

# Epigenetic factors and cardiac development

Jan Hendrick van Weerd<sup>1,2,3†</sup>, Kazuko Koshiba-Takeuchi<sup>1,2†</sup>, Chulan Kwon<sup>4</sup>,  
and Jun K. Takeuchi<sup>1,2,5\*</sup>

<sup>1</sup>Cardiovascular Regeneration, Institute of Molecular and Cellular Biosciences, the University of Tokyo, Tokyo, Japan; <sup>2</sup>Biological Sciences, Graduate School of Sciences, the University of Tokyo, Tokyo, Japan; <sup>3</sup>Heart Failure Research Center, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; <sup>4</sup>Division of Cardiology, Johns Hopkins University School of Medicine, Baltimore, MD, USA; and <sup>5</sup>JST PRESTO, Understanding Life by iPS Technologies, Japan

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## Abstract

Congenital heart malformations remain the leading cause of death related to birth defects. Recent advances in developmental and regenerative cardiology have shed light on a mechanistic understanding of heart development that is controlled by a transcriptional network of genetic and epigenetic factors. This article reviews the roles of chromatin remodelling factors important for cardiac development with the current knowledge of cardiac morphogenesis, regeneration, and direct cardiac differentiation. In the last 5 years, critical roles of epigenetic factors have been revealed in the cardiac research field.

## Keywords

Epigenetics • Chromatin remodelling factors • Baf60c • Cardiac transcription factors • Cardiac cell fate • Cardiac reprogramming

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## 1. Introduction: cardiac morphogenesis and diseases

The heart is the first functional organ to form in developing embryos, and cardiogenesis takes place in a highly conserved manner from insects to vertebrates. After the formation of three germ layers (ectoderm, endoderm, and mesoderm), pre-cardiac mesodermal cells arise bilaterally from the nascent mesoderm. These cells migrate into the midline and differentiate, giving rise to the contractile heart.

In mammals and birds, the bilateral cardiogenic mesodermal cells migrate and merge at the anterior midline to generate the cardiac crescent, a crescent-shaped heart-forming region at the cranial border of the embryonic disc.<sup>1–3</sup> Upon folding of the embryonic disc, the cardiac crescent positions towards a developing neck area of the embryo and the edges of the cardiac crescent migrate to fuse and form the primitive heart tube.<sup>4</sup> The resulting heart tube undergoes the process of looping and chamber formation, accompanied by the activation of specific cardiac gene expression programmes required for the differentiation and maturation of pre-cardiac cells to generate the myocardium of the atrial and ventricular chambers, as well as the inflow tract, atrioventricular canal, and outflow tract.<sup>5,6</sup>

Two distinct pools of pre-cardiac fields were identified, both of which contribute to heart formation.<sup>7</sup> The first heart field (FHF) cells are derived from the cardiac crescent and give rise to the

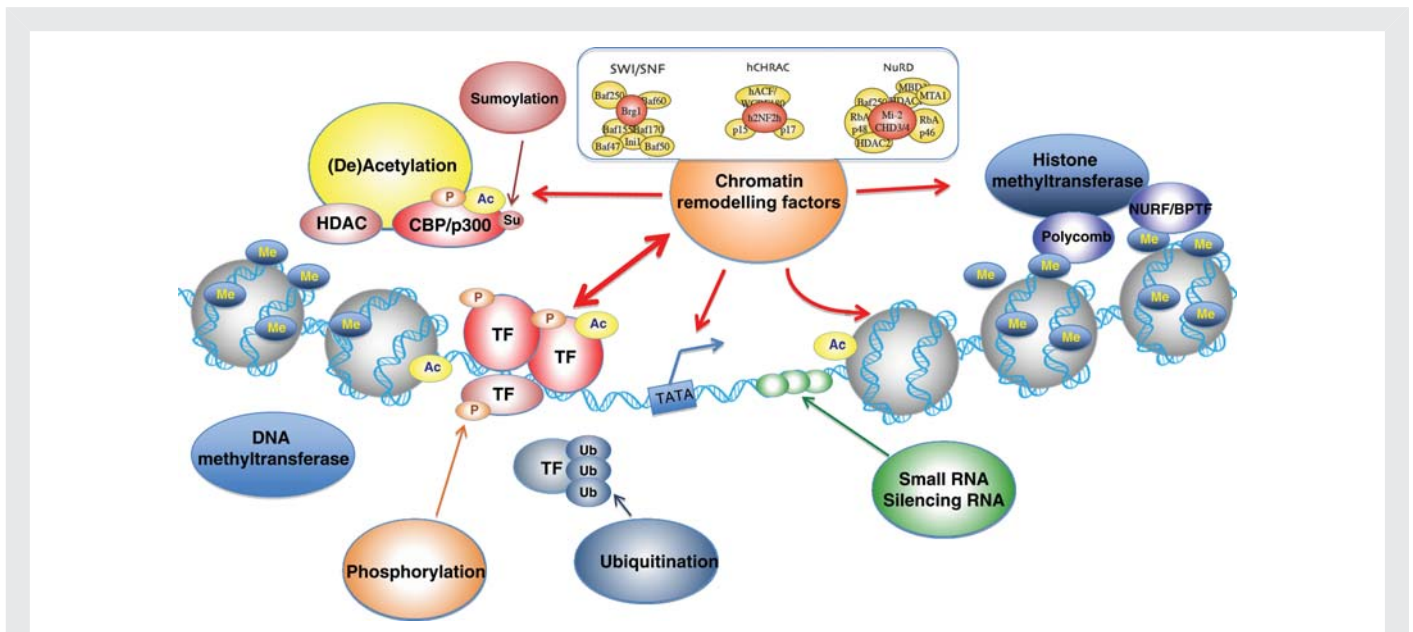
formation of the left ventricle and a part of the atria.<sup>8</sup> Cells from the second heart field (SHF), described by Kelly *et al.*,<sup>9</sup> are located in the pharyngeal mesoderm dorsal to the heart tube, giving rise to the outflow tract and right ventricle myocardium at the arterial pole of the heart.<sup>9–11</sup>

