a significant increase in the number of cardiomyocytes. Interestingly, none of the top miRs increased proliferation of rat cardiac fibroblasts. Synthetic miR-590-3p and miR-199a-3p were subsequently tested in vivo, either injected directly into the heart of neonatal rats, or AAV9 vectors expressing either miR were injected intraperitoneally in neonatal mice or intracardially in adult mice. In all cases, an increase in cardiomyocyte proliferation was observed. Finally, adult mice were treated with AAV vectors expressing the two miRs after a MI. After 60 days, the size of the infarcts were significantly reduced and beneficial effects on cardiac function were observed.

In another recent study, Boon et al. compared miR expression profiles from the hearts of young or aged mice and identified a number of miRs that play a role in cardiac pathophysiology, including the miR-34 family, which is upregulated in aged hearts.[66] MiR-34a was shown to be the predominant family member in the heart, with higher levels in cardiomyocytes compared with noncardiomyocytes. As miR-34a had previously been shown to induce apoptosis,[67] 18-month old mice were treated with an antimiR-34a antagomir molecule, Ant34a. After one week, Ant34a showed a reduced number of dead cells compared to controls. The impact of miR-34a after acute MI was assessed by injecting antagomirs or LNA-based antimiRs of miR-34a to mice after acute MI. Two weeks later, cardiac contractile function was significantly improved and there was a reduction in the number of dead cells, both cardiomyocytes and noncardiomyocytes. Subsequent investigations showed additional favorable effects on other heart-cell types, including endothelial cells.

By comparing miR expression profiles in mouse cardiac ventricles at postnatal days 1 and 10, Porrello et al. have shown that members of the miR-15 family are upregulated in the mouse heart shortly after birth, and that these miRs contribute to the repression of cell cycle genes, including checkpoint kinase 1, and postnatal cell cycle arrest.[68] LNA-modified antimiRs of the miR-15 family were subsequently administered by subcutaneous injection to postnatal mice to inhibit the expression of the miR-15 family until adulthood.[69] After 21 days, a MI was induced and the treatment group showed an increase in cardiomyocyte proliferation and, over time, an improvement in left ventricular systolic function. These studies also showed induction of noncardiomyocyte proliferation; the significance of these effects need to be further understood. The results indicate that postnatal inhibition of the miR-15 family with the corresponding chemically modified antimiRs promotes myocardial regeneration.

An attractive approach for the regeneration of an injured heart is the reprogramming of cardiac fibroblasts into cardiomyocytes.[70] Recently, Jayawardena et al. highlighted the power of utilizing more than one miR to redirect cell fate.[8] Six miRs were selected based on their roles in the development of cardiac muscles, and differentiation and synthetic mimics of these miRs were transfected into mouse cardiac fibroblasts both individually and in all possible double and triple combinations. MiR-1 and various combinations changed the expression of fibroblastic genes to cardiomyocyte genes. To test the concept in the adult heart, a lentivirus-encoding miR-1 or a combination of lentiviruses encoding the miRs 1, 133, 208, and 499 were injected intramyocardially directly after induced myocardial injury. After four weeks, new cells, characteristic of cardiomyocytes, were observed. The results indicate that miR-1 alone is sufficient to induce the expression of cardiac markers in fibroblasts. However, the combination of miRs 1, 133, 208, and 499 was able to enhance the maturation of the converted fibroblasts to cells with functional properties characteristic of cardiomyocytes. Interestingly, the addition of the small molecule JAK inhibitor 1 (**29**),[71] an inhibitor of JAK1, 2, and 3 (Figure 11), significantly increased the efficiency by further inducing the cardiac marker α-MHC and the expression of cardiac ion channels. While the mechanism of the increased efficiency generated with JAK inhibitor 1 is not clear, this result provides intriguing future opportunities for combining miR and small-molecule modalities.

