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REVIEW

# An epigenetic perspective on the failing heart and pluripotent-derived-cardiomyocytes for cell replacement therapy

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**Abstract** **As life expectancy rises, the prevalence of heart failure is steadily increasing, while donors for organ transplantation remain in short supply (Zwi-Dantsis and Gepstein, 2012). Indeed, myocardial infarction represents the foremost cause of death within industrialized nations (Henning, 2011) and further, approximately 1% of all newborns harbor a congenital heart defect. Although medical interventions allow > 80% of those with cardiac defects to survive to adulthood, there are often extreme emotional and financial burdens that accompany such congenital anomalies, and many individuals will remain at a heightened risk for myocardial infarction throughout the remainder of their lives (Verheugt et al., 2010; Amianto et al., 2011). In this review, we will discuss the nature of the failing heart and strategies for repair from an epigenetic standpoint. Significant focus will reside on pluripotent-to-cardiomyocyte differentiation for cell replacement, epigenetic mechanisms of cardiomyocyte differentiation, epigenetic "memories," and epigenetic control of cardiomyocyte cell fate toward translational utility.**

**Keywords** heart failure, pluripotent, cardiomyocytes, epigenetics, DNA methylation, lncRNA

## The failing heart

Despite tremendous improvement in our understanding of heart failure (HF), deciphering the complex series of events leading to HF and generating novel strategies for reversing failure remain daunting. Typically, and often following myocardial infarction (MI), HF is described as inadequate systemic delivery of blood due to enlargement of the heart and deteriorating cardiac function. Problematic events associated with HF are collectively termed "cardiac remodeling" and occur at the organ, tissue, cellular, and molecular level. Although numerous cell types are involved in the remodeling process, the cardiomyocyte (CM) is considered the primary cellular culprit. In fact, MI results in losses of up to 1 billion CMs (Laflamme and Murry, 2011), followed by replacement of these lost CMs with fibroblasts and corresponding fibrosis, and therefore a drastic reduction in contractile capacity (Kehat and Molkentin, 2010; Krenning et al., 2010; Laflamme and Murry, 2011). HF, MI, and CM along with other common abbreviations used in this article are summarized in Table 1. As cardiac remodeling is progressive, therapeutic strategies aimed primarily at correcting diminished cardiac output or improving blood flow, are typically ineffective in slowing HF or reducing mortality (Koitabashi and Kass, 2012). However, by targeting key components of cardiac remodeling at the molecular, cellular, and tissue engineering levels, researchers may be able to slow or reverse many aspects of the remodeling process. Clinical therapies aimed at reversing pathological remodeling are summarized in an excellent review by Koitabashi and Kass (Koitabashi and Kass, 2012).

Cardiac remodeling is marked initially by necrotic tissue repair, major changes in heart appearance, primarily in terms of left ventricular mass and volume, and ultimately large-scale reductions in cardiac function (Cohn et al., 2000; Kehat and Molkentin, 2010; Koitabashi and Kass, 2012). At the cellular level, remodeling is marked by CM necrosis, apoptosis, and hypertrophy accompanied by increases in fibroblast proliferation and collagen fibril deposition (Cohn et al., 2000; Kehat and Molkentin, 2010; Krenning et al., 2010; Fan et al., 2012; Koitabashi and Kass, 2012). At the molecular level, virtually endless factors are at play during cardiac remodeling. Yet, global transcriptional, genetic, and

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**Table 1** Common abbreviations within article

| Term | Abbreviation |
|---|---|
| heart failure | HF |
| myocardial infarction | MI |
| cardiomyocyte | CM |
| cardiac progenitor | CP |
| ventricular cardiomyocyte | VM |
| pluripotent-derived-cardiomyocyte | pluripotent-CM |
| hESC-derived-cardiomyocyte | hESC-CM |
| chromatin remodeling complex | CRC |
| polycomb remodeling complex 2 | PRC2 |
| histone methyl transferase | HMT |
| histone deacetylase | HDAC |
| histone acetyltransferase | HAT |
| X-chromosome inactivation | XCI |
| non-coding RNA | ncRNA |
| microRNA | miRNA |
| long non-coding RNA | lncRNA |

epigenetic studies have begun to decipher the complex mechanisms at work here and have already led to a number of promising therapeutic strategies (Koitabashi and Kass, 2012; Chow et al., 2013a; Lin and Pu, 2014).

## Basic epigenetic mechanisms

It is widely appreciated that epigenetic flaws constitute an important component and often primary source for human diseases including imprinting disorders, X-chromosome inactivation (XCI) disorders, aging, cancer, multifactorial idiopathic disorders such as schizophrenia, and as focused on next, cardiomyopathies (Robertson, 2005; Gopalakrishnan et al., 2008; the ENCODE Project Consortium, 2012). Generally defined as heritable factors that influence the interpretation of the genetic sequence without amending the sequence itself, epigenetic factors include, histone modifications, DNA methylation, chromatin remodeling, and non-coding RNAs (ncRNAs). These epigenetic features as related to chromatin accessibility and function are described in Fig. 1. Although it is virtually impossible to illustrate all potential cross-talk between unique epigenetic components, it is important to recognize that dynamic networks of epigenetic players are constantly working to regulate the daily life of a cell. Many epigenetic features exist in a hierarchy toward locus-specific and regional control of nucleosome positioning and chromatin architecture. By coordinating these chromatin modifications at key regulatory regions and networks of regulatory regions (i.e.: promoters, enhancers, etc.) cells can globally regulate coding and non-coding transcription and downstream processing events such as alternative splicing (Kim et al., 2009; Luco et al., 2011). For example, chromatin remodeling complexes (CRCs) and transcription co-factors interpret patterns of histone modifications/DNA methylation and can be further guided by ncRNA species (whose own transcription is under epigenetic regulation) for site-specific manipulation or reinforcement of existing epigenetic patterns (Fig. 1).

