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Review

Epigenetics and microRNAs in cardiovascular diseases

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ABSTRACT

Cardiovascular diseases are among the leading causes of mortality worldwide. Besides environmental and genetic changes, these disorders can be influenced by processes which do not affect DNA sequence yet still play an important role in gene expression and which can be inherited. These so-called 'epigenetic' changes include DNA methylation, histone modifications, and ATP-dependent chromatin remodeling enzymes, which influence chromatin remodeling and gene expression. Next to these, microRNAs are non-coding RNA molecules that silence genes post-transcriptionally. Both epigenetic factors and microRNAs are known to influence cardiac development and homeostasis, in an individual fashion but also in a complex regulatory network. In this review, we will discuss how epigenetic factors and microRNAs interact with each other and how together they can influence cardiovascular diseases.

1. Introduction

Epigenetics is defined as heritable changes in the expression of genes that are not due to alterations to the DNA sequence itself and includes processes such as DNA methylation, histone modifications, and ATP-dependent chromatin remodeling enzymes. Gene expression is also regulated by non-coding RNAs, such as microRNAs (miRNAs), which influence gene expression post-transcriptionally [1]. While the epigenetic status of the cell and miRNAs levels are highly regulated under normal conditions, their dysregulation can lead to a wide host of ailments ranging from cancer to drug addiction [2,3]. Cardiovascular diseases, which represent a major cause of morbidity and mortality in the world [4], are also determined by such alterations. Starting in the embryonic heart, an intricate network of genes needs to be expressed in a precisely controlled fashion to ensure proper development, homeostasis and stress response. Changes to this concerted program can be caused by an aberrant epigenetic regulation and can result in a range of malignant phenotypes such as cardiomyopathy, fibrosis, hypertrophy and heart failure (HF) [5,6]. In this review, we focus on the interplay between epigenetic factors and miRNAs and how this influences cardiovascular diseases.

1.1. Epigenetics

The basic element of eukaryotic chromatin, a complex of proteins and DNA, is the nucleosome, which consists of a protein core of eight histone subunits around which 147 base pairs of DNA are coiled [7]. The condensation state of the chromatin affects gene transcription by making parts of the genome more or less accessible [8]. This chromatin remodeling can occur locally to facilitate expression of specific genes, but also on a larger scale from chromosome domains to entire chromosomes [8].

One class of epigenetic mechanisms that can influence chromatin accessibility is the post- translational modification of histones (Fig. 1). Histones H2A, H2B, H3, and H4 are the core proteins that compose the nucleosome, while histone H1 is known as the linker histone and contributes to the formation of higher order chromatin structures by interacting with DNA and the other histones [9]. Each core histone subunit occurs twice in the nucleosome and has an amino-terminal tail extending out from the nucleosome. A wide variety of post-translational modifications to the histones have been described, most often occurring in the tail region, including methylation, acetylation, glycosylation, ubiquitination and phosphorylation, among others [10]. A nomenclature has been defined to describe the particular histone being altered as well as the nature and position of the modification [11]. The first element to be specified is the histone subunit involved; then, the modified amino acid and its relative position and, finally, the type and level of modification. For example, H3R17me2s indicates that histone H3 underwent a symmetrical dimethylation on the arginine at position 17. The complex pattern of modifications at different positions of one

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histone is called the 'histone code' and can result in either charge neutralization or specific interaction with reader proteins [12]. While the former consists in the compaction of chromatin due to neutralization of the negative electrostatic charge of DNA by the positively charged histone, the latter refers to the recognition of histone modifications by reader proteins. Different reader proteins can recognize specific modifications and not only can they function as transcription factors or other epigenetic regulators, but they can also influence the chromatin state [12].

1.1.1. *Histone acetylation and deacetylation*

Histone acetylation involves the addition of an acetyl group to a lysine residue in the histone tail, resulting in the neutralization of the lysine's positive charge, leading to more open and accessible chromatin [13]. The enzymes that catalyze this process are called histone acetyltransferases (HATs), while those that remove the acetyl group are referred to as histone deacetylases (HDACs). H3K27ac is an example of a histone acetylation that marks active enhancer regions [14]. Additionally, acetylation of this lysine blocks methylation at the same position (H3K27me3), showing that multiple types of modifications can compete for the same position [15].

1.1.2. *Histone methylation and demethylation*

Histone methylation consists in the addition of one or more methyl groups to a lysine or arginine by histone methyltransferases (HMTs) [16]. These enzymes transfer the methyl group from S-adenosyl-L-methionine to the amino acid residue and are grouped into two categories based on whether they methylate arginine or lysine [17]. While methylation was originally thought to be irreversible, in 2004 Shi et al. identified an amine oxidase that could demethylate lysine 4 on histone H3, an epigenetic mark often enriched near the transcription start sites of active promoters which enhances gene transcription [18].

Unlike acetylation, histone methylation does not neutralize the electrostatic charge of the amino acid residue. It can both enhance transcription (such as for H3K4me3) or repress it (such as for H3K27me3), depending on the histone and the residue being methylated.

1.1.3. *ATP-dependent chromatin remodeling*

Other classes of epigenetic changes orchestrate chromatin remodeling without directly modifying the histones. Among them, ATP-dependent chromatin remodeling enzymes modify chromatin status by altering the interactions between histones and DNA through ATP hydrolysis (Fig. 1) [19]. Three types of remodeling are known: sliding of a nucleosome to expose/cover a region of DNA, exchanging one histone subunit for another variant, and removing or assembling a nucleosome along a stretch of DNA [19]. As a result, this remodeling changes the local accessibility of DNA and influences genomic organization. Members of four major subfamilies of remodelers can interact cooperatively or competitively with each other, the genome itself and other epigenetic marks or proteins to facilitate precise control over gene expression and genomic organization.

1.1.4. *DNA methylation*

DNA methylation consists in the reversible addition of a methyl group to a nucleotide, usually to the 5C of a cytosine (Fig. 1) [20]. Methylation of promoter regions is associated with silencing of gene expression by lowering the accessibility of chromatin, by inhibiting the binding of transcription factors, or by attracting histone modifiers [20,21]. The enzymes responsible for methylating DNA are called DNA methyltransferases (DNMTs). DNMT1 is responsible for the maintenance of existing methylation patterns during DNA replication, while DNMT3A and DNMT3B catalyze *de novo* methylation [22].