These examples provide exciting data that could lead to both cardiac-regeneration therapies and to the design of drugs targeting some of the proteins whose expression is affected by these miRs. In addition, a LNA-modified antimiR-122 molecule, miravirsen, has recently been shown to be effica-

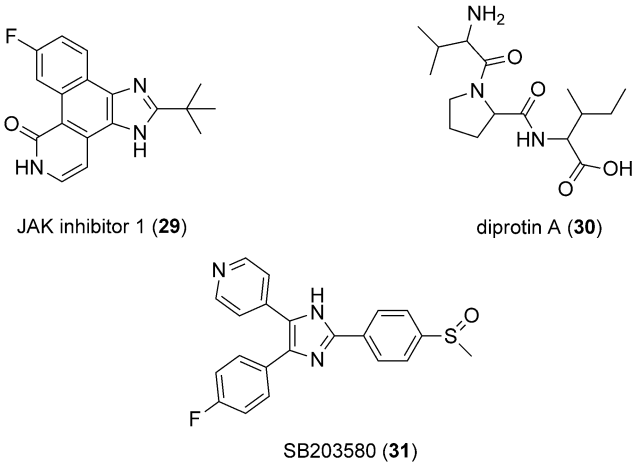


Figure 11. Chemical structures of JAK inhibitor 1, diprotin A, and SB203580—small molecules used in combination with additional modalities to enhance effects on cardiac regeneration.

cious and well-tolerated in humans with hepatitis C virus, showing promise for this class of chemicals.[72] While still in its infancy, examples of small-molecule inhibitors of miRs are starting to appear and it will be interesting to monitor the developments in this area to discover small-molecule inhibitors of miRs that are relevant for regeneration of the heart.[73]

2.3. Peptides and Proteins

Peptides and protein drugs are becoming more accessible and important as therapeutic agents.[74] Advances in biotechnology are increasing the accessibility, administration options, and range of opportunities with these therapeutic modalities. In addition, scientific breakthroughs are allowing modifications to circumvent previous issues, including immunogenicity of proteins and rapid plasma degradation leading to short half-lives in vivo. Commonly used chemical modifications include the attachment of glycans or poly(ethylene glycol) (PEG) chains to proteins, often by covalent linkage of chemically activated glycans or activated PEG derivatives (e.g. PEG benzotriazole carbonate) to a lysine or N-terminal amino group, respectively.[75] Glycosylation or PEGylation have been shown to increase the stability of proteins by reducing their rate of clearance and hence provide longer half-lives.[76] However, multiple reactive sites in proteins often allow low control of the position of PEG or glycan addition, and reduced biological activity is sometimes observed. Further developments in chemistry are now enabling site-specific post-translational modification of proteins.[77] Recently, Dumas et al. described the use of the Suzuki–Miyaura reaction to selectively attach PEG chains to proteins containing halogenated amino acids by using PEG/boronic acid derivatives in the presence of a water-soluble Pd^{II} salt and no external ligands to provide up to 70% conversion into PEGylated protein.[77a]

Drugs based on naturally occurring proteins or peptides have the advantage of modulating fundamental signals and the potential to be highly efficacious and selective for their molecular targets. However, these pathways are also present in many organs other than the heart, and hence, cardiac specificity will often be required to avoid potential adverse effects. Modulation of signaling pathways can affect many biological processes, and a thorough understanding of biological mechanisms and PK–PD profiles as well as the identification of suitable biomarkers in order to establish effective and safe dosing regimens will be required. In addition, despite the progress in manufacturing and methods of delivery, the ease and cost to produce these compounds are, in general, higher than with small molecules, and the compounds often need to be delivered by a non-oral route.

Peptides, ranging from synthetic peptides of about 30 amino acids in length to full-length therapeutic proteins, are investigated as tools to understand biological mechanisms as well as their use as potential therapeutics for cardiac regeneration.[78,79] A number of growth factors have been identified based on the understanding of signaling pathways involved in cardiac development, growth, and differentiation.[12b] These growth factors can exert a diverse range of cardiovascular effects, including inducing angiogenesis, reducing apoptosis, and increasing the recruitment and proliferation of stem cells in the myocardium. They bind to their receptors, which include protein tyrosine kinases, cytokine receptors, and GPCRs, or act in a paracrine (released from the cell in which it is produced and binds to its receptor on nearby cells) fashion to trigger a number of biological processes. The modulated signaling pathways are highly complex and the mechanistic understanding is not thoroughly understood. While a number of growth factors exist that exhibit cardioprotective effects, this review will focus on those that have been shown to induce regeneration in terms of progenitor cell recruitment and proliferation, cardiomyocyte proliferation, or reprogramming of another cell type to cardiomyocytes. Additional excellent reviews are available giving thorough descriptions of growth factors with alternative cardioprotective effects.[80]