## DNA methylation, chromatin remodeling, and histone modifications

DNA methylation in mammals refers to the covalent attachment of a methyl group to the C5 position in cytosine (forming 5′ methylcytosine (5mc)), most often symmetrically across the double helix at CpG dinucleotides. In recent years, especially in a developmental context, non-CG methylation and hydroxymethylation (hMC) have both garnered considerable attention. Although outside the scope of this review, it is important to note that like CG methylation patterns, both non-CG methylation and hMC have tissue-specific distribution and roles in governing transcription (Laurent et al., 2010; Stroud et al., 2011; Guo et al., 2014). hMC in particular, is an important component of DNA demethylation pathways, which involves iterative oxidation of mC to hMC via ten-11 translocation (TET) enzymes and either passive loss over cell division or base excision repair activities by thymine DNA glycosylase (TDG) (Fig. 1) (Kohli and Zhang, 2013). Some evidence has implied activation-induced deaminase (AID)/APOBEC activity in active demethylation pathways, especially during embryogenesis, but contrary evidence implies a limited role (for review see (Kohli and Zhang, 2013)). Maintenance CpG methylation is carried out by DNA methyl transferase I (DNMT1) and *de novo* methylation is catalyzed by DNMT3A and DNMT3B. Although the majority of individual cytosines are methylated genome-wide, short stretches of high CG density often found at gene promoters ("CpG islands") are typically unmethylated. However, hypermethylation of gene promoters can facilitate methyl binding domain (MBD) association and corresponding recruitment of repressive histone modifiers and chromatin remodelers for persistent gene silencing (Kim et al., 2009).

DNA, whether methylated or unmethylated, is typically wrapped around an octamer of histone proteins. This primary unit of chromatin, the nucleosome, is made up of 8 histone core proteins (two of each of H3, H4, H2A, and H2B) bound by 147 bp of DNA (Luger et al., 1997). The incorporation, positioning, and removal of nucleosomes, as well as the restructuring of histone components (i.e.: histone variants) and/or histone-DNA interactions using ATP hydrolysis is collectively referred to as chromatin remodeling. Such activity can expose or restrict DNA accessibility and profoundly influence transcriptional levels. Four main families of chromatin remodeler complexes (CRCs) exist based on sequence similarity and structure. These include SWI/SNF, chromodomain helicase DNA binding, ISWI, and INO80 complexes (Ho and Crabtree, 2010). As discussed later in the context of HF, the vertebrate ortholog of the quite

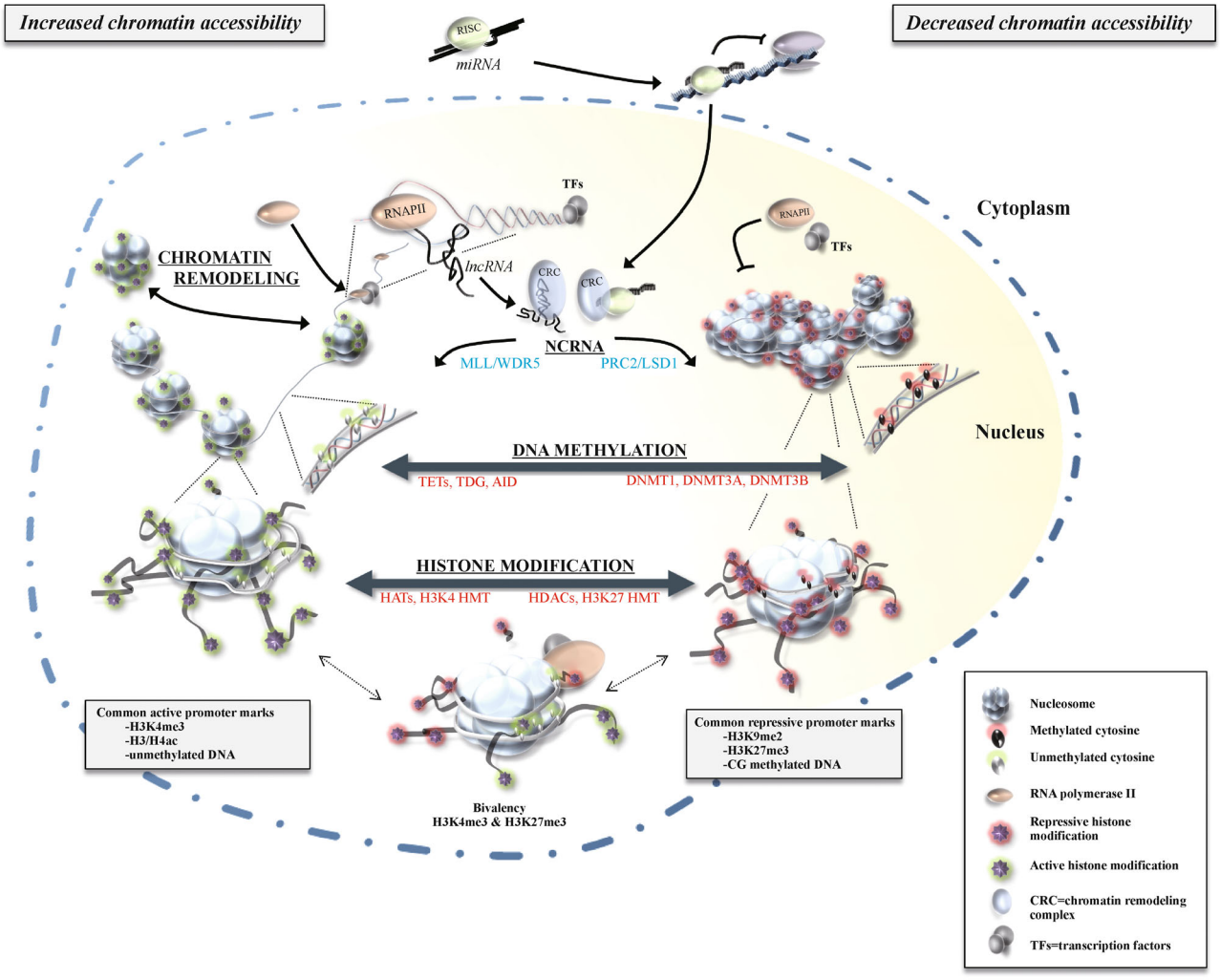


**Figure 1** Basic epigenetic mechanisms that govern chromatin accessibility and transcription activity. As shown, patterns of histone modifications, DNA methylation, ncRNA expression, and chromatin remodeling (including local nucleosome density) all can influence levels of chromatin compaction and ultimately transcription at a typical promoter (Arya et al., 2010; Bannister and Kouzarides, 2011; Fisher and Fisher, 2011; Handy et al., 2011; Huang and Li, 2012; the ENCODE Project Consortium, 2012; Grote and Herrmann, 2013; Chen and Dent, 2014). Common enzymes that control the deposition or removal of epigenetic marks are displayed in red. Complexes that ncRNAs may associate with toward chromatin modulation are shown in blue. miRNA is displayed post-transcription, processing, and nuclear export.

famous SWI/SNF CRC is the brahma-associated factor (BAF), which have brahma or brahma-related gene 1 (Brg1) ATPase subunits (Hang et al., 2010; Bannister and Kouzarides, 2011; Chen and Dent, 2014). As with most CRCs a considerable variety of BAF subunits exist, are expressed in a cell-type or tissue-specific manner, and are functionally interdependent on local epigenetic states (Reisman et al., 2009). This collectively allows for a high degree of specificity in the type, location, and nature of chromatin remodeling.