1.2. *MicroRNAs*

Besides the mechanisms detailed in Section 1.1, gene expression can also be influenced by microRNAs. These non-coding RNAs exert their function post-transcriptionally and as such they differ from the classical epigenetic factors previously described, which regulate gene expression at the chromatin level, thus representing a separate class of gene regulators. MiRNAs are small (~22 nucleotides) single-stranded noncoding RNA molecules with high evolutionary conservation [23] (Fig. 2). MiRNA genes derive from intergenic, intronic, exonic or polycistronic loci and are transcribed by RNA polymerase II into a long primary miRNA transcript (pri-miRNA) [24]. This pri-miRNA is subsequently cleaved by the Drosha ribonuclease to form the pre-miRNA, a 70 nucleotide (nt) long hairpin structure with 2 nt 3′ overhangs, and exported to the cytoplasm. There another ribonuclease, Dicer, processes the pre-miRNA to form a 22 nt long miRNA:miRNA* duplex. The two

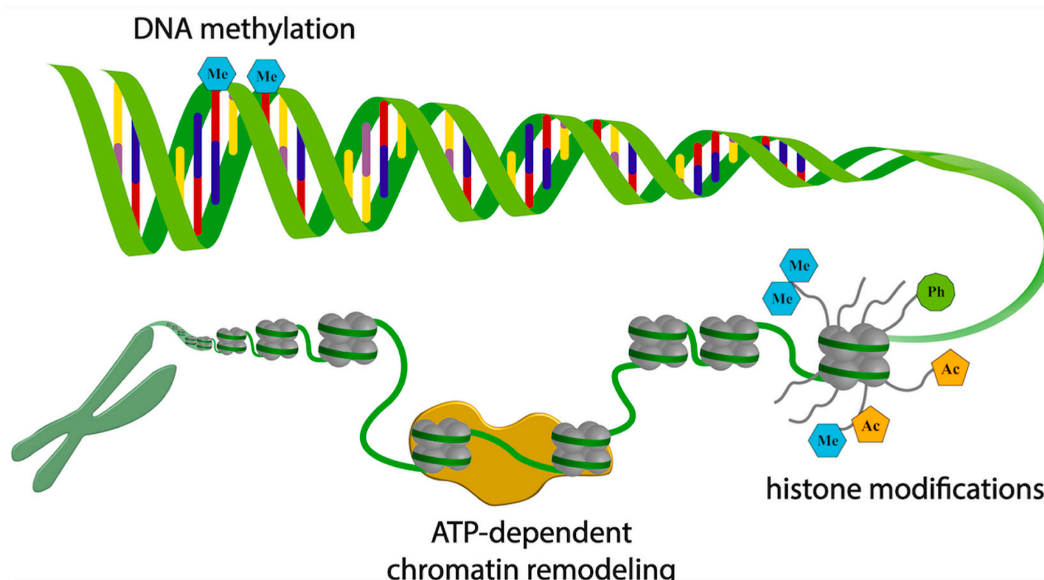

Fig. 1. epigenetic modifications affecting chromatin status. Different transient chemical modifications acting on the DNA or histones can activate or repress gene expression. See text for further details.
Me, methylation; Ac, acetylation; Ph, phosphorylation.

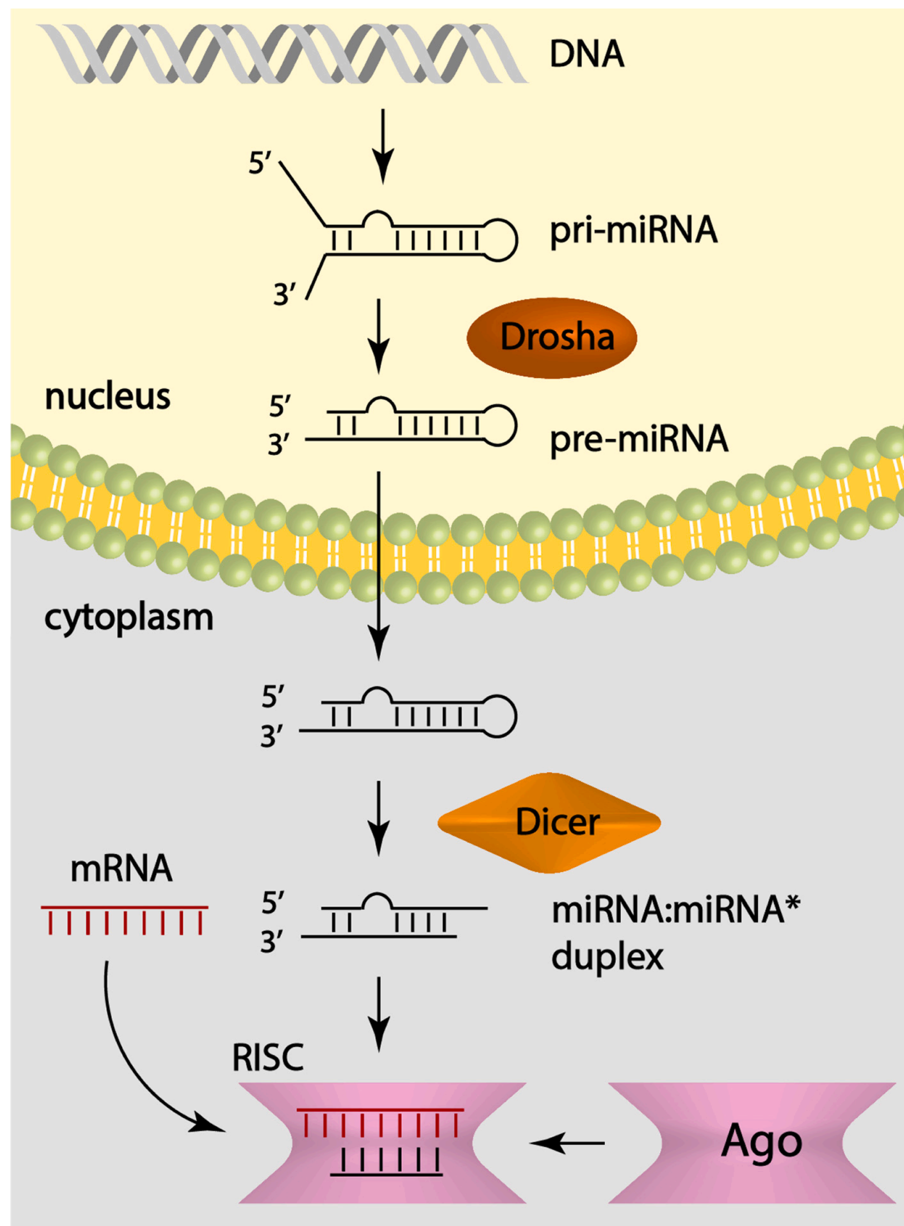

Fig. 2. microRNA biogenesis.
The microRNA (miRNA) gene is initially transcribed into a long transcript that is sequentially processed by ribonucleases to a 22 nucleotides long transcript. See text for further details.

strands of this duplex are imperfectly complementary to each other and, after loading on the RNA-induced silencing complex (RISC) by combining with the Argonaute (AGO) protein, one of the two strands, the passenger, is degraded, while the other, the guide, is delivered to its mRNA target [25].

Interestingly, both miRNA strands can regulate different processes in the same tissue. For example, while miR-21-5p was found upregulated in different models of heart failure [26,27], fibroblast exosomal-derived miR-21-3p was detected as a paracrine-acting miRNA that positively regulates cardiomyocyte hypertrophy [28]. Similarly, experimental findings support the role of miR-126-5p in atheroprotective endothelial regeneration, while its sister strand miR-126-3p, derived from apoptotic endothelial cells, exerts a protective role at predilection sites, where the capacity of endothelial cells is insufficient upon hyperlidemic stress [29].

The recognition of the mRNA target is determined by the miRNA's seed sequence, which usually covers the nucleotides in positions 2–7 of the mature miRNA [30]. Due to the small size of the seed sequence or the imperfect nature of the base-pairing, multiple mRNA targets can be regulated by a single miRNA, and, *viceversa*, a single miRNA can recognize several mRNAs. As a result of mRNA targeting, miRNAs regulate gene expression by either repressing translation of the transcript, in case of incomplete base-pairing, or promoting its degradation, in case of full seed sequence complementarity [31].