One growth factor shown to increase survival and proliferation of cardiac stem and progenitor cells is IGF-1.[81] IGF-1 is a 72 kDa polypeptide made up of 70 amino acids that binds to its tyrosine kinase receptor IGF-1R and activates both the PI3K-Akt and MEK1/2-Erk1/2 signaling pathways.[82] IGF-1 is a key protein involved in muscle development,[83] and significant alterations in the IGF-1 system have been reported in patients after an acute MI. Lower IGF-1 levels upon acute MI are associated with worse outcomes, namely higher mortality and development of severe congestive heart failure.[84] A second growth factor involved in the recruitment and differentiation of cardiac stem cells is the hepatocyte growth factor (HGF). HGF, which is a heterodimeric molecule that consists of a 69 kDa α-chain and a 34 kDa β-chain linked by a disulfide bond, was originally discovered as a growth factor for hepatocytes and subsequently shown to have both anti-apoptotic and chemotactic properties with different cell types.[85] HGF signals through its tyrosine kinase receptor, c-Met, and different studies have shown that dosing HGF either intravenously or intramyocar-

dially to mice led to improved cardiac function after MI.[86] It is apparent that HGF exerts its effects through different mechanisms, including progenitor cell recruitment, reduction in apoptosis, and angiogenesis.

Because of the contributions of different mechanisms with both IGF-1 and HGF and with both signaling pathways being present in cardiac stem cells, Ellison et al. administered a combination of IGF-1 and HGF as a single dose by an intracoronary injection to pigs after an acute MI.[87] This led, in a dose-dependent manner, to significant activation and subsequent proliferation and differentiation of endogenous cardiac stem cells to form cardiomyocytes and regenerate the microvasculature. Cardiac function was improved two months after the dose was given, and the positive effects on survival and growth were still measurable. The authors reported that the generated cardiomyocytes were unlikely to be fully mature and hence unable to generate the full force of mature cardiomyocytes. Hence, further understanding of the factors that drive cardiomyocyte maturation is required.

Another protein involved in the recruitment of progenitor cells, in this case endothelial progenitor cells, is the chemokine stromal cell derived factor-1 (SDF-1). SDF-1, which is a protein that contains 68 amino acids, binds to the CXCR4 receptor and acts as a chemotactic agent for endothelial progenitors and hence, contributes to the regeneration of the vasculature.[88] SDF-1 is upregulated after MI, however, it is rapidly degraded by dipeptidyl peptidase-4 (DPP-IV) and matrix metalloproteinases including MMP2.[89] The use of recombinant SDF-1 is limited by its short half-life and low concentrations in injured areas of the heart.

However, elegant strategies have been designed to circumvent these issues. Segers et al. developed a protease-resistant SDF-1 variant (S-SDF-1(S4V)), in which a valine residue at position 4 was replaced with a serine to protect against MMP-2 cleavage; DPP-IV cleavage was reduced by adding a second serine residue at the N terminus, thus leading to increased stability.[90] S-SDF-1(S4V) was administered with self-assembling peptide nanofibers, to increase the concentration in the heart, in a rat MI model and resulted in increased cardiac function and density of blood vessels. In addition, treatment of mice after MI with the DPP-IV inhibitor diprotin A (**30**, Figure 11) also increased the concentration of SDF-1 in the heart, thus leading to an increased recruitment of progenitor cells.[91]

Separately, Ziegler et al. constructed the bispecific molecule SDF1-GPVI, consisting of SDF-1 and a recombinant form of the soluble platelet collagen receptor glycoprotein VI (GPVI).[92] GPVI binds to collagen at exposed extracellular matrix, hence preferentially binding to lesions with damaged vasculature and targeting SDF-1 to the site of action. The SDF-1-GPVI fusion protein delivered higher concentrations of SDF-1 to the injured regions of the heart and enhanced the accumulation of recruited cells, with evidence that angiogenesis is promoted and cell survival is sustained. SDF1-GPVI then significantly reduces the infarct size and preserves myocardial function in mice after MI.[92] This is an elegant approach to target the required molecule to the site of action and reduce systemic effects.

One further approach aimed for a controlled release of SDF-1 directly at the infarct site in the heart using a "patch". This was investigated by binding recombinant mouse SDF-1α covalently to fibrinogen through a PEG linker.[93] Briefly, fibrinogen was PEGylated using the benzotriazole carbonate derivative of PEG (BTC-PEG-BTC), followed by addition of murine SDF-1α and then thrombin to form a fibrin gel. Addition of the gel patch directly to the infarct area led to improved heart function and no expansion of the scar area.

