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Review

Epigenetic Regulation of Cardiomyocyte Differentiation from Embryonic and Induced Pluripotent Stem Cells

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Abstract: With the intent to achieve the best modalities for myocardial cell therapy, different cell types are being evaluated as potent sources for differentiation into cardiomyocytes. Embryonic stem cells and induced pluripotent stem cells have great potential for future progress in the treatment of myocardial diseases. We reviewed aspects of epigenetic mechanisms that play a role in the differentiation of these cells into cardiomyocytes. Cardiomyocytes proliferate during fetal life, and after birth, they undergo permanent terminal differentiation. Upregulation of cardiac-specific genes in adults induces hypertrophy due to terminal differentiation. The repression or expression of these genes is controlled by chromatin structural and epigenetic changes. However, few studies have reviewed and analyzed the epigenetic aspects of the differentiation of embryonic stem cells and induced pluripotent stem cells into cardiac lineage cells. In this review, we focus on the current knowledge of epigenetic regulation of cardiomyocyte proliferation and differentiation from embryonic and induced pluripotent stem cells through histone modification and microRNAs, the maintenance of pluripotency, and its alteration during cardiac lineage differentiation.

Keywords: epigenetic markers; cardiomyocyte; proliferation; differentiation; induced pluripotent stem cell; embryonic stem cell

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1. Introduction

During fetal development, the proliferation of cardiomyocytes increases the fetal heart size, but after birth, proliferation is limited and terminal differentiation of the cells results in hypertrophy [1–3]. Upregulation of cardiac-specific genes in adults and exit of the cardiomyocytes from the permanent cell cycle causes terminal differentiation in adult cardiomyocytes [1]. The proliferation of adult cardiomyocytes is blocked by silencing the *E2F* genes (encoding a family of transcription factors) that regulate G2/M (mitosis control) and cytokinesis. Epigenetic mechanisms mediate cell cycle gene silencing and cardiac-specific gene upregulation in adults [4].

Regeneration of myocardial tissue in the infarcted wall for the treatment of cardiomyocyte loss is a potential treatment strategy. To achieve this goal, studies are being conducted on pluripotent cells that can differentiate into cardiomyocytes, such as embryonic stem cells and induced pluripotent stem cells. The best stem cell candidate for cell-based therapy of myocardial diseases in regenerative medicine should have two important characteristics: the ability to override immunological rejection and plasticity to differentiate into desired cardiovascular cells.

Non-proliferative characteristics of adult cardiomyocytes and the difficulty of mesenchymal stem cells to differentiate into cardiomyocytes make pluripotent stem cells the best candidate for the production of human cardiomyocytes. Human embryonic stem cells were the first type of pluripotent stem cells to be used in cell therapy for differentiation into cardiomyocytes [5]. However, the most important concerns regarding the use of embryonic stem cells for tissue engineering are the immunogenicity potential and ethical limitations of the use of human embryonic stem cells [6]. These concerns, however, do not exist in the case of induced pluripotent stem cells, which are produced by nuclear reprogramming of somatic cells and can be used as a source for cardiomyocyte production for therapeutic purposes [7]. Direct injection of embryonic stem cells into the heart increases the risk of teratoma formation; therefore, it is necessary to differentiate them into cardiomyocytes prior to implantation [8].

Currently, there are three established methods for differentiating pluripotent stem cells into cardiomyocytes [9]. The first method involves the coculturing of pluripotent stem cells with mouse visceral endoderm-like (*end-2*) stromal cells or culturing in the *end-2*-conditioned medium. The differentiation mechanism is poorly understood in the *end-2*-based method, but it is simpler and inexpensive compared to the embryoid body-based method [10]. Moreover, due to the addition of a MAPK inhibitor, the cardiomyocyte yield efficiency of this method is higher than that of the embryoid body-based method [11]. The second method is the use of embryoid bodies in suspension. Chemical and physical circumstances that mimic early embryonic development together activate molecular pathways for the differentiation of pluripotent stem cells into cardiomyocytes. Small, spherical aggregates of pluripotent stem cells form embryoid bodies. The formation of such embryoid bodies depends on the protocol used; when cultured, 70–97% of these embryoid bodies differentiate into cardiomyocytes [12,13]. Fully defined culture conditions are the most important advantages of this method (Table 1). The disadvantages of this method include the difference in the number of beating cardiomyocytes between different embryoid bodies, low yield efficiency, and immature phenotype of cardiomyocytes [14]. The third method, which was recently developed, involves the addition of small molecules and growth factors into pluripotent stem cell culture medium [9,15]. The earlier studies of a two-dimensional monolayer differentiation were compared with the recent studies of the three-dimensional culture based on the formation of embryoid bodies and spheroids based on the formation of embryoid body and spheroid [16,17]. The advantages of this method include a large scalable quantity of cell differentiation, production of more matured cardiomyocytes, higher cardiomyocyte yields (85–95%), and cost reduction as a result of fewer media components [18].

Table 1. Cardiac differentiation protocols from human pluripotent stem cells.