Besides the canonical mechanism, very recently, alternative aspects on miRNA function emerged, expanding the known spectrum of post-transcriptional gene regulation. One of these mechanisms is based on the 8-oxoguanine (o^8G) modification, induced by reactive oxygen species [32]. In miRNAs, such modifications can occur in specific positions of the seed region and serve as a means of mRNA regulation through o^8G-A base pairing. This epitranscriptional regulatory mechanism has been proven for miR-1 in mice, in which treatment with an adrenergic agonist resulted in generation of reactive oxygen species, which in turn caused the introduction of o^8G in position 7 of the seed sequence in the miRNA.

As a consequence, miR-1 could now target novel mRNAs, resulting in cardiac hypertrophy.

In another non-canonical mechanism, miRNAs regulate specific cell processes through a direct miRNA-protein interaction. This mechanism was studied in endothelial cells, where miR-126-5p was shown to form a ternary complex with Ago2 and the RNA binding protein Mex3a, a shuttle between the nucleus and the cytoplasm [33]. Specifically, while Ago2 interacts with the 5′ region of the miRNA, Mex3a binds preferentially to the 3′ part of miR-126-5p, rather than to the complementary strand, through its K homology domains. Despite the mechanisms directing the miRNA to the nucleus being elusive, it is clear that in this compartment, miR-126-5p acts as an aptamer by directly binding caspase-3 with its seed-sequence and preventing apoptosis.

Currently, it is estimated there are ~2300 miRNAs in the human genome [34], affecting up to 60% of human protein coding genes in the pathophysiology of different tissues [35]. They can both play a direct role, by targeting mRNAs crucial for the tissue phenotype, or be indirect influencers, by regulating intermediate processes, such as the epigenetic status of relevant genes [36].

2. Interactions between epigenetic factors and miRNAs

MiRNAs modulating the expression of enzymes that drive epigenetic processes are known as 'epi-miRNAs' and can target, directly or indirectly, epigenetic modulators such as DNMTs, HDACs, HMTs or TETs [1,37]. The first epi-miRNA, miR-29b, was discovered by Fabbri et al. and was found to be downregulated in lung cancer [38]. This miRNA can target DNMT3A and DNMT3B, two enzymes frequently upregulated in lung cancer and linked to aberrant DNA methylation in this disease. Overexpressing miR-29b in lung cancer cells subsequently restored normal DNA methylation patterns, leading to renewed expression of tumor suppressor genes that were silenced due to methylation and lowered tumorigenicity [38].

Conversely, epigenetic processes can also influence the expression of miRNAs [37,39]. As with protein-coding genes, DNA methylation can have an effect on the expression of miRNAs through methylation of CpG islands near their promoters. One analysis by Weber et al. estimated that approximately half of all miRNA genes are associated with CpG islands [40]. Methylation of these dinucleotides occurred at high frequencies for miRNA genes, in contrast to the pattern for protein-coding genes. An example of this process is the silencing of miR-203 by aberrant methylation of its promoter, which in turn promotes ABL1 and BCR-ABL1 oncogene expression [41]. MiRNAs expressed from a host gene can have their own promoters or share them with their host genes [1]. In the latter case, methylation of the promoter influences expression of both the host gene and its associated miRNA. Histone modifications can also influence miRNA expression. Recently, Li et al. showed that inhibition of Enhancer of zeste homolog 2 (EZH2), a methyltransferase which can trimethylate H3K27, led to upregulation of miR-34a expression in pancreatic ductal adenocarcinoma cells [42]. This study also identified HOTAIR, a long noncoding RNA (lncRNA), as necessary to guide EZH2 to the promoter region. This demonstrates that some interactions between miRNAs and epigenetic processes require additional factors to occur.

Finally, miRNAs and epigenetic factors can also affect each other in a feedback loop. An example of this is the interaction between protein arginine methyltransferase 7 (PRMT7) and miR-24-2. A study using mouse embryonic stem cells (ESC) showed that PRMT7 represses expression of *Mir-24-2* gene, which encodes for both miR-24-3p and miR-24-2-5p, through upregulation of H4R3me2s [43]. These miRNAs in turn target the 3′ untranslated region (UTR) of *Prmt7* to inhibit its expression. MiR-24-3p and miR-24-2-5p induce differentiation of mouse ESCs by repressing *Klf4* and *c-Myc*, with miR-24-3p additionally downregulating *Oct4* and *Nanog*. In addition to promoting pluripotency of ESCs, these 4 genes induce *Prmt7* expression, providing a secondary feedback loop between *Mir-24-2* and *Prmt7* through intermediary transcription factors.

3. Interactions between epigenetic changes and miRNAs in cardiovascular diseases

The influence of individual epigenetic factors and miRNAs on cardiovascular health and disease is increasingly being recognized. More recently, interactions between epigenetic factors and miRNAs in the same context have also been described (Fig. 3, Table 1).

3.1. MicroRNAs and histone acetylation in cardiovascular diseases

As mentioned in Section 1.1.1, histone acetylation is an epigenetic mark associated with enhanced transcription due to formation of a more open and accessible chromatin state. In addition to this, reader proteins can recognize and bind to acetylated histones directly, such as the bromodomain and extraterminal (BET) protein family [84]. Bromodomain Containing 4 (*BRD4*) is a member of this family and can interact with the positive transcription elongation factor b (P-TEFb) complex, known to phosphorylate RNA polymerase II and thereby able to enhance gene transcription in the heart [84]. A study by Stratton et al. found that miR-9 can target *Brd4* in neonatal rat ventricular cardiomyocytes [44]. However, following stimulation with phenylephrine to induce hypertrophy, miR-9 expression was downregulated, with a concomitant increase in BRD4 on the protein level. A luciferase reporter assay further confirmed that miR-9 targets *Brd4*. Moreover, a specific subset of super enhancer (SE) sites were enriched in BRD4 binding sites. SEs are regions of the genome comprised of multiple enhancers which can act at long range in cell state- and cell type-specific transcription programs [85]. Introducing a miR-9 mimic repressed BRD4 binding to these sites but did not affect binding to constitutively expressed cardiac genes [44].

A study using diabetic mice found that expression of *p66*^{Shc}, a gene that plays a role in regulating apoptosis and oxidation state of cells [86], was increased in diabetic mice hearts [45]. In these hearts, there was an increase of acetylation of histone H3 as well as a decrease of DNA methylation of CpG islands at the *p66*^{Shc} promoter due to downregulation of the *Sirt1* and *Dnmt3b* genes respectively, leading to constitutive expression of *p66*^{Shc}. While glycaemic control on its own failed to reverse the upregulation of *p66*^{Shc} and increased oxidative stress, knocking down expression of *p66*^{Shc} using a small interfering RNA (siRNA) concurrent with glycaemic control led to decreased production of ROS and improved cardiac function. The downregulation of *Sirt1* and *Dnmt3b* were due to upregulation of miR-34a and miR-218 respectively in the hearts of these diabetic mice [45]. The link between miR-34a and SIRT1 was corroborated in the work by Tabuchi et al. who investigated the effects of statins on miR-34a and SIRT1 in endothelial progenitor cells (EPCs) isolated from coronary artery disease (CAD) patients [46]. After confirming that overexpression of miR-34a reduced SIRT1 protein levels in cultured EPCs, the authors found that CAD patients had increased levels of miR-34a and concomitant reduced SIRT1 compared to healthy controls. Atorvastatin treatment resulted in the decreased expression of miR-34a and increased SIRT1, however rosuvastatin, another type of statin, had no such effect [46]. Whether miR-34a is influenced by *Dnmt3b* also in CAD, however, still remains to be determined.