The promise of SDF-1 recently gained further support with the disclosure of the results of a clinical phase I study with a nonviral DNA plasmid that encodes human SDF-1 (JVS-100).[94] JVS-100 was delivered by an endomyocardial injection to patients with ischemic cardiomyopathy, and improvements in clinical end points were observed after four months.

Neuregulin-1 (NRG-1), a member of the epidermal growth factor family of proteins, is widely expressed, including in the heart.[95] NRG-1 binds to the ErbB4 receptor, leading to its dimerization with the co-receptor ErbB2 and activating various signaling cascades, including the mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways. The involvement of NRG-1 in heart failure was first hypothesized following reports of clinical cardiotoxicity of the ErbB2 antibody trastuzumab, which is used as a treatment against breast cancer.[96] In addition, expression of NRG-1 is reduced in the later stages of heart failure.[97]

Administration of a recombinant human NRG-1 (rhNRG-1) containing 61 amino acids has shown beneficial effects on cardiac function and survival in models of chronic heart failure.[98] Different mechanisms have been implicated for its action, including increased cardiomyocyte cell cycle activity and proliferation through ErbB4 signaling,[5b] in both adult rat ventricular myocytes and adult mice. Our own recent data showed that NRG-1 increased incorporation of EdU in hiPSC-derived cardiomyocytes (Figure 7), demonstrating that NRG-1 treatment also promotes cell cycle entry in human cardiomyocytes (unpublished work). Clinical trials are currently performed with rhNRG-1 in patients with stable chronic heart failure, and encouraging results from two studies have been disclosed.[99] For the first study, rhNRG-1 was administered as daily intravenous infusions for ten days and, although not statistically significant, showed an increase in left ventricular ejection fraction and reductions in end-systolic and end-diastolic volumes at 90 days, suggesting beneficial effects on the long-term reversal of cardiac remodeling.[99a]

Studies with members of the fibroblast growth factor (FGF) family of proteins have shown their cardioprotective effects.[100] In addition, p38 MAPK (p38) induces cell cycle exit of many cell types and Engel et al. used a p38 inhibitor, SB203580 (**31**, Figure 11),[101] and showed that this molecule regulates the expression of genes that are important for mitosis in cardiomyocytes.[102] A combination of FGF-1 and SB203580 caused proliferation of adult rat ventricular cardiomyocytes at a level significantly greater than for FGF-1 or SB203580 alone. Thus, p38 inhibition, specifically inhibition of p38α, is required for FGF-mediated induction of the cell cycle and enhancement of the proliferative capacity of

cardiomyocytes. Treatment of rats after an acute MI with the combination led to reduced scarring and improved cardiac function, whereas p38 inhibition alone failed to rescue heart function and FGF-1 alone improves angiogenesis.[103] These studies show another example of potential synergies with combinations, consisting of agents from different modalities.

Thymosin β4 (Tβ4) is an endogenously occurring peptide consisting of 43 amino acids.[78] Administration of Tβ4, either intracardially or intraperitoneally, in a murine model after MI dramatically restored cardiac function over four weeks. A number of modes of action for Tβ4 have been proposed, including activation of integrin-linked kinase and Akt,[104] and stimulation of neovascularization by inducing the formation of endothelial and smooth-muscle cells.[105] Most recently, Smart et al. reported that pretreatment of mice with Tβ4 prior to inducing MI activated a population of cardiac progenitor cells known as epicardium-derived cells (EPDCs) and increased the number of EPDCs positive for the cardiac progenitor marker, Wilm's tumor gene 1 (Wt1 +).[106] The increase in EPDC population subsequently contributed to new cardiomyocyte formation and neovascularization, ultimately leading to improvements in functional parameters such as the ejection fraction and scar volume, which was reduced.

JI-38, a synthetic peptide agonist (consisting of 29 amino acids) of growth hormone releasing hormone (GHRH), was discovered in the search for more potent and stable derivatives than the shortened fragment, human GHRH-(1-29)-NH_2, of human GHRH.[107] Replacement of a number of the amino acid residues to reduce enzymatic degradation led to JI-38, a more potent agonist with a longer effect compared with native GHRH. Recently, JI-38 treatment following MI in rats led to improved cardiac function, and reduced infarct size and cardiac fibrosis compared to rat recombinant growth hormone (rrGH).[79] The proliferation of endogenous cardiac c-kit$^+$ progenitor cells was also increased. Interestingly, the effects exerted by JI-38 appear not to involve the GH/IGF-1 axis, as the circulating levels of these hormones are not increased upon treatment, in contrast to the treatment with rrGH. The mechanism behind the difference is unclear.