The induction, expansion, and differentiation of pre-cardiac cells are controlled by various signalling molecules, including bone morphogenetic proteins (BMPs),<sup>12</sup> fibroblast growth factors (FGFs),<sup>8</sup> and wingless-related MMTV integration site (Wnt) proteins.<sup>13</sup> BMP and FGF signals are important for the induction and differentiation of cardiogenesis.<sup>12,14,15</sup> BMPs play a key role in the specification of FHF cells by activating the expression of cardiac transcription factors such as Nkx2–5, Gata4, and Tbx5.<sup>16,17</sup> SHF progenitors, on the other hand, require Wnt/ $\beta$ -catenin signalling for their proper development.<sup>13,18,19</sup> Wnt/ $\beta$ -catenin signals positively regulate the expansion of SHF progenitors and affect the expression of *Islet1* (*Isl1*), a marker for multi-potent cardiac progenitor cells (CPCs).<sup>18,20,21</sup>

The mammalian heart consists of various cell types, including atrial and ventricular cardiomyocytes, fibroblasts, endocardial cells, epicardial cells, cells from the conduction system (sinoatrial node, atrioventricular node, purkinje fibers), smooth muscle cells making up the aorta and (coronary) arteries, and cells from the autonomous nervous system. Formation of the functional heart requires proper development of these cardiac cells through tight transcriptional regulation of cardiac genes. The fact that congenital cardiac anomalies occur at a high

<sup>†</sup> These authors contributed equally to the work presented here.

\* Corresponding author. Tel: +81 3 5841 8488; fax: +81 3 5841 8487, Email: junktakeuchi@iam.u-tokyo.ac.jp (or) takeuchi.jab@m.titech.ac.jp



**Figure 1** Chromatin structure and remodelling factors. DNA is organized in chromatin composed of condensed nucleosomes: units of 146 bp of DNA wrapped twice around an octamer of two copies of each histone protein H2A, H2B, H3, and H4. The flexible amino-terminal tails of the histones, protruding outward from the nucleosome, allow for post-translational modifications through (de)acetylation, phosphorylation, ubiquitination, methylation, and sumoylation. Such covalent modifications alter DNA–histone interactions, affecting accessibility of transcription factors.

frequency [ $\sim 1$ –2% of human live births suffer from a form of congenital heart defects (CHDs)] and mutations in numerous transcription factors can cause CHDs indicates the complexity of cardiac development.<sup>22–24</sup> At the epigenetic level, transcription factors are regulated by the assembly of DNA in higher-order chromatin structures (Figure 1). In this review, we will focus on epigenetic chromatin remodelling factors that are important for cardiac development, and discuss how these factors can be exploited to regulate the directed differentiation of non-cardiac cells towards fully functional cardiomyocytes in the search for new therapies against human CHDs.

## 2. Epigenetic factors and their roles in cardiac development

### 2.1 Epigenetic regulation: chromatin remodelling and DNA methylation

Eukaryotic development requires epigenetic mechanisms to control gene transcription for cell specification and differentiation. Chromatin remodelling is one of the essential epigenetic mechanisms for gene regulation (Figure 1). Chromatin is a multifaceted complex that serves to efficiently pack the large amount of DNA in the 5  $\mu\text{m}$  cell nucleus and to regulate gene transcription.<sup>25–27</sup> It consists of nucleosomes that are formed by wrapping of DNA around a core of histones.<sup>28</sup> Condensation of nucleosomes enables the packing of all the genomic molecules into the relatively small nucleus.<sup>26</sup> This compact, higher-order organization of chromatin requires regulatory mechanisms to allow the access of transcription factors to the DNA.<sup>29–32</sup> The chromatin state often determines gene activation and repression. ‘Open chromatin’ (euchromatin) refers to a lightly packed form of DNA that allows active gene transcription, whereas

‘closed chromatin’ (heterochromatin) is a tightly packed form of DNA in which transcription is repressed.<sup>28,33,34</sup>

