CONTEMPORARY REVIEW

Epigenetics in Congenital Heart Disease

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ABSTRACT: Embryonic heart development is an intricate process that mainly involves morphogens, transcription factors, and cardiac genes. The precise spatiotemporal expression of these genes during different developmental stages underlies normal heart development. Thus, mutation or aberrant expression of these genes may lead to congenital heart disease (CHD). However, evidence demonstrates that the mutation of genes accounts for only a small portion of CHD cases, whereas the aberrant expression regulated by epigenetic modification plays a predominant role in the pathogenesis of CHD. In this review, we provide essential knowledge on the aberrant epigenetic modification involved in the pathogenesis of CHD. Then, we discuss recent advances in the identification of novel epigenetic biomarkers. Last, we highlight the epigenetic roles in some adverse intrauterine environment-related CHD, which may help the prevention, diagnosis, and treatment of these kinds of CHD.

Key Words: biomarkers ■ cardiac development ■ congenital heart disease ■ epigenetics

The heart is one of the first organs to develop during embryogenesis, and its development depends on faithful and precise expression of various genes in a temporal and spatial manner. Morphogens, transcription factors, and cardiac genes are involved in early heart development and form a hierarchical regulatory relationship. For example, morphogens Nodal (Nodal growth differentiation factor), Wnts (Wnt family members), bone morphogenic proteins, Sonic hedgehog, and retinoic acid regulate transcription factors mesoderm posterior bHLH transcription factor 1, NK2 homeobox 5 (NKX2.5), GATA binding protein 4 (GATA4), ISL LIM homeobox 1 (ISL1), and T-box transcription factor 1 (TBX1), which in turn transcriptionally activate their target cardiac genes. In addition, transcription cofactors including Yes1 associated transcriptional regulator, Tafazzin, and vestigial like family member 4 interact with DNA binding transcription factors including TEA domain transcription factor, which activates the expression of target genes that regulate cardiac cell proliferation and embryonic heart development. However, mutation or aberrant expression of these genes during cardiogenesis may induce congenital heart disease (CHD).

CHD, the most common type of birth defect, is characterized by congenital malformation of heart walls, valves, or blood vessels, which can be divided into several phenotypes including atrial septal defect, ventricular septal defect (VSD), atrioventricular septal defect, tetralogy of Fallot (TOF), and hypoplastic left heart syndrome. CHD affects ≈1% of live births and accounts for 30% of fetal deaths. Because of its high morbidity and mortality, extensive studies have been conducted to identify the origins of CHD; however, the causes of the majority of cases remain elusive. Single gene mutation of specific morphogens, transcription factors, or cardiac genes in cardiogenesis is sufficient to cause only about 10% of all CHD cases, which suggests that extragenomic factors governing gene expression may play a predominant role in the pathogenesis of CHD.

Epigenetic modification is the extragenomic mechanism, which does not involve alterations in the DNA sequence but is capable of regulating gene expression by influencing transcription or inhibiting translation. Growing evidence has demonstrated the association of epigenetics with cardiac development and diseases. In this review, we mainly focus on aberrant patterns of DNA methylation, histone modification, ATP-dependent chromatin remodeling, and microRNA (miRNA) involved in the pathogenesis of CHD (Figure 1), discuss recent advances in identification of novel epigenetic biomarkers, and highlight epigenetic roles in some adverse intrauterine environment-related CHD, providing essential...
knowledge for the understanding of CHD pathogenesis, and the diagnosis and treatment of CHD (Figure 2).

**DNA METHYLATION**

DNA methylation is the process of adding a methyl group into the 5’ carbon of cytosine, which alters the structure of DNA molecules and then interferes with the binding of transcription factors, resulting in possible changes in gene expression patterns. In mammals, DNA methylation mainly occurs in dinucleotide CpG (DNA methylation on cytosines followed by guanine residues) sites, which constitute ≈1% of the genome, and most of them are methylated. CpG islands are regions rich in CpG sites, most of which are not methylated in gene promoters, and these CpG islands play critical roles in the regulation of gene expression. DNA methylation pattern is maintained by DNMT1 (DNA methyltransferase 1) during the course of somatic cell division, whereas de novo DNA methylation is modulated by DNMT3A (DNA methyltransferase 3A) and DNMT3B (DNA methyltransferase 3B). DNA demethylation is regulated by TET (ten-eleven translocation) enzymes, which oxidize 5mC (5-methylcytosine) to 5hmC (5-hydroxymethylcytosine) and promote locus-specific DNA demethylation. TET is also involved in the process of cardiac progenitor specification by maintaining the hypomethylated status and expression level of NKX2-5.