Extending out from the nucleosome core, the N-terminal tales of histones are subjected to extensive post-translation modification. Histone modifications are wide-ranging and include: acetylation, methylation, phosphorylation, sumoylation, and ubiquitination. Their collective distribution constitute the histone code of gene regulation (Jenuwein and Allis, 2001; Shilatifard, 2006; Kim et al., 2009). Perhaps the most extensively studied histone modifications are acetylation and methylation, which occur on lysines or lysines and arginines, respectively. As with these 2 types of modifications, there is extensive cross-talk between individual types of modifications. For example, H4 acetylation regulates arginine methylation deposition (Feng et al., 2011). Key enzymes regulating this process are histone acetyltransferase (HATs) and histone deacetylases (HDACs), which facilitate the opening or tightening of DNA interactions with histones, respectively. In general, extensive histone acetylation is associated with more open chromatin structures and higher transcriptional activity. As with HATs and HDACs, a variety of histone methyltransferases (HMTs) and methylases are now known to exist and differ by substrate specificity (Kim et

al., 2009). Rather than a simple adjustment to the electrostatic interactions between the histones and DNA, as is the case with histone acetylation, transcriptional effects of histone methylation depend to a great extent on the actual amino acid modified. As shown in Fig. 1, a common marker of active gene transcription is H3K4me3 (Santos-Rosa et al., 2002), whereas H3K27me3 marks gene repression (Kim et al., 2009). The addition of mono-, di-, or tri-methylation to lysines or mono- or di-methylation to arginines is catalyzed by a variety of HMTs typically containing the catalytic SET domain, but unique in their targeting. For example, H3K4me3 is catalyzed by mixed-lineage leukemia 4 (MLL4) HMT, whereas H3K27me3 is catalyzed by enhancer of zeste homolog 2 (EZH2) HMT (Sarma et al., 2008), the latter being a polycomb repressive complex member (PRC2). Demethylation is also highly substrate specific and typically involves the activity of catalytic jumonji domains inherent to most demethylases (Bannister and Kouzarides, 2011). The resulting pattern of histone modifications generates binding sites for specific protein including recruitment of chromatin remodeling activity and transcription factors. For example bromodomain-containing transcription factors and CRCs specifically bind to acetylated lysines (Mujtaba et al., 2007). Highlighting the interdependency of DNA methylation, histone modifications, and chromatin remodelers, a number of CRCs contain multiple epigenetic modulating activities. For example, the nucleosome remodeling and deacetylation complex (NuRD) possesses chromatin remodeling activity, histone deacetylase activity via components HDAC1 and HDAC2 (which may lead to PRC2 recruitment), and is functionally linked to DNA methylation and hMC through its methyl binding domain 3 (Mbd3) (Reynolds et al., 2012; Cai et al., 2014; Chen and Dent, 2014).

Figure 1 also illustrates that nucleosomes can simultaneously harbor both active and inactive histone modifications (ie: H3K4me3 and H3K27me3). These so called “bivalent states” are typically found at developmental genes maintaining a silent, but poised state (RNAPII already recruited) in pluripotent cells. Upon differentiation, these domains are resolved to a monovalent active or repressed state in a tissue-specific fashion. Debate on this hypothesis can be found in Chen et al., (Chen and Dent, 2014). As depicted in figure, 1 recent evidence indicates that H3K27me3 and H3K4me3 marks may coexist within the same nucleosome, but likely not the same N-terminal tail (Voigt et al., 2012).

## ncRNAs

We now understand that the majority of the eukaryotic genome is pervasively transcribed and under the control of classic epigenetic mechanisms described above (the ENCODE Project Consortium, 2012). In fact, ~60% of processed transcripts lack protein-coding potential and therefore constitute a wide array of non-coding RNAs (ncRNAs) (Djebali et al., 2012). Two primary classes of ncRNAs will be discussed here: long-noncoding RNAs (lncRNAs) and microRNAs (miRNAs). However, most research to date has been centered on the latter. As implied by their names, miRNAs (typically 21-25 bp) are smaller than lncRNAs ( $>200$ bp). Both are endogenously encoded and immature transcripts are subjected to typical mRNA processing mechanisms (5′ capped, spliced, and polyadenylated) (Cai et al., 2004; Nie et al., 2012). Conventional processing of a pri-miRNA transcript also includes Drosha/Pasha, Exportin 5, and Dicer activities resulting in a cytoplasmic mature miRNA bound by the RNA induced silencing complex (RISC). Although miRNA-mRNA sequence complimentarily ultimately guides RISC (and central RISC component Argonaute (AGO)) toward translational inhibition or mRNA destruction, growing bodies of evidence imply miRNA to have nuclear functions as well (for review (Huang and Li, 2012)) (Fig. 1). In particular, miRNAs have the ability to both activate (transcriptional gene activation (TGA)) and silence (transcriptional gene silencing (TGS)) target promoters via assisted recruitment of RNAPII and involving altered promoter epigenetic states (Huang and Li, 2012). In short, mature miRNAs bound by RISC, can be shuttled into the nucleus and be guided by the innate binding specificity nuclear miRNA has with other RNA species (including lncRNAs) and/or DNA (Huang and Li, 2012; Salmanidis et al., 2014). Though the exact molecular mechanisms underlying TGS/TGA and epigenetic reconfiguration will require considerably more investigation (Salmanidis et al., 2014), we know that TGS is associated with diminished RNAPII promoter localization, PRC2 recruitment toward H3K27 trimethylation, and increased heterochromatin formation (Kim et al., 2006; Kim et al., 2008; Kavi and Birchler, 2009; Huang and Li, 2012).

Similarly, lncRNAs can have both gene activating and silencing functions, and although their origins and finer details of action are distinct from miRNAs, both ncRNA species can operate to control gene expression via protein interactions that site-specifically influence chromatin architecture. lncRNAs, in particular, are often transcribed antisense from a protein-coding gene and can function as *cis*- and *trans*-acting regulators of gene expression. Accumulating evidence supports a common theme in which lncRNAs create functional scaffolds to orchestrate the activity of a number of CRCs (Fig. 1). For example lncRNA HOTAIR has a 5′ and 3′ domain that can direct the PRC2 and LSD1/CoREST/REST chromatin modifying complexes, respectively. This couples H3K27 tri-methylation (by PRC2) and H3K4 demethylation (by LSD1) repressive activities to common loci (Tsai et al., 2010). In contrast, multiple lncRNAs can promote MLL/WDR5 mediated H3K4me3 and corresponding activation of chromatin. (Yang et al., 2014). Lastly, and to complicate matters, lncRNAs can serve as sponges to diminish miRNA regulation of mRNA abundance (Hansen et al., 2013) and recent evidence indicates some lncRNAs

may in fact have small peptide coding functions (Cohen, 2014).