3.2. MicroRNAs and histone deacetylation in cardiovascular diseases

In mouse models of cardiac hypertrophy (CH) generated either through transverse aortic constriction (TAC) surgery or angiotensin-II (Ang-II) treatment, expression of miR-21-3p was found decreased compared to mice exposed to sham surgery or vehicle administration [47]. Adeno-associated virus-mediated overexpression of the miRNA could rescue the hypertrophic phenotype after TAC surgery as well as reduce the levels of phosphorylated protein kinase B (phospho-AKT) and phosphorylated glycogen synthase kinase-3 beta (phospho-GSK3β).

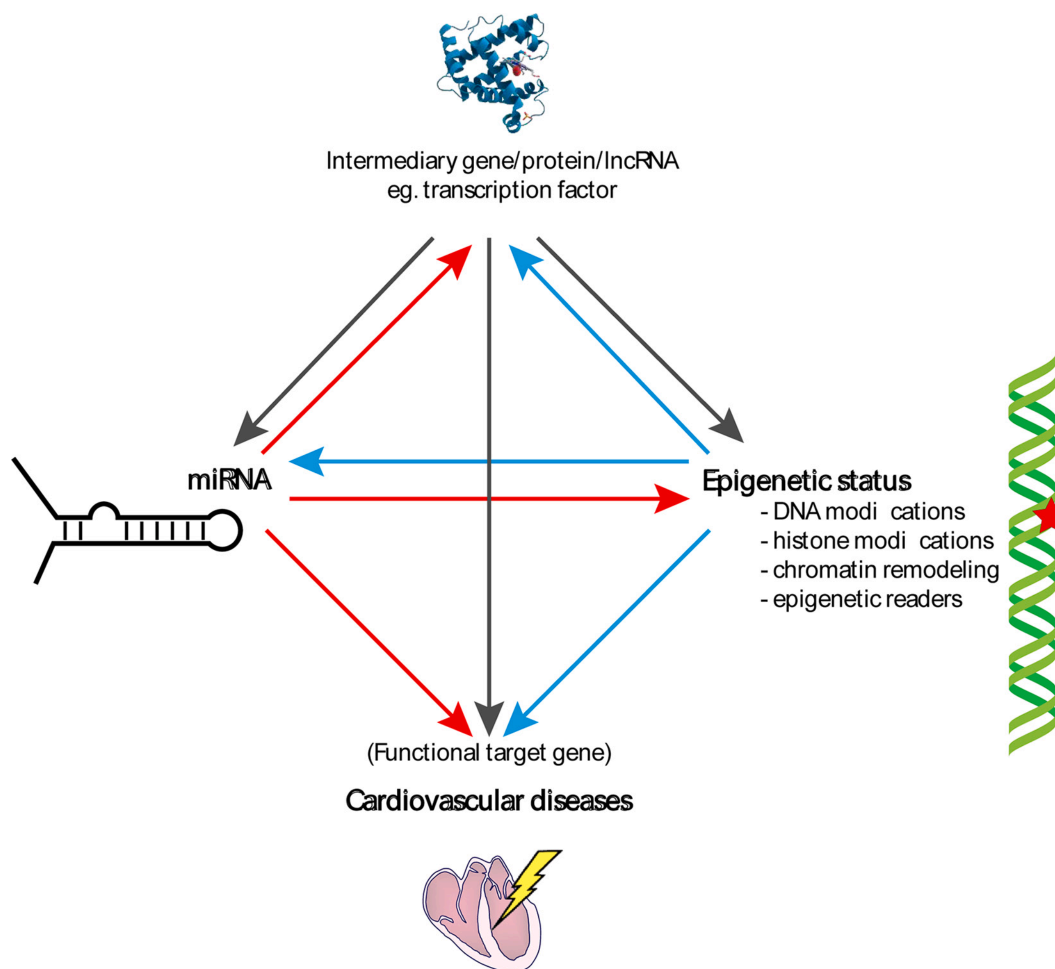

Fig. 3. interactions between miRNAs, epigenetics and cardiovascular diseases.
MiRNAs (red arrows) can affect the expression of epigenetic proteins influencing the epigenetic status of a cell, while these epigenetic processes (blue arrows) can in turn regulate miRNA expression. Other biomolecules, such as transcription factors or long noncoding RNAs (lncRNA) (grey arrows), can act down- or upstream or as intermediaries within the miRNA-epigenetic axis. This intricate crosstalk can have an effect of cardiac health.

Interestingly, the Akt/Gsk3β pathway is known to play a role in CH [87]. *Hdac8*, a member of the Class I histone deacetylases, was confirmed as a direct target of miR-21-3p and its overexpression in TAC mice could reverse the cardioprotective effect of miR-21-3p and increased levels of phospho-AKT and phospho-GSK3β [47].

Another study using a TAC mouse model for CH found that treatment with the Class I and IIb HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) prevented the downregulation of miR-133a in cardiomyocytes normally seen 2 weeks post TAC [48]. SAHA treatment also reduced left atrial diameter and cardiac fibrosis, though it did not impact CH or systolic function. Analysis of miR-133a enhancer regions using chromatin immunoprecipitation revealed that both HDAC1 and HDAC2 were present, suggesting the crosstalk among these epigenetic elements.

A study from Zhang et al. showed that multiple factors can regulate CH in parallel [49]. The long non-coding RNA MEG3 was found upregulated in both heart of mice that underwent TAC and in cardiomyocytes treated with angiotensin-II. Coupling bioinformatic and functional analyses revealed that the transcription factor STAT3 positively regulates MEG3, which in turn acts as a sponge reducing the levels of miR-361-5p in hypertrophic cardiomyocytes. Among the putative targets of miR-361-5p, HDAC9 was downregulated upon miRNA overexpression *in vitro* and its expression was rescued when cells were transfected with MEG3 mimic. Taken together, these data suggest that, when activated by STAT3, MEG3 promotes CH *via* miR-361-5p/HDAC9 axis.

Treatment with the pan-HDAC inhibitor trichostatin A (TSA) resulted in miR-129-5p overexpression in both H9c2 cells and mice [50]. H9c2 cells treated with miR-129-5p antagomir showed increased cell proliferation, compared with cells treated with TSA. Protein levels of cyclin-dependent kinase 6 (CDK6), a known miR-129-5p target and cell cycle regulator [88], were reduced in TSA-treated H9c2 cells, but not in TSA-treated cells expressing the miR-129-5p antagomir, indicating that the phenotypic effect of the global HDAC inhibitor was mediated by miR-129-5p through its target CDK6.