The spatiotemporal expression of cardiac genes is orchestrated perfectly during heart development, and studies have been conducted to elucidate the regulatory role of DNA methylation involved in this process. A mouse model was used to compare the difference in DNA methylation in embryonic hearts between E11.5 and E16.5.

### Figure 1. Schematic diagram of mechanisms of epigenetic modification.

Epigenetic modification usually includes DNA methylation, histone modification, ATP-dependent chromatin remodeling, and microRNA (miRNA). H3K4me3 and H3K9ac as representative histone marks are illustrated here; however, there are some other histone marks that are not shown. CpG indicates DNA methylation on cytosines followed by guanine residues; H, histone; H3K4me3, indicates the tri-methylation at the 4th lysine residue of the histone H3 protein; H3K9ac, indicates the acetylation at the 9th lysine residue of the histone H3 protein; 3′-UTR, the three prime untranslated region mRNA, messenger RNA.
and E14.5, and researchers found 181 genes were developmentally regulated, and 79 genes had correlative changes between their methylation and expression. Of these genes, Has2 (hyaluronan synthase 2), which is required for heart valve formation, was downregulated at E14.5, and its elevated methylation was dependent on DNMT3B. Another study analyzed DNA methylomes from highly purified cardiomyocytes of neonatal, adult healthy, and adult failing hearts, and identified large genomic regions that were differentially methylated during cardiomyocyte development and maturation, suggesting that dynamic DNA methylation modulates cardiomyocyte development, maturation, and disease.

Case-control studies have been extensively performed to uncover the role of abnormal DNA methylation of cardiac genes, cardiac transcription factors, and related signaling pathway molecules in CHD. One study determined the genome-wide DNA methylation pattern in myocardial biopsies obtained from patients with TOF or VSD and controls, and found a remarkable overlap of hypermethylated promoters and downregulated genes. Of note, the authors identified a novel hypermethylated developmental CpG island in the promoter of SCO2 encoding a protein that is required for the assembly of cytochrome c oxidase, which may drive the metabolic state of cardiac cells toward glycolysis, retarding their terminal differentiation and facilitating cardiomyopathy. Another study assessed gene-specific DNA methylation difference in peripheral blood lymphocytes from 180 mothers with nonsyndromic CHD-affected pregnancies (cases) and 187 mothers with unaffected pregnancies (controls). Using a multifactorial statistical model, researchers observed that majority of differentially methylated CpG sites between the 2 groups were hypermethylated in cases and located within CpG islands, and that the genes of interest were enriched in multiple biological processes involved in fetal development by gene set enrichment analysis. A third study searching for abnormal DNA methylation profiles in heart tissue of fetuses with syndromic and nonsyndromic CHD found multiple hypermethylated CpGs in the cardiac transcription factor GATA4 gene body and higher expression of GATA4 transcript coexisted in fetuses with syndromic and nonsyndromic CHD compared with healthy controls, suggesting this epigenetic modification likely contributed to the pathogenesis of the malformation. A fourth study investigated the methylation status of RXRA (retinoid X receptor α), an important component of the retinoid acid signaling pathway, in the pathogenesis of TOF. Researchers indicated that the methylation status of RXRA promoter region was significantly higher in right ventricular outflow tract myocardium of patients with TOF, whereas the mRNA level of RXRA was decreased.

Figure 2. Representative diagram of genetic and epigenetic origins, adverse intrauterine environment inducer of congenital heart disease (CHD), and epigenetic biomarkers and potential epi-drugs for CHD. For each panel, examples are given to represent the current knowledge associated with CHD. Representative heart defect, tetralogy of Fallot, is depicted here: (1) Ventricular septal defect. (2) Right ventricular hypertrophy. (3) Overriding aorta. (4) Pulmonary stenosis. For the genetic mutation panel, the reference that includes NKX2-5, GATA4, TBX5, TBX1, NODAL, and NOTCH1 was cited but not discussed in the article. Dotted arrow indicates epi-drugs are still not investigated for the treatment of CHD but may have the therapeutic potential. HDAC indicates histone deacetylase enzyme; miR, microRNA.
simultaneously, which may help decipher the pathogenesis of TOF and provide a new candidate target for therapy. The modifiers and modifications of DNA methylation are summarized in Table 1.