The state of chromatin structure can be regulated by ATP-dependent chromatin remodelling complexes or modifications of histone tails.<sup>32,35</sup> The ATP-dependent chromatin remodelling complexes use the energy of ATP hydrolysis to modify chromatin structure. They can be classified into the complexes of SWI/SNF, ISWI, nucleosome remodelling and deacetylase complex (NuRD), and INO80 on the basis of their catalytic ATPase subunits.<sup>31,35–38</sup> Modification of histone tails is often enzymatically reversible<sup>39–41</sup> and results in an alteration of the interaction between chromatin and DNA. These modifications include acetylation,<sup>42</sup> methylation,<sup>43</sup> phosphorylation,<sup>44</sup> sumoylation,<sup>45</sup> and ubiquitination.<sup>46</sup>

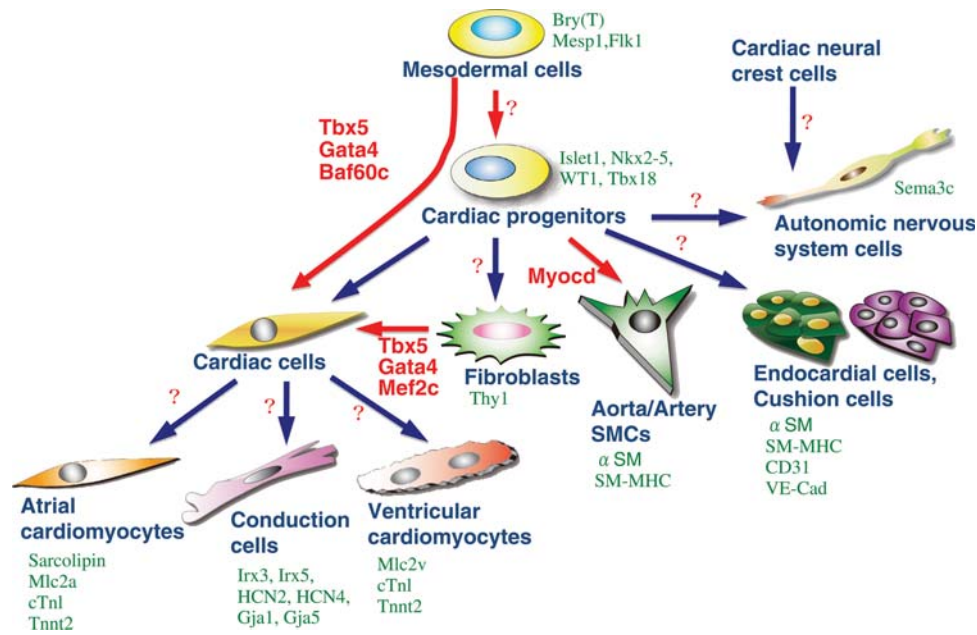
Another epigenetic mechanism that regulates gene transcription, besides histone modification, is DNA methylation.<sup>47,48</sup> DNA methylation typically occurs at CpG sites that contain cytosine-guanine nucleotides in a linear sequence. CpG-rich islands, short stretches of DNA with a relatively high frequency of CpG sites,<sup>48</sup> are often found at promoters of mammalian genes, and the extent of methylation at these sites is well correlated with the transcription status of corresponding genes. DNA methylation functions to stably silence gene transcription.<sup>47</sup>

### 2.2 Chromatin remodellers for cardiac development and CHDs

#### 2.2.1 Brg1/Brm-associated factor complex

The Brg1/Brm-associated factor (BAF) chromatin remodelling complex is the mammalian SWI/SNF complex composed of at least 11 subunits, and their variable arrangements contribute to distinct functions during development.<sup>31,49</sup>

The ATPase subunit of the BAF complex is encoded either by homologous genes *Brg1* (Brahma-related gene 1) or *Brm*, but Brg1 is the



**Figure 2** Cardiac cell types derived from multipotent progenitors. Differentiated cardiac cell types are marked by indicated genes (green). Recently, several factors have been defined as master regulators for cardiomyogenesis (red). The combination of Tbx5, Gata4, and Baf60c induces direct differentiation of mesodermal cells into ectopic beating myocytes, bypassing the cardiac progenitor state.<sup>55</sup> Tbx5, Gata4, and Mef2c together can also induce cardiomyocytes from fibroblasts.<sup>91</sup> Factors for direct induction of other cardiac cell types are currently unknown (question marks).

indispensable ATPase of the BAF complex.<sup>31,49,50</sup> Brg1 acts in the BAF complex to increase promoter accessibility for transcription factors, but it can also directly bind to transcription factors such as Gata proteins to regulate gene transcription.<sup>51</sup> Mice heterozygous for *Brg1* deletion exhibit cardiac morphogenetic defects, suggesting haploinsufficiency of Brg1 in heart development.<sup>52</sup> It turns out that the proper dosage of Brg1 is critical for normal heart development, as the disruption of the balance between Brg1 and CHD-causing cardiac transcription factors such as Tbx5, Tbx20, and Nkx2-5 leads to severe cardiac anomalies.<sup>52</sup> In mouse embryos, Brg1 activates  $\beta$ -myosin heavy chain ( $\beta$ -MHC, expressed primarily in foetal myocytes) while repressing  $\alpha$ -MHC expressed in adult myocytes.<sup>53</sup> Although silenced in adult mice, Brg1 is reactivated upon cardiac stress in adult myocytes and induces an  $\alpha$ -MHC to  $\beta$ -MHC shift, suggesting its role in maintaining myocytes in an embryonic state.<sup>53,54</sup>

