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Reawakening Atlas: Chemical Approaches To Repair or Replace Dysfunctional Musculature

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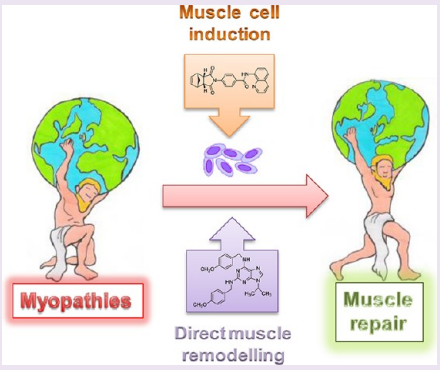
ABSTRACT: Muscle diseases are major health concerns. For example, ischemic heart disease is the third most common cause of death. Cell therapy is an attractive approach for treating muscle diseases, although this is hampered by the need to generate large numbers of functional muscle cells. Small molecules have become established as attractive tools for modulating cell behavior and, in this review, we discuss the recent, rapid research advances made in the development of small molecule methods to facilitate the production of functional cardiac, skeletal, and smooth muscle cells. We also describe how new developments in small molecule strategies for muscle disease aim to induce repair and remodelling of the damaged tissues *in situ*. Recent progress has been made in developing small molecule cocktails that induce skeletal muscle regeneration, and these are discussed in a broader context, because a similar phenomenon occurs in the early stages of salamander appendage regeneration. Although formidable technical hurdles still remain, these new advances in small molecule-based methodologies should provide hope that cell therapies for patients suffering from muscle disease can be developed in the near future.



The human musculature is composed of three tissue types: skeletal, cardiac, and smooth muscle. Unfortunately, a large number of diseases compromise muscle function and significantly impact on human health. For example, ischemic heart disease is a leading cause of death.[1] Other types of diseases include early onset genetic disorders, *e.g.*, Duchenne muscular dystrophy,[2] and late onset diseases with environmental elements dictating progression, *e.g.*, cardiovascular disease.[3,4] Muscle diseases also impact on developed countries because of their demographic aging.[5] For example, muscle wasting (sarcopenia) decreases skeletal muscle mass by roughly 40% between 20 and 60 years of age and compromises the ability of aged people to live independently (reviewed in ref 6).

Cell therapy is an attractive option to treat muscle diseases *via* the delivery of cells that participate in functional recovery of the dead or degenerating tissue.[7] The potential for cell therapy to be successful has already been demonstrated for other diseases, and the most prominent example is the transplantation of multipotent hematopoietic stem cells (derived from bone marrow) for certain types of chemotherapy-resistant leukemia,[8] reviewed in ref 9. However, for effective cell therapies to be developed for muscle diseases, an ample and reliable cell source is required. Stem cells are an attractive source for cell therapy because they have the potential to differentiate into the three muscle types or can divide to produce more stem cells (in the context of muscle disease, cell therapies are reviewed in refs 10−12). There are many different classifications of human stem cell, such as embryonic, fetal, amniotic, adult, and induced pluripotent stem cells (reviewed in 13−17). Thus, there are a number of stem cell sources with potential application as cell therapy approaches for muscle disease.

The successful application of stem cells to treat muscle disease relies on the development of rigorous and, ideally, simple protocols for stem cell culture and differentiation into "functional" muscle cells suitable for clinical application. This is an area where chemical biologists can make a significant impact, because small molecules are an attractive tool for modulating cell behavior (to be discussed below). An important example is the rapid modification of the 2006 seminal study reporting induced pluripotent stem cells (iPSCs) from mouse adult fibroblasts, by Takahashi and Yamanaka.[18] These "first generation" iPSCs relied on forced expression of four transcription factors linked to the maintenance of stem cell properties (Oct4, Sox2, c-Myc, and Klf4; now known as the Yamanaka factors[19]). Rapid progress by chemical biologists led to the development of protocols that can replace one or more of the Yamanaka factors with small molecules. For example, Professor Shen Ding, at the Gladstone Institute of Cardiovascular Disease, demonstrated that adult human epidermal keratinocytes can be converted to iPSCs by the forced expression of Oct4 and a two-step treatment with small molecule inhibitors of cell signaling pathways and regulatory mechanisms, such as transforming growth factor-β signaling, glycogen synthase kinase 3-dependent signaling, and histone acetylation.[20] Thus, small molecules have the potential to

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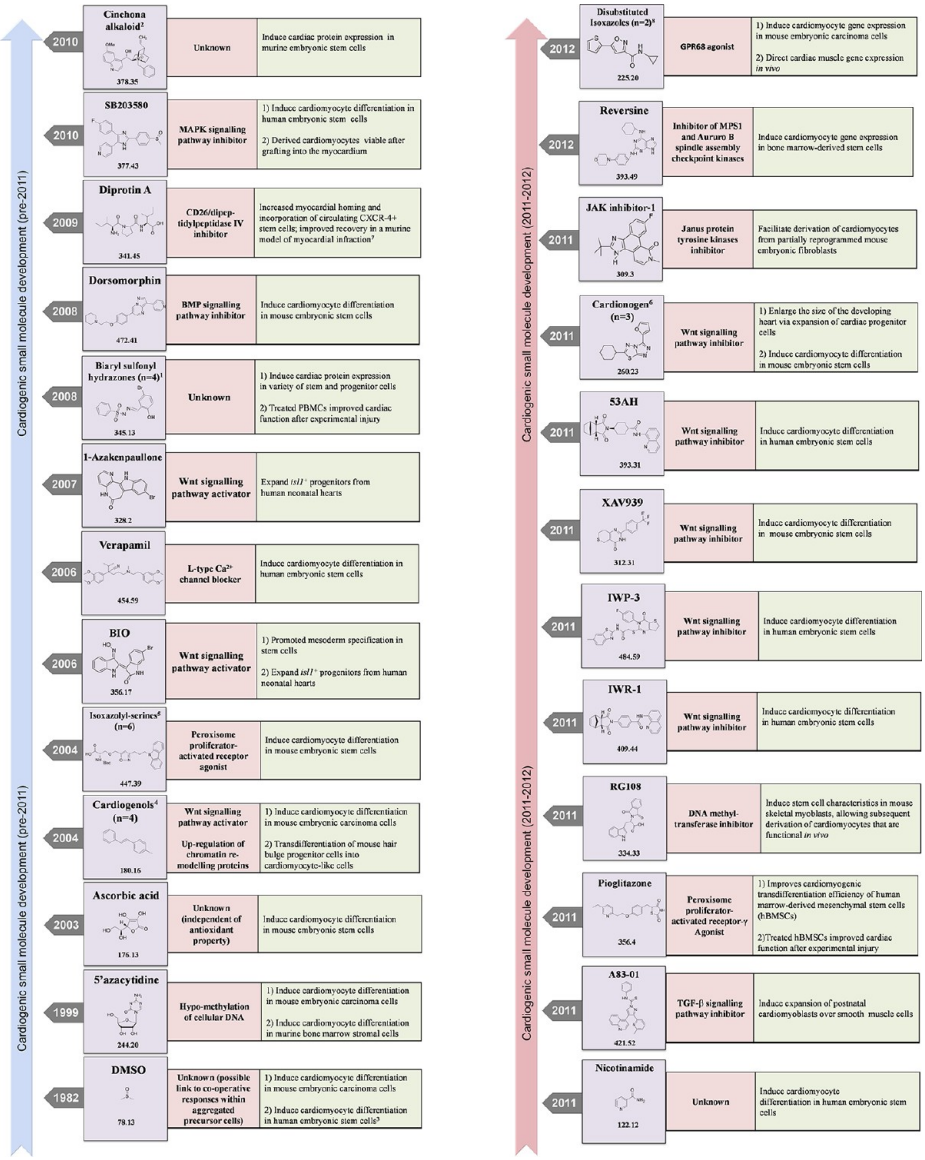

Figure 1. Timeline showing the discovery of small molecule inducers of cardiogenesis. Rapid research progress has been made in the years 2011 and 2012. The purple box shows the small molecule structure and molecular weight. The pink box shows the small molecule effect in cells (when known). The green box shows analyses of the cardiogenesis effect for the small molecule. Notes: [1]structure of sulfonyl hydrazone-1 is shown; [2]structure of the active alkaloid IV-8 is shown; [3]used in combination with ascorbic acid and 5-azacytidine; [4]structure shown is cardiogenol D (highest activity); subsequent analysis has indicated that cardiogenol C is inactive in mouse embryonic carcinoma cells;[164] [5]structure shown is isoxazolyl-serine 1a (highest cardiogenesis activity); [6]structure shown is cardionogen-1 (most characterized in the published study[74]); [7]used in combination with G-CSF-based stem cell mobilization; [8]these are a subset of previously described "hit" molecules for induction of cardiac gene expression.[58] The corresponding references for each molecule described in the figure are as follows: 5-azacytidine,[56,165,166] ascorbic acid,[132] cardiogenols,[167] isoxazoyl serines,[168] BIO,[53] verapamil,[169] 1-azakenpaullone,[170] biaryl sulfonyl-hydrazones,[58] dorsomorphin,[171] diprotin A,[75] SB203580,[172] cinchona alkaloid,[173] nicotinamide,[174] A83-01,[175] pioglitazone,[64] RG108,[62] IWR-1,[42] IWP-3,[42] XAV939,[45] 53-AH,[42] cardionogen,[74] JAK inhibitor-1,[176] reversine,[177] and disubstituted isoxazoles.[68]

substitute for "master"[21] transcription factors that determine cell fate.