Given the interplay between all types of epigenetic features discussed, it is not surprising that epigenetic components of HF include disruptions of normal histone modifications and chromatin remodeling activity, as well as perturbations to DNA methylation and ncRNA profiles. These relationships, in the context of HF, are discussed next.

## Epigenetic mechanisms at the molecular level of HF

### Histone modifications and DNA methylation in HF

At the molecular level, HF is often marked by irregular calcium sensing and cycling within CMs, disrupted excitation-contraction coupling, increased hypertrophic signaling, impaired β-andrenergic responses, improper extracellular matrix-CM cross-talk, altered apoptotic, developmental, and metabolic signaling networks, and defective calcium/calmodulin-dependent kinase II (CaMKII) function, among others (Cohn et al., 2000; Zhang et al., 2002a; Backs et al., 2006; Bossuyt et al., 2008; Fan et al., 2012; Koitabashi and Kass, 2012; Awad et al., 2013; Ferrara et al., 2014). The latter, CaMKII is directly involved in activating myocyte enhancing factor 2 (MEF2) target genes through post-translational phosphorylation of HDACs, namely, HDACs 4, 5 and 9. CaMKII mediated phosphorylation of HDACs promotes dissociation of HDAC bound MEF2, subsequent cytoplasmic shuffling of HDACs, and the release of MEF2 transcriptional activity. This often involves coordinating efforts with p300 HAT (Chang et al., 2004; Backs et al., 2006; Zhang et al., 2007; Bossuyt et al., 2008). Activation of MEF2 and other pro-hypertrophic transcription factors also occurs through CaMKII targeting of histone H3 and concomitant chromatin remodeling, of which overactivation of CaMKII is associated with hypertrophic phenotypes (Awad et al., 2013). p300 also serves as a co-activator of critical CM developmental transcription factor GATA-4 and was shown to promote ventricular remodeling after MI (Miyamoto et al., 2006). In fact, CM hypertrophy usually involves upregulation of fetal cardiac genes and decreased expression of adult cardiac genes, of which histone acetylation (Zhang et al., 2002a; Trivedi et al., 2007) and H3K9me3 (Majumdar et al., 2008) are thought to have defining influences on the reprogramming steps. Misregulation of histone modifications at central CM specific transcription factors and/or key signaling pathways involved in normal cardiac function has now been widely documented within MI/HF (Zhang et al., 2002a; Chang et al., 2004; Backs et al., 2006; Miyamoto et al., 2006; Trivedi et al., 2007; Zhang et al., 2007; Bossuyt et al., 2008; Majumdar et al., 2008; Movassagh et al., 2011; Awad et al., 2013;). With regards to DNA methylation, global methylation differences clearly exist between healthy and cardiomyopathic left ventricles, in which cardiac disease samples were hypomethylated at promoters and hypermethylated in gene bodies (Movassagh et al., 2011). In patients with dilated cardiomyopathy, altered DNA methylation was correlated with lymphocyte antigen 75 (LY75) and HER3 cell surface receptor expression, both of which may have important roles in HF (Haas et al., 2013). Differential DNA methylation at angiogenic genes and loss of H3K4 methylation at key arrhythmogenic genes are also likely contributors to HF incidence (Movassagh et al., 2010; Stein et al., 2011). Lastly, irreversible DNA methylation at Notch-responsive promoters disrupts the activation of this CM proliferation promoting pathway following MI (Felician et al., 2014). Collectively, these studies imply an important role for these classic epigenetic modifications in cardiomyopathies.

### lncRNAs and miRNAs in HF

ncRNAs are increasingly being appreciated for their roles in normal cardiac development, in MI, and in HF. To date, only a handful of studies have directly considered lncRNAs and MI/HF, but given tissue-specific expression of these RNAs (Grote et al., 2013; Klattenhoff et al., 2013) and associations of specific lncRNAs expression levels or polymorphisms with MI (Ishii et al., 2006; Ahmed et al., 2013; Papait et al., 2013; Kumarswamy et al., 2014; Matkovich et al., 2014), it is expected that confirmed lncRNA contributions to cardiac remodeling will significantly increase. Indeed, Han et al. very recently investigated a lncRNA cluster, *Myheart*, which is transcribed antisense from the myosin heavy chain (Myh7) promoter, operates by antagonizing Brg1/BAF-Hdac-Parp stress induced chromatin repressive activity, and ultimately provides a cardioprotective effect from pathological hypertrophy (Han et al., 2014). Prior research on chromatin remodeler Brg1 had indicated that hypertrophic stimuli induced Brg1 expression (normally expressed in embryonic heart only), corresponding Brg1/BAF-Hdac-Parp complex formation, and a shift from adult MHC to fetal MHC forms (Hang et al., 2010). Taken together, these studies illustrate the dynamic interplay between multiple epigenetic factors (ncRNA, chromatin remodeling, histone modifiers) and the induction of fetal cardiac gene expression during hypertrophy.

Numerous miRNAs are now considered biomarkers or candidates of biomarkers for HF. miR133b and miR499 were shown to be upregulated in MI patients and animal models of MI (Wang et al., 2010); whereas, miR134, miR370, miR192, miR340, and miR624 were elevated and significantly correlated with cardiovascular disease (Hoekstra et al., 2010; Sondermeijer et al., 2011). miR24, miR125b, miR195, miR199a, and miR214 were all upregulated in mouse models of cardiac hypertrophy and confirmed in HF patients (van Rooij et al., 2006). Intriguingly, a number of distinct cardiomyopathies have unique miRNA signatures (van Rooij et al., 2006; Ikeda et al., 2007; Sucharov et al.,

2008; Hoekstra et al., 2010; Jaguszewski et al., 2014). Further, and well in line with the re-emergence of fetal cardiac programs during cardiac hypertrophy, miRNA signatures exhibited during HF are similar to those in fetal cardiac tissue (Ikeda et al., 2007). In addition to correlative studies between miRNA expression and disease risk, manipulation of miRNAs represents a viable therapeutic strategy. For example, overexpression of miR21 significantly decreased MI size and left ventricle hypertrophy in animal models. Such CM protection was associated with miR21 targeting of programmed cell death 4 and activator protein 1 signaling (Dong et al., 2009). miR22 deletion, on the otherhand, prevented cardiac hypertrophy and remodeling in mouse models subjected to isoproterenol infusion and calcineurin transgene stressors (Huang et al., 2013). Sucharov et al. demonstrated that miR100 overexpression in neonatal rat CMs promoted β-adrenergic associated cardiac gene repression. However, miR133b restricted amendments to β-adrenergic receptor mediated expression, suggesting that miRNA modulation could be utilized to improve β-adrenergic mediated responses toward reverse cardiac remodeling (Sucharov et al., 2008). With regards to stimulating resident CM progenitors (CPs) to contribute to infarct healing, miR-17-92 overexpression was shown to promote cardiac regeneration following MI in adult mice via CP cell proliferation (Chen et al., 2013). Collectively, these studies coupled with and a multitude of basic epigenetic/epigenomic studies lead one to the conclusion that numerous epigenetic factors, dynamically interacting with one-another, regulate key aspects of cardiac remodeling. Furthermore, this loss of proper epigenetic regulation usually involves reactivation of fetal cardiac gene networks, and represents a common origin for a number of human cardiomyopathies.