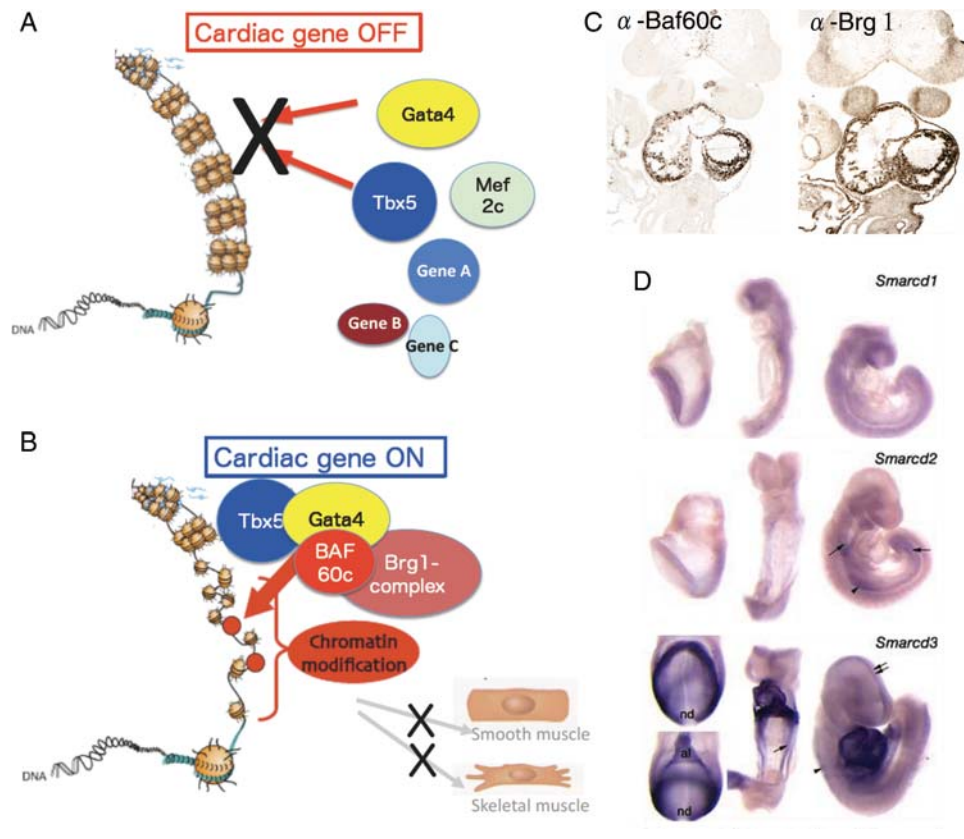
Baf60c is the cardiac-specific subunit of the BAF complex during early development and required for the ectopic induction of cardiomyocyte differentiation in combination with Gata4 and Tbx5.<sup>55</sup> (Figure 2, Figure 3A and B). Baf60c is encoded by the gene *Smarcd3*, whose mRNA is initially restricted to the developing heart from mouse embryonic day (E) 7.5.<sup>50,56</sup> Its subfamily members, *Smarcd1* and *Smarcd2*, which encode Baf60a and Baf60b, respectively, are not expressed in the developing heart at these stages, indicating the tissue specificity of Baf60c in embryonic development (Figure 3C and D).<sup>50,57</sup> Baf60c cooperates with Tbx5 to initiate their target gene activation for FHF formation.<sup>55,58,59</sup> Baf60c deficiency leads to outflow tract shortening, hypoplastic right ventricles and atria, and lack of atrioventricular canal.<sup>56,57</sup> Baf60a plays a role in linking the glucocorticoid receptor to the BAF complex, and is involved in c-fos/c-jun-mediated transcriptional activity. The precise role of Baf60b is

unclear, but it is specifically ubiquitinated by Unkempt, a RING finger protein partner of Rac GTPase. Although the significance of this ubiquitination is not completely understood, it is thought to be involved in maintaining the stoichiometry of the SWI/SNF complex.