Culture Type	Method	Induction Condition	References
3D	EB formation	RPMI 1640 + human serum albumin, phosphoascorbate, ITS, lipid mix, Y-27632, DS-I-7	[12]
	EB formation	RPMI1640 + B27 + CHIR99021	[13]
	EB formation	KO-DMEM + 20% FBS (+DMSO)	[19]
	EB formation	KO-DMEM + 20% FBS (+DMSO)	[16]
	EB formation	Stempro34 w/Activin A, BMP4, bFGF, VEGF, DKK1	[20]
	EB formation	KO DMEM + 15% FBS w/Wnt3a	[21]
	EB formation or monolayer	LI-APEL + Matrigel w/Activin A, BMP4, bFGF, VEGF, SCF, WNT3A	[22]
	EB formation	Stempro34 w/Activin A, BMP4, bFGF, VEGF, DKK1, TGFβi, BMPi	[23]
	EB formation	Stempro34 w/Activin A, BMP4, bFGF, IWR-1, triiodothyronine	[24]
	EB formation	BPEL w/Activin A, BMP4, CHIR99021, SCF, VEGF	[25]
	EB formation	Stempro34 w/BMP4, Activin A, bFGF, IWP2, VEGF, BMP4, RA, bFGFi, TGFβi	[17]
2D	Coculture with visceral endoderm-like cells (END2)	DMEM + 20% FBS	[10]
	Monolayer	RPMI1640 + B27 + MatriGel w/Activin A, BMP4	[26]
	Monolayer	RPMI1640 + B27 + w/Activin A, BMP2	[15]
	Monolayer	RPMI1640 + B27 w/Activin A, BMP4, bFGF, Noggin, RA/RAi, DKK1	[27]
	Monolayer (matrix sandwich)	RPMI1640 + B27 + Matrigel w/Activin A, BMP4, bFGF	[28]
	Monolayer	RPMI1640 + B27 w/CHIR99021, IWP2	[29]
	Monolayer	CDM3 w/CHIR99021, Wnt-C59	[18]
	Monolayer (multilayer plates)	RPMI1640 + B27 + Fibronectin or collagen type I w/CHIR99021, BMP4, IWR-1	[30]

Nuclear reprogramming via epigenetic modifications in cells for the specification and differentiation of different cell types is a complex process, which is performed by regulating the chromatin structure [31,32]. In induced pluripotent stem cell biology, epigenetic and chromatin modifications are critical, and are reversible and dynamic processes [33]. Several epigenetic factors play important roles in cardiomyocyte differentiation via transcription factors and signaling pathways. These epigenetic factors or regulators include modifications of histone, adenosine triphosphate (ATP)-dependent chromatin remodeling complexes, DNA methylation, and microRNAs (miR) [34,35]. They affect the suppression or expression of a gene by changing the availability of DNA sequences for DNA-binding proteins, inhibiting translation, or cleaving the complementary target messenger RNAs [36]. These alterations result from the modification of DNA-histone covalent interactions, which leads to an increase or decrease in the accessibility of DNA by loosening or tightening the chromatin, respectively, and by post-transcriptional regulation of gene expression. Epigenetic regulation of cardiomyocyte development from embryonic stem cells and induced pluripotent stem cells is the focus of this review.

2. The Different Types of Stem Cells: Embryonic Stem Cells and Induced Pluripotent Stem Cells

Protocols to induce cardiac differentiation in human pluripotent stem cells have been previously developed in some studies (Figure 1). The characteristics of embryonic stem cells are different from that of induced pluripotent stem cells. The inherent plasticity of embryonic stem cells could be a potential advantage in their application in regenerative medicine. Moreover, somatic cell nuclear transfer using embryonic stem cells can produce pluripotent stem cells that have the patient's nuclear genome that can differentiate into cardiomyocytes and repair heart damage. Although this strategy has been used in studies involving animal cardiac repair [37,38], there are various limitations to its applicability in humans, including low efficiency of somatic nuclear transfer, insufficient pluripotency of produced lines, abnormalities encountered in cloned cells, high cost, and the ethical debate surrounding the need for super-ovulated volunteers.

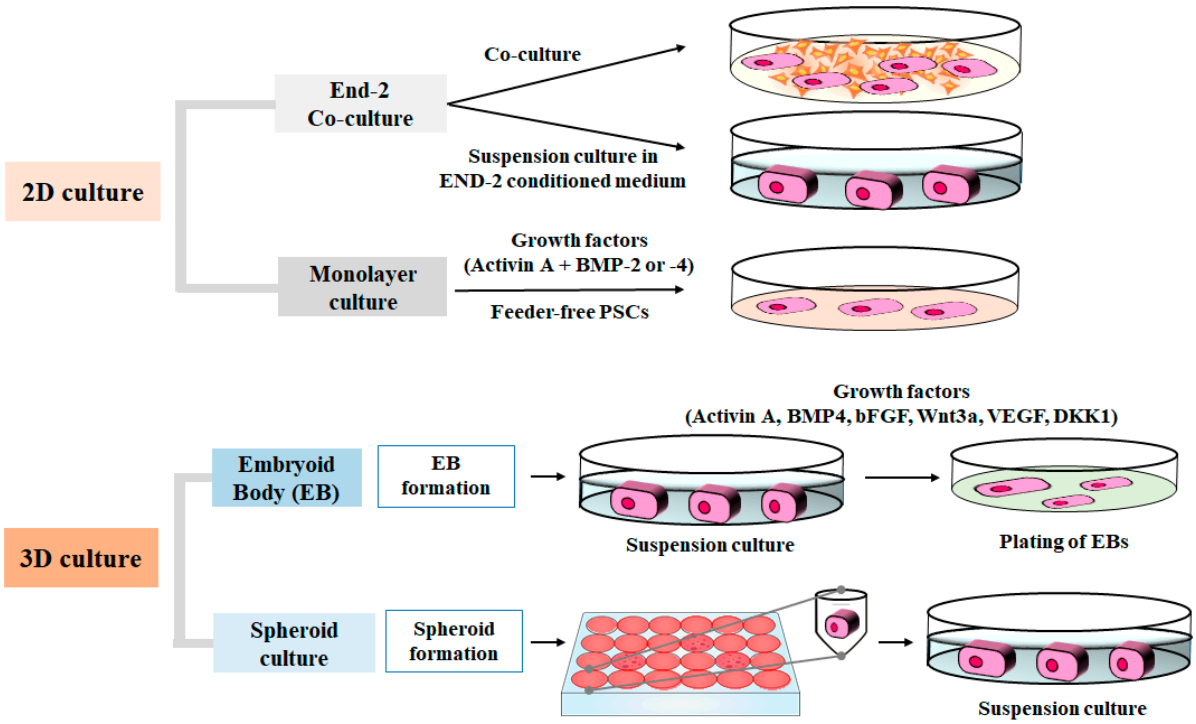

Figure 1. Differentiation induction into cardiomyocytes from pluripotent stem cells (PSCs) using two-dimensional (2D) or three-dimensional (3D) culture.