In this review, we discuss small molecule-based methodologies that facilitate the development of cell therapies for muscle disease. In the past five years, great strides have been achieved in the application of small molecules to produce functional muscle cells from various stem cell sources. Our review will also discuss new small molecule approaches that directly induce muscle repair and regeneration. For skeletal muscle, this field has also produced significant developments in the past year, and because of similarities with urodele amphibian limb regrowth after amputation, this work will be discussed in a broader context of tissue regeneration. Our review will discuss the challenges of developing cell therapy for each muscle type (cardiac, skeletal, smooth), along with examples of diseases for which small molecules-based methodologies are contributing toward the development of therapies. Importantly, we will also describe the technical hurdles that remain to be addressed before a functional cure can be developed. However, before we discuss these topics, we would like to provide a summary describing why small molecules can be considered such useful tools for cell biology research.

SMALL REGULATORS OF BIG BIOSYSTEMS: WHY ARE SMALL MOLECULES SUCH AN ATTRACTIVE OPTION FOR CONTROLLING CELL BEHAVIOR?

Small molecule regulators possess distinct advantages over alternative methods, such as genetic manipulation. Small molecules have the potential to modulate multiple, specific effects within a cell, *via* simultaneous modulation of specific protein functions or by acting across different protein families (for example, retinoic acid, which targets nuclear receptors, or BIX 01294, which targets histone-modifying enzymes). Small molecules can also induce epigenetic mechanisms, which is an important consideration for manipulating cell fate. Small molecules are the probes for the field of chemical genetics, which have provided many important biological discoveries (example references are 22−24). The effects of small molecules are often reversible and can produce a high level of temporal control over target protein function, *via* the induction of rapid activation or inhibition. This allows flexible regulation of complex signaling networks. A high degree of control can also be achieved by fine-tuning the treatment concentration. These advantages facilitate the synergistic production of multiple effects to achieve an overall change in cell phenotype. Synthetic chemistry now provides almost limitless structural and functional diversity for small molecules, which gives huge potential for controlling molecular interactions that can be investigated by structural design or screening. This is an important advantage for clinical development. The upper mass limit for small molecules (around 800 Da) is also significant, because it allows the possibility of free diffusion across the cell membrane and is the upper limit for oral bioavailability. Small molecules are ideally suited for biomedical research, because they are cheap to purchase, have relatively simple storage or quality control requirements (compared to protein agents, such as growth factors), and are easy to use. However, small molecule methodologies are not faultless. For example, off-target events affecting proteins of a similar conformation can be problematic. Nevertheless, small molecule-based methodologies are gaining importance as regulators of cells fate and facilitating the development of approaches for regenerative medicine (this addressed in more detail in refs 25−27). Next, in this review we will discuss the impact of these methodologies on strategies to develop cell therapy for cardiac disease.

MENDING A BROKEN HEART: CHEMICAL TOOLS THAT ENHANCE THE PRODUCTION OF NEW CARDIAC CELLS OR FACILITATE CARDIAC REGENERATION

Cardiomyocytes are the muscle cells of the heart. Once thought incapable of regeneration, heart muscle has been recently shown to possess regenerative capacity to replace cardiomyocytes lost by natural turnover.[28] However, this potential for regeneration is much less than that of skeletal muscle, and catastrophic injuries, such as a heart attack (myocardial infraction), result in the ischemic loss of approximately 20% of the cardiomyocyte population, on the order of billions of cells. Cardiomyocytes in the ventricle are especially affected,[29] and tough, fibrotic scar tissue forms in their place. The inability to adequately replace these lost cardiomyocytes is a major contributory factor to the observation that patients suffering from a cardiac infarction (MI) generally progress to clinical heart failure.[29,30] Thus, cell therapy to deliver new cardiomyocytes to the infraction site is an attractive option for preventing this disease progression. Due to the significant impact of MI on the health of our society, the majority of novel small molecule-based methodologies for cardiomyocyte cell therapy aim to treat the consequences of this disease.

Figure 1 shows a timeline and overview delineating the discovery of small molecules that modulate cardiomyocyte differentiation. It can be seen that the pace of discovery is accelerating to become an important component of research in the derivation of cardiomyocytes from various cell sources or the characterization of cellular signaling pathways governing cardiomyocyte differentiation. These advances can be viewed in relation to the rapid progress made in combinatorial chemistry in the 1990s, which facilitates the generation of diverse small molecule libraries based on known bioactive molecules, to provide very large collections of structurally related compounds for drug discovery (in the order of thousands or tens of thousands; reviewed in refs 31−33). In addition, this decade also featured rapid progress in the development of small molecule screening technologies (reviewed in refs 34 and 35), and a prominent example, zebrafish-based screening, is discussed below. In this next part of the review, we will discuss the recent research advances that utilize these small molecules to generate cardiomyocytes from different cell sources, using specific examples from the research literature.

Small Molecule Methodologies for Cardiomyocyte Differentiation in Induced Pluripotent and Embryonic Stem Cells. The first indicator that chemicals can regulate cardiomyocyte differentiation was reported in 1982, with the finding that simple 1% DMSO treatment for 5 days can induce mixed cultures of cardiac and skeletal muscle cells to differentiate from mouse embryonic carcinoma cells (a cell line used at that time to model stem cell behavior[36]). Since then, embryonic stem cells or iPSCs have become available for researchers and the signaling pathways controlling cardiomyocyte differentiation during embryonic development have been characterized;[18,37] cardiomyocyte differentiation is reviewed in depth in ref 38. These advances have resulted in the production of ventricular cardiomyocytes (rather than atrial or nodal cardiomycoytes, which are also found in the heart) for cell therapy approaches to treat MI. Small molecule approaches are an integral part of this process, either in the development of a standardized culture protocol for cardiomyocyte differentiation, the search for new methods to simplify cardiomyocyte differentiation from stem cells, or the discovery of new agents that modulate this differentiation process. Interesting, recently published examples of each of these research approaches are discussed below.

Cardiomyocyte differentiation from stem cell cultures aims to repeat cardiomyogenesis that occurs during early embryonic development. Mouse-based studies have shown that cardiomyogenesis is initiated in the embryonic mesoderm, which forms by gastrulation at embryonic day 5 (E5) and involves the Nodal signaling pathway (reviewed in refs 39 and 40). Nodal signaling maintains the expression of bone morphogenetic protein 4, which induces Wnt pathway signaling in the proximal epibalsts. At E5.75, Wnt signaling induces the expression of meso-endodermal markers, and these in turn induce expression of mesoderm posterior protein 1 (MESP1), which is a "master regulator" of cardiac cell specification in the developing cardiac mesoderm.[41] MESP1 inhibits Wnt signaling to initiate cardiomyocyte specification *via* the expression of a number of cardiogenic regulatory genes, such as Nkx2-5 and Isl1. Thus, cardiomyocyte differentiation is an ordered, stepwise process

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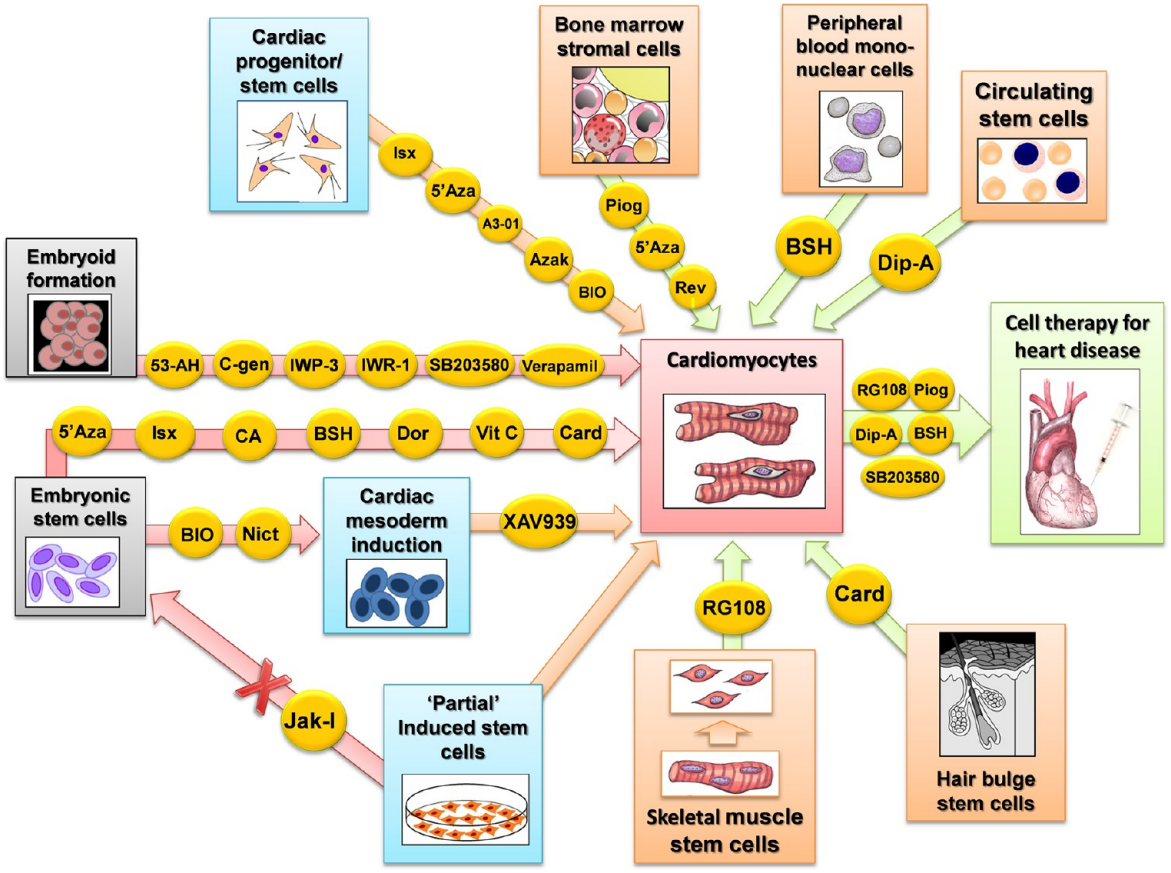