A recent study by Liu et al. found that miR-132-3p, which is downregulated in primary murine cardiomyocytes following H_2O_2 induced oxidative stress, targets *Hdac3* [51]. These cells showed upregulation of HDAC3 and concomitant reduced levels of H3K9Ac at the promoters of oxidation-related genes such as *Bcl-xL*, *Prdx2*, and *Hsp70*. Administrating a miR-132-3p mimic improved the viability following oxidative stress and reduced reactive oxygen species (ROS) production. TUG1, a lncRNA known to target miR-132-3p [89] and upregulated after H_2O_2 treatment, was shown to function upstream of the miR-132-3p-HDAC3 axis by sponging the miRNA [51]. These results were recapitulated in an *in vivo* ischemia-reperfusion (I/R)-induced myocardial infarction (MI) mouse model. The infarcted area showed increase levels of TUG1 and decreased levels of miR-132-3p. Administering an adenovirus expressing either an miR-132-3p mimic or a siRNA construct to reduce TUG1 levels before I/R led to reduced infarct size and reduced ROS production.

Table 1
microRNAs and epigenetic processes involved in cardiac health and disease.

miRNA	Epigenetic gene/ process	Cardiovascular disease/process	Reference
miR-9	*Brd4* Histone acetylation (reader)	Hypertrophy	[44]
miR-34a	*Sirt1* Histone acetylation	Diabetic heart	[45]
miR-34a	*Sirt1* Histone acetylation	Coronary artery disease	[46]
miR-21-3p	*Hdac8* Histone deacetylation	Hypertrophy	[47]
miR-133a	*Hdac1/Hdac2* Histone deacetylation	Hypertrophy	[48]
miR-361-5p	*Hdac9* Histone deacetylation	Hypertrophy	[49]
miR-129-5p	Histone deacetylation	Hypertrophy/proliferation	[50]
miR-132-3p	*Hdac3* Histone deacetylation	Myocardial infarction	[51]
miR-1	*Hdac4* Histone deacetylation	Ischemia/reperfusion injury	[52]
miR-1	*Hdac4* Histone deacetylation	Cardiac hypertrophy	[53]
miR-182	*Hdac9* Histone deacetylation	Atherosclerosis	[54]
miR-43a	*Hdac1* Histone deacetylation	Atherosclerosis	[55]
miR-208b	*Ezh2* Histone methylation	Cardiac hypertrophy	[56]
let-7c	*Ezh2* Histone methylation	Congenital heart defects due to Down syndrome	[57]
miR-26a	*Ezh2* Histone methylation	Myocardial infarction	[58]
miR-217	*Ehmt1/ Ehmt2* Histone methylation	Cardiac hypertrophy	[59]
miR-29a and miR-29c	*Suv4-20 h1* and *Suv4-20 h2* Histone methylation	Cardiac aging	[60]
miR-25	PRC2/*Ezh2* Histone methylation	Pressure overload-induced cardiac hypertrophy and heart failure	[61]
miR-1 and miR-133a	*Kdm6A, Kdm6B* and *Ezh2* Histone demethylation	Cardiac fibroblast reprogramming	[62]
miR-22-3p	*Kdm3a* Histone demethylation	Myocardial infarction	[63]
miR-99/100 and let-7a/c	*smarca5* ATP-dependent chromatin remodeling	Myocardial infarction	[64]
miR-99a	*Smarca5* ATP-dependent chromatin remodeling	Congenital heart defects due to Down syndrome	[57]
miR-218	*Dnmt3b* DNA methylation	Diabetic heart	[45]
miR-184	*Dnmt1* DNA methylation	Arrhythmogenic cardiomyopathy	[65]
miR-193a	*Dnmt1/Dnmt3b* DNA methylation	Cardiac stem cell proliferation and migration	[66]
miR-128	DNA methylation	Atherosclerosis	[67]
miR-127	DNA methylation (imprinting)	Atherosclerosis	[68]
miR-29 family	*Dnmt1/Dnmt3a* DNA methylation	Cardiac aging/hypoxia	[69]
miR-133a	*Dnmt1/Dnmt3* DNA methylation	Cardiac hypertrophy	[70]
miR-133a	*Dnmt1/Dnmt3a/ Dnmt*3b DNA methylation	Diabetic cardiomyocytes	[71]
miR-133a	*Dnmt1* DNA methylation	Hyperhomocysteinemia	[72]
miR-22	*MeCP2* DNA methylation (reader)	Ischemia	[73]
miR-212/132 cluster	*MeCP2* DNA methylation (reader)	Pressure overload-induced cardiac hypertrophy and heart failure	[74]
miR-539	DNA methylation (imprinting)	Anoxia Myocardial infarction	[75] [76]
miR-410 and miR-495	DNA methylation (imprinting)	Myocardial infarction, cardiac hypertrophy, muscular dystrophy and cardiac hypertrophy	[77,78]
miR-154	DNA methylation (imprinting)	Cardiac hypertrophy	[79]
miR-541	DNA methylation (imprinting)	Cardiac hypertrophy	[80]
miR-433	DNA methylation (imprinting)	Cardiac fibrosis	[81]
miR-30c-1 (among others)	DNA (hydroxy) methylation	Dilated cardiomyopathy	[82]
miR-24-1 and miR-155	DNA methylation	Heart failure (hypertrophic obstructive cardiomyopathy, ischemic cardiomyopathy and dilated cardiomyopathy)	[83]

While an earlier study had shown the protective effects of hydrogen sulfide (H_2S) upon activation of mTOR, after myocardial hypoxia-reoxygenation (HR) in neonatal rat cardiomyocytes (NRCM) [90], a recent paper linked this protective effect to the miR-1-*Hdac4* pathway [52]. After establishing that H_2S preconditioning attenuated apoptosis following HR in NRCMs, the expression of miR-1 and *Hdac4* was assessed. The upregulation of miR-1 observed after HR was attenuated in the H_2S pretreated group, while the HR-induced downregulation of *Hdac4* was inhibited by H_2S. Similar results were found following I/R injury in rats *in vivo*. A causal link between miR-1 and *Hdac4* was established after transfecting cardiomyocytes with miR-1 mimics, which repressed HDAC4 protein levels in a dose-dependent manner. This result was corroborated by the fact that *Hdac4* has two miR-1 target sites in its 3'UTR [52]. Taken together, these results support that mTOR, miR-1 and *Hdac4* might be involved in the same pathway. Another study confirmed the link between miR-1 and *Hdac4* in both NRCM and in rat, using them as thyroid hormone-induced CH models [53]. In both cases, mir-1 expression was reduced following thyroid hormone treatment, while *Hdac4* was increased. Overexpression of miR-1 in NRCM cells or suppression of HDAC4 using TSA prevented the hypertrophic response.

HDAC9 is known to play a role in atherosclerosis [91–93] and has been shown to be regulated by miRNAs also in this context. Overexpression of miR-182 in apolipoprotein E (ApoE) knock-out mice caused the development of severe atherosclerotic lesions showing increased levels of CD68 and macrophage-derived lipoprotein lipase (LPL), a key molecule in the development of atherosclerosis [54]. Moreover, HDAC9 was identified as a target of miR-182 and its down-regulation resulted in blocking LPL core protein deacetylation and

subsequent LPL overexpression in macrophages.