Exciting opportunities lie ahead for these peptides and proteins. Initial data in both animals and early clinical trials are encouraging, showing the potential to regenerate the heart after injury (Table 2). However, prolonged administration of growth factors will need careful monitoring for side effects such as cancer development or progression. In addition, an improved understanding of the mechanisms by which these compounds, for example, NRG-1, illicit their cardioprotective effects along with improved understanding of PK-PD and biomarkers will be needed.

An alternative, elegant strategy to the dosing of proteins and toward increasing their exposure in vivo is the administration of modified mRNA (modRNA) that encodes the protein of choice.[108] The mRNA can then be translated to produce the protein in situ using a cell's own protein synthesis machinery. Pioneering work of Karikó et al. has shown that replacement of uridine-5′-triphosphate with pseudouridine-5′-triphosphate during an in vitro transcription reaction provides modRNA, in which each uridine (**32**) is replaced with pseudouridine (**33**), a naturally occurring modified

Table 2: Heart repair and regeneration with miRNAs, small molecules, peptides, or proteins in models of myocardial infarction.

Strategic approach	Target cell type[a]	Species	Mechanism of action	Functional improvement	Follow up	Reference
Cardiomyocyte proliferation						
miR-15 family inhibitors[b]	CM	mouse	Proliferation	yes	21 days	[68]
miR-590/199a mimics[c]	CM	mouse	Proliferation	yes	60 days	[5c]
Neuregulin-1	CM	mouse	Proliferation	yes	14 weeks	[5b]
Epicardium-derived cells						
Thymosin β4	EPDC	mouse	expansion and differentiation to ECs and CMs	yes	28 days	[106]
Isx-1 & 2	EPDC	mouse	cardiomyogenic differentiation	yes	7 days	[13d]
c-kit$^+$ cardiac progenitor cells						
IGF-1/HGF[d]	CPC	mouse, pig	proliferation and myogenic differentiation	yes	21 days	[87]
JI-38	CPC	rat	Proliferation	yes	28 days	[79]
In vivo reprogramming of somatic heart cells						
miR1/133/208/499[e]	CF	mouse	conversion into CM	not determined	28 days	[8]
Bone marrow derived progenitor cells						
SDF-1[f]	BMC	rat	Recruitment	yes	28 days	[90]

[a] BMC: bone marrow derived progenitor cells; CF: cardiac fibroblast; CM: cardiomyocyte; CPC: cardiac progenitor cell; EC: endothelial cell; EPDC: epicardium-derived cell. [b] Subcutaneous injection of LNA-modified antimiRs. [c] Intramyocardial injection of AAV9 vectors expressing the miR. [d] Intracoronary injection of two proteins. [e] Intramyocardial injection of lentiviruses encoding miRs. [f] Intramyocardial injection of nanofibers.

nucleotide (Figure 12).[109] This modRNA has been shown to suppress the RNA-mediated immune activation and also enhance the translational capacity of the mRNA.[109]

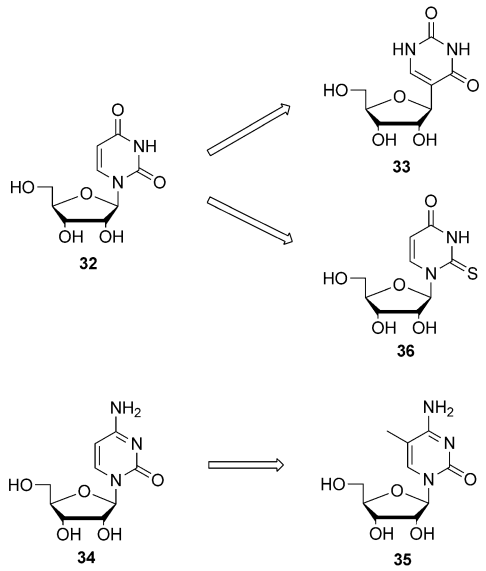


Figure 12. The modified RNA is synthesized using an in vitro transcription reaction with a T7 polymerase.[110] In this reaction the pyrimidine bases, uridine and cytidine, can be replaced.