Somatic cells can be reprogrammed into induced pluripotent stem cells via retroviral transduction of *OCT4*, *SOX2*, *KLF4*, and *c-MYC* [7,39–41], plasmid transfection without using *c-Myc* [42], using recombinant proteins [43], adenovirus vectors [44], the PiggyBac transposon system [45], cell and transgene-free embryonic stem cell protein extracts [46], or Sendai virus vectors [47]. Induced pluripotent stem cells can differentiate into all three germ lineages. Deriving from autologous sources could be the most valuable aspect of induced pluripotent stem cells. Further, induced pluripotent stem cells are syngeneic. However, there are several limitations to its applicability in humans, including the low efficiency of deriving induced pluripotent stem cells, the long process for therapeutic purposes, and the tendency to form teratomas [48].

The epigenetic mechanisms responsible for the induction of pluripotency in somatic cells have not been fully characterized. Mechanistic insights into the reprogramming and retention of induced pluripotency of these cells are crucial for their efficient clinical application. Modifications in the epigenetic makeup of a cell can directly affect gene repression or expression. Epigenetics of embryonic stem cells and induced pluripotent stem cells are extremely complex. To be pluripotent (continuous proliferation, undifferentiation, and differentiation into a particular cell lineage), these cells should have the following three important characteristics: (1) their epigenetic code is continuous and active; (2) genes in the undifferentiated cells remain active, and those in the developing cells are repressed; and (3) they have sufficiently loosened chromatins once they start to differentiate. Modifications of epigenetic enzymes and factors play important roles in all these steps [49–57] (Figure 2).

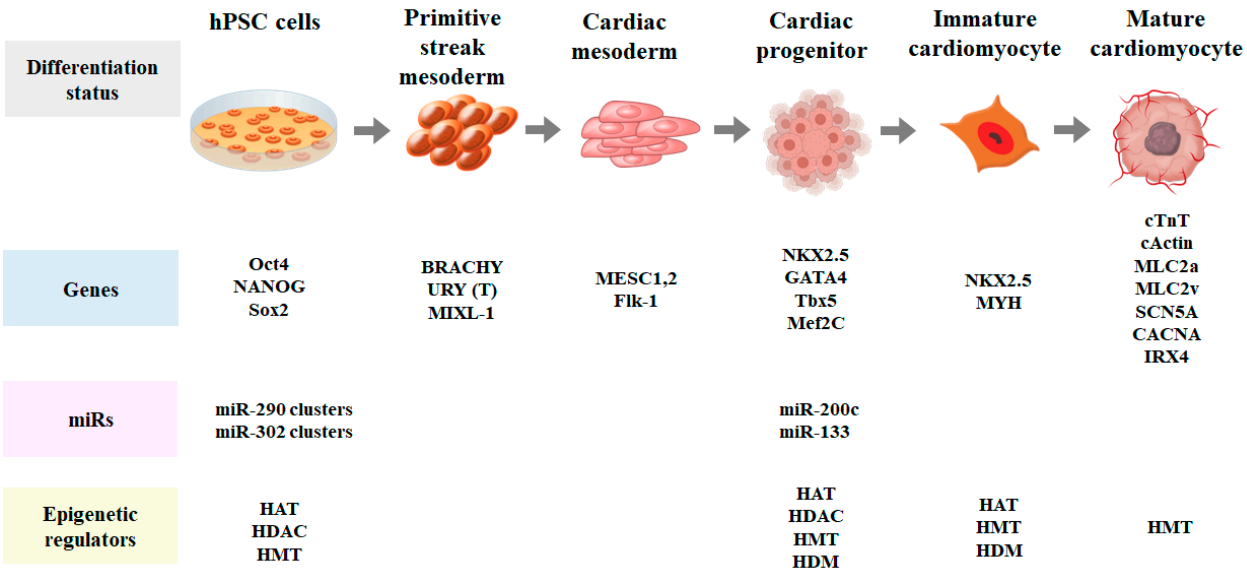

Figure 2. Differentiation of pluripotent stem cells (PSCs) into cardiomyocytes according to stage-specific gene expression and epigenetic regulation.

3. Epigenetic Regulation of Gene Expression and Silencing

Epigenetic changes regulate gene expression and silencing without changes in a DNA sequence. This is enabled by several modification mechanisms, such as histone protein modifications (methylation, acetylation, phosphorylation, sumoylation, ubiquitination, deamination, ribosylation, and proline isomerization), DNA modifications (DNA methylation), modifications using adenosine triphosphate (ATP)-dependent chromatin remodeling complexes, and microRNAs [31] (Table 2). The activation of cell-cycle inhibitors and cardiac-specific genes and repression of cell cycle progression and non-cardiac genes are critical for cardiac differentiation [58,59] (Figure 3).

Table 2. Epigenetic histone markers in cardiac-specific gene expression.