## Pluripotent cell-derived CMs for cardiac regenerative medicine

Working under the paradigm that reversal of cardiac remodeling offers the most promising avenue for curing the failing heart, a number of clinical therapies are being employed to promote such reversal (Koitabashi and Kass, 2012). Renin-angiotensis-aldosterone system antagonists, blockages of β-adrenergic-receptors, cardiac resynchronization therapy, and left ventricular assist devices have all showed promising results toward reverse remodeling. Inflammation control to minimize fibrosis, stimulation of angiogenesis, and the induction of cardiomyogenesis are actively being explored to manage initial stages ischemic tissue injury (Dimmeler et al., 2005; Laflamme and Murry, 2005; Passier et al., 2008; Segers and Lee, 2008). For the latter, there is evidence to support endogenous cardiomyogenesis in mice and humans (Bergmann et al., 2009; Senyo et al., 2013) including MI induction of CMs to re-commence cell division in mice (Porrello et al., 2011; Senyo et al., 2013). Further, recent work has established the presence of, and isolation protocols for, CPs existing in specific niches, capable of proliferation and CM differentiation (Leri et al., 2011; Chimenti et al., 2012). Despite these encouraging results, endogenous CM sources for cell replacement currently remain hugely inadequate in the face of large-scale cellular losses that accompany acute MI and progressive remodeling during HF (Zwi-Dantsis and Gepstein, 2012). As such, significant focus has been placed on identifying scalable sources for new functional CMs for use in cell replacement based regenerative medicine.

Multiple stem cell and progenitor cell types, including skeletal myoblasts, hematopoietic stem cells, mesenchymal stem cells, human embryonic stem cells (hESCs), and induced pluripotent stem cells (iPSCs) were initially explored for their capacity to develop into functional CMs. Although all have demonstrated improved cardiac function in rodent MI models, pluripotent-derived-CMs (pluripotent-CMs) were shown to contribute directly to contractility through electrical coupling and are highly favored for scalability (Rubart et al., 2003; Dimmeler et al., 2005; Laflamme and Murry, 2005; Xue et al., 2005; Halbach et al., 2007; Segers and Lee, 2008; Gepstein et al., 2010; Carpenter et al., 2012; Zwi-Dantsis and Gepstein, 2012). In fact, hESC-derived-CMs (hESC-CMs) were quite recently used for the first time to promote cardiac regeneration in non-human primate hearts (Chong et al., 2014). Transplanted CMs became host vascularized, developed electromechanical connections with host CMs, and widespread remuscularization of the damaged primate hearts was observed. Additionally, Chong et al. used heat shock and pro-survival cocktail treatments of CMs coupled with the mattress suture strategy to improve engraftment and there was no evidence of teratoma formation in this study. Poor CM survival post-transplant and the innate teratoma forming properties of pluripotent cells are typical concerns for pluripotent-CM transplant strategies and have been the focus of earlier studies (Caspi et al., 2007a; Laflamme et al., 2007; Nussbaum et al., 2007; Robey et al., 2008). Though these primate studies illustrate the tremendous potential of pluripotent-CMs for cardiac repair, CM immaturity (also seen in (Chong et al., 2014)) and heterogeneity (not addressed in (Chong et al., 2014)) remain major concerns.

### Phenotypes of pluripotent-CMs

Evidence indicates that pluripotent-CM's are functionally immature and composed of mixtures of atrial, nodal (pacemaker), and ventricular myocyte subtypes (Laflamme et al., 2007; Porrello et al., 2011), of which the latter constitutes the predominant CM required for infarct repair (Ng et al., 2010). Although pluripotent-CM's and subtypes exhibit many CM features, these cells display less defined rod shapes, minimal multinucleation (Snir et al., 2003), and immature sarcomere and myofibril organization (Snir et al., 2003; Yoon et al., 2006). Further, observations of automaticity, a relative

depolarized resting potential, and delayed action potential upstroke suggest immature contractile behavior (Brito-Martins et al., 2008; Porrello et al., 2011). Some evidence indicates immature CMs may have improved survivability post-engraftment and accompanies intriguing ideas of in-culture biomechanical force training and endothelial cell coculture to coax further maturation (Reinecke et al., 1999; Caspi et al., 2007b; Sartiani et al., 2007; Rajala et al., 2011; Tulloch et al., 2011; Chong et al., 2014; Hirt et al., 2014); but the hypertrophic and arrythmogenic capacity that is inherent to heterogeneous and immature CMs is a major concern (Zhang et al., 2002b; Ng et al., 2010; Papait and Condorelli, 2010). For example, though nodal cells may be essential for creating biologic pacemakers, transplant of such protracted pacemaker activity with distinct neurohormonal response characteristics could intensify the already heightened arrhythmia risk following MI (Zhang et al., 2002b; Yi et al., 2009). Interestingly, Chong et al. reported significant incidence of ventricular arrhythmias post-engraftment in primate models, attributing the most likely cause to be large heart size and the relative immaturity of hESC-CMs which may slow conduction during ventricular depolarization and ultimately lead to arrhythmia (Chong et al., 2014). However, prepared CMs were 73% cTnT positive on average and not defined by CM subtype, so CM heterogeneity may in fact be a basis for observed arrhythmias. In contrast, a prior study by the group actually showed hESC-CMs to decrease arrhythmias in guinea-pigs, which further indicates that host heart size and beat rate are important considerations for CM based cardiac regeneration (Shiba et al., 2012). It is likely that optimized CM maturity, homogeneity/heterogeneity, grafting strategy, and graft size may need to be tailored for individual classes of infarcted or failing hearts.