Further work from Warren et al. on reprogramming human cells to pluripotency and directing differentiation showed that complete substitution of uridine with pseudouridine and replacement of cytidine (**34**) with 5-methylcytidine (**35**) further reduced the immune response and increased protein expression.[110] Kormann et al. also showed that replacement of 25% of uridine and cytidine with 2-thiouridine (**36**) and 5-methylcytidine, respectively, decreased mRNA binding to Toll-like receptors (TLRs) TLR3, TLR7, and TLR8 and the RNA-responsive immune sensor, retinoic acid inducible protein I (RIG-I); also, the activation of the innate immune system substantially decreased and the stability of the mRNA simultaneously increased, allowing prolonged protein expression.[111]

Very recently, Zangi et al. used modRNA that encodes human vascular endothelial growth factor-A (VEGF-A), an angiogenic factor that signals through its receptor KDR.[112] Intramyocardial injection of VEGF-A modRNA to mice gave improved myocardial outcome after MI, in part because of improved formation of functional vessels in the injured heart. Interestingly, the cardiac progenitor marker, Wt1, was dramatically upregulated by VEGF-A modRNA, thus showing that the pool of Wt1 + EPDCs was increased and the differentiation of these progenitor cells to endothelial cells was enhanced. A small increase in cardiomyocyte differentiation was also observed, although this increase alone is unlikely to translate to therapeutically meaningful results. This study highlights modRNA as an exciting new modality for the transient expression of proteins in vivo, and additional improvements in mRNA stability, further enhancing protein expression and delivery to the tissue (or even cell type) of choice could increase the opportunities further.

3. Challenges in Drug Discovery

Because of the complex architecture and function of the heart, many challenges need to be overcome for each modality before effective and safe pharmaceuticals are delivered. Despite progress that is made to elucidate the mechanisms behind many of the agents highlighted, there are still many unknowns on how different agents are eliciting their biological effects. For example, a number of the growth factors display multiple effects and the miRs, by nature, or antimiRs act on many genes and potential pathways. In addition, the physiology of adult stem cells is still poorly understood, which further complicates our understanding of where a given population of adult stem cells exists within the heart, how it is activated and/or mobilized, and how it is amplified and differentiates. Hence, much research is needed to understand the molecular mechanisms involved, and thus to provide new molecular targets and pathways for drug discovery. In addition, greater opportunities could lie with more phenotypic and polypharmacological approaches, as suggested by the efficacy of a number of combination therapies.

3.1. Translational Understanding

Advancing a lead compound through the drug discovery and development process requires progression from in vitro biochemical studies and cell assays, through efficacy studies in preclinical models, safety in animal studies, and translation into human patients. Translating in vitro activity into therapeutically beneficial in vivo efficacy is one of the greatest hurdles. Methods and techniques need to be developed to enable success within this area, including new imaging and diagnostics and predictive efficacy and safety biomarkers. An important caveat is that therapies routinely working in rodent disease models do not necessarily translate to the same effects in humans. Historically however, animal experiments have made a significant contribution to the understanding of human disease and encouraging data is available for some of the therapeutic modalities described. However, they need to be continually improved to better predict the effectiveness of treatment strategies in clinical trials.[113] Several animal species are used as models for cardiac regeneration. Non-rodent species provide advantages including large size, similarity to human physiology and pathology, and longer life span. For example, the size and anatomy of a pig heart more closely replicates that of a human heart and the disease state can be recapitulated more closely.

Translating from animals to humans requires an in-depth understanding of biological signaling pathways across species and species-specific properties. Differences in manipulating signaling cascades can give rise to opposite effects, which may complicate translational understanding. Notably, p38 MAPK negatively regulates the generation of hESC-derived cardio-

myocytes, whereas it promotes the formation of cardiomyocytes from mouse ESC.[114]

3.2. Timing of Treatment After a Myocardial Infarction

Whilst beating heart cells, albeit perhaps not fully mature, can already be generated in vitro, the understanding of the factors that guide the activation of cardiomyocyte progenitor cells and repair of the heart during development or after injury is developing. This knowledge will be needed to ensure the timely and effective control in stimulating the heart's regenerative capacity. For maximal effects, a thorough understanding of the PK–PD relationship of the agent will be crucial in order to establish the dosing regimen that results in the required drug concentration in the heart and thus the effective response. Small molecules have the potential to be precisely delivered to the heart and, with good understanding of PK–PD relationships, the doses can be titrated according to the response, offering an advantage in a dynamic clinical setting.