Polybromo (BAF180), the prominent subunit of the BAF-related PBAF complex, is also involved in cardiogenesis by potentiating transcriptional activation mediated by nuclear receptors, such as RXR $\alpha$ , VDR, and PPAR $\gamma$ .<sup>60</sup> Deletion of Baf180 does not lead to early embryonic lethality, but, similar to RXR $\alpha$  deletion, results in a very thin cardiac wall with diminished trabeculae.<sup>61</sup> BAF180 is expressed in the epicardium and holds a non-redundant function to that of Baf60c in the respect that it mediates late aspects of cardiac chamber maturation and coronary development.<sup>60</sup> Ablating other subunits of the BAF complex (Baf47, Baf155, or Baf250) cause embryonic lethality at pre-implantation (Baf47, 155) or E6.5 (Baf250) in mice, indicating an essential role of the BAF complex for early embryonic development.<sup>31,62,63</sup>

## 2.2.2 NuRD and histone deacetylase complex

The NuRD complex contains histone deacetylases that function as transcriptional repressors.<sup>64</sup> Similar to the BAF complex, the NuRD complex exhibits diverse functions as a result of variable assemblies. Their ATPase activity resides in the two Mi-2 proteins, CHD3 and/or CHD4.<sup>65</sup> NuRD complexes mediate gene repression and regulate cell patterning and differentiation during early development.<sup>66,67</sup> The NuRD complex associates with Whsc1 (Wolf-Hirschhorn syndrome candidate 1) methyltransferase<sup>68</sup> and interacts with the Spalt-family zinc-finger transcription factor Sall4,<sup>69</sup> which is involved in interventricular septum development,<sup>70</sup> suggesting that the complex may play a role in heart development.



**Figure 3** Chromatin remodelling factor-mediated regulation of cardiac transcription factors. (A) In the absence of Baf60c, the cardiac transcription factors Gata4, Mef2c, and Tbx5 may not have access to their target genes. The highly condensed euchromatin in which the DNA is tightly wrapped around histones makes transcription factors inaccessible to regulatory DNA, thereby represses transcription. (B) Chromatin remodelling factors modify chromatin organization by unwinding DNA from the histones, making target sequences accessible for transcription factors. Baf60c acts as a bridge to bring the Brg1 complex together with the transcription factors Tbx5 and Gata4 in a tissue-specific manner. (C) Heart-restricted expression of Baf60c and Brg1 shown by immunostaining. (D) Expression of Smarcd3 (encoding Baf60c) restricted to cardiogenic mesoderm and the developing heart shown by *in situ* hybridization (adapted from Lickert et al.<sup>56</sup>).

### 2.2.3 Histone methyltransferase

Whsc1 is a histone methyltransferase that regulates activation of Nkx2–5, a homeobox protein critical for cardiac morphogenesis.<sup>68</sup> Whsc1 physically associates with Nkx2–5 and is required for the negative regulation of Nkx2–5 and its target genes, possibly through histone H3 trimethylation at lysine 36 H3K6me3.<sup>68</sup> Similar to Nkx2–5 mutations, Whsc1 mutations cause CHD, including atrial and ventricular septum defects in mice and human.<sup>68,71</sup>

Smyd1 (SET and MYND domain 1), a member of the lysine methyltransferase family, is specifically expressed in muscle tissue and acts as a transcriptional repressor by catalysing histone methylation through the SET domain.<sup>72,73</sup> Smyd1-deleted mouse embryos exhibit severe cardiac defects, including loss of the right ventricle with disrupted cardiomyocyte maturation.<sup>72</sup> In zebrafish, Smyd1 is essential for cardiac muscle contraction and myofiber maturation,<sup>74</sup> suggesting a conserved role of Smyd1 for cardiac development.

### 2.2.4 High mobility group chromatin protein

The high mobility group (HMG) of nuclear proteins exerts its function by architectural remodelling of the chromatin structure and by forming multi-protein complexes with promoter/enhancer sites, leading to transcriptional activation of their target genes.<sup>75</sup> The cardiac HMG member,

HMGA2, was shown to play important roles for cardiac differentiation *in vitro* and *in vivo*. Overexpression or siRNA-mediated knockdown of HMGA2 enhances or blocks cardiomyocyte differentiation *in vitro*, respectively. In *Xenopus* embryos, normal heart formation is blocked upon morpholino-mediated knockdown of HMGA2.<sup>75</sup> The fact that ‘HMGA2 is abundantly expressed during embryogenesis, whereas its expression is almost undetectable in adult tissues’ further indicates its role for embryonic heart development.<sup>75</sup> Furthermore, Nkx2–5 appears to be a target of HMGA2; in the presence of BMP, HMGA2 forms a protein complex with Smad1/4 and synergistically up-regulates promoter activity of Nkx2–5 in the presence of BMP stimulation through Smad- and HMGA2-binding elements. Moreover, promoter activity of Nkx2–5 requires a conserved HMGA2-binding site.<sup>75</sup>

## 3. Cell fate specification and epigenetic signalling

### 3.1 Transcription factors with instructive roles for cardiac differentiation

Cardiac transcription factors play critical roles in the early processes of cardiac cell specification and lineage determination. A number of