Another study supports the regulation of HDAC1 by miR-34a in atherosclerosis [55]. MiR-34a was found highly expressed in atherosclerotic plaques and in human aortic endothelial cells (HAECs) treated with oxidized low-density lipoproteins (Ox-LDL). On the contrary, HDAC1 was downregulated in the same samples. Reverse genetic functional studies showed that miR-34a negatively influences HAEC viability and promotes atherosclerosis by inhibiting HDAC1 and subsequently modulating apoptosis-related genes.

3.3. MicroRNAs and histone methylation in cardiovascular diseases

Several studies focused on the crosstalk between miRNAs and histone methylation in cardiac pathophysiology. EZH2 is the functional component of the polycomb repressive complex 2 (PRC2) associated with H3K27 methylation and subsequent transcriptional silencing and it was shown to play a role in normal murine cardiac development [94] and disease [95]. One study on CH using a murine TAC model found that EZH2 interacts directly with pri-miR-208b to regulate the expression of pro-hypertrophic genes, such as Natriuretic Peptide A (*Nppa*) and B (*Nppb*) [56]. Increased interaction between EZH2 and the intergenic bidirectional promoter (bdP) of the α- and β-MHC genes after TAC surgery led to transcriptional suppression of antisense β-MHC and α-MHC. In the healthy heart, the expression of α-MHC dominates over that of β-MHC, but this is reversed during hypertrophy [96]. This effect was attenuated by administration of TSA, which induced the release of the EZH2/pri-miR-208b complex from the bdP and the reduction of H3K27me3 in favor of increased acetylation of the histones at the bdP. On the other hand, EZH2 binding to the hypertrophic marker genes *Nppa* and *Nppb* was reduced after TAC surgery, resulting in the overexpression of these genes [56]. This effect was not reversed after TSA treatment.

In addition to direct protein-miRNA interactions, other miRNAs target *Ezh2* mRNA, resulting in its inhibition. One such miRNA is let-7c. Transgenic mouse ESC overexpressing let-7c showed increased disposition toward mesoderm formation and cardiac differentiation [57]. *Ezh2* mRNA levels inversely correlated with let-7c expression in WT ESC throughout the differentiation process toward cardiac cells and, accordingly, a luciferase reporter assay confirmed that let-7c directly targets *Ezh2*. Chromatin immunoprecipitation revealed that let-7c overexpressing ESC had reduced H3K27me3 marks in the promoters of cardiac transcription factors *Nkx2.5*, *Mef2c*, and *Tbx5*, and an increase of H3K4me3, a histone mark associated with transcriptional activation [97]. Interestingly, comparison of human euploid fetal heart tissue to heart tissue with trisomy 21 (Down syndrome), a genetic affliction often leading to congenital heart defects, showed that let-7c was upregulated in the latter group, coupled to a reduction in *EZH2* levels [57].

Another study compared the transcriptional response after cardiac injury in mouse and zebrafish, the latter having a higher capacity for cardiac regeneration [58]. Following global gene profiling of both the border zone of the infarcted mouse heart and the regenerative tissue in zebrafish heart after ventricular resection, 45 miRNA-dependent networks were found to be differentially regulated. Among them, miR-26a, targeting *Ezh2*, was found downregulated in zebrafish heart following the insult, but remained stable in mouse heart. Inhibition of this miRNA in neonatal cardiomyocytes using miR-26a-specific oligonucleotides led to overexpression of *Ezh2* and subsequent repression of *Ezh2* target genes such as *Cdkn1a*, *Cdkn2a* and *Cdkn2b*, negative cell cycle regulators, eventually resulting in increased cardiomyocyte proliferation [58].

A study conducted by Thienpont et al. on a rat model of CH assessed the levels of transcriptionally repressive histone marks H3K9me2 and H3K27me3, two modifications usually found in large homogeneous genome segments [59]. Reduced H3K9me2 methylation was found in a subset of these segments, including the pro-hypertrophic fetal cardiac development genes *Nppa*, *NppbI* and Myosin Heavy Chain 7 (*Myh7*), which were all overexpressed. The authors attributed the reduction in H3K9me2 levels to lowered expression of Euchromatic Histone Lysine Methyltransferase 1 (*Ehmt1*) and 2 (*Ehmt2*) genes, responsible for H3K9 dimethylation. In turn, this reduction might be caused by an increase in miR-217, a miRNA which can target both *Ehmt1* and *Ehmt2*. Accordingly, overexpression of *Ehmt2* or repression of mir-217 provided protection against the hypertrophic phenotype. Interestingly, analysis of hypertrophic hearts from deceased patients revealed the same pattern of elevated miR-217 expression, reduced *EHMT1/2* mRNA and lower H3K9 methylation levels, supporting the role of the crosstalk between miRNAs and histone methylation in regulating the cardiac phenotype [59].

One study on senescent mouse embryonic fibroblasts found an increase in miR-29a and miR-29c levels upon activation of the canonical TGF-β signaling pathway [60]. These miRNAs are members of the miR-29 family, known to play a role in cardiovascular diseases [98] and were both found to target *Suv4-20 h1* and *Suv4-20 h2* methyltransferases, leading to a decrease in H4K20me3 [60]. Moreover, hearts of older mice showed an increase in the amount of miR-29 as well as reduced H4K20 methylation. Interfering with this process through TGF-β signaling inhibitors resulted in reduced cardiac miR-29, increased H4K20me3 and improved cardiac function.

Finally, a recent study by Oh et al. supported the regulation of sarcoendoplasmic reticulum $Ca2^{+}$ ATPase (*Serca2a*) expression by the PRC2-Six1-miR-25 signaling axis [61]. Reduced expression of the *Serca2a* gene, which is transcriptionally repressed by miR-25 in the heart [99], is associated with HF [100]. In hearts from both dilated cardiomyopathy patients and mice that underwent TAC surgery, both the transcription factor *Six1* and miR-25 were upregulated [61]. The former can enhance expression of the latter through binding to the promoter of the Minichromosome Maintenance Complex Component 7 (*Mcm7*) gene, which encodes miR-25 in its 13th intron. Lastly, expression of all the PRC2 components was reduced in hearts of TAC mice, leading to lowered trimethylaton of H3K27 at the *Six1* promoter and its subsequent derepression. Taken together, these data suggest the hypothesis in which cardiac stress hinders normal repression of *Six1* by PRC2, leading to increased miR-25 expression followed by decreased levels of *Serca2a*.

3.4. MicroRNAs and histone demethylation in cardiovascular diseases

Compared to histone methylation, fewer studies have reported on the role of histone demethylation and miRNAs in cardiac processes. A study by Dal-Pra et al. discovered that a combination of 4 miRNAs, miR-1, miR-133a, miR-208, and miR-499 ('miR combo') is capable of reprogramming fibroblasts into cardiomyocyte-like cells both *in vitro* in mouse cardiac fibroblasts and *in vivo* in ischemic mouse myocardium [101]. Furthermore, demethylation of H3K27me3 was shown to be essential for inducing reprogramming by the miR combo [62]. Accordingly, the levels of several histone methyltransferases and demethylases were altered after treatment with the miR combo. The authors found that miR-1 and miR-133a increased expression of histone demethylases *Kdm6B* and *Kdm6A* respectively, and that both also decreased the expression of *Ezh2*. Despite these interesting results, further studies are required to discern the mechanistic events linking miR-1 and miR-133a, *Ezh2*, *Kdm6B* and *Kdm6A* [62].