We need to be aware that lack of functional efficacy and even safety concerns may be a matter of timing. For example, administering a therapy immediately after MI may enhance efficacy of cardiac muscle regeneration and limit the fibrotic response. However, the strength of the heart wall needs to be sufficient to withstand the pressure of a beating heart and hence some fibrotic repair may be required. Eulalio et al. showed that the timing of miR expression is critical; the best result was achieved when the miRs were injected immediately after the MI, but not when administered several days later.[5c] In addition to timing, the length of treatment is likely important. Dosing rhNRG-1 for ten days improves function for at least 90 days.[99a] However, some treatments are likely to illicit an effect in an acute manner, while other agents may have a greater effect on long-term remodeling, and prolonged continuous therapy may indeed be required to observe an improvement in heart structure such as volume and shape of the ventricular chamber, functional endpoints, or a survival benefit.[9]

3.3. Safety Challenges

As many of the highlighted options are targeting fundamental developmental pathways, concerns have understandably been raised over the safety of regenerative therapies, including the potential for genetic instability, leading for instance to proliferative disorders including tumor formation and growth. The dosing of any compound will need to be understood, controlled, and their effects on other tissues carefully monitored. The development of methodologies to identify potential safety hazards, such as quantitative preclinical and clinical imaging technologies, will be fundamental to gain insight into the safety implications of stimulating repair of damaged tissue from endogenous stem cell sources. In addition, detailed gene expression profiling and bioinformatics analysis will be important to monitor gene expression patterns; this knowledge can also be utilized to improve drug candidates to accomplish desirable attributes, while eliminating or minimizing side effects.

There is also potential for undesirable epigenetic changes, which could have profound effects on cells, as the biology of normal adult stem cells is regulated by epigenetic programming.[115] Thus, it will be important to study the global epigenetic changes associated with treatment. Characterizing the relationships among the epigenome, pluripotency, and tumorigenicity should benefit the development of safe regenerative medicines.[116]

A strategy to reduce the potential of adverse events in noncardiac tissues is the targeting of the heart specifically and perhaps even the border zone (myocardial tissue of intermediate injury adjacent to the infarct) following a MI (Figure 13).[117] Several techniques can be used, including direct intramyocardial injection during open-heart surgery and catheter-based methods.[2b] However, for longer-term therapies, less invasive treatment will be necessary. Following a MI, the injured heart often expresses unique proteins that differentiate it from the surrounding healthy tissue. Conjugation of a drug molecule to a ligand for one of these proteins can then be used to target the molecule to the injured heart. One example is the SDF1-GPVI fusion protein, for which the GPVI protein is the targeting molecule which delivers SDF1 to the damaged vasculature.[92] Another is platelet (P)-selectin, a receptor on endothelial cells that mediates the rolling of cells, which is upregulated in ischemic myocardium. Scott et al. used a systemic injection of a P-selectin antibody conjugated with immunoliposomes carrying VEGF and showed improvements in cardiac function that were not observed with nontargeted VEGF.[118] These

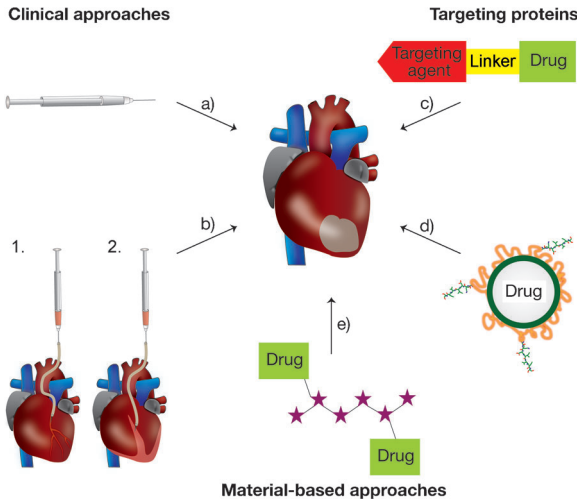


Figure 13. Strategies for targeting the heart with cardiac regenerative therapies: a) intramyocardial injection during open heart surgery; b) catheter-based approaches, such as, 1) intracoronary injection, and 2) intramyocardial injection; c) bispecific conjugates with a component to target the injured heart specifically, joined by an appropriate linker to the therapeutic agent; d) liposomes that contain drug molecules, coated through a linker with antibodies or peptides to target proteins or receptors in an injured heart; e) biopolymers as a drug delivery technique to locate therapeutic agents in the injured regions.

approaches highlight the potential to target different therapeutic modalities to an injured heart after systemic injections.