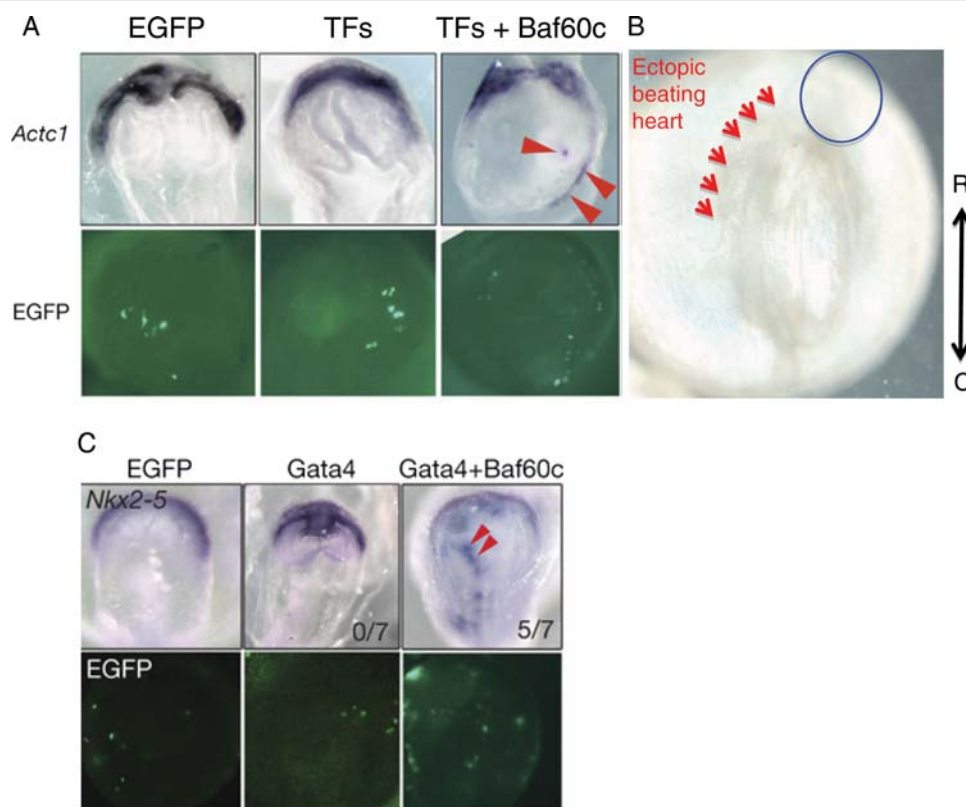
gain-of-function experiments have been carried out to identify factors to induce cardiac differentiation. For instance, the ectopic overexpression of Gata5, a zinc-finger transcription factor essential for proper heart and endoderm development, induces the expression of several cardiac genes (*Nkx2-5*, *Gata4*, *Gata6*) in zebrafish.<sup>76</sup> Gata4 possesses a similar potential in *Xenopus*.<sup>77</sup> However, the observed ectopic heart tissues appear to be formed as a secondary effect, as the overexpression of Gata genes causes additional axis-formation along the rostro-caudal axis.<sup>76,77</sup> Conditional deletion of  $\beta$ -catenin in the early endoderm layer leads to ectopic heart formation with *Nkx2-5* expression, and this phenotype is attributed to blockage of the inhibitory role of the Wnt pathway on cardiac differentiation.<sup>78,79,80</sup> In *Xenopus*, overexpression of myocardin was sufficient to induce ectopic expression of  $\alpha$ -SMA,  $\alpha$ -cardiac actin, and *Nkx2-5*. However, myocardin alone appears to be insufficient for establishing beating heart cells.<sup>81</sup> One of the key regulators for early heart development is Tbx5, a T-box transcription factor.<sup>58,82,83</sup> Tbx5 specifies the left-right identity of the cardiac chambers and the development of the ventricular septum.<sup>84</sup> Mice heterozygous for Tbx5 exhibit mal-formed cardiac chambers with an abnormal inter-ventricular septum, and homozygous deletion of Tbx5 alleles results in the absence of the left ventricle.<sup>58,59</sup> Similarly, human mutations in Tbx5 cause the Holt–Oram syndrome, which is characterized by atrial septal defects, upper

limb defects, and anomalies of the digits.<sup>58,85,86</sup> The importance of Tbx5 in heart development is also exemplified by the fact that its role is evolutionarily conserved among species.<sup>70,87</sup> Although overexpression of Tbx5 affects cardiac septum morphogenesis, it is not enough to induce cell differentiation into cardiomyocytes. Given that no single transcription factor so far has been shown to sufficiently induce cardiomyocytes, the developmental programme of cardiogenesis might be activated through multiple factors.

### 3.2 Master regulators for cardiomyogenesis

'Master regulators' control multiple genes to direct cell differentiation and are sufficient to activate an entire developmental programme. In 1988, Davis and colleagues demonstrated that overexpression of MyoD, a basic-helix-loop-helix (bHLH) transcription factor, is sufficient to convert fibroblasts to skeletal muscle cells.<sup>88,89</sup> Similarly, another bHLH-type transcription factor, myocardin, is sufficient to activate the developmental programme of smooth muscle differentiation.<sup>81,90</sup> However, as described earlier, no single transcription factor is known to act as a master regulator for cardiomyogenesis.