Epigenetic Factors		Affected Gene	Cardiac Tissue Effect	References
HAT				
	p300	αMHC and αSA	Development	[60,61]
		GATA4	Development	[62]
		Homeobox protein Nkx-2.5	Differentiation	[63]
		Mef2c	Morphogenesis, myogenesis, and vascular development	[64]
	MOF		Down-regulation of cardiac hypertrophy and cardiomyopathies	[65]
	CBP		Unkown	[66,67]
	Gcn5		Differentiation	[68]
HDAC				
	HDAC1,2	Hopx, GATA4	Proliferation	[69,70]
	HDAC3	Suppression of Mef2 and CDK inhibitor 1, 1B, 1C, 2C, 2B	Lack: increased cardiac hypertrophy Expression: cardiac hyperplasia without hypertrophy	[71]
		Suppression of Tbx5	Cardiac hypertrophy	
	HDAC4	Mef2 suppression	Cardiac hypertrophy prevention	[72,73]
	HDAC5,9	Mef2 suppression	Cardiomyocytes abnormality, thin-walled myocardium, and ventricular septal defects	[74,75]
	SIRT6	Oct4, Sox2 and Nanog	Differentiation	[76]
HMT				
	PTIP	Kcnip2	Electrical conduction	[77]
	Smyd1	skNAC	Muscle-specific transcription activator	[78]
		Hand2	Muscle-specific transcription activator	[79]
		Irx4	Muscle-specific transcription activator	[80]
	Smyd2		Neonatal cardiomyocytes histone methyltransferase	[81]
	Wolf-WHSC1	Nkx2.5	Lack: congenital cardiac defects Activity: transcription repression	[82]
	PRC2	Oct4, Sox2, and Nanog	Maintain pluripotency	[51]
		EZH1	Silencestage-specific gene	[83]
		EZH2	Executing pluripotency	[84]
		Eed	Differentiation	[4]
		RbAp46/48	Differentiation	[85]
		Suz12	Differentiation	[85]
	G9a and GLP		Non-cardiac gene silencing	[86]
	Suv39h1		Cell cycle exit	[4]
HDM				
	KDM4a	ANP	Up-regulation	[73]
		BNP	Up-regulation	[87]
	UTX	ANP, MLC2, and a-CA	Differentiation	[88]
		TFs, Nkx2.5 Tbx5, GATA4, SRF, Brg1-associated factor Baf60c	Increase cardiac-specific genes activation	[88]
	JmjC		Development	[89]
	Jmjd6		Development	[89]
miR				
	let-7		Dedifferentiation	[90]
	miR-200c	GATA4, SRF, and TBX5	Repressed differentiation and maturation	[91]
	302-367 cluster	Nanog, Oct3/4, Sox2, and Rex	Development	[92]
	miR-520		Dedifferentiation	[90]