Another study focused on the interaction between *Kdm3a*, which demethylates H3K9me1/2, and miR-22-3p, which targets this demethylase and in turn is targeted by lncRNA H19 following MI in rats [63]. Overexpression of the lncRNA with an adenoviral vector pre-MI reduced the infarct size, reduced fibrosis and inflammation and improved heart performance. Similarly, knocking down expression of miR-22-3p or overexpressing *Kdm3a* with adenoviral vectors improved the cardiac phenotype after MI [63]. This study is an example of an intricate interaction of non-coding RNAs and epigenetic factors, highlighting the complexity of CVD regulation.

3.5. MicroRNAs and ATP-dependent chromatin remodeling in cardiovascular diseases

Also ATP-dependent chromatin remodeling enzymes have been shown to interact with miRNAs in cardiovascular diseases. One study using zebrafish sought to determine whether the inability of mammals to regenerate heart tissue following damage is due to lacking activation of the required molecular mechanisms, or rather to evolutionary loss of these mechanisms [64]. MiRNAs miR-99/100 and let-7a/c were found to be downregulated during regeneration in zebrafish heart following amputation of the ventricular apex while also being evolutionarily conserved across vertebrates. Concurrently, expression of miR-99/100 target genes beta subunit of farnesyl-transferase (*fntβ*) and SWI/SNF-related matrix associated actin-dependent regulator of chromatin subfamily a, member 5 (*smarca*5), an ATP-dependent chromatin remodeling enzyme of the SWI/SNF family, were upregulated. Injecting miR-99/100 mimics into zebrafish hearts following amputation inhibited cardiac regeneration, with reduced cardiomyocyte proliferation, while miR-99/100 inhibitors injection into normal hearts led to enlarged organs. These results indicate that miRNAs play a functional role in adult zebrafish heart regeneration by regulating chromatin accessibility. Conversely, adult murine and human hearts, which under normal circumstances highly express miR-99/100 but no FNTβ or SMARCA5, fail to downregulate miR-99/100 and let-7a/c. This lack of transcriptional response following the injury could partially explain the failure of adult mammal hearts to adequately regenerate compared to zebrafish. However, artificial inhibition of these miRNAs using anti-miRs in an *in vivo* mouse model for MI improved the functional outcomes and reduced the size of the infarcted area and fibrotic scarring [64]. Histological analysis revealed more cardiomyocytes positive for FNTβ and SMARCA5 in anti-miR treated animals post injury compared to animals treated with scrambled controls, as well as increased DNA synthesis and phosphorylation of histone H3. The involvement of miR-99a in cardiac regeneration was corroborated in a different study showing that overexpression of this miRNA repressed cardiac differentiation in mouse ESC through modulation of the Nodal/Smad2 signaling pathway [57]. Moreover, miR-99a overexpression led to an increase of H3K27me3 histone marks at promoters of cardiac transcription factors, further supporting the tight interaction among different classes of epigenetic factors.

3.6. MicroRNAs and DNA methylation in cardiovascular diseases

The mutual influence of miRNAs and DNA methylation occurs either through targeting the *DNMT* mRNA by a miRNA, or through the differential methylation of miRNA promoters. An example of the latter mechanism was found in the study by Gurha et al. in which the plakophilin 2 gene (*Pkp2*) was knocked down (KD) in HL-1 cells to serve as a model for arrhythmogenic cardiomyopathy [65]. MiR-184 was found to be the most downregulated miRNA out of the 750 that were screened. The cause of this downregulation was shown to be twofold: firstly, the repression of transcription factor E2F1 (which normally enhances miR-184 expression). Secondly, the hypermethylation of the upstream region of miR-184, due to recruitment of DNMT1 to its promoter region. Suppression of this miRNA led to increased adipogenesis in wild type HL-1 cells, while its overexpression in *Pkp2* KD HL-1 cells reduced the number of cells containing fat droplets, rescuing the adipogenic phenotype, a hallmark of the disease [65].

In another study, a regulatory loop between c-kit, DNMT and miR-193a in mouse c-kit positive cardiac stem cells (CSC) was identified [66]. Treatment of c-kit positive CSC with insulin-like growth factor (IGF)-1 increased expression of c-kit and enhanced both proliferation and migration through activation of the phosphoinositol 3-kinase (PI3K)/RAC-alpha serine/threonine-protein kinase (AKT-1)/DNMT signaling pathway. In turn, activation of this pathway led to hypermethylation of CpG islands in the *Mir-193a* promoter region, inhibiting its expression [66]. Accordingly, given the known repression of *c-Kit* expression by *Mir-193a* [102], this closes a negative feedback loop between c-kit, PI3K/AKT/DNMT and the miRNA which regulates CSC proliferation and migration.

The mutual influence of miRNA and DNA methylation was also observed in a study focused on vascular smooth muscle cells (VSMCs), whose abnormalities are associated with atherosclerosis, restenosis and aneurysm [67]. After a screening to identify miRNAs modulated by altered DNA methylation in VSMCs, miR-128 was selected and modulated *in vitro* and *in vivo* to study the underlying mechanisms. Not only was miR-128 found to regulate VSCM proliferation and migration, but also its overexpression in VSMCs resulted in altered levels of Dnmts and Tets, an abnormal DNA methylation profile, and demethylation of Myh11, a VSMC differentiation marker. Interestingly, miR-128 was also shown to regulate the stem cell pluripotency gene KLF4, a regulator of VSMC differentiation known to modulate DNA methylation as well. Altogether, these data support the hypothesis that miR-128 controls through KLF4 the methylation status of Myh11, which in turn prevents VSMC dedifferentiation and atherosclerosis. Another study focused on genome wide methylation patterns in atherosclerotic lesions identified a hypomethylation peak on the 14q32.2 locus [68]. Genes in this region are subject to imprinting regulation and include the paternally expressed *Rtl1* gene, essential for fetal capillary maintenance as well as for intra-plaque *de novo* capillary formation in adults. Also miR-127, mapping on the 14q32.2 locus, was found to be hypomethylated in the same samples and, furthermore, both the *Rtl1* gene and miR-127 were overexpressed in atherosclerotic lesions. Despite miR-127 being previously found to be upregulated in atherosclerotic plaques [103], this is an unexpected yet interesting result, as miR-127 is known to target *Rtl1*, thus suggesting a possible intricate epigenetic mechanism orchestrating atherosclerosis.

Also the turquoise killifish (*Nothobranchius furzeri*), a model for cardiac aging, was used to show a connection between miRNAs and DNA methylation [69]. This study showed an increased oxidative stress in the aging heart, accompanied by deregulation of 41 miRNAs. Members of the miR-29 family were among the most upregulated ones. *Dnmt* genes are known targets of this miRNA family, and, accordingly, *dnmt1* and *dnmt3a* were found downregulated in the aged heart together with a global decrease in DNA methylation [69]. These results were confirmed in a transgenic zebrafish miR-29 knockdown model, in which *dnmt1, dnmt3a* and *dnmt3b* were more expressed than in wild type controls and the global DNA methylation level was higher. The relationship between miR-29 family and methylation genes was further proved in human cardiac fibroblasts subjected to hypoxic conditions which showed in parallel miR-29a and b downregulation and increased DNMT activity.