Recently, various combinations of cardiac transcription factors were used in an attempt at the directed transdifferentiation of non-cardiac cells into the cardiomyocyte lineage.<sup>55,57</sup> In this study, developmentally critical cardiac transcription factors (*Gata4*, *Nkx2-5*,



**Figure 4** Ectopic induction of cardiomyogenesis by defined factors (Tbx5, Gata4, and Baf60c). (A) Ectopic induction of cardiac tissues by co-overexpression of TFs (Tbx5, *Nkx2-5*, and *Gata4*) and Baf60c. The early cardiomyocyte marker *Actc1* was used to monitor the induction of cardiomyocytes. The chromatin remodelling component Baf60c is required for the induction. (B) Beating heart tissues (arrowheads) are observed in non-cardiogenic mesoderm upon overexpression of Tbx5, *Gata4*, and Baf60c. At this stage, the endogenous heart cells do not beat, indicating accelerated cardiac differentiation by the defined factors. (C) Whole-mount *in situ* hybridization showing that *Gata4* requires Baf60c to induce ectopic expression of *Nkx2-5*. EGFP expression indicates transfected cells (adapted from Takeuchi and Bruneau<sup>55</sup>).

**Table 1** Combinatorial activation of beating heart by Gata4, Tbx5, and Baf60c +, transfection of DNA; O, cardiac marker induction or beating heart induction

Actc1, Myl7 induction	Beating heart induction	Tbx5	Gata4	Gata1	Nkx2-5	Baf60c	Baf60b
×	×	+	-	-	-	-	
×	×	-	+	-	-	-	
×	×	-	-	+	-	-	
×	×	+	+	-	-	-	
×	×	+	-	+	-	-	
×	×	-	-	-	+	-	
×	×	+	-	-	+	-	
×	×	-	+	-	+	-	
×	×	+	+	-	+	-	
○	×	-	+	-	-	+	-
○	×	-	+	-	-	-	+
○	×	-	-	+	-	+	-
×	×	-	-	+	-	-	+
○	○	+	+	-	-	+	-
○	○	+	+	-	+	+	-

and Tbx5) were introduced into mesodermal cells of developing mouse embryos in different combinations. However, any combination of Gata4, Nkx2-5, and/or Tbx5 did not fully induce cardiomyocyte differentiation, suggesting that these transcription factors are not sufficient for cardiomyogenesis (Figure 4A, Table 1). Surprisingly, the addition of Baf60c, a cardiac-specific subunit of BAF chromatin remodelling complexes,<sup>56</sup> led to ectopic differentiation of mesodermal cells into beating cardiomyocytes.<sup>55</sup>

Chromatin modification is a dynamic process required for the proper function of transcription factors, allowing them to have access to their target loci (Figure 3A and B). Genome-wide screening revealed the presence of cardiac-specific chromatin remodelling factors,<sup>56</sup> indicating their potential involvement in directed transdifferentiation. Indeed, expression dosage of Baf60c allowed Gata4 to access its target genes by modifying their chromatin structures, leading to the ectopic expression of cardiac genes. Tbx5 overexpression promoted cardiomyogenesis by repressing the activation of non-cardiac mesodermal genes.<sup>55</sup> Chromatin immunoprecipitation assays confirmed these findings by showing the presence of the heart-specific Baf60c-remodelled chromatin. Furthermore, the binding of Gata4 and Tbx5 to the cTnT promoter region required Baf60c-mediated chromatin remodelling, suggesting that the combination of Gata4, Tbx5, and Baf60c acts as a master regulator for cardiomyocyte differentiation from mesodermal cells (Figure 3).<sup>55</sup>

More recently, Ieda et al.<sup>91</sup> demonstrated that combinatorial overexpression of developmentally critical transcription factors is sufficient to the direct reprogramming of cardiac fibroblasts into functional cardiomyocytes. Interestingly, Gata4 and Tbx5 were also required for the reprogramming, although Mef2c was used instead of Baf60c (Figure 2). The induced cardiomyocytes expressed the cardiac-specific markers Actc1, Myh6, Ryr2, and Connexin43, whereas Col1a2—a marker for fibroblasts—was markedly down-regulated. Strikingly, they exhibited a global gene expression profile similar to that of cardiomyocytes, with cardiomyocyte-like chromatin patterns on several genes, indicating epigenetic resetting. H3K27me3 (trimethylated histone H3 of lysine 27) and H3K4me3 (trimethylated

histone H3 of lysine 4) mark transcriptionally inactive or active chromatin, respectively.<sup>92</sup> Further methylation analyses of induced cardiomyocytes revealed decreased levels of H3K27me3 and increased levels of H3K4me3 in the promoters of cardiomyocyte genes.<sup>91</sup>

Curiously, Baf60c was not required for the reprogramming of cardiac fibroblasts. This is likely due to cell-type differences between embryonic mesodermal cells and fibroblasts. It is reasonable to speculate that cardiac or dermal fibroblasts share similar chromatin patterns with cardiogenic cells, so that overexpression of cardiac chromatin remodellers may not be necessary for the event. Also, Mef2c may regulate expression of chromatin remodelling factors required for cardiac reprogramming.