**Generation and enrichment of pluripotent-CMs**

Following spontaneous embryoid body-based CM derivation systems (Narazaki et al., 2008; Haase et al., 2009), protocols for generating pluripotent-CMs have relied on existing knowledge of mesoderm formation and subsequent cardiac development, including Wnt, nodal, and transforming growth factor (TGF) β signaling (Gadue et al., 2006; Lindsley et al., 2006; Kattman et al., 2011). For some time, routine protocols have involved treatment of pluripotent cells with TGFβ member Activin A and bone morphogenic protein 4/basic fetal growth factor (BMP4/bFGF) to stimulate mesoderm and cardiac mesoderm commitment, respectively, followed sometimes with Wnt inhibition using Dickkopf-1 (DKK1) to enhance cardiomyogenesis (Xue et al., 2005; Narazaki et al., 2008; Rai et al., 2012). Resulting populations typically contain 30%–50% CMs, which can be further enriched. However, the molecular details that govern this pathway, especially CPs spontaneous transition into beating CMs, require significantly more investigation to fully exploit puripotent-CM's in cardiac regeneration (Naito et al., 2006; Ueno et al., 2007; Yang et al., 2008; Rajala et al., 2011). For example, by understanding temporal patterning of Wnt signaling, including both cardiac development enhancing and repressive functions, Lian et al. developed a small molecule based differentiation protocol that effectively induces high yields of functional CMs (80%–98%) across many pluripotent cell lines (Lian et al., 2013).This serum and growth factor free system represents a milestone for improving CM differentiation from pioneering efforts (2001) that yielded $< 1\%$ CMs in final differentiation products (Kehat et al., 2001). Additional promising techniques for improving CM differentiation include the double layer Matrigel "sandwich" method (Zhang et al., 2012a) and inhibition of p38 mitogen-activated protein kinase pathways (Graichen et al., 2008).

Purification strategies of CMs include labor intensive microdissection (Kehat et al., 2001; Mummery et al., 2003; Caspi et al., 2007a), genetic selection strategies, flow cytometry for cell sorting, and Percoll density gradients, which take advantage of the unique buoyancy of CMs (Xu et al., 2002; Laflamme et al., 2005). Transgenic approaches typically involving inducible expression of cardiac-specific promoters for drug and/or fluorescent-based selection of cells have significantly increased purity of derived CMs, but involve the usual risks of insertional mutagenesis and elevated oncogenic potential (Kolossov et al., 2005; Anderson et al., 2007; Shiba et al., 2009). Due to the general lack of CM and CM-subtype-specific cell surface markers, scalable flow cytometry based isolation of CMs will require additional exploration (Hattori et al., 2010; Pascut et al., 2011). However, pluripotent-CMs have now been enriched by SIRPA (Dubois et al., 2011), VCAM1 (Uosaki et al., 2011), and EMILIN2 (Van Hoof et al., 2010) cell surface markers, additionally by Raman micro-spectroscopy (Pascut et al., 2011), and recently by unique metabolic differences between CMs and non-CMs post-differentiation (Tohyama et al., 2013). While many of these strategies are quite practical for *in vitro* applications, it remains unclear which approach(es) will be routinely employed to obtain sufficient quantities and enrichment of clinical grade pluripotent-CMs. Ultimately, a defined and xeno-free differentiation and enrichment strategy, yielding necessary purity and cellular maturity of CMs toward functional engraftment will be needed. Cardiomyopathy-specific CMs (e.g.: chamber-specific CMs for distinct locations of ischemic tissue damage) will likely constitute the next round of evolution in this field.

Though transplant strategies themselves are outside the scope of this review, it is important to highlight that continued advances in controlling pluripotent-CM differentiation toward functional integration with the host (i.e.: vascularization, electro-mechanical coupling) will undoubtedly be coupled to, and often directly enhanced by, tissue engineering and grafting strategies. These include the use of engineered scaffolds or decellurized heart scaffolds for carrying cells (both CMs and intentional cell mixtures) and extracellular

matrix to failing heart sites along with the enhancement of vascularization at engraftment sites (Laflamme et al., 2005; Caspi et al., 2007b; Laflamme et al., 2007; Robey et al., 2008; Stevens et al., 2009; Lesman et al., 2010; Kreutziger et al., 2011; Lu et al., 2013; Nunes et al., 2013; Radisic and Christman, 2013). With proper design, engineered scaffolds can promote neovascularization (or be pre-vascularized) (Laflamme et al., 2005; Laflamme et al., 2007; Lesman et al., 2010; Kreutziger et al., 2011), improve organization of and within individual CMs (Tulloch et al., 2011), and be integrated with electrical field stimulation (Nunes et al., 2013), all aimed at facilitating CM structural and electrophysiological maturation toward efficient engraftment and integration with host tissue. Although there is no doubt that cardiac tissue engineering strategies have a critical role to play in cardiac regenerative medicine, global transcriptional and epigenetic studies on CMs infused into, or differentiated within artificial microenvironments, will provide unprecedented insight into the fundamental components of CM development and the influence environment plays on cardiomyopathy development. Indeed, the microenvironment itself may need to be as dynamic as the changing identity of a cell on the journey from pluriopotency-to-CM cell fate.

## Epigenetic influences on CM cell fate. "Memories" and lineage propensity.

Histone modifications, DNA methylation, and chromatin remodeling working in conjunction with ncRNA transcripts dynamically alter chromatin structure and chromatin signaling at millions of "switches" genome-wide in a concerted effort to control gene expression, cellular behavior, and ultimately cell identity (the ENCODE Project Consortium, 2012). CM differentiation from pluripotent cell sources has already significantly improved our understanding of transcriptional and epigenetic programs involved in human cardiomyogenesis (Bar-Nur et al., 2011; Kattman et al., 2011; Rajala et al., 2011; Paige et al., 2012; Xu et al., 2012; Zwi-Dantsis and Gepstein, 2012; Chow et al., 2013a; Lian et al., 2013; Gu et al., 2014). Extensions of findings from transcriptional studies have resulted in major improvements in CM yield by recapitulation/enhancement of normal differentiation programs (Kattman et al., 2011; Rai et al., 2012; Lian et al., 2013), but what role do epigenetic and epigenomic studies play? In addition to improving our understanding of epigenetic programs in cardiomyogenesis and in HF, it is important to consider that global and targeted manipulation of the epigenome may be used toward improving CM differentiation and perhaps toward correcting epigenetic origins of cardiac disease.