**HISTONE MODIFICATION**

Histone octamers containing 2 copies of each of the core histones H2A, H2B, H3, and H4 are wrapped by a segment of DNA to form a nucleosome, which not only stores compacted DNA but also regulates gene expression. Histone modification influences chromatin architecture and then changes the accessibility of transcription factors and initiation complexes, causing gene activation or silencing. Histone modification mainly comprises well-studied methylation and acetylation, as well as phosphorylation, ubiquitination, and sumoylation. The nomenclature of histone modifications depends on the specific type of histone protein, amino acid type and location, and type of modification. For instance, H3K4me3 indicates a trimethylation at the lysine 4 residue of histone H3. The maintenance of histone modification generally includes 3 types of factors: writers (eg, methyltransferase, acetyltransferase), erasers (eg, demethylase, deacetylase), and readers (eg, effector proteins recognizing specific banding sites), each of which is essential for cardiac development, and their aberrant patterns are associated with CHD. In a case-control study comparing the incidence of de novo mutations in severe CHD cases and controls by analysis of exome sequencing, researchers found a pronounced excess of de novo mutations in genes involved in writing, erasing, or reading of H3K4 (4th lysine residue of the histone H3 protein) methylation or H2BK120 (120th lysine residue of the histone H2B protein) ubiquitination that is required for H3K4 methylation, revealing a potential pathogenic role of aberrant histone methylation in CHD. Knockout studies further showed the regulatory role of different histone modifiers in cardiac development and CHD. DOT1 like histone lysine methyltransferase (DOT1L), H3K79 (79th lysine residue of the histone H3 protein) methyltransferase, is involved in normal mammalian development. Dot1L knockout is lethal in embryonic mice, because it is essential for the proliferation of mouse embryonic stem cells and cardiac development. LSD1 (lysine-specific demethylase 1) is the first of a group of enzymes discovered to have lysine-specific demethylase activity. A study used Cre-lox technology to generate 2-point mutations in the Lsd1 allele in mice, which resulted in the reduced

| Table 1. DNA Methylation, Histone Modification, and ATP-Dependent Chromatin Remodeling in CHD |
|---|---|---|---|---|
| **Modifiers** | **Modification** | **Target genes** | **Disease phenotype** | **References** |
| DNMT3B | Hypermethylation | H3K4me3, H3K9me3 | CHD, Kabuki syndrome, impaired | 25, 26 |
| ... | Hypermethylation | MEF2C, Cx43, Anp and β-MHC | Impaired EMT, proliferation and differentiation, increased apoptosis | 27, 28 |
| MLL2 | H3K4me3 | Nkx2.5, Tbx5, Mef2c | CHD, Kabuki syndrome, impaired differentiation of ESCs | 24 |
| EZH2 | H3K27me3 | Six1, Hey2 | TOF | 19 |
| UTX | H3K27me3 demethylation | GATA4 | TOF or VSD | 21 |
| DPF3 | BAX complex recruitment | GATA4, EBAF | Impaired ectoderm and mesoderm | 23 |
| P300 | H3K4, H3K9, H3K27, H4 acetylation | Six1, Hey2 | Incomplete cardiac looping, severely reduced | 29, 30 |
| HDAC3 | Deacetylation | TGF-β, Tbx5 | Ventricular contractility | 24 |
| HDAC2 | Deacetylation | GATA4 | Cardiac-induced cardiac dysplasia | 25, 26 |
| G9a | H3K9me3 | Mef2c, Cx43, Anp and β-MHC | Alcohol-induced cardiac damage | 25, 26 |
| BRG1 | Chromatin remodeling | GATA4, Tbx5, Tbx20, Nkx2.5 | Cardiac anomalies, trabeculation defects | 25, 26 |
| CHD7 | Chromatin remodeling | Nkx2.5, PlexnA2 | CHD, CHARGE syndrome | 25, 26 |

... indicates not investigated; BAX, BCL2 associated X, apoptosis regulator; BRG1, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4; CHD, congenital heart disease; CHD7, chromodomain helicase DNA-binding 7; DNMT3B, DNA methyltransferase 3B; DPF3, double PHD fingers 3; EMT, endothelial-to-mesenchymal transition; ESCs, embryonic stem cells; EP300, E1A binding protein p300; EZH2, enhancer of zeste 2 polycomb repressive complex 2 subunit; HDAC3, histone deacetylase3; HDAC2, histone deacetylase2; H3K4, the 4th lysine residue of the histone H3 protein; H3K4me3, the tri-methylation at the 4th lysine residue of the histone H3 protein; H3K9me3, indicates the tri-methylation at the 9th lysine residue of the histone H3 protein; TOF, tetralogy of Fallot; UTX, Utx histone demethylase; and VSD, ventricular septal defect.
interaction between the LSD1 protein and known binding partners as well as decreased enzymatic activity. Mice homozygous for this allele died perinatally because of heart defects, with the majority of mice suffering from VSD.51 WD repeat domain 5 (WDR5) is a core subunit of the human lysine methyltransferase (MLL) and SET domain containing 1, histone lysine methyltransferase (SET1) histone H3K4 methyltransferase complexes, defined as the reader of H3K4 methylation.52 The deletion of Wdr5 in Xenopus tropicalis resulted in substantial pericardial edema that altered the structure of the heart, impeding accurate determination of its cardiac looping.53

In the following section, we mainly focus on the involvement of histone methylation and acetylation in CHD.