Figure 2. Functional grouping of small molecule inducers of cardiogenesis. It can be seen that the majority of reported small molecules induce cardiomyocyte formation directly in monolayer embryonic stem cells or in embryoid bodies, which are spheroid aggregates of embryonic stem cells, allowing differentiation into tissue types that are similar to embryonic tissue structures.[44] For simplicity, technical differences in the derivation of particular cell types have been omitted (for example, cardiomyocytes derived from bone marrow stromal cells treated with 5-azacytidine was achieved by rounds of subcloning cells and repeated treatment[56]). Small molecules on the arrow toward "Cell therapy for heart disease" induced the derivation of cardiomyocytes that improve heart function in animal models of cardiac infarction. Abbreviations: BSH, biaryl sulfonyl hydrazones; Azak, 1-azakenpaullone; CA, ainchona alkaloid; Dor, dorsomorphin; Vit C, ascorbic acid; 5′Aza, 5-azacytidine; Card, cardiogenol; Isx, isoxazoles; C-gen, cardionogen; Dip-A, diprotin A; Jak-I, JAK inhinitor-1; Piog, pioglitazone; Nict, nicotinamide; Rev, reversine.

involving the finely timed induction of various regulatory factors.

There are a number of different methods to induce cardiomyocyte differentiation from stem cell cultures, although they comprise a similar overall, stepwise approach: predifferentiation culture, differentiation format, addition of mesoderm induction factors, addition of cardiac specification factors (which commit mesodermal cells to the cardiomyocyte differentiation pathway), and addition of cardiac differentiation factors (reviewed in ref 40). Thus, small molecule approaches that can substitute for one of more of these steps would be advantageous for the generation of a simple, consensus, defined protocol for optimal cardiomyocyte differentiation. During cardiogenesis, it is apparent that Wnt pathway signaling plays an important, biphasic role during embryonic cardiogenesis. In the past year, two reports have shown that small molecule inhibitors of the Wnt pathway can be applied to cardiomyocyte differentiation from stem cells.[42,43] The first study, by Willems *et al.*, screened 550 small molecule modulators of cell signaling in MESP1-positive embryoid bodies (three-dimensional cell aggregates that respond to similar cues that direct embryonic development[44]) derived from human embryonic stem cells (hESCs) that express the mCherry red fluorescent protein under control of the myosin, heavy chain 6 (MYH6) cardiac-specific gene promoter. Small molecules were screened 4 days after embryoid body formation, with 6 days treatment and image analysis of mCherry expression after an additional 4 days. A "hit" compound found to significantly increase cardiogenesis was inhibitor of Wnt response 1 (IWR-1). Cardiomyocyte differentiation was confirmed by RT-PCR analysis of specific marker expression. The importance of Wnt signaling pathway inhibition was confirmed using two other small molecule inhibitors, 53AH (which is a more potent, structural analogue of IWR-1) and XAV939 (a tankyrase inhibitor that is cardiogenic in mouse embryonic stem cells[45]). This was an important confirmation, because it suggests that the effect of IWR-1 is not due to nonspecific effects. Of note, this approach illustrates an important, general technique for small molecule target validation in chemical genetics. Confirmation that a cellular regulatory pathway controls a phenotypic process can be achieved using multiple small molecules targeting the same pathway modulator, but possessing different, nonspecific activities. Moreover, the usefulness of small molecule-based approaches was shown by the finding that inhibitor of Wnt production 3 (IWP-3) also promotes cardiomyocyte differentiation. IWP-3 blocks endogenous Wnt signaling and indicates the importance of controlling endogenous Wnt production, rather than just controlling exogenously added

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factors. The importance of developing small molecule inducers of cardiogenesis was also confirmed by the finding that Dickkopf-related protein 1(DKK1), a regularly used cardiac differentiation factor, which mediates MESP1-induced Wnt signaling inhibition during cardiogenesis,[46−48] was over 40-fold less effective than these small molecule regulators.

The usefulness of small molecule inhibition of Wnt signaling was confirmed in a concomitant study published by Ren *et al.*[43] Employing 2 day small molecule blockade of Wnt signaling by IWR-1 or IWP-1 in 4 day, BMP-4 treated embryoid bodies derived from human ESCs or human iPSCs, this study provided a more detailed analysis of the phenotypic properties in differentiated cardiomyocytes. The differentiation efficiency of both adult and neonatate-derived iPSCs showed an approximately 8-fold improvement compared to previous reports (*e.g.*, ref 49) and reached nearly 40%, as measured by observing beating embryoid bodies. RT-PCR analysis of the atrial (MCL2A), ventricular (MLC2V), and nodal-like (HN4) marker expression suggested that differentiated cultures contained a mixed population of cardiomyocytes. Functional analyses, such as calcium transient duration and action potential duration, suggested that these cardiomycoytes possessed typical electrophysiological function and pharmacologic responsiveness. However, a caveat in this study is the finding that ESCs and neonatal iPSC show different efficiencies of beating embryoid body formation after treatment with IWR-1 or IWP-1. This suggests the need for further studies into unraveling the signaling networks that control cardiogenesis.

Figure 2 shows a classification of small molecules used in the derivation of cardiomyocytes from various stem cell sources. It can be seen from Figure 2 that a number of small molecule regulators of various cellular processes have been employed in cardiomyocyte differentiation. Unfortunately, a discussion of all of these small molecules is beyond the scope of this review, but we would like to point out a couple of additional, interesting examples. Kempf *et al.* have recently developed a serum-free, chemically defined differentiation protocol for cardiomyocyte differentiation from a human ESC cell line, based on small molecule inhibition of mitogen-activated protein kinases (MAPK) activity using SB203580.[50] Reducing the use of serum in culture is advantageous for cell therapy approaches, because serum can promote nonspecific differentiation and there is potential for contamination by serum-derived infectious agents or xenoantigens, which could cause an immunogenic response after transplantation.[51,52] Treatment of 1 day embryoid bodies with 5 μM SB203580 for up to 14 days could induce up to 80% beating embryoid bodies in the culture. In addition, Kempf *et al.* use their small molecule as a probe to extensively characterize the role of MAPK signaling during cardiogenesis. However, a limitation of this study is that cardiomyocytes were analyzed by the expression of specific markers; there was no assessment of electrophysiological properties.

Cardiomycoyte specification *in vivo* involves biphasic Wnt signaling (as described above). Thus, in addition to small molecule Wnt inhibitors, small molecule Wnt signaling activators have also been exploited for cardiomyocyte differentiation from human ESCs.[53] Naito *et al.* treated embryoid bodies at day 0 with BIO (a small molecule activator of Wnt signaling *via* inhibition of glycogen synthase kinase-3β) for 3 days and observed an increase in beating embryoid bodies at day 15. Thus, BIO treatment induces cardiomyocyte lineage commitment in the embryoid body (equivalent to the mesoderm stage) but should be removed for cardiomyocyte differentiation to proceed (as confirmed by the finding that Wnt signaling activation at day 5 inhibits cardiomyocyte differentiation and enhances the expression of hematopoietic and vascular cell-specific genes[53]). Thus, it can be envisaged that a small molecule-based protocol can be developed, based on Wnt signaling activation or inhibition at early or late stages, respectively.

Unfortunately, there are problems associated with potential cell therapy applications using ESCs, such as ethical issues,[54] and iPSCs may induce immunogenicity.[55] Therefore, the use of alternative cell sources for cardiomyocyte generation has become an attractive research option and small molecule approaches are making important contributions to this endeavor. We will discuss some interesting examples in the next section of this review.

Small Molecules That Induce Cardiomyocyte Differentiation in Tissue-Specific Stem Cells. The first report that a small molecule can induce cardiomyocyte differentiation in tissue-specific stem cells was by Makino *et al.*, in 1999, with the demonstration that 7 day treatment of immortalized murine bone marrow cells with 3 μM 5-azacytidine (an inhibitor of DNA methyltransferase, which induces epigenetic changes in gene expression patterns) could induce beating in approximately 30% of the cells and differentiation into fetal ventricular cardiomyocytes.[56] As shown in Figures 1 and 2, some interesting small molecule inducers of cardiogenesis in tissue-specific stem cells and progenitor cells have been published, such as the induction of cardiomyocyte marker expression in murine hair bulge progenitor cells by treatment with 5 μM Cardiogenol C for 4 days, which inhibits Wnt signaling[57] and the ability of a family of sulfonyl-hydrazones to induce cardiac mRNA and protein expression in human mobilized peripheral blood mononuclear cells 4 days after 72 h treatment.[58] Two recently published articles illustrate the potential of this approach for developing cell therapies for MI and these are discussed below.