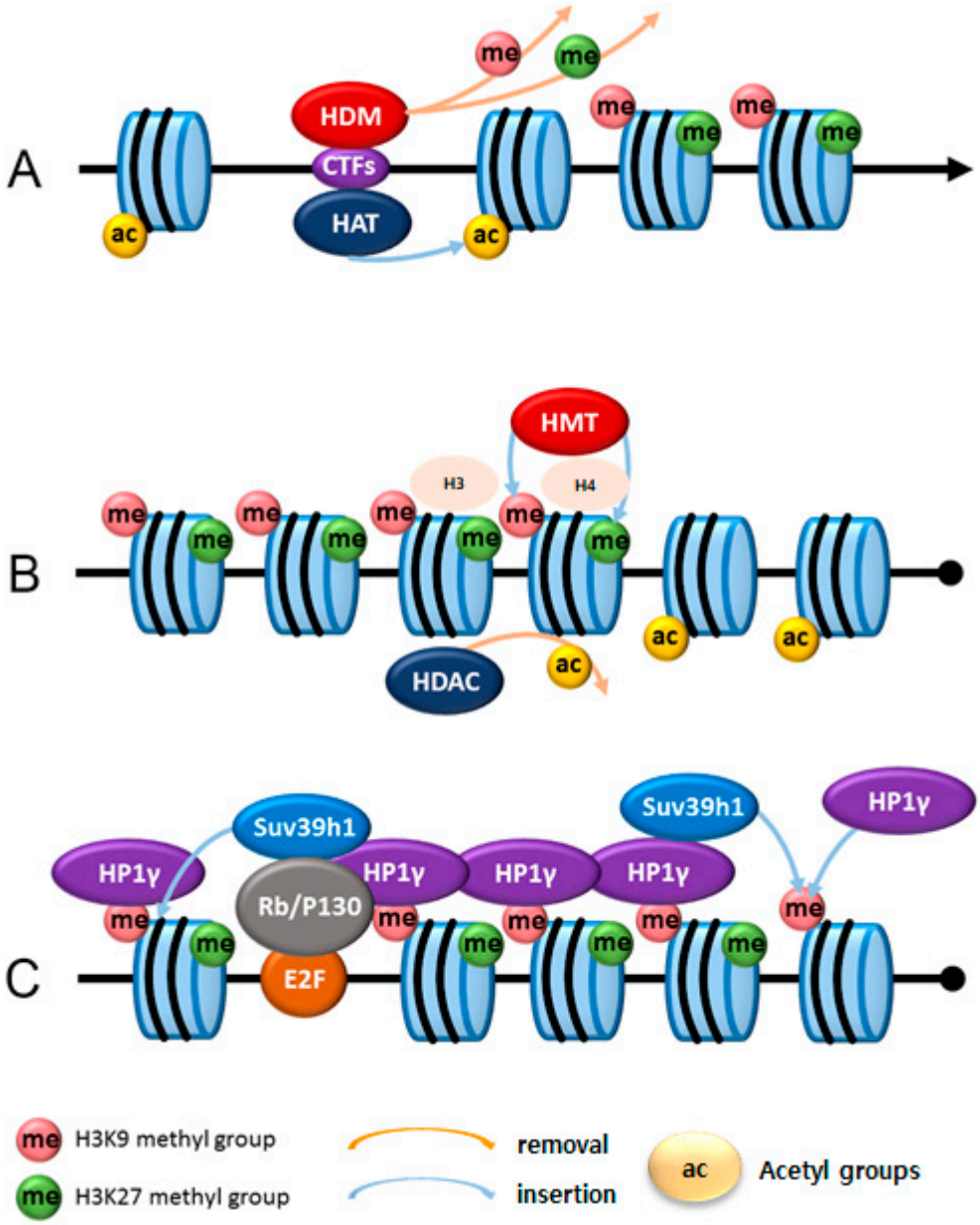

Figure 3. Model of epigenetic gene regulation in cardiomyocytes. (**A**) Activation of cardiac-specific genes: cardiac-specific transcription factors (CTFs) activate cardiac-specific genes by recruiting transferring of acetyl groups to histone H3 and/or H4 histone using acetyltransferases (HAT). Also, they recruit histone demethylases (HDM) to remove silencing methyl marks from H3K9 and H3K27. (**B**) Silencing of non-cardiac genes: histone deacetylases (HDACs) remove acetyl groups (AC) from H3 and/or H4, and histone methyltransferases (HMT) put methyl groups on H3K9 and H3K27 to suppress and silence non-cardiac genes. (**C**) By recruiting HP1γ to E2F responsive promoters, Rb directly promotes permanent cell cycle exit that has undergone methylation of H3K9 (modified from [93]).

αMHC, α-myosin heavy chain; αSA, α-sarcomeric actin; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CBP, cAMP response element-binding protein; CDK, cyclin-dependent kinase; Eed, embryonic ectoderm development; Ezh, catalytic subunit enhancer of Zeste; GATA4, critical transcription factor for cardiac development; Hand2, heart- and neural crest derivatives-expressed protein 2; HAT, histone acetyltransferases; HDAC, histone deacetylases; HDM, histone demethylase; HMT, histone methyltransferase; Hopx, homeodomain-only protein; Irx4, iroquois homeobox 4; Kcnip2, Kv channel-interacting pro-

tein 2; Mef2, myocyte-specific enhancer factor 2; miR, microRNA; MOF, males absent on the first; PRC2, polycomb repressive complex 2; PTIP, PAX interacting protein 1; RbAp46/48, retinoblastoma protein-associated protein 46/48; skNAC, skeletal nascent polypeptide-associated complex alpha; SRF, serum response factor; Suz12, suppressor of Zeste 12; TBX5, T-box transcription factor 5; UTX, ubiquitously transcribed tetratricopeptide repeat, X chromosome.

3.1. Histone Modifications

Gene transcription is affected by several histone modifications such as acetylation, methylation, ubiquitination, pho-phorylation, and sumoylation that occur on lysine and arginine residues of histone tails. Based on our present knowledge of the N-terminus of histones, there are eight acetylate lysine positions, H3K9, H3K14, H3K18, H3K23, H4K5, H4K8, H4K12, and H4K16. H3 and H4 acetylation by histone acetyltransferase and deacetylation by histone deacetylase regulates gene activation and silencing, respectively.

Moreover, six lysine residues of histones can be methylated: H3K4, H3K9, H3K27, H3K36, H3K79, and H4K20. Depending on which amino acids in the histones are methylated, and the number of attached methyl groups, they can either increase or decrease the transcription of DNA. Methylation of H3K4 and H3K27 was found to discriminate between genes that are expressed, poised for expression, or stably repressed; therefore, they could reflect the state and lineage potential of embryonic stem cells [53]. Methylation of H3K36 facilitates gene annotation by marking primary coding and noncoding transcripts [53]. H3K9 and H4K20 methylation are detected in active, telomeric, and satellite long-terminal repeats. Active chromatin has methylated H3K4, whereas inactive chromatin is marked by methylation of H3K9 [94].

Histone acetyltransferases, histone deacetylases, histone methyltransferases, and histone demethylases control these post-translational modifications. Moreover, transcription factors have access to euchromatin, the looser form of chromatin that promotes gene expression, whereas the dense structure, heterochromatin, prevents gene expression [95].

Histone methyltransferases add methyl groups to lysine and arginine residues, while histone demethylases remove methyl groups. Histone methylation forms heterochromatin, and histone acetylation forms euchromatin. Mono, di, or trimethylation of and types of amino acid residues in the histone affect transcriptional activation (for example, by methylation of H3K4, H3K36, and H3K79) or gene repression (for example, by methylation of H3K9 and H3K27) [31]. The trimethylation of H3K9 by recruiting heterochromatin protein 1 (HP1) induces heterochromatin stability [96]. Moreover, trimethylation of H3K27 represses and poises genes [88,97].

3.2. Histone Acetyltransferases

p300 histone acetyltransferase activity is expressed in the embryonic myocardium and is required for cardiac development [98,99]. p300 enhances the expression of the α-myosin heavy chain (αMHC) and α-sarcomeric actin (αSA) [60,61]. It also interacts with GATA4 (a transcription factor critical for cardiac development), homeobox protein Nkx-2.5 (a transcription factor critical for regulating tissue differentiation and determining the patterns of temporal and spatial development), and myocyte-specific enhancer factor 2C (MEF2C is involved in cardiac morphogenesis, myogenesis, and vascular development) [62–64]. 'Males-absent on the first' (MOF) protein, another histone acetyltransferase, plays a critical role in the down-regulation of cardiac hypertrophy in mice and human cardiomyopathies [65]. cAMP response element-binding protein (CBP) is a histone acetyltransferase expressed in the embryonic heart; however, its deficiency does not affect heart formation [66,67]. GCN5, a histone acetyltransferase, plays a role in in vitro cardiac differentiation [68].