**HISTONE METHYLATION**

Histones can be methylated at lysine and arginine residues; generally, lysine residue methylation has been more extensively studied, including K4, K9, K27, K36, and K79 in histone H3, and K20 in histone H4.54 Lysine residues can be methylated to varying degrees, including mono (me1)-, di (me2)-, or trimethyl (me3) groups,55 which adds another layer of complexity to their regulatory mechanisms. Histone methylation or demethylation can cause either gene activation or silencing depending on the methylated lysine residue sites and the degree of methylation.56

Studies have demonstrated that histone lysine methylation is a pivotal epigenetic regulator in cardiac development, whereas their aberrant patterns may cause cardiac anomalies. Trimethylation of H3K4 is usually associated with transcriptional activation of target genes, which is primarily catalyzed by TrxG (trithohaxotide group) proteins.57 A population-based study revealed an association between lysine methyltransferase 2D (MLL2) (a TrxG member) and CHD in patients with Kabuki syndrome.26 CHD was diagnosed in 19 out of 27 (70%) patients with the MLL2 variant, and the anatomic types of CHD included aortic coarctation, bicuspid aortic valve, perimembranous subaortic ventricular septal defect, atrial septal defect of the os- tum secundum type, and conotruncal heart defects.26 Mll2 was also found to be essential for the differentiation of mouse embryonic stem cells in a study.26 When it was deleted, the differentiation of embryonic stem cells into cardiac lineage was completely abolished; in addition, the levels of H3K4me3 and mRNAs of core cardiac transcription factors (Nkx2.5, Tbx5, and Mef2c) were significantly decreased. Trimethylation of H3K27 (27th lysine residue of the histone H3 protein) (the trimethylation at the 27th lysine residue of the histone H3 protein [H3K27me3]) induces robust gene silencing.20 which is regulated by PcG (polycomb group) proteins comprising PRC1 (polycomb repressive complex 1) and PRC2 (polycomb repressive complex 2).58 The catalytic subunit enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2), a histone methyltransferase in PRC2, is critical for the catalyis role. A previous study indicated that deletion of Ezh2 in cardiac progenitors triggered postnatal myocardial pathology and unstable cardiac gene expression.27 Notably, the homeodomain transcription factor gene Six1 was activated, which induced cardiomyocyte hypertrophy and skeletal muscle gene expression, suggesting that ezh2-mediated repression of Six1 in differentiating cardiac progenitors is essential for stable postnatal heart gene expression and homeostasis. In a mouse model in which Ezh2 was specifically ablated in the mouse heart, researchers observed that hypoplastic endocardial cushions, impaired endothelial-to-mesenchymal transition process, decreased cardiomyocyte proliferation and increased apoptosis.28 In addition, they found that the Hey2 gene is a downstream target of Ezh2, and that the regulation of Hey2 expression by Ezh2 may be independent of Notch signaling activity.

The maintenance of histone methylation patterns requires the close collaboration of writers, erasers, and readers. UTX (ubiquitously transcribed tetratricopeptide repeat, X chromosome), a specific histone demethylase of H3K27me3, was found to be involved in embryonic heart development in a knockout study, because Utx-null embryos had reduced somite counts, neural tube closure defects, and heart malformation between E9.5 and E13.5.29 Another knockout study also found that Utx was required for the establishment of ectoderm and mesoderm; however, this contribution was independent of the catalytic activity of UTX.30 Double PHD fingers 3 (DPF3) is a member of the highly conserved d4 protein family, which can bind methylated lysine residues of H3K4, functioning as a reader in histone modification.31 A group showed that DPF3 was remarkably upregulated in the right ventricular myocardium of patients with TOF in a genome-wide gene expression study.32 They further found that morpholino knockdown of dpf3 in zebrafish resulted in incomplete cardiac looping and severely reduced ventricular contractility, which confirmed that DPF3 is a key epigenetic factor for heart and muscle development.31