Skeletal myoblasts (SMs) are muscle stem cells that reside beneath the basal lamina that surrounds muscle fibers.[59] SMs were the first cell therapy approach for treating MI.[60] However, reported improvements in ventricular function may not be sustained, and grafted SMs are not electrically integrated, which may cause arrhythmias.[61] However, skeletal muscle can regenerate readily after biopsy, and SMs represent a useful source of tissue-specific stem cells. Pasha *et al.* have recently reported the induction of stem cell characteristics in murine SMs by treatment with RG108, a DNA methyltransferase (DNMT) inhibitor.[62] SMs were shown to express three of the four Yamanaka factors: Sox 2, Klf4, and cMyc. SMs were harvested from Oct3/GFP-transgenic mice, treated with 500 μM RG108 for 5 days, and then cultured on mouse embryonic fibroblast feeder cells for 3 weeks. SMs that expressed GFP (termed SiPs) were isolated and induced to undergo embryoid body formation. By day 5, beating bodies were observed and isolated for further analysis. Cardiomyocyte differentiation was shown by analysis of early and late specific marker expression, and importantly, the clinical potential of these cells was assessed in a murine, immunocompetent model of acute MI. Thirty thousand differentiated cells were injected into the left ventricle 3−4 times at 10 min after MI induction. Four weeks later, grafted cells were shown to form intercalated discs and gap junctions with the host cardiac cells, suggesting that electrical coupling had taken place. In addition, fibrosis was

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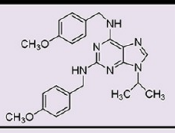
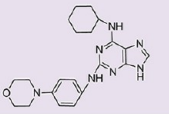
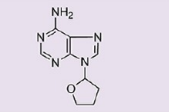
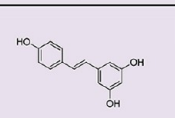
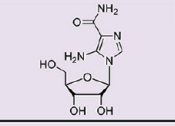
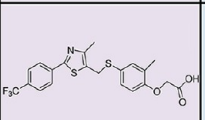
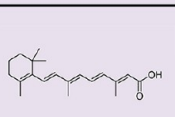
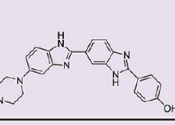
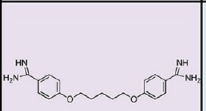
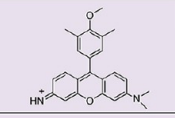
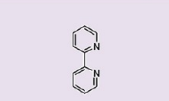
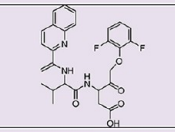
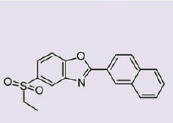
significantly reduced and cardiac function was improved, as shown by increased left ventricle ejection fraction and left ventricle fractional shortening. Overall, this study seems to offer an attractive small molecule-based method for inducing cardiogenesis in SMs, although further studies should address issues such as electrophysiological properties of differentiated cardiomyocytes and whether treatment with RG108 can induce similar responses in human SMs.

Bone marrow cells contain two tissue-specific stem cell populations: hematopoietic (HSCs) and mesenchymal (MSCs).[10] Cell therapy with patient-derived bone marrow cells has been studied in clinical trials of MI, but the results have been mixed (reviewed in ref 63). Thus, approaches to increase the performance of these stem cells in animal models of MI may lead to new strategies for clinical trials. Shinmura *et al.* have recently reported that transdifferentiation of MSCs in cardiomyocytes can be enhanced by treatment with the small molecule pioglitazone, an activator of perosixome proliferators-activated receptor-γ (PPAR- γ) signaling that increases the expression of mitochondrial genes.[64] It is known that cardiomyocytes rely on free fatty acids as a major energy source and have distinct mitochondrial morphology.[65] Thus, the rationale for this study was that small molecule stimulation of PPAR- γ signaling would enhance the reported low level of cardiomyocyte transdifferentiation in MSCs.[64,66] Human bone marrow MSCs were treated with 3 μM pioglitazone for 2 weeks before co-culture with murine cardiomyocytes. This approach produces a dramatic increase in cardiomyocyte transdifferentiation after two weeks of co-culture, as shown by an approximately 10-fold increase in the number of cells expressing the cardiomyocyte marker, troponin-I. Importantly, transdifferentiated cells were evaluated in a rat immunodeficient MI model. One to two million cells were injected into the ischemic border zone of the ventricle two weeks after MI. Interestingly, daily 2.5 mg/kg pioglitazone was also administered orally to one experimental group. Analysis was carried out two weeks later and revealed that cardiac parameters, such as fibrosis volume and left ventricle systolic pressure, were improved after transplantation. However, surviving MSC-derived cardiomyocytes were rare, suggesting that these improvements in cardiac function may be caused by factors secreted from the transplanted cells, which could produce anti-apoptotic effects or inhibit post left ventricular remodeling after MI.[64] A most interesting finding from the study was that oral pioglitazone treatment significantly increased the number of surviving, transplanted cells and blood vessel formation in the infarction zone. Although the precise mechanism of orally delivered pioglitazone on transplanted MSC-derived cardiomyocytes was unknown, and cardiac parameters were not improved compared to transplantation with MSC-derived cardiomyocytes alone, this study does show the potential for small molecule methods to enhance tissue-specific stem cell transplantation into cardiomyocytes and influence the cardiac cellular environment post-MI. Recent advances in these remodeling-based strategies have been reported, and we will discuss some pertinent examples in the next section of this review.

Small Molecules That Directly Remodel the Cardiac Ventricular Environment after MI. Recently, there has been increasing appreciation of the inherent ability of heart tissue to undergo regeneration, and resident cardiac stem cell populations, such as epicardium-derived stem cells, are known to participate in cardiac remodeling after MI.[67] Given the difficulties associated with cardiomyocyte transplantation approaches for treating MI, such as cell death after grafting, new small-molecule based methods are being developed that directly modulate the heart's own regenerative response post-MI. Russell *et al.* have recently published two studies that further characterize the cardiogenic effects of a family of 3,5-disubstituted isoxazoles (Isx), which were initially discovered as enhancers of myocardial repair by murine p19 embryonic carcinoma cells.[68] This study utilized TNR transgenic mice, which allows monitoring of progenitor cell activation by Notch signaling, which has been shown to regulate cardiac progenitor cell (CPC) differentiation.[69] Animals received daily IP treatment with Isx for 7 days, with or without induction of MI. Analysis of the CPC population in the epicardium showed that Isx treatment activated these cells to proliferate and drove specification toward the cardiomyocyte lineage, as demonstrated by increased expression of specific markers, such as Nkx2-5. However, induction of MI immediately before administration of Isx overrode this effect by day 21 and induced CPC differentiation toward fibrotic and angiogenic cell lineages, as assessed by expression profiling. Thus, higher efficacy Isx small molecules will be required for increasing regenerative repair after MI. However, the ability of Isx to induced cardiogenesis in this CPC population is a new indicator of the usefulness of developing small molecule modulators of cardiogenesis, and this was supported by a subsequent study by Russell *et al.* that characterized the cellular mechanism of Isx action.[70] As the authors' state, using Isx as a chemical "black box" to discover novel drug mechanisms in an unbiased manner, mechanistic studies of calcium release in murine epicardial CPCs led to the conclusion that G(q) protein-coupled receptor (G(q)PCR) receptors were candidate targets for Isx. Subsequent analysis identified the extracellular proton/pH-sensing GPCR GPR68 as the target and analysis of murine hearts after MI showed that epicardial CPCs expressing GPR68 formed a zone of proton-sensing cells around the site of MI (where pH is dropping as acidic metabolic by products accumulate (such as lactate)). Isx pharmacologically regulated gene expression (mRNAs and miRs) in this GPR68-enriched border zone, driving cardiomyogenic and pro-survival transcriptional programs *in vivo*. We believe this study represents a powerful example of small molecule based approaches, which allow both the characterization of cell signaling pathways (*via* chemical genetics) and the development of new pharmaceuticals.