3.3. Histone Deacetylases

Inhibition of histone deacetylase undergoes acetylation of H3 and H4 and differentiation of cardiomyocytes in vitro [64,100]. Cardiac-specific deletion of histone deacetylase 1 and 2 genes causes neonatal mortality, dilated cardiomyopathy, and cardiac arrhythmias [101]. The interaction of histone deacetylase 2 and HOPX (homeodomain-only protein) limits cardiomyocyte proliferation via GATA4 deacetylation and decreases its transcriptional activity [69,70]. Histone deacetylase 3 suppresses TBX5 activity [102]. Cardiac-specific deletion of histone deacetylase 3 in mice intensifies cardiac hypertrophy [103]. In contrast, overexpression of cardiac histone deacetylase 3 intensified cardiac hyperplasia by the suppression of cyclin-dependent kinase inhibitor 1 ($p21^{cip1}$), 1B ($p27^{kip1}$), 1C ($p57^{kip2}$), 2C ($p18^{inc4c}$), and 2B ($p15^{inc4b}$) without causing hypertrophy [71]. Histone deacetylase 4 prevents cardiac hypertrophy by suppressing *Mef2* [72,73]. The cardiac development functions of histone deacetylase 5 and histone deacetylase 9 overlap and involve the suppression of *Mef2*, and a simultaneous lack of both causes cardiomyocyte abnormality, thin-walled myocardium, and ventricular septal defects [74,75].

Histone deacetylases are recruited by binding of *Hey* proteins close to transcription start sites, leading to deacetylation of histones, condensation of chromatin, and repression of target genes. In cardiomyocytes, the binding of cardiac activators recruits histone acetylases, thereby counteracting *Hey* proteins [104].

Acetylation of H3K56 is associated with the transcriptional activation of pluripotent genes in embryonic stem cells [105]. The NAD-dependent histone deacetylase SIRT6 targets acetylated H3K56 in mouse embryonic stem cells [106]. SIRT6 directly regulates the expression of the core pluripotent genes, *OCT4*, *SOX2*, and *NANOG*, via deacetylation of H3K56, which in turn controls embryonic stem cell differentiation through Tet-mediated oxidation of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) [76].

3.4. Histone Methyltransferases

Histone methylation has an important role in regulating cardiac development [78,82,88,107–110]. De novo mutations in genes that modify H3K4 and H3K27 cause congenital heart disease [111]. PAX interacting protein 1 (*PTIP*), an H3K4 histone methyltransferase subunit, regulates the expression of genes involved in electrical conduction in the heart, such as Kv channel-interacting protein 2 (*KCNIP2*) [77]. Cardiac-specific knockout of *PTIP* altered the regulation of sodium and calcium handling. H3K4 histone methyltransferase does not affect myosin heavy chain beta (*MHC-β*) and atrial natriuretic peptide (*ANP*), which are genes associated with cardiac hypertrophy. SMYD1, a muscle-specific transcription activator, methylates H3K4 in vitro [79,112,113]. SMYD1 plays an efficient role in cardiomyocyte maturation and development of the right ventricle by interacting with skeletal nascent polypeptide-associated complex alpha (skNAC) via upregulation of heart- and neural crest derivatives-expressed protein 2 (HAND2) and iroquois homeobox 4 (IRX4) [78–80]. However, the relationship between the suppression of *SYMD1* expression and histone methyltransferase activity in cardiac development is not clear [113,114]. The interaction of *SYMD1* with sarcomere proteins plays a role in myosin protein methylation [114,115]. Moreover, *SYMD1* interacts with histone deacetylases as a transcriptional repressor [78,81]. SMYD2 is a neonatal cardiomyocyte histone methyltransferase of H3K4 and H3K36, and its deficiency can be compensated by other histone methyltransferases [81]. Another histone methyltransferase is Wolf-WHSC1, which mono-, di-, and tri-methylates H3K36. Patients with Wolf–Hirschhorn syndrome have a WHSC1 deletion, which causes congenital cardiac defects [116]. WHSC1 deficiency leads to ventricular and atrial septal defects [107]. Occupation of *Nkx2.5* target genes with *Nkx2.5* and WHSC1 interaction via tri-methylation of H3K36 leads to transcriptional repression [82].

During embryonic stem cell differentiation, polycomb repressive complex 2 (PRC2), a histone methyltransferase complex, activates and occupies target genes *OCT4*, *SOX2*, and *NANOG* in order to maintain pluripotency [51]. PRC2 is recruited by methylated H3K27 [94]; the silence-stage-specific gene *EZH1* mediates the methylation of H3K27 and

complements *EZH2* in maintaining stem cell identity and executing pluripotency [83,84,117]. The H3K27 methyltransferase *EZH2* plays an important role in the regulation of gene expression and is related to heart development [118,119]. In female mammalian somatic cells, methylated H3K27 is prominent in the inactivated X-chromosome [94]. An increase in trimethylated H3K27 induces stable recruitment of PRC2 and leads to the differentiation of cardiac progenitor cells into cardiomyocytes [118]. Trimethylated H3K4 fully activates promoters, while H3K4 dimethylation correlates with the basal transcription-permissive state [120]. A pattern of monomethylated H3K4 deposition at the transcription start site of a select group of genes precedes transcriptional activation, acquisition of trimethylated H3K4, and recruitment of RNA polymerase II phosphorylated at serine 5 (RNAP) [59]. This preactivation is important for genes that are not regulated by polycomb complexes.