**HISTONE ACETYLATION**

Histone acetylation is linked to gene transcription activation, because the acetylation of the N-terminal tails of the lysine residues on histones can loosen chromatin spatial structures and thus facilitate the binding of transcription factors to DNA, initiating gene transcription.59 Histone acetylation is catalyzed by HATs (histone
acetyltransferases), which are traditionally classified into 2 distinct classes (type A and type B) based on their subcellular localization. E1A binding protein p300 (P300) is an extensively studied type A HAT that has an important role in embryonic cardiogenesis, and its ectopic expression is related to CHD. A study indicated the acetylation of H3K4, H3K9 (the 9th lysine residue of the histone H3 protein), and H3K27 mediated by P300 plays a crucial role in the regulation of Gata4 expression in cardiogenesis. Another study focused on the regulatory mechanism of EBAF (Left-right determination factor 2). It is also called LEFTY2), which is a NODAL pathway inhibitor that plays a critical role during mammalian cardiac development. The authors found that P300 was involved in the transcriptional activation of EBAF by inducing the hyperacetylation of histone H4 at the EBAF promoter. A third study showed that the recruitment of P300 to enhancers requires MLL4 (enhancer H3K4me1/2 methyltransferase) during the differentiation of embryonic stem cells. The deacetylation of histones, mediated by HDAC (histone deacetylase) enzymes, is associated with gene silencing. Studies have already shown their epigenetic regulatory roles in heart development and CHD. A research group found murine embryos lacking Hdac3 in the second heart field exhibited a variety of anomalies (eg, ascending aortic dilatation, outflow tract malrotation, a double outlet right ventricle, a bi-cuspid aortic valve, VSD), and elevated transforming factor 5 (TBX5) target genes. Researchers further addressed HDAC3 physically interacting with TBX5 and catalyzing its acetylation to suppress TBX5-dependent activation of cardiomyocyte lineage-specific genes. Another study revealed that HDAC2, along with HOP homeobox (HOPX) (a small homeodomain factor), mediates deacetylation of Gata4 during embryonic development, whereas the absence of Hdac2 and Hopx in mouse embryos results in Gata4 hyperacetylation and cardiac defects. The modifiers and modifications of histone are summarized in Table 2.

### ATP-DEPENDENT CHROMATIN REMODELING

In addition to DNA methylation and histone modification, ATP-dependent chromatin remodeling is another type of epigenetic regulation of gene expression at the chromatin level. ATP-dependent chromatin remodeling complexes (remodelers) move, eject, or restructure nucleosomes by using the energy of ATP hydrolysis, which can alter the accessibility of DNA molecules to transcription factors and regulate gene transcription. These remodelers are mainly divided into 4 groups on the basis of their composition and activities, including SWI/SNF (switching defective/sucrose nonfermenting), ISWI (imitation SWI), NURD (nucleosome remodeling and deacetylation)/Mi-2/CHD (chromodomian, helicase, DNA binding), and INO80 (inositol requiring 80). All these remodelers share a common ATPase domain, whereas their functions are specific because each remodeler complex has a unique protein domain. The role of distinct remodelers during mammalian cell differentiation and organogenesis has recently been well reviewed.

Growing evidence demonstrates that remodelers are closely related to embryonic cardiogenesis and CHD. BRG1 (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4) is the ATPase subunit of the BAF...
Brg1/− factors causes severe cardiac anomalies. For example, the balance between Brg1 dosage-dependent manner, whereas disrupting the expression of CHARGE-like phenotypes including heart septal defects. Another study indicated that inactivation of CHD7 (chromodomain helicase DNA-binding 7) causes a hypoplastic left ventricle and dilated atria. CHD7 (chromodomain helicase DNA-binding 7) is another well-studied ATP-dependent chromatin remodeler that is associated with CHD. Haploinsufficiency for the CHD7 gene is the leading cause of CHARGE (coloboma, heart defects, atresia choanae [also known as choanal atresia], growth retardation, genital abnormalities, and ear abnormalities) syndrome characterized by a specific pattern of defects including CHD. A study analyzed the information on cardiac defects in 299 patients with pathogenic CHD7 mutations, and found that 74% of them had a type of CHD. Notably, atrioventricular septal defect and conotruncal heart defects were overrepresented. A knockout study indicated that mice homozygous for Chd7 died at E10.5, whereas those heterozygous for Chd7 developed multiple CHARGE-like phenotypes including heart septal defects. Another study indicated that inactivation of Chd7 (chromodomain helicase DNA-binding 7) causes multiple embryonic heart defects. Interestingly, the authors found that CHD7 binds to enhancer regions of the cardiac transcription factor Nkx2.5 in a bone morphogenetic protein–dependent pattern to directly govern the expression of Nkx2.5 during cardiogenesis. A third study revealed the cooperation of CHD7 and BRG1 in the regulation of the expression of PlexinA2 that encodes a receptor for semaphorin to guide neural crest cells migration into the outflow tract. Researchers found that the BRG1–CHD7 complex was formed on the PlexinA2 promoter in E9 cardiac neural crest tissues, and that the coexpression of Brg1 and Chd7 had an >2-fold synergetic effect on the promoter activation of PlexinA2 in an in vitro study. The modifiers of ATP-dependent chromatin remodeling are summarized in Table 2.