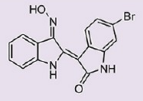
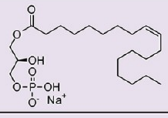
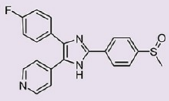
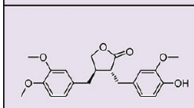
As mentioned above, the 1990s witnessed rapid progress in the development of small molecule screening technologies. For example, the zebrafish system was developed as a powerful, vertebrate-based system for chemical library screening.[71,72] The maintenance cost is less than 1% that of mice, they can grow in multiformat plates, and chemical treatments produce very similar toxicological and teratological effects in zebrafish and humans. Zebrafish are also useful for cardiovascular research because they possess a closed cardiovascular system, rapid development, and suitability for genetic analysis. Mutated zebrafish have been generated with different cardiovascular phenotypes that closely correlate with human disease (reviewed in ref 73). Small molecule screening in zebrafish larvae led to the recent report by Ni *et al.*, describing the discovery of Cardionogen 1-3, thiadiazole compounds that can remodel the zebrafish heart during the first 72 h of development.[74] Employing embryos transgenic for GFP under the control of the cardiac myosin light chain 2 promoter, Ni *et al.* showed that

Table 1. Development of Small Molecules That Induce Skeletal Muscle Dedifferentiation, Enhance Myopathic Muscle Repair, or Improve Its Functional Performance

Structure	Name & M.W.[a]	Mechanism	Analyses[b]	Laboratory & year[c]
	Myoseverin[e] 432.5	Inhibition of microtubule polymerization	Induce myotube cellularization and decrease CDKI expression(*121, 178*)	**Prof. S Schultz** Department of Chemistry, The Scripps Research Institute, CA (2000)
	Reversine 393.49	Inhibitor of MPS1 and Aururo B spindle assembly checkpoint kinases	Induce pluripotent behaviour in cellularized myotubes[d](*119*) and myoblasts(*179*)	**Prof. S Ding** Department of Chemistry, The Scripps Research Institute, CA (2004)
	SQ22536 205.2	Adenylyl cyclase inhibitor	Induce pluripotent behaviour in cellularized myotubes(*118*)	**Prof. D Williams** Gwangju Institute of Science and Technology, South Korea (2012)
	Resveratrol 228.2	Activation of SIRT1 and PGC-1alpha	Enhance muscle endurance(*85*)	**Prof. J Auwerx** Institut de Génétique et de Biologie Moléculaire et Cellulaire, France (2006)
	AICAR (Acadesine) 258.23	AMPK agonist	Exercise mimetic that enhance muscle endurance(*83*)	**Prof. RM Evans** Gene Expression Laboratory, Salk Institute, CA (2008)
	GW1516 (GW-501516) 453.498	PPARβ/δ agonist	Enhance muscle endurance synergistically with exercise(*83*)	**Prof. RM Evans** Gene Expression Laboratory, Salk Institute, CA (2008)
	Retinoic acid 300.4	Ligand for DNA-bound retinoic acid receptor	Induce skeletal myogenesis in mouse embryonic stem cells(*180, 181*)	**Prof. I Skerjanc** Department of Biochemistry, The University of Western Ontario (2009)
	Hoechst derivatives 379.31	Binds expanded CTG trinucleotide repeats in *DMPK* mRNA	Inhibits MBNL1 protein binding, reducing slicing defects(*105*)	**Prof. MD Disney** Department of Chemistry, University at Buffalo (2009)
	Pentamidine 310.27	Binds expanded CTG trinucleotide repeats in *DMPK* mRNA	Inhibits MBNL1 protein binding, reducing slicing defects(*104*)	**Prof. JA Berglund** Department of Chemistry and Institute of Molecular Biology, University of Oregon (2009)
	B25 349.28	NF-κB Activator	Induce myotube cellularization into viable muscle cells(*182*)	**Prof. YT Chang** Laboratory of Bioimaging Probe Development, National University of Singapore (2010)
	BpV(bipy) 380.3	Tyrosine phosphatase inhibitor	Induce myotube cellularization into myoblasts capable of muscle repair[f] (*120*)	**Prof. IM Conboy** Department of Bioengineering, University of California (2011)
	Q-VD-OPH 513.8	Anti-apoptosis caspase inhibitor	Induce myotube cellularization into myoblasts capable of muscle repair[g] (*120*)	**Prof. IM Conboy** Department of Bioengineering, University of California (2011)
	SMTC1100 (BMN195) 322.27	Unknown	Upregulates utrophin expression and alleviates symptoms in Duchenne muscular dystrophy models (*98*)	**Prof. KE Davies** MRC Functional Genomics Unit, University of Oxford (2011)

dx.doi.org/10.1021/cb3003368 | *ACS Chem. Biol.* 2012, 7, 1773−1790

Table 1. continued

Structure	Name & M.W.[a]	Mechanism	Analyses[b]	Laboratory & year[c]
	BIO 356.17	Wnt signaling pathway activator	Induce pluripotent behaviour in cellularized myotubes(*118*)	Prof. D Williams Gwangju Institute of Science and Technology, South Korea (2012)
	Lysophos-phatidic acid 436.52	Activation of G-protein-coupled receptors	Induce pluripotent behaviour in cellularized myotubes(*118*)	Prof. D Williams Gwangju Institute of Science and Technology, South Korea (2012)
	SB203580 377.43	MAPK signaling pathway inhibitor	Induce pluripotent behaviour in cellularized myotubes(*118*)	Prof. D Williams Gwangju Institute of Science and Technology, South Korea (2012)
	Arctigenin 372.4	Increases AMPK phosphorylation	Enhances muscle endurance[h](*84*)	Prof. X Shen State Key Laboratory of Drug Research, Chinese Academy of Sciences, Shanghai(2011)

[a]Molecular weight. [b]Number(s) in brackets refer to the relevant reference for each small molecule. [c]Refers to the date of the first published report. [d]In conjunction with siRNA knock-down of the cyclin-dependent kinase inhibitor (CDKI), p21. [e]A structurally related compound, myoseverin B, is also available.[121] [f]Used in conjunction with the apoptosis (Q-VD-OPH) inhibitor. [g]Used in conjunction with the tyrosine phosphtase (BpV(bipy)) inhibitor. [h]Potential synergistic effect with exercise training was not assessed.

10 μM Cardionogen treatment at 5 h post fertilization (hpf) induced myocardial hyperplasia at 72 hpf, which was due to increased cardiac cell numbers in both the atrium and ventricle, resulting from Cardionogen-induced expansion of cardiac progenitor cells. Interestingly, further analysis demonstrated that 1 μM Cardionogen treatment for 8 days could induce cardiomyocyte differentiation in 4 d embryoid bodies derived from murine ESCs, although the subtype (ventricular, atrial, or nodal) or electrophysiological properties were not assessed. Encouragingly, the mechanism for Cardionogens was assessed, and these molecules were found to be inhibitors of Wnt signaling. Further testing of Cardionogen in adult zebrafish may also be rewarding, but the route of administration will have to be considered, because an advantage of zebrafish larvae for screening is that small molecules can be simply added to the fish water. This report of a small molecule inducer of myocardial hyperplasia and subsequent studies in mammalian models of cardiac function could be of great interest.

Recently, some other examples of small molecules that influence cardiac tissue remodeling, either during development or after MI, have been published.[75−77] Unfortunately, a discussion of all of these is beyond the scope of this review. However, we would like to point out the studies by Zaruba *et al.* and Theiss *et al.*, which report an interesting application for Diprotin A, a small molecule inhibitor of dipeptidylpeptidase activity.[75,78] Stromal cell-derived factor 1α (SDF-1α) is the major chemokine attracting endogenous endothelial progenitor homing to the heart. However, SDF-1α can be cleaved by dipeptidylpeptidase IV. Zaruba *et al.* demonstrated that combined administration of granulocyte-colony stimulating factor, a chemokine that functions to mobilize progenitor cells, including endothelial progenitors, from bone marrow and Diprotin A *via* intraperitoneal administration enhanced the recruitment of progenitor cells to the myocardium and improved cardiac function after MI, *via* increased angiogeneis, leading to increased survival. The authors of this review believe that this approach, which directly modulates the ischemic environment after MI, may be a profitable direction for future research because it avoids some of the technical limitations associated with cell transplantation (which are discussed in more detail in the conclusion section of this review).

In summary, a large number of cardiogenic small molecule modulators are now available. Current research is focusing on developing new, functionally superior small molecule regulators of the key pathways governing cardiomyocyte differentiation, such as Wnt signaling. Moreover, previously characterized bioactive small molecules are being applied to cardiomyocyte differentiation protocols (*e.g.*, the MAPK inhibitor, SB203580). In addition, cardiomyocyte differentiation from tissue-specific stem cells is being facilitated by small molecule epigenetic modulators (*e.g.*, the DNMT inhibitor, RG108). Finally, the MI zone itself has become a direct target for remodeling by small molecules to make this environment more permissive for functional recovery by resident cardiogenic cells (*e.g.*, Isx or pioglitazone). Thus, small molecules are now an integral component of the diverse, exciting strategies to combat progression to clinical heart failure.

REGAINING LOST STRENGTH: SMALL MOLECULE APPROACHES TO COMBAT SKELETAL MUSCLE DISEASE

Skeletal muscle comprises approximately 40% of an adult's body mass and consists of approximately 600 individual muscles.[79] Unfortunately, cell therapy using intramuscular injection results in only limited migration from the injection site (in the order of millimeters; reviewed in refs 80 and 81). Non-muscle stem cell sources possessing myogenic potential and homing after systemic delivery have been identified, but this approach has not yet produced a viable therapy for myopathies.[82] Thus, small molecule-based approaches may provide an alternative strategy to induce muscle repair.