Embryonic ectoderm development (Eed), retinoblastoma protein-associated protein 46/48 (RBAP46/48), catalytic subunit enhancer of Zeste 1 (EZH1/EZH2), and suppressor of Zeste 12 (SUZ12) are four components of PRC2 [85]. *EZH1* and *EZH2* are predominantly expressed in adult and embryonic hearts [4] and stabilize cardiac differentiation by gene silencing [118,119]. Moreover, *EZH2* binds to and methylates GATA4 and reduces its interaction with p300 and its transcriptional activity, and suppresses α-myosin heavy chain expression in embryonic cardiomyocytes [121]. In addition, G9a and GLP, which are major mono- and di-methyltransferases of H3K9 in cardiomyocytes, play a role in non-cardiac gene silencing during cardiac differentiation [86]. During cardiac differentiation, tri-methylation of H3K9, mediated by *Suv39h1*, regulates cell cycle exit [4]. Therefore, suppressive marks such as di- and trimethylated H3K9 and trimethylated H3K27, which suppress transcriptional activity, lead to cell cycle exit and non-cardiac gene silencing.

3.5. Histone Demethylases

Histone methylation is known to be irreversible because the half-life of histones is approximately equal to the half-life of methylated histone [122]. However, the discovery of histone demethylases that remove methyl groups from histones suggests a novel cellular regulatory process. KDM4a is a histone demethylase of trimethylated H3K9 and tri-methylated H3K36 [123]. KDM4a is upregulated and enriched in the atrial natriuretic peptide ANP and brain natriuretic peptide (BNP) promoters [73,87].

The jumonji family proteins have histone demethylase activity and demethylate mono-, di-, and tri-methylation and play an essential role in the development of cardiomyocytes. Jumonji is encoded by the *JARID2* gene and consists of a DNA-binding domain, an AT-rich interaction domain (ARID), and two conserved domains (*JmjN* and *JmjC*) [124]. The JmjC domain is essential for histone demethylation [124,125]. Another member of this family, JMJD6, is a histone demethylase of H3 and H4 arginine, which plays an important role in cardiomyocyte development [89].

Ubiquitously transcribed tetratricopeptide repeat gene on the X chromosome (UTX) is another JMJC protein and an H3K27 demethylase that is encoded by the X chromosome [126–128]. UTX plays an important role in the differentiation of embryonic stem cells into cardiomyocytes and the expression of cardiac-specific genes (*ANP*, *MLC2*, and *a-CA*) by demethylation of trimethylated H3K27 [88]. The interaction of UTX with *TFS*, *NKX2.5*, *TBX5*, *GATA4*, serum response factor (SRF), and *Brg1*-associated factor BAF60C promotes cardiac-specific gene activation [88]. In addition, UTX demethylates H3K4 for cardiac enhancer activation [88].

3.6. DNA Methylation

DNA methylation is an essential epigenetic mediator for regulating cell development, which is a reversible process critical for embryonic stem cell differentiation [129]. DNA methyltransferase enzymes (DNMT1, DNMT3a, and DNMT3b) attach a methyl group to 50-CpG-30 dinucleotides. DNA methylation occurs at the fifth carbon position in the cytosine residue. The formation of CG dinucleotides establishes a repressed chromatin state and inhibits gene expression [130,131]. DNA methylation plays a role in X-chromosome

inactivation, cell differentiation, changing chromatin structure, tumorigenesis, genomic imprinting, tissue-specific gene expression, and induction of pluripotency in somatic cells [130,132]. During the process of demethylation of genes that are involved in the induction of pluripotency in somatic cells and reprogramming them into induced pluripotent stem cells, a cytidine deaminase molecule, AID, plays a key role [133]. The Fe^{2+}- and α-ketoglutarate-dependent dioxygenases, and TET enzymes (TET1, TET2, and TET3) catalyze DNA methylation [134,135]. These enzymes revert the methylation status of DNA by successive oxidation of 5mC into 5hmC, 5-carboxy cytosine (5caC), and 5-formyl cytosine (5fC), which are intermediates in an active DNA demethylation mechanism [136,137]. Embryonic stem cell pluripotency is maintained by increasing the levels of 5hmC, TET1, and TET2 [138–140]. During the differentiation stage, TET1 and TET2 expression decreases, which leads to repression of pluripotent genes and activation of developmental genes [135,141–144].

Bivalent domains consist of two near regions: large regions of methylated H3K27 and small regions of methylated H3K4, which were found to silence and equilibrate developmental genes in embryonic stem cells [49]. To induce lineage differentiation of induced pluripotent stem cells into cardiomyocytes, certain small molecules are used to manipulate epigenetic regulators. They include BIX01294 as a histone methyltransferase inhibitor [145], RG108 and 5-azacytidine as DNA methyltransferase inhibitors [146,147], and valproic acid, a histone deacetylase inhibitor [148].

3.7. ATP-Dependent Chromatin Remodeling Complexes

ATP-dependent chromatin remodelers use the energy of ATP hydrolysis to disrupt or alter the histone-DNA association, thus providing DNA accessibility [149] via the repositioning of nucleosomes (sliding, twisting, or looping). They include four families, of which the switching-defective and sucrose non-fermenting families are the ones studied more extensively with respect to cardiomyocyte development [149]. The Brahma-related gene 1 (*Brg1*)/Brahma (Brm)-associated factor complex interacts with several cardiac transcription factors, including NKX2-5, GATA4, TBX5, and TBX20 [150–152]. BRG1 promotes cardiomyocyte proliferation through BMP10 stimulation, activates the β-myosin heavy chain in the fetal cardiomyocytes, and represses the α-myosin heavy chain in adult cardiomyocytes [153]. Overexpression of TBX5, GATA4, and the BRG1/BRM-associated factor subunit BAF60C promotes differentiation of non-cardiac mesoderm into cardiac tissue [154].