### Epigenetics of pluripotent-CM differentiation

Recently, attempts have been made to determine the chromatin signatures that accompany CM differentiation from mammalian pluripotent cells. Paige et al. (Paige et al., 2012) were confronted with heterogeneous populations throughout their cardiac differentiation time course, but nevertheless were able to identify essential players in mammalian cardiovascular development. They observed that CM differentiation is not merely a simple resolution of bivalency. Bivalency resolution refers to the transition of dual H3K4me3 (active)/H3K27me3 (repressive) marked developmental genes to a silenced or active state over development (Fig. 1). In fact, in their study a number of critical mesodermal genes were decorated heavily with repressive H3K27me3, yet highly expressed. Although developmental cardiac transcription factors did display bivalent resolution toward active H3K4me3, cardiac structural genes increased H3K4me3 upon CM commitment without H3K27me3 marks at earlier time points. Additional studies on bivalent histone marks through CM differentiation have shown that CMs electrophysiology is epigenetically regulated through H3K27me3 and H3K4me3 dynamics and that derived CMs may be primed epigenetically for further maturation (Chow et al., 2013b). Pair-wise promoter DNA methylation studies on hESCs and CMs have shown that DNA hypomethylation also occurs at structural genes during cardiomyogenesis (Gu et al., 2014). In our laboratory, recent work has confirmed CM structural gene promoter hypomethylation, which appears to be progressive over multiple stages of differentiation (manuscript in preparation). We further observed thousands of stage-specific differentially methylated regions corresponding to critical mesodermal and CM developmental transcription factors and essential signaling pathways (i.e.: WNT signaling pathways). Like Paige et al. and others, we observed pluripotent differentiation to be far from a simple resolution of gene activation or silencing. CM epigenomic programming appears to be dynamic, and likely involves multiple global chromatin states (Paige et al., 2012; Wamstad et al., 2012; Chen and Dent, 2014).

ncRNAs have roles in XCI, imprinting, polycomb repressive complex recruitment, and as discussed earlier, cardiomyogenesis and MI/HF (van Rooij et al., 2006; Ishii et al., 2006; Ikeda et al., 2007; Zaratiegui et al., 2007; Sucharov et al., 2008; Wang et al., 2010; Hoekstra et al., 2010; Sondermeijer et al., 2011; Papait et al., 2013; Ahmed et al., 2013; Jaguszewski et al., 2014; Kumarswamy et al., 2014; Matkovich et al., 2014). A number of studies have established lncRNA and miRNA expression to occur in a developmental-specific manner including numerous ncRNAs uniquely expressed or upregulated in CM differentiation (Fu et al., 2011; Grote and Herrmann, 2013; Grote et al., 2013; Klattenhoff et al., 2013; Matkovich et al., 2014; Zhu et al., 2014). However, functional validation of ncRNA candidates in CM differentiation has been lacking until recent years. Klattenhoff et al. recently demonstrated that depletion of *Braveheart* (Bvht) lncRNA, disrupted normal activation of critical cardiac transcription factors, and significantly decreased numbers of contracting embryoid bodies during

differentiation (Klattenhoff et al., 2013). Like *Bvht*, lncRNA *Fendrr* contributes to cardiomyogenesis through interactions with other epigenetic regulators of cardiac development (Grote et al., 2013). Both *Bvht* and *Fendrr* have been shown to bind PRC2 machinery, and by extension depletion of *Fendrr* and *Bvht* disrupts normal deposition/resolution of H3K4me3 or H3K27me3/H3K4me3, respectively, at a subset of cardiac developmental genes. In an analogous manner, miRNA let-7c was shown to promote pluripotent-CM differentiation through targeting of PRC2 member EZH2, and removal of repressive H3K27me3 at cardiac developmental genes (Coppola et al., 2014). As miR-99a is transcribed from the same miRNA cluster, it is intriguing that this miRNA, exerted opposite effects on CM differentiation. Overexpression of miR99a attenuated Nodal signaling via repression of *Smarca5* chromatin remodeling activity and cardiac gene promoters were shown to have increased H3K27me3 levels. A variety of additional studies have functionally confirmed miRNA roles in pluripotent-CM differentiation including miR199a-3p, miR590-3p (91), miR363 (Gupta and Rao, 2014), miR499 (Li et al., 2013) miR200b (Yao et al., 2013), miR1, miR499 (Fu et al., 2011), and miR-133 (Takaya et al., 2009). However, additional work will be needed to determine ncRNA control of and regulation by, other epigenetic regulators of chromatin architecture through CM differentiation. To this end, differentiation studies that track dynamic networks of epigenetic changes will continue to prove instrumental not only to our understanding of cardiac biology, but for our fundamental understanding of the nature of epigenetic marks and their control of global transcription.

## Epigenomic landscapes and lineage bias in CM differentiation

We know that pre-existing epigenomic landscapes, or crude adjustments to these landscapes, have major influences on pluripotent cell fate. For example, simply treating ESCs with HDAC inhibitor trichostatin A (TSA) increases GATA4 acetylation and promotes ESC differentiation toward CMs (Kawamura et al., 2005), and valproic acid treatment (another HDAC inhibitor) on hESC-derived-CMs enhances physical CM maturation (Chow et al., 2013b). Likewise, ESCs treated with demethylating agents 5-aza-cytidine or less toxic zebularine significantly improves CM differentiation and facilitates expression of key cardiac developmental genes (ex; Nkx2.5) (Choi et al., 2004; Yoon et al., 2006; Horrillo et al., 2013). Zeburaline was also shown to enhance H4 acetylation, reduce H3K9me1, and increase H3K4me3, highlighting the integration of a number of epigenetic marks toward common chromatin structural changes (here increased accessibility and expression activity). Even pluripotency itself, without epigenomic manipulation, can be described as a continuum of several stem cell potency states, unique in transcriptional and epigenetic make-up, and innately distinct in differentiation propensity (Osafune et al., 2008; Hough et al., 2009; Kim et al., 2010; Sullivan et al., 2010; Bar-Nur et al., 2011; Gafni et al., 2013). Epigenetically naïve pluripotency (the ground state) is defined by global hypomethylation and decreased repressive H3K27me3 on developmental gene promoters, as well as a pre-XCI state (Marks et al., 2012). In mice, such naïve cells can drift toward a primed pluripotent state reminiscent of the post-implantation epiblast, which in many ways is more similar to typical hESC cultures (Brons et al., 2007). That is, both murine and human primed ESCs (most hESC lines), relative to naïve cells, exhibit more heterogeneous expression profiles, often have underwent XCI in female lines, have increased *de novo* methyl transferase expression, increased class II enhancer marks (poised for activation during differentiation), and a non-uniform differentiation propensity (Hanna et al., 2010; Gafni et al., 2013). So although pluripotency is classically tested as the ability of a stem cell to differentiate into all 3 germ layers and more stringently to form a blastocyst chimera, epigenomic differences whether through crude manipulation of the epigenome (Choi et al., 2004; Yoon et al., 2006; Chow et al., 2013b; Horrillo et al., 2013), adjustments to the microenvironment (Horton et al., 2009; Tompkins et al., 2012), or progressive accumulation of epigenetic changes over passaging (Tanasijevic et al., 2009; Tompkins et al., 2012), can all have defining influences on cell fate.