A list of small molecules that modulate the regenerative capacity of muscle tissue is shown in Table 1. These molecules can be broadly subdivided into two groups: (a) those that directly enhance muscle repair and (b) those that reduce the differentiated phenotype of skeletal muscle, allowing the

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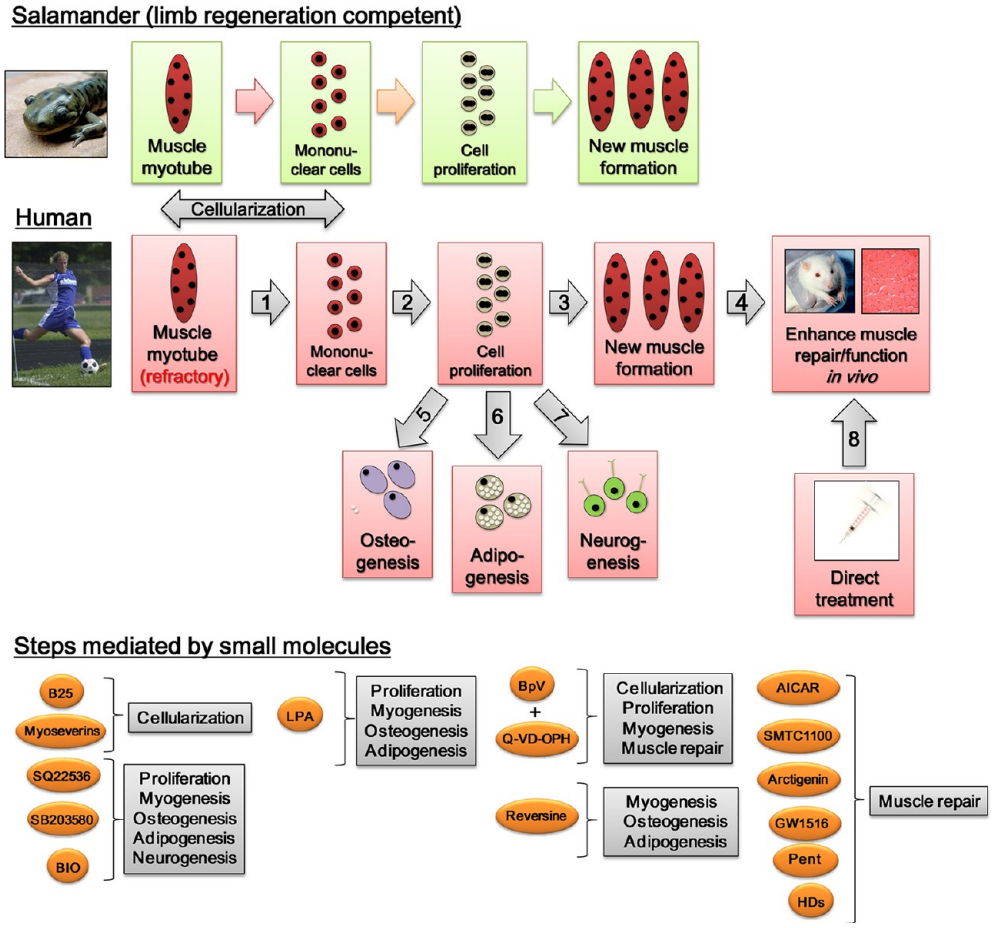

Figure 3. Small molecule-based strategies to induce skeletal muscle dedifferentiation or enhance muscle function and repair. Urodele amphibians, such as the salamander, undergo muscle dedifferentiation (cellularization and proliferation) as part of a limb regeneration process. In contrast, mammalian skeletal muscle is refractory. However, recent advances have shown that small molecule treatments can induce muscle dedifferentiation in mammalian cultured muscle. This approach also has the potential to induce regeneration of diseased muscle in humans or provide alternative, useful cell types, such as neurons, adipocytes, or osteoblasts (by subsequent cell culture with appropriate differentiation media). Moreover, small molecules have been developed that directly correct muscle disease gene defects or enhance muscle performance to combat age-related muscle degeneration, such as sarcopenia.

derivation of stem cells. This categorization is shown in Figure 3, which also shows how these molecules affect muscle regeneration. In this review we will first discuss the direct enhancement of muscle repair.

Small Molecules That Directly Enhance Skeletal Muscle Repair. Small molecules that enhance repair can be further classified into two groups. Members of the first group enhance the functional performance of skeletal muscle and can be classified as exercise mimetics.[83−85] These molecules (resveratrol, a plant product produced in response to pathogens, and arctigenin, a plant product found in the greater burdock (*Arctium lappa*); AICAR and GW1516) have interesting therapeutic potential, because they may be suitable for development as therapeutic agents to inhibit or even reverse sarcopenia. It should be noted that these compounds were shown to modulate muscle gene expression in a pattern similar to the effects of exercise, rather than just acting as performance-enhancing stimulants (as reviewed in ref 86). In addition, the discovery of these small molecule exercise mimetics has aided an understanding of the key components of the cellular signaling pathways that trigger exercise-induced transcriptional remodeling of skeletal muscle. An example was the finding that inclusion of resveratrol in dietary intake activates silent information regulator two protein-1 (SIRT1) in murine skeletal muscle.[85] SIRT1 is a deacetylase that targets a large number of proteins implicated in the regulation of cellular processes, such as fat and glucose metabolism, in response to environmental cues (the various effects of SIRT1 in cells are reviewed in ref 87). The exercise mimetic effect of resveratrol was linked to SIRT1-mediated deacetylation and activation of peroxisome proliferator-activated receptor (PPAR) γ coactivator (PGC-1α), which is a master regulator of mitochondrial biogenesis and a transcriptional coactivator of numerous genes linked to energy metabolism (reviewed in ref 88). Moreover, PGC-1α has been shown to be activated in human skeletal muscle by endurance training.[89] The exercise mimetic AICAR is a nucleoside that enters the cells and converts to a ligand for AMP-activated protein kinase (AMPK), which forms a complex with PGC-1α. Intraperitoneal AICAR treatment for 4 weeks was found to increase exercise endurance in mice on its own, while another small molecule, GW1516, could increase endurance only in combination with exercise training.[83] GW1516 activates PPARδ, which is part of a transcriptional complex that recruits PGC-1α to target gene promoters. Recently another natural product insulin mimetic has been reported by Tang *et al.*, who show that daily arctigenin treatment in mice by intraperitoneal

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injection increased AMPK activity in skeletal muscle *via* the calmodulin-dependent protein kinase kinase and serine/threonine kinase 11(LKB1) signaling pathways. These effects lead to the upregulate expression of PGC-1α to exert an exercise mimetic effect, as determined by increased performance in a treadmill test.[84] In summary, these small molecule exercise mimetics have made a valuable contribution to our understanding of exercise-induced muscle remodeling and they also possess therapeutic potential as candidate agents to treat muscle wasting disorders, such as sarcopenia.

The second group of small molecules that enhance muscle repair are designed to overcome genetic mutations that produce myopathy. Duchenne muscular dystrophy (DMD) is caused by mutations in the dystrophin gene. It is an X-linked recessive, lethal myopathy with an incidence of around 1 in 3,600 boys.[90] Death generally occurs in the third decade. The dystrophin gene is one of the longest genes in humans and encodes a protein of over 35,000 amino acids.[91] In normal skeletal muscle fibers, dystrophin forms part of a protein complex that connects the cytoskeleton to the extracellular matrix. Loss of dystrophin eventually leads to muscle fiber death due to calcium influx through the cell membrane (sarcolemma) and mitochondrial rupture.[92,93] Genetic therapies for DMD are hampered because recombinant viral vectors that efficiently transduce muscle cannot carry the full-length dystrophin gene. Muscle-forming cells that can carry the dystrophin gene are technically challenging to produce in numbers sufficient to adequately replace the diseased musculature (these approaches are reviewed in ref 81). Antisense molecules designed to make ribosomes skip premature stop codons in the dystrophin gene, which affects around 10−15% of DMD patients, have shown restoration of dystrophin expression in clinical trials.[94] However, optimizing this approach to treat all DMD patients would require the use of viral vectors, such as AAV, but this is hampered by latent immunity in the general population.[95]

An attractive, small molecule-based alternative approach to antisense strategies for DMD therapy aims to use molecules that can upregulate expression of the gene, utrophin, which localizes to the muscle membrane sarcolemma in fetal muscle but is replaced by dystrophin during development.[96] Utrophin can compensate for dystrophin deficiency in the skeletal muscles of murine models of DMD.[97] Significantly, Summit plc, in conjunction with the MRC Functional Genomics Institute at the University of Oxford, has recently reported SMTC1100, the first small molecule that can upregulate utrophin expression.[98] SMTC1100 orally administered for 4 weeks produces an approximately 20% increase in utrophin mRNA expression in the diaphragm of a mouse model of DMD. Moreover, these mice showed improved performance in the ability to resist fatigue after forced exercise, which is analogous to the 6 minute walk test used as a the pivotal outcome measure in DMD clinical trials. Interestingly, a dramatic synergistic performance increase was achieved by co-administration of the anti-inflammatory drug prednisone, which is a clinically approved agent for DMD. Thus, SMTC1100 is now ideally placed to enter clinical trials for DMD. However, optimism should be cautious, in light of the recent clinical trials failure of Ataluren, a small-molecular agent designed to make ribosomes become less sensitive to, or possibly ignore premature stop codons, despite promising preclinical data.[98,99]

Myotonic dystrophy type 1 (DM1) is the most common form of muscular dystrophy diagnosed in adults and also causes cataracts, heart defects, endocrine problems, and poor muscle relaxation.[100] DM1 is caused by expanded trinucleotide repeats in the 3′ untranslated region (3′UTR) of the mRNA of dystrophia myotonica protein kinase (DMPK), which binds and sequesters the protein muscle blind-like1 (MBL1) and produces mRNA slicing defects in a number of genes.[101−103] Small molecule approaches have been developed to specifically disrupt the DMPK 3′UTR-MBL1 complex and reduce the pathogenesis of DM1. For example, two classes of small molecules, pentamidine and Hoechst derivatives, have been shown to rescue mRNA splicing in mice expressing expanded CUG repeats[104,105] Moreover, a Hoechst derivative (bis-benzimidazole) has recently been shown to alleviate DM1 splicing defects in a mouse model.[106] In summary, small molecules are in development that target the genetic defects underlying skeletal muscle disease. Although they are still in the preclinical stage of development, the interesting nature of their mechanisms illustrates the creative approaches being taken by chemical biologists to tackle these incurable disorders.