MicroRNA

miRNA is a small noncoding RNA molecule with a length of ~22 bp, which can bind the three prime untranslated region (3′UTR) of target mRNAs to cause cleavage or translational repression. Efficient translation of miRNAs requires a loop structure resulting from the interaction between PABPC (cytoplasmic polyA-binding protein) and eIF4G (eukaryotic translation-initiation factor 4G). miRNAs are part of the argonaute RISC component (AGO) complex and can guide the Ago complex to target mRNAs by binding to partially complementary target sites, which causes deadenylation of the target mRNAs, thus leading to translational repression. A study predicted that >1000 miRNAs collectively regulate over one-third of human genes. A single miRNA can regulate different kinds of target mRNAs, and a single mRNA can be regulated by many different miRNAs. miRNA biogenesis depends on Dicer, which cleaves precursor miRNAs into short double-stranded RNA fragments. The cardiac-specific deletion of Dicer triggered embryonic death from heart failure by E12.5 because of a poorly developed ventricular myocardium, suggesting that miRNAs collectively play an important role in cardiogenesis. Gain- and loss-of-function studies also indicated the contribution of individual microRNAs (miRNAs) to cardiac development. For example, excess miR-1 in the developing heart suppressed ventricular myocyte proliferation, whereas targeted deletion of muscle-specific miR-1-2 triggered nearly half of mouse embryos to die as weaning, and some mice exhibited ventricular septal or conduction system defects at later stages.

miRNAs often play important roles in cardiac development by regulating feedback loops. A pair of studies showed that miR-130 and miR-133 act as necessary linkages in the control of fibroblast growth factor 8 Mus musculus (house mouse) (Fgf8) signaling in early cardiac specification. Specifically, BMP2 (bone morphogenic protein 2) induces miR-130 and miR-133 expression, and their overexpression results in a decrease in fibroblast growth factor 8 Homo sapiens (human) (FGF8) expression, which subsequently leads to the increased expression of BMP2 and cardiac-specific markers NKK2.5 and GATA4.
Two other studies investigated the involvement of miR-1 in the regulation of cardiac and skeletal muscle proliferation and differentiation.\textsuperscript{63,64} miR-1 controls the balance between cardiomyocyte proliferation and differentiation by targeting Hand2, and promotes myogenesis by repressing HDAC4. The repression of histone deacetylase4 (HDAC4) enhances the activity of the myocyte enhancer factor 2A (MEF2) transcription factor which, in turn, facilitates the expression of miR-1.\textsuperscript{63,64}

**MIRNA PROFILES IN TOF**

TOF is the most common type of cyanotic CHD, accounting for 5% to 8% of all CHDs.\textsuperscript{67} Extensive studies have been conducted to investigate the role of miRNA expression implicated in the pathogenesis of TOF. A study sought to illustrate the potential role of miRNAs regulation of Cx43 (connexin 43) expression in the pathology of TOF.\textsuperscript{65} The authors detected the expression of Cx43 and related miRNAs in the myocardium from patients with TOF and controls, and found that the increased levels of Cx43 mRNA and protein negatively correlated with decreased expression of miR-1 and miR-206, suggesting that miR-1 and miR-206 might contribute to the pathology of TOF by targeting Cx43.\textsuperscript{65} Another study identified 18 deregulated miRNAs in right ventricular outflow tract tissues from infants with nonsyndromic TOF.\textsuperscript{66} These miRNAs were predicted by bioinformatic analysis to target a network of genes involved in heart development and CHD. Among these miRNAs, miR-424/424* was found to promote cell proliferation and inhibit cell migration in primary embryonic mouse cardiomyocytes by directly targeting NF1 and HAS2, which are involved in cell migration, cardiac morphogenesis, and cardiac outflow tract septation. A third study examined the expression of miRNAs in the right ventricular myocardium from infants with TOF and healthy controls. Thirty-three miRNAs were found to be altered significantly, which were negatively correlated with 44 genes that regulate cardiac development. Focusing on the contribution of miR-421 to TOF, the authors indicated that altering the expression of miR-421 in primary cells derived from infant heart tissue had an inverse impact on the expression of SRY-box transcription factor 4 (SOX4), a key regulator of the Notch pathway, supporting a role for miR-421 in the regulation of SOX4.\textsuperscript{67} A fourth study found that miRNA-940 was the most downregulated among 75 dysregulated miRNAs in human heart tissues from patients with TOF compared with its level in healthy controls. Functional analysis further indicated that reduced miRNA-940 expression affected the proliferation and migration of progenitor cells in the secondary heart field by targeting JARID2, a gene with a potential effect on cardiac outflow tract development.\textsuperscript{68} The miRNAs involved in heart development and CHD are summarized in Table 2.