### 3.3 Approaches for cardiac regeneration

CHDs are the most common birth defects in humans, and heart disease remains the leading cause of human death worldwide. The high morbidity and mortality is largely attributed to the limited regenerative capacity of the heart. Recent research has focused on developing new strategies, especially cell-mediated therapies, to treat damaged hearts. One approach is to utilize pluripotent stem cells (PSCs) such as embryonic stem cells (ESCs) or induced PSCs (iPSCs). These cells are highly plastic and can expand and differentiate into most of existing cells, including functional cardiomyocytes.<sup>75,93,94</sup> After birth, the heart itself is insufficient in its regenerative response upon damage, such as from infarcts, as most cardiomyocytes are terminally differentiated and do not proliferate. Therefore, ESCs or iPSCs may hold potential for treating cardiac defects. In addition, iPSC transplantation is advantageous over whole-organ transplantation in that these cells can be directly obtained from patients to avoid immune rejection.<sup>95</sup> However, transplantation of undifferentiated iPSCs into the mouse heart has resulted in teratoma formation.<sup>96</sup> Analysis of these teratomas revealed cell types from all three embryonic germ layers, indicating that existing cardiac cells do not guide iPSCs to differentiate into cardiac cells.<sup>96,97</sup> As described earlier, cardiogenesis takes place in a highly coordinated fashion by interactions of multiple factors,<sup>12,98-101</sup> and it will therefore be

important to understand the cardiogenic mechanisms of these factors for iPSC-mediated cardiac therapy.

CPC-based therapy offers a better approach for heart regeneration. CPCs are committed pre-cardiac cells with a potential to differentiate into multiple cardiac cell types, including cardiomyocytes, smooth muscle cells, and endothelial cells.<sup>20,21,102</sup> The identification of Isl1<sup>+</sup> cells led to the discovery of the undifferentiated CPCs, which has advanced our knowledge of multi-potent CPCs.<sup>20,103</sup> They are also marked by Nkx2-5 or Flk1 and can be isolated from early developing embryos or differentiating pluripotent cells.<sup>21,102,104</sup> A recent trial of embryonic CPC transplantation in post-myocardial infarcted hearts of non-human primates showed successful engraftment with myocardial differentiation,<sup>105</sup> suggesting that CPCs can be used as an effective source for heart regeneration. Understanding the mechanisms of lineage-specific differentiation of CPCs will accelerate the CPC-mediated cardiac therapeutics.

Hattori *et al.*<sup>106</sup> recently introduced a novel approach to isolate cardiomyocytes. By use of tetramethylrhodamine ethyl ester perchlorate (a fluorescent dye specific to mitochondria), they successfully isolated embryonic and neonatal cardiomyocytes (>99% purity) by fluorescence-activated cell sorting. Moreover, transplantation of these purified cardiomyocytes did not induce teratoma formation, and their aggregation resulted in long-term survival of the transplanted myocytes *in vivo*.<sup>106</sup> Further studies will be necessary to test their effects on damaged hearts and large animals. Induced cardiomyocytes from directed differentiation also have tremendous therapeutic potential to treat heart disease. However, the differentiation method will need to be optimized before a clinical trial. For example, the differentiation efficiency needs to be improved with quantitative studies and more rigorous functional assays should be carried out *in vitro* and *in vivo*. In addition, it will be important to test whether endogenous cells such as cardiac fibroblasts can be directly differentiated into cardiomyocytes *in vivo*.

## 4. Future perspectives

Numerous genetic and epigenetic factors regulating cardiac morphogenesis, differentiation, and maturation have been identified through decades of progress in developmental cardiology. The knowledge gained from the developmental studies led to the recent breakthrough discoveries of defined factors, whose co-overexpression is sufficient to instruct non-cardiac cells to convert into cardiomyocytes. The defined factors (Gata4 and Tbx5 with Baf60 or Mef2c), also essential for cardiac development, are transcription and chromatin remodelling factors that act cooperatively with others, highlighting the importance of a mechanistic understanding of transcriptional and chromatin regulation. It would be interesting to see whether direct differentiation of other cardiac lineages such as smooth muscle, endothelial, or conduction cells also occurs through defined factors. As illustrated in Figure 2, different types of cardiac cells express distinct gene products, but it is mostly unknown if these cell types can be directly obtained from multi-potent progenitors by defined factors. It would be important to identify factors that can activate the programmes for individual cardiac lineage determination.

Although cardiac transcription factors have been extensively studied for their roles and targets, the mechanisms by which chromatin remodellers modulate activation or repression of specific signalling and transcriptional networks are not well understood. Further investigation will be required to elucidate the roles of cardiac epigenetic

factors for a better understanding of the process of cardiogenesis, as well as directed cardiac differentiation or reprogramming.

Given that the mammalian heart has limited regeneration capacity, direct reprogramming is emerging as a novel form of potential cardiac therapeutics along with CPC-mediated transplantation.<sup>105,106,107</sup> We are rapidly entering into a new era of cardiac regenerative medicine that combines knowledge from the diverse fields of heart biology, including developmental, molecular and cellular cardiology, and cardiac physiology. This integrative approach and effort should accelerate novel discoveries for future cardiac therapeutics as well as preventive strategies for CHD.

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