Small Molecule Cocktails That Induce Skeletal Muscle Dedifferentiation and Regeneration. Skeletal muscle possesses a relatively greater regenerative capacity compared to cardiac muscle, because individual skeletal muscle fibers are associated with muscle stem cells, called satellite cells.[107] Satellite cells proliferate in response to natural muscle turnover or injury and eventually differentiate to form new muscle fibers.[108] However, satellite cell effectiveness at muscle repair decreases with age, and fat tissue can replace the lost muscle fibers.[109] An attractive approach for providing a replacement source of muscle satellite cells is to induce muscle fiber dedifferentiation into mononuclear, proliferating muscle stem cells. As shown in Figure 3, this phenomenon occurs naturally in animals capable of appendage regeneration, such as the salamander (reviewed in ref 110) but is not thought to occur in mammals.[110,111] Of interest, it should be noted that a recent report does describe at least some capacity for muscle dedifferentiation in mice after muscle injury.[112] In contrast, it is known that isolated mammalian skeletal muscle myotubes are refractory (*i.e.*, do not enter the cell cycle) when exposed to stimuli that induce salamander myotube nuclei to enter the cell cycle.[113] However, it has been shown that mammalian myotubes can be induced to dedifferentiate by the forced expression of viral oncogenes,[114] genes linked to embryonic development[115] or cellular differentiation.[116] This may also be achieved by inhibiting the expression of the gene myogenin, which maintains skeletal muscle differentiation.[117] From the viewpoint of developing therapies that may induce human skeletal muscle dedifferentiation and regeneration, the development of small molecule effectors would be advantageous.[25,118,119]

Within the past year, two research papers report the development of small molecule methods to induce skeletal muscle dedifferentiation.[118,120] Both studies showed that small molecules could induce muscle myotube cellularization into proliferating mononuclear cells that could redifferentiate to produce new muscle tissue. However, there are also important differences in these two studies. One approach, which was carried out in the authors' laboratory, employed a stepwise approach of individual small molecule treatments to achieve muscle dedifferentiation. A tubulin-binding molecule, myoseverin, was used to induce muscle myotube fission into single cells. A number of tubulin-binding small molecules exist,[121] but we selected myoseverin because it has relatively low toxicity for cells[122] and can also reduce the expression of cyclin-dependent

kinase inhibitor proteins, which inhibit cell cycle progression.[121] Thus, myoseverin treatment may "prime" myotube nuclei for cell cycle re-entry. To induce cell cycle re-entry, we selected the small molecules lysophosphatidic acid (LPA; pleiotropic activator of G-protein-coupled receptors), SQ22536 (adenylyl cyclase inhibitor), SB203580 (p38 mitogen-activated protein (MAP) kinase inhibitor), and BIO (glycogen synthase-3 kinase inhibitor). LPA and SQ22536 were selected as potential inducers of cellulate proliferation because they can reduce p21 expression in senescent human fibroblasts. p21 is a "gatekeeper" of skeletal muscle terminal differentiation. SB203580 was selected because p38 MAP kinase inhibition blocks muscle differentiation and reduces p21 stability. BIO was selected because it can induce dedifferentiation in terminally differentiated mammalian cardiomyocytes. Using this approach we found that an intriguing cell type could be derived from differentiated muscle, which showed characteristics of multipotent (LPA treatment) or, possibly, pluripotent (SB203580, SQ22536, or BIO treatment) cells and that can be redifferentiated into neuronal, adipogenic or osteogenic cells.[123] We believe that this work offers exciting further opportunities for developing small molecule therapeutics for enhancing tissue regeneration, because a fish model of appendage regeneration suggests that multipoint stem cells are produced after injury.[124] Moreover, mice with digit amputation and exogenous treatment with extracellular matrix molecules showed recruitment of multipotent cells to the injury site, which have the potential to promote a regenerative response.[125] Thus, it can be speculated that these active small molecules could be developed as a therapeutic application to enhance multiple types of tissue regeneration, including skeletal muscle.

The second report demonstrating small molecule methods to induce dedifferentiation adopted a different approach.[120] Muscle differentiation is associated with the increased expression of tyrosine phosphatase enzymes, and muscle dedifferentiation induced by viral oncogenes is associated with apoptosis.[120,126] The authors of this report speculated that the inhibition of both apoptosis and tyrosine phosphatases would induce muscle myotube differentiation. This was achieved using the tyrosine phosphatase inhibitor BpV and the anti-apoptosis caspase inhibitor Q-VD-OPH (Table 1). Importantly, the mononuclear cells derived after combined treatment with these two small molecules could form new skeletal muscle tissue *in vivo*.[120]

We believe that the aforementioned advances in small molecule development for skeletal muscle dedifferentiation are important research advances. Achieved within just the past year, the novel biological applications for these small molecules should advance research in regenerative medicine, facilitating the development of pharmacologically based clinical strategies to enhance muscle regeneration and combat age- or disease-related muscle loss. Next in this review, we would like to discuss small molecule-based methodologies that are facilitating the derivation of smooth muscle cells (SMCs) for cell therapy approaches.

RETIGHTENING A LOOSENED GRIP: SMALL MOLECULES EMPLOYED IN THE DERIVATION OF SMOOTH MUSCLE CELLS

Similar to skeletal muscle, involuntary smooth muscle tissue is also distributed throughout the body. While individual skeletal muscles can be composed of different fiber types (such as "fast" twitch or "slow" twitch), smooth muscle tissue can form integral components of different tissues, such as blood vessels, bladder, gastrointestinal tract, uterus, reproductive and respiratory tracts, pili, and the iris.[127] Smooth muscle cell (SMC) differentiation can be induced in a number of different cell types, such as stem cells obtained from adipose tissue, bone marrow cells, and skeletal muscle cells.[128] One major therapeutic application is cell-based therapy for urinary incontinence, which affects about 17 million people in the United States, although many incidences go unreported.[129] One cause is smooth muscle deterioration in the intrinsic sphincter and the extrinsic sphincter of the bladder, which is linked to aging.[130] Thus, periurethral injection of smooth muscle cells may be able to restore sphincter integrity.[128] Stem cells represent an attractive source for differentiation into smooth muscle for cell therapy.

However, a survey of the research literature by the authors suggests that, compared to cardiac and skeletal muscle, there are relatively few examples of small molecules that are used to induce smooth muscle differentiation. However, small molecules are being utilized in the direct derivation of SMCs from various cell sources or as modulators of stem cell differentiation into different embryonic lineages, which can be further differentiated into specific SMC lineages. Some interesting examples of these approaches are discussed below.

Small Molecule-Based Methodologies for the Direct Induction of Smooth Muscle Differentiation. Small molecules have been used to direct smooth muscle differentiation from adult skin precursor cells and bone marrow-derived mesenchymal stem cells.[131] In both cases, ascorbic acid (which has been used to induce cardiomyocyte differentiation in mouse embryonic stem cells[132]) was used as part of the differentiation media, which also included transforming growth factor-β.[131,133] The goal of this study was to produce smooth muscle cells for generating blood vessel grafts or promoting angiogenesis *in vivo*, which was confirmed in an animal model. Retinoic acid has been used to direct smooth muscle differentiation from embryonic stem cells as part of the Wobus protocol, although the efficiency has been deemed too low to generate sufficient numbers of functional muscle cells.[131,134,135] Interestingly, 5-azacytidine has been used to derive muscle precursor cells from adipose tissue-derived stem cells.[136,137] Three months after periurethral injection into incontinent rats, bladder capacity and leak point pressure was improved and the grafted cells were found to express alpha-smooth muscle isoform of actin, a component of the contractile apparatus. Another interesting example is the recent study by Kim *et al.*, which employs the small molecule GW1516 (which was shown to enhance muscle endurance synergistically with exercise[83]) to modulate smooth muscle cell behavior. GW1516 treatment was shown to reduce senescence in murine vascular smooth muscle cells, which have been shown to contribute to oxidative stress in atherosclerotic plaques.[138] This study suggests that future approaches could be developed to directly control smooth muscle behavior *in vivo*.