**RECENT ADVANCES**

**Epigenetic Biomarkers**

Aberrant DNA methylation patterns have been investigated for use as biomarkers for CHD diagnosis. A group performed a genome-wide methylation assay in newborn blood from nonsyndromic TOF cases and matched controls, and identified 64 differentially methylated genes, of which 25 genes showed high predictive accuracy for TOF based on the area under the receiver operating characteristics curve. Multiple differentially methylated genes, including ABCB1, PPP2R5C, TLR1, SELL, SCN3A, CREM, RUNX, and LHx9, were linked to heart development and postnatal heart diseases.\textsuperscript{68} The same group also compared the DNA methylation difference in placental tissue between isolated VSD cases and controls by genome-wide DNA methylation assay.\textsuperscript{69} It revealed that a total of 80 CpG sites in 80 genes were highly accurate in the prediction of VSD by the area under the receiver operating characteristics curve. These genes are previously known to be associated with heart development or disease, such as HEY2 and ISL1 in cardiac ventricle development, SRF in heart looping, ACTC1 and HEY2 in cardiac muscle cell differentiation, ISL1 in cardiac septum development, and SRF, HEY2, ISL1, and HEYL in heart morphogenesis.

Circulating miRNAs were also recently investigated for use as biomarkers in the diagnosis and prediction of CHD.\textsuperscript{69} Researchers screened 84 candidate miRNAs related to cardiovascular development in plasma from atrial septal defect and healthy children. Intriguingly, the expression profiles of hasa-let-7a and hasa-let-7b in children with atrial septal defect were similar to those of their mothers. Area under the receiver operating characteristics curve analyses revealed that these 2 miRNAs in patients or in their mothers are valuable for atrial septal defect diagnosis or prediction, respectively. Another study also explored the plausibility of prenatal prediction for fetal CHD with a panel of maternal serum miRNAs.\textsuperscript{70} Researchers found 4 miRNAs (miR-19b, miR-22, miR-29c, and miR-375) were remarkably upregulated in pregnant women who had fetuses with CHD at 18 to 22 weeks of gestation. Moreover, according to a multiple logistic regression analysis, the combination of the 4 miRNAs exhibited high efficiency for the early diagnosis of fetal CHD.

To date, studies exploring the use of epigenetic biomarkers for CHD diagnosis are still limited, and specific and reliable biomarkers have not yet been identified. Further research is needed to accumulate more data and confirm their usefulness in the clinical practice for the diagnosis of CHD.
ADVERSE INTRAUTERINE ENVIRONMENT-RELATED CHD

Diabetes

Fetal hyperglycemia associated with maternal diabetes is a well-known teratogen that raises the risk of having an infant with CHD by 3- to 5-fold. A study sought to examine whether epigenetic factors are the underlying contributor of diabetes-associated CHD. Hyperglycemia was found to increase chromatin accessibility at the endothelial NO synthase (Nos3) locus, leading to decreased NO synthesis, which subsequently increased the transcription of JariID2 (jumonji and AT-rich interaction domain containing 2) (a regulator of histone methyltransferase complexes), and the upregulation of JARIID2 directly resulted in the inhibition of Notch1 expression, further affecting normal heart development. Our group previously analyzed the expression features of miRNAs in embryonic hearts from nondiabetic or diabetic dams, and found that 149 miRNAs were significantly altered. The majority of the potential miRNA target genes were predicted to be associated with cardiac development-related pathways (including STAT3 and IGF-1) and transcription factors (Cited2, Zeb2, Mef2c, Smad4, and Ets1). Interestingly, overexpression of the antioxidant enzyme superoxide dismutase 1 restored maternal diabetes-altered miRNAs, suggesting that oxidative stress is responsible for the dysregulation of these miRNAs.

Hypoxia

Hypoxia during gestation imposes profound adverse effects on cardiac development, which increases the occurrence of CHD. A recent study revealed the underlying mechanism of how hypoxic stress determines cardiac progenitor cell (CPC) fate and contributes to CHD. The authors found that hypoxia regulates CPC proliferation and differentiation and restrains cardiomyocyte maturation. Further study showed that hypoxia upregulates miR-210 expression in Sca-1+ (Calcium-transporting ATPase) CPC and hinders the cell differentiation. The blockage of miR-210 expression significantly promotes the differentiation of Sca-1+CPCs into cardiomyocytes. These findings provide clear evidence that hypoxia alters CPC fate, and reveal a novel mechanism of miR-210 in hypoxia-associated CHD.

Alcohol

Maternal alcohol consumption during gestation is closely related to the occurrence of CHD. A recent study indicated that abnormal histone methylation is involved in alcohol-induced cardiac dysplasia. Histone H3K9me3 (tri-methylation at the 9th lysine residue of the histone H3 protein) was decreased because of the reduced expression of histone methyltransferase G9a. Moreover, the expression of cardiomyogenesis-related genes (Mef2c, Cx43, Anp, and β-MCH) were downregulated in alcohol-exposed fetal mouse hearts, suggesting that aberrant H3K9me3 mediated by histone methyltransferase G9a may account for the alcohol-induced abnormal expression of cardiomyogenesis-related genes during pregnancy. Histone acetylation inhibitors have been approved by the Food and Drug Administration for the treatment of cancers, which also demonstrate remarkable therapeutic potential for heart failure. A group investigated the protective effect of curcumin (a natural histone acetylation inhibitor) on alcohol-induced cardiac damage during pregnancy. They found that alcohol increased the acetylation of H3K9 and the expression of the transcription factors GATA4 and MEF2C in cardiac progenitor cells; however, these alterations can be reversed by the addition of curcumin. They further revealed that curcumin treatment may rescue alcohol-induced fetal cardiac apoptosis through restoring H3K9 acetylation pattern near the promoter regions of apoptosis-related genes.

DISCUSSION AND FUTURE PERSPECTIVE

The causes of CHD are multifactorial. Gene mutation accounts for only a small portion of CHD cases, with the pathogenesis of the majority of cases remaining unknown. Aberrant epigenetic patterns have been identified in patients with CHD, and animal and in vitro studies further validate that epigenetic dysregulation is, at least in part, responsible for the pathogenesis of CHD. However, epigenetic regulation is complicated and multilevel, and the dysregulation of DNA methylation, histone modification, ATP-dependent chromatin remodeling, or miRNAs in heart development can result in CHD. Thus, integrating all data, including that on different types of epigenetic alterations, may help to reveal the regulatory role of epigenetics in the formation of CHD. Moreover, integrating all genome and epigenome data will certainly be useful for elucidating the pathogenesis of CHD more comprehensively.

Clinically, the evaluation and modulation of epigenetics manifest as promising prospects in the diagnosis and treatment of CHD. Aberrant CpG methylation patterns at specific loci are regarded as the most successful epigenetic biomarkers, and they have previously been investigated for use in the diagnosis of CHD. Circulating miRNAs in pregnant mothers or infants show high accuracy and availability for the diagnosis of CHD. Epi-drugs have been extensively investigated, and some of them have reached the market. For instance, DNA
methyltransferase inhibitors have been approved by the Food and Drug Administration for the treatment of hematological malignancies,100 and HDAC inhibitors are not only approved for use in oncology but are also showing promising roles for use in treating adult heart failure.96 These kinds of inhibitors may also have potential value in the treatment of CHD and need further investigation.

Although tremendous progress has been made in understanding of involvement of epigenetic modifications in heart development and CHD, some specific knowledge gaps remain to be filled. For example, it is not fully understood how cardiac transcription factors interact with histone modifiers or chromatin remodelers, how DNA methylation interacts with histone lysine methylation, or how different histone modifiers interact with each other. Fortunately, recent advances in high-throughput DNA sequencing technology may enable the identification of the binding sites of cardiac transcription factors, histones, and DNA modifiers at a genome-wide level, which may help with understanding of these interactions. In addition, prenatal exposure to environmental risk factors such as alcohol, hypoxia, malnutrition, therapeutic drugs, or maternal diabetes may cause CHD,105; however, only limited studies have focused on the involvement of epigenetic regulation in this process; therefore, further investigations are still needed. In summary, improving and integrating fundamental research data of epigenetic modifications in heart development and CHD to identify highly specific and reliable epigenetic biomarkers and to establish epigenetics-based therapeutics for CHD are the main challenges ahead for scientists.

ARTICLE INFORMATION
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Disclosures
None.

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