Perhaps the most elegant examples of a pre-existing epigenomic landscapes influencing cell fate are those involving pluripotent epigenetic "memories." Studies on histone modifications and DNA methylation in iPSCs and their somatic tissues of origin established the existence of residual epigenetic marks in the reprogrammed stem cells. iPSCs harboring epigenetic "memories" exhibit increased propensity to differentiate toward the somatic lineage from which they were originally derived, presumably due to the pre-existing set of partial instructions epigenetically retained within the iPSCs (Kim et al., 2010; Sullivan et al., 2010; Bar-Nur et al., 2011). Drawing focus on cardiac cell "memories," Sanchez-Freire et al., identified human CMs derived from cardiac progenitor-iPSCs to be generated with greater efficiency than fibroblast-iPSC-derived-CMs (Sanchez-Freire et al., 2014). They noted fibroblast somatic "memory" to include retained Nkx2.5 promoter methylation and corresponding restricted expression. However, when examining successfully derived CMs from either source, they appeared to be functionally equivalent (electrophysiology, calcium handling, therapeutic benefit in mouse MI model). Rizzi et al., similarly demonstrated enhanced CM differentiation efficiency for cardiac-derived-iPSCs in mice, but also observed functional improvement relative to CMs from fibroblast-iPSCs (calcium handling) (Rizzi et al., 2012). Besides the obvious differences in species (human vs mouse), Sanchez-Freire et al. ultized CPs rather than CMs for initial iPSC generation. As CP cells are multipotent precursors to CMs, derived iPSCs would not be expected to harbor epigenetic

"memories" reminiscent of terminally differentiated CMs. This may explain why CP-iPSCs can differentiate toward CMs with greater efficiency, but lack CM functional improvements relative to fibroblast-iPSC-derived-CMs. Further support that epigenetic "memories" may influence not only CM differentiation efficiency, but also CM function, can be seen in iPSC-to-CM subtype differentiation. To this end, iPSCs from murine ventricular cardiomyocytes (VMs) were shown to differentiate into VMs with higher efficiency and decreased CM heterogeneity as compared to fibroblast-iPSCs or genetically matched ESCs (Xu et al., 2012). As implied by formally describing VM-iPSC derived CMs as ventricular in nature, not only did VM-iPSCs produce increased CPs earlier in differentiation, but resulting CMs were electrophysiologically identical to VMs. Such clear lineage and ultimately CM functional bias was tied to residual VM DNA methylation signatures in VM-iPSCs. Although a number of studies have demonstrated that prolonged iPSC passaging can diminish epigenetic "memories, " such lineage bias may be useful for deriving certain difficult to generate cell types including CM subtypes (Kim et al., 2010; Sullivan et al., 2010; Bar-Nur et al., 2011; Xu et al., 2012; Chow et al., 2013b). Perhaps more important from an epigenomic perspective, however, is that cellular developmental histories encoded in epigenomic landscapes may directly shape induced differentiation outcomes.

**Looking forward**

From a HF and CM differentiation standpoint, proper epigenetic regulation of cardiac developmental programs is critical. As cardiac remodeling involves reactivation of fetal CM gene networks, it is not surprising that several common epigenetic factors are at work in pathological remodeling as well as in normal CM development (Miyamoto et al., 2006; Takaya et al., 2009; Papait and Condorelli, 2010; Kehat and Molkentin, 2010; Wang et al., 2010; Fu et al., 2011; Handy et al., 2011; Paige et al., 2012; Wamstad et al., 2012; Li et al., 2013). By extension, studies on polycomb repressive activity in HF and CM hypertrophy may prove to be particularly fruitful. Further parallels exist between the use of global epigenetic modifiers, such as HDAC inhibitor TSA, toward improving CM differentiation and the use of these agents to improve hypertrophic phenotypes (Antos et al., 2003; Kawamura et al., 2005). Though counterintuitive at first glance, it may be that TSA unlocks CM developmental genes in pluripotency (Karamboulas et al., 2006), but activates antihypertrophic pathways in the failing heart (Antos et al., 2003), and/or promotes transcriptional activity and endogenous cardiomyogenesis/angiogenesis toward repair (Zhang et al., 2012b; Zhang et al., 2012c). Regardless, these rather non-specific epigenomic landscape modifiers radically influence cell behavior and differentiation both in disease states and for *in vitro* cell differentiation. Therefore, more tailored control of the epigenome, it seems, would deliver novel approaches to correcting epigenetic components of human cardiomyopathies and also provide a basis to efficiently differentiate target cells and subtypes from pluripotent sources. Establishing pluripotent epigenomic landscapes that are most conducive to generating desired cell types for cell replacement based regenerative medicine will certainly require additional work. Yet, it may be possible to generate epigenomes inherently more suited for CM differentiation and ultimately mature and chamber-specific CM differentiation. Somatic cell epigenomic "memories" in iPSCs have already established that a pre-existing epigenomic landscape appears to guide cells toward unique cell fates (Kim et al., 2010; Sullivan et al., 2010; Bar-Nur et al., 2011; Xu et al., 2012; Chow et al., 2013b) and global signatures may already provide working maps for further epigenetic manipulation. To this end, and perhaps in complement to global epigenetic modifiers, locus-specific epigenetic control represents an essential evolution in molecular medicine. Though such technology is still within its infancy, it will likely ride on the heels of transcription activator like effector (TALE) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 systems that allow for unprecedented DNA binding control (Nature Methods editorial, 2012; Jinek et al., 2012), and be inspired by fusions of epigenetic catalytic domains to MBD/MLL and zinc fingers (Santillan et al., 2006; Fukushige et al., 2008; Sera, 2009). CRISPR, in particular, allows for multiple simultaneous Cas9 DNA binding events through guide RNA expression arrays (Nissim et al., 2014). The CRC guiding potential of ncRNAs coupled with the relative simplicity to introduce many small RNA species simultaneously may also facilitate new breakthroughs in modulating the epigenome. Regardless of the exact road taken, studies on the specificity and heritability of site-specific epigenetic modifiers, and ultimately the promise to influence cellular differentiation or correct epigenetic sources of pathogical remodeling and other cardiac diseases, will be exciting future developments.

## Compliance with ethics guidelines

Joshua D. Tompkins, PhD and Arthur D. Riggs, PhD declare they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

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