Small Molecule-Based Modulation of ESC Differentiation into Appropriate Cell Lineages for Smooth Muscle Cell Derivation. A recent study has made significant progress toward the stepwise generation of lineage-specific smooth muscle cells, with small molecules forming an integral part of the differentiation protocol. Cheung *et al.* reports the establishment of a highly defined, stepwise protocol for the induction of vascular smooth muscle differentiation from

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human pluripotent stem cell lines (embryonic stem cells and induced pluripotent cells).[139]Vascular smooth muscle can be considered as a mosaic tissue derived from distinct lineages during embryonic development (reviewed in ref 140), which may explain why diseases of the blood vessel wall, for example, aneurysms and calcification, occur at specific sites in the vasculature, even though most risk factors, (such as hypertension and diabetes) produce systemic effects. In this study, small molecule modulators of cellular signaling were used in the derivation of three embryonic lineage tissues (neuroectoderm, paraxial mesoderm, and lateral plate mesoderm progenitors) that were subsequently differentiated into vascular smooth muscle cells. To induce neuroectoderm differentiation in the stem cells, one of the factors used was the small molecule SB431542, which inhibits the nodal cellular signaling pathway (that plays a key role in pattern formation during embryonic development (reviewed in ref 141) by inhibiting nodal type I receptor ALK7 signaling.[142] To induce mesoderm subtype differentiation from the neuroectoderm culture, a reversible small molecule inhibitor of the phosphoinositide 3-kinase cellular signaling pathway (LY294002) was included in the differentiation culture media. This pathway regulates diverse functions such as cell motility, proliferation, and differentiation (reviewed in ref 143). This approach allowed the derivation of specific vascular smooth muscle subtypes that correlate to different arterial regions, which could facilitate the development of bioengineered vascular grafts for specific disease regions of the vasculature. This recent advance also provides another important example of the roles small molecules can play in the development of standardized protocols for the production of highly differentiated, high "quality" cells for regenerative medicine.

OVERALL CONCLUSIONS, TECHNICAL LIMITATIONS, AND POSSIBLE FUTURE DIRECTIONS

This is an exciting time for chemical biologists working in the field of regenerative medicine. Within the past year, major breakthroughs have been achieved. For example, 12 of the 25 small molecule modulators of cardiogenesis described in this review were reported in the years 2011 or 2012. In addition, the past year has witnessed the first developments of small molecule inducers of skeletal muscle myotube dedifferentiation. This impressive progress has been reflected in other areas of regenerative medicine, such as small molecule induction of pancreatic and neuronal differentiation (please refer to refs 144 or 145 for an extensive review on these topics). These small molecules have helped us to gain great insight about which cellular signaling pathways are the key regulators of muscle differentiation. However, further advances remain before small molecule-modulated muscle cells can be applied for patient therapy. From the prospect of enhancing skeletal muscle regeneration, the development of small molecule cocktails that induce skeletal muscle regeneration are undoubtedly exciting, but there is currently no strategy to directly apply these in animal models of muscle degeneration. Improvements in drug delivery systems and their targeting to specific tissues, such as skeletal muscle,[146−148] offers hope that these small molecule modulators of muscle regeneration can be further developed as promising therapeutics. From the perspective of small molecule approaches for treating cardiac disease, it can be seen that from the relatively large number of small molecule inducers of cardiogenesis, only five have been shown to be effective in animal models of heart disease (Figure 2). Thus, for the majority of the reported small molecule inducers of cardiomyocyte differentiation, further validation is required to show their potential effectiveness as tools to generate therapeutically relevant cardiomyocyte populations. Moreover, it should be noted that cardiac tissue is a complex syncytium of various cell types. Cardiomyocytes comprise only 30−40% of the heart cell population;[149] additional cells, such as endothelial cells and fibroblasts, provide the level of vascularization and appropriate robustness required to cope with the high workload (a 300 g organ producing 100,000 Joules output per day to pump approximately 180 million liters of blood over a lifetime[29]). Thus, the optimal cell transplantation therapy for myocardial regeneration should comprise a natural mixture of these various cardiac cells. Electrical conductivity and endocrine responsiveness in the regenerative cardiac tissues should also be addressed. Tissue engineering strategies are being developed that may allow fully functional engraftment of small molecule-derived cardiomyocytes, such as the use of 3-dimensional matrices based on extracellular matrix components (for an example, please see refs 150 and 151) or the development of cell sheet technology (for examples, please see refs 152 and 153) or patches composed of biological polymers, which may be interlaced with conductive nanowires (example references are 154 and 155). Large numbers of pure cardiomyocyte populations may need to be purified from small molecule-treated cell cultures. Although much recent progress has been made to improve the efficiency of cardiomyocyte differentiation, unwanted or potentially harmful subpopulations of cells may remain within the cardiomyocyte culture. For example, small molecule approaches to induce cardiomyocyte differentiation from human embryonic stem cells or human induced pluripotent stem cells would require purification before transplantation, because undifferentiated cells that remain within the culture possess the potential to form teratomas (benign tumors containing derivatives of all three germ layers) in the recipient. In addition, many differentiation techniques produce heterogeneous cell populations with cardiomyocyte numbers varying from 1% to ∼50% of the total cells (this issue is addressed in more detail in a recent review[156]). Approaches are being developed to address this, including the use of fluorescence-activated cell sorting or magnetic bead sorting, using cell surface markers, such as vascular cell adhesion molecule-1 and signal receptor protein-α,[157] or the mitochondrial dye tetramethylrhodamine methyl ester perchlorate, which only labels highly differentiated cardiomyocytes possessing high mitochondrial density.[158]

Thus, there is now a plethora of small molecules available for researchers to modulate cardiogenesis to develop therapies for heart disease or gain further insights into the cellular signaling pathways regulating cardiac cell development. In fact, there are still potential opportunities to find novel muscle differentiation regulators. For example, the induction of cardiogenesis in embryonic stem cells involves down-regulation of Notch1 signaling,[159] which would suggest that small molecule inhibitors of Notch, *e.g.*, DAPT,[160] may facilitate cardiomyocyte generation. Recent reports that cardiac fibroblasts can be directly reprogrammed to cardiomyocytes by the retroviral deliver of cardiac reprogramming factors after MI also offers an interesting opportunity for chemical biologists.[161,162] Improving the delivery of reprogramming factors, using small molecules and epigenetic modulators, can overcome the limitations of

retroviral delivery, which are unsuitable for clinical applications due to genomic integration.

It is the opinion of the authors of this review that recent advances in small molecule approaches that directly target the cell population in the diseased muscle, to remodel the regeneration process, represent a potentially fruitful avenue for future endeavor. However, caution is also warranted here, because the examples described in this review are either unable to enhance cardiac function after MI (*i.e.*, Isx) or have only been tested in rodent models (*e.g.*, pioglitazone). A similar situation also arises in skeletal muscle diseases, because the transplanted cells only migrate a few millimeters from the injection site, meaning that an unacceptable number of cell injections would be required (for example, grafting one billion grafted cells would require a cell mass of 50 g[29]). Thus, small molecule approaches to remodel diseased skeletal muscle appear to be advantageous, although results in humans are still lacking (*e.g.*, SMTC1100) or there is some controversy (*e.g.*, resveratrol[163]). Moreover, small molecules appear to have been underutilized in the derivation of smooth muscle cells, relative to cardiac and skeletal muscle, and epigenetic modulators, such as RG108 (discussed above) may have the potential to be applied to smooth muscle derivation from nonmuscle cells sources. Nevertheless, the authors of this review hope that the dramatic progress and attractive future avenues of research provided by small molecule modulators of cell behavior has confirmed the importance of these chemical tools in biomedical research. Overall, in light of the recent development of so many novel small molecule-based approaches for combating the diverse spectrum of muscle diseases, this progress should provide patients with hope that new or improved therapies will soon be entering clinical trials.

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Notes

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KEYWORDS

Cardiogenesis: For this review, cardiogenesis is defined as the induction of cardiomyocyte differentiation or the induction of cardiomyocyte precursor cells for less differentiated cells, such as embryonic stem cells.

Differentiation: A cellular process in which a less specialized cell becomes a more specialized cell type. For example, during prenatal development, mesenchymal cells differentiate to give rise to bone, cartilage, muscle, fat, tendons and ligaments.

Dedifferentiation: A cellular process in which a partially or terminally differentiated cell reverts to an earlier developmental (or less specialized) stage. All species possess a natural capacity for dedifferentiation as part of their ability to regenerate tissues, but taxonomically primitive vertebrates retain a greater capacity for dedifferentiation and regeneration.

Induced pluripotent stem cell (iPSC): A type of pluripotent stem cell derived from a nonpluripotent cell using experimental techniques, such as viral-mediated expression of genes that regulate stem cell behavior.

Multipotent: A type of stem cell that can differentiate into multiple cell lineages, but cannot give rise to the entire spectrum of cell types in the human body. For example, hematopoietic bone marrow cells are multipotent cells that can produce all classes of blood cell.

Musculature (muscle): This is the contractile tissue of animals. Muscle is derived from the mesodermal layer of embryo tissue and is classified as skeletal, cardiac, or smooth muscle tissue.

Myopathy: A disease affecting skeletal muscle, resulting in muscle weakness of varying extents.

Pluripotent: A type of stem cell that can differentiate into any of the cells types in fetal or adult humans.

Small molecules: A low molecular weight organic compound, with an upper weight limit of approximately 800 Da, which allows for the possibility to rapidly diffuse across cell membranes and reach intracellular sites of action. The 800 Da weight limit is also one condition for oral bioavailability.

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