3.8. MicroRNAs

MicroRNAs (miRs) are emerging as key players in the reprogramming and differentiation of induced pluripotent stem cells. miRs are small noncoding RNAs that are transcribed from both the intragenic and intergenic regions. They play critical roles in a variety of different processes, and we are beginning to understand their role in pluripotent cells. The role of miRs in pluripotency has been studied using Dicer-null and DGCR8-null ES cells, which lack mature miRs [90,155–157]. A subset of miRs controls the expression of DNA methyltransferases, histone deacetylases, and polycomb group genes [158]. When differentiation of Dicer-null ES cells is induced by embryoid body formation, the cells show only a slight decrease in OCT4 levels and a slight increase in the expression of early differentiation genes [90]. This indicates the role of miRs in the early differentiation of these cells. Additionally, miRs seem to play an important role in pluripotency. Two miRs clusters (clusters 290 and 302) have binding sites on their promoter regions for pluripotency-associated genes. Moreover, members of these clusters are regulated by pluripotency-associated genes such as OCT4, SOX2, and NANOG [92,159]. Expression of pluripotency-associated miRs clusters (miR-520) and inhibition of tissue-specific miRs (let-7) during reprogramming can increase the efficiency of the dedifferentiation process [90]. miR-200c represses the differentiation and maturation of human embryonic stem cells into cardiomyocytes by reducing the mRNA levels of GATA4, SRF, and TBX5 [91]. Figure 4 shows various miRs

and their roles in the expression or suppression of genes associated with the differentiation and proliferation of cardiomyocytes.

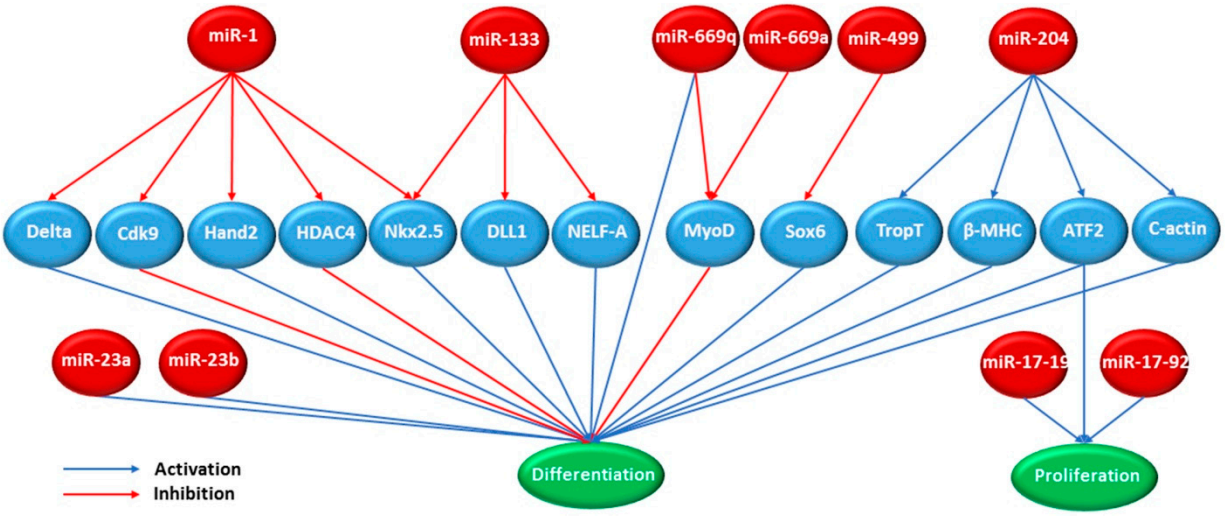

Figure 4. Various miR subtypes (red circle) role in inhibition (red arrows) and activation (blue arrows) of specific transcription factors (blue circle), which are responsible for differentiation and proliferation of cardiomyocytes (modified from [160]). For example, increased miR-1 induces differentiation of cardiac progenitor cells by repressing the translation of cdk9. Conversely, increased miR-133 inhibits this process by repressing the translation of DLL1, a transcription factor that promotes the expression of cardiac mesoderm genes.

4. Terminal Differentiation of Cardiomyocytes

For cardiac lineage differentiation, inducing signals from other lineages and non-cardiac genes is a prerequisite [161]. The cardiac lineage arises from the lateral plate mesoderm during development [162]; BMP signaling is the main signaling pathway associated with cardiac lineage differentiation. Retinoid signaling, and BMP2 signal neighboring the visceral endoderm, are involved in the specification of the cardiac lineage [163,164]. These specific signaling pathways direct early mesodermal lineage-specific genes, Brachyury [165], and the mesendodermal transcription factor GATA4, which is essential for the activation of the cardiac signaling cascade [166,167].

Heterochromatin condensation is a characteristic feature of fully differentiated cells and shows an irreversible exit from the cell cycle [4,168–170]. This feature prevents the accessibility of transcriptional factors to heterochromatic loci [171]. Very low histone acetylation and high trimethylation of H3K9 are characteristics of heterochromatic loci. Embryonic cardiomyocytes have high levels of acetylated H3K9/14, H3K18, and H3K27; however, after they are fully differentiated, the level of acetylated histones decreases and trimethylation of H3K9 and H3K27 increases, which leads to repression of the transcription of related genes [4]. These epigenetic processes are mediated by histone deacetylases, histone methyltransferases, Rb family proteins, and HP1 family proteins.

Among the histone deacetylase family members, histone deacetylase 1 plays an important role in cell types other than cardiomyocytes, including retinal cells and oligodendrocytes [172–174]. However, there is little information about the role of histone deacetylases in the heterochromatin assembly of differentiated cardiomyocytes. Histone deacetylase 1 plays a critical role in the differentiation, termination, and downregulation of