3.4. Need for Broad Interdisciplinary Collaboration

The outlined challenges and opportunities will require significant cross-disciplinary collaborations and for researchers to cross over into novel areas (Figure 14). As examples,

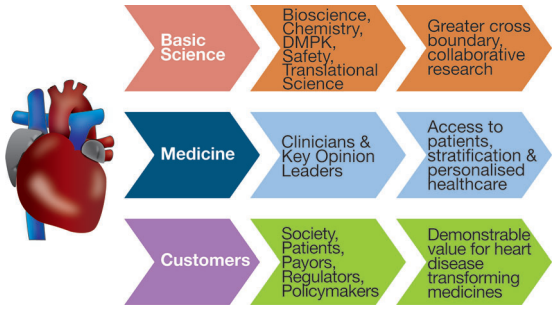


Figure 14. Significant cross-disciplinary research and collaborations will be required. DMPK = drug metabolism and pharmacokinetics.

there will likely be opportunities for combination therapy using different therapeutic modalities and a need to develop novel delivery techniques. Large steps need to be taken in understanding stem cell and developmental biology. In addition, many aspects of chemistry, drug metabolism and pharmacokinetics, and safety need to work across the borders of large and small molecules, work closely with experts in delivery techniques and device technology as well as in close collaboration with biologists and clinicians experienced in cardiovascular medicine. Many of these advances will be driven by highly productive collaborations between scientists in academic labs and industry, and will provide innovative breakthroughs for tackling this major challenge.

4. Conclusion and Outlook

Few therapeutic areas have enjoyed the enormous growth seen recently in the field of cardiac regenerative medicine. However, the long-term therapeutic potential remains uncertain. Studies with different chemical and therapeutic modalities are helping to build knowledge and understand the complex biology, and examples have shown positive effects on cardiomyocyte formation, reduction in the size of the damaged area after MI, and heart function. Some agents, such as NRG-1, showed beneficial effects in early clinical trials, leading the way to discover novel therapies. The use of small molecules to regenerate a damaged heart is less advanced, however, examples are starting to appear. In addition, recent years have seen the approval of the first small-molecule regenerative drug, eltrombopag, which acts in situ on resident progenitor cells in patients with thrombocytopenia.[119]

The development of novel screening methods, including phenotypic approaches, and access to relevant human cells and disease models are enabling the discovery of new agents. While it has been possible to deduce a number of signaling pathways and endogenous signals that drive the desired biological effects, one of the challenges is the deconvolution of the actual molecular target or targets. While this is a developing area, partly enabled by the discovery of powerful mass spectrometry techniques,[120] it is clear that new target deconvolution techniques will be required. These developments in the area of cardiac regeneration, including application to many of the examples discussed herein, may reveal interesting biological targets for intervention with alternative chemical modalities.

In-depth understanding of the modulated signaling pathways will be required both from an efficacy and safety perspective. This includes translation of the effects from animal models to humans and identification of any species differences that can affect the desired outcome. In addition, the timing of treatment will be important and hence, understanding PK–PD profiles as well as identification of suitable biomarkers in order to establish effective and safe dosing regimens will be required. Developments in chemistry and applications in biotechnology are enabling the synthesis, modification, and optimization of different therapeutic modalities to provide molecules with variable ADME and efficacy properties, thus allowing options to generate optimal PK–PD properties.

The effects of regenerative treatment on other tissues will need careful monitoring to ensure reduced side effects. This will be aided by developing methodologies such as quantitative imaging technologies, detailed gene expression profiling, and powerful bioinformatics analysis to monitor gene expression patterns. A strategy to reduce the potential of adverse side effects in noncardiac tissues is specific non-invasive targeting of the heart. Interesting examples are appearing, such as bispecific conjugates and immunoliposomes, and novel techniques, including their application to devices such as biopolymers, will allow exciting opportunities for targeting the heart after systemic administration. Many of these advances will be driven by highly productive collaborations between scientists in academia and industry.

As outlined herein, there is an increasing body of evidence to suggest that both endogenous adult stem cell modulation and induction of cardiomyocyte proliferation, as well as reprogramming of lineage-committed cells (e.g. cardiac fibroblasts) to produce functional cardiomyocytes with both small and large molecules is achievable. In addition, novel opportunities will be created to utilize chemistry in different contexts spanning across the traditional borders of small and large molecules. Although formidable technical hurdles still remain, the outlined regenerative therapeutic strategies could circumvent the scarcity of heart donors and human cardiomyocytes, and, most importantly, offer a transformatory impact in the treatment of heart failure.

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