In addition to intrinsic processes, also environmental factors can influence epigenetic mechanisms. For example, exposure to phenanthrene, a polycyclic aromatic hydrocarbon pollutant linked to cardiac dysfunction [104], was shown to induce CH *in vivo* in rats as well as *in vitro* in H9C2 cells and primary rat cardiomyocytes [70]. This was attributed to enhanced expression of *Dnmt1* and *Dnmt3* which led to increased global DNA methylation. In particular, hypermethylation of the region near the miR-133a transcription start site led to a reduction in miR-133a expression. This miRNA is known to play a critical role in CH [105]. The relationship between miR-133a and *Dnmt1*, *Dnmt3a* and *Dnmt3b* genes was further confirmed in a study by Chavali et al. in diabetic cardiomyocytes [71]. Accordingly, in a separate research on cardiomyocytes subjected to hyperhomocysteinemia, the same group observed a decrease in *Dnmt1* expression, possibly due to miR-133a activity [72].

MiRNAs can also target epigenetic reader genes which recognize methylated DNA. The methyl CpG binding protein 2 (MeCP2) binds methylated DNA and can function as a transcriptional repressor or, to a lesser extent, activator [106]. The mechanism behind the repression is thought to occur, at least partially, through MeCP2 interaction with a protein complex involving HDAC, corroborating that different epigenetic elements can interact with each other. MeCP2 was also shown to

influence transcription of various miRNAs, and in turn can be targeted by them [106]. For example, miR-22 was found to target *Mecp2* in *in vitro* murine cardiomyocytes, where it reduced ischemia-induced apoptosis [73]. Interestingly, miRNA-22 originated from exosomes secreted by mesenchymal stem cells that underwent ischemic preconditioning. This shows that the connections between miRNAs and epigenetic factors can occur across different cell types, making the possible range of interactions even larger.

A more recent study found that miR-212/132 cluster can also target *Mecp2* [74]. Using mice that underwent reversible TAC surgery as a model for chronic left ventricular pressure overload, Mayer et al. found that *Mecp2* was significantly downregulated on both mRNA and protein level but normalized again following removal of the aortic stenosis. This reversible downregulation was observed also in biopsies from failing human hearts examined before and after cardiac unloading [74]. At the same time, miR-212/132 cluster was upregulated after either TAC surgery or upon activation of α1- and β1-adrenoceptors and was proven to be able to target *Mecp2* using a luciferase assay. Using a cardiomyocyte-specific transgenic mouse model in which *Mecp2* expression could be regulated with doxycycline, the authors found that *Mecp2* overexpression led to worsened hypertrophy, fibrosis, and contractile dysfunction after TAC [74]. Conversely, ablation of the epigenetic reader strengthened cardiac recovery after TAC surgery, but did not influence the DNA methylation of its target genes, which were identified as mitochondrial genes and their transcriptional regulators.

A particular group of miRNAs implicated in cardiac diseases lie in the evolutionary conserved *Dlk1-Dio3* noncoding RNA locus between the Delta-like homolog 1 (*DLK1*) and type III iodothyronine deiodinase (*DIO3*) protein coding genes [107,108]. Following the *DLK1* gene there are two differentially DNA-methylated regions (DMR), IG-DMR and Gtl2-DMR, subject to epigenetic imprinting. The methylation status of the IG-DMR region determines gene expression within the *DLK1-DIO3* locus: the methylated paternal allele expresses the protein-coding genes, while the unmethylated maternal allele is responsible for expressing the noncoding RNAs. The *MiR-539* gene maps in the *Dlk1-Dio3* locus and was found upregulated both *in vitro* in anoxic conditions [75] and *in vivo* following MI [76]. Other miRNAs in the same locus are miR-410 and miR-495, which promote cardiomyocyte proliferation *in vitro* and are upregulated in murine models for MI, CH and muscular dystrophy [77,78]. Another *Dlk1-Dio3* miRNA playing a role in CH is miR-154. In a TAC mouse model, which featured upregulation of miR-154, knock down of this miRNA improved negative cardiac remodeling and fibrosis following the surgical procedure [79]. Conversely, miR-541 was downregulated in an *in vitro* CH model featuring Ang-II-treated cardiomyocytes, while overexpression of this miRNA in mice led to reduced hypertrophy after Ang-II treatment [80]. The same locus also contains miR-433, found to be increased in three different models of cardiac fibrosis [81]. Inhibition of the miRNA with an antagomir yielded improved preservation of cardiac function and less fibrosis [81].

One recent study analyzed DNA methylation and hydroxymethylation in a *Mybpc3* mutant mouse model of dilated cardiomyopathy and found that, in addition to protein-coding genes, several miRNA genes had differential (hydroxy)methylation status compared to healthy controls [82]. Among these, miR-30c-1, which causes dilated cardiomyopathy in transgenic mice overexpressing it [109], was hyper-hydroxymethylated.

Finally, a study analyzed the global DNA methylation profile of HF patients suffering from different cardiovascular diseases, such as hypertrophic obstructive cardiomyopathy, ischemic cardiomyopathy and dilated cardiomyopathy, compared to control patients [83]. Two particularly interesting differentially methylated miRNAs, miR-24-1 and miR-155, were detected. In both cases, the methylation status showed the same trend as the expression pattern change in HF patients. MiR-24-1 was hypermethylated and underexpressed, while miR-155 was hypomethylated and overexpressed.

4. Conclusion and future perspectives

This review highlights the central and intricate role of epigenetic factors in the etiology of cardiovascular diseases. These factors feature an intricate crosstalk between themselves and potentially with inherited rare genetic variations or polymorphisms. As a consequence, this complex network represents an additional level of cardiac phenotype regulation and can determine a patient's susceptibility to a given disease and the response to a treatment regimen.

On the other hand, several studies demonstrated how environmental factors, such as pollution, smoking, psychosocial context, diet and drugs, influence cardiovascular diseases by modulating both epigenetic factors [110–112] and miRNAs [113–115] thus increasing the complexity of the mechanisms underlining a pathogenic phenotype.

While being intricate, elucidating the network connecting epigenetics, miRNAs and environmental factors could not only allow better understanding of their cardiovascular effects and guide lifestyle changes but also assist in developing more personalized therapies. Available treatments for cardiovascular diseases have failed to be equally effective in all patients, suggesting that interindividual variability is an aspect playing an important role in personalized therapy. Besides genome mapping, existing and emerging technologies such as RNA-sequencing and high-throughput profiling of miRNAs, methylation or acetylation patterns could assist in determining the (*epi*)genetic make-up defining an individual's risk profile and finding novel therapeutic epigenetic targets. In this context, the utility of small molecules as epigenetic or miRNA modulators has been proven to overcome ischemia/reperfusion injury by targeting HDAC [116,117]. Several studies showed that statins can regulate both histone and DNA methyltransferases, resulting in serum cholesterol reduction, or miRNAs, improving CAD or hyperlipidemia [46,118–121] and highlight how future statin therapies could aim to not only affect one side of the miRNA-epigenetic effector network, but instead modulate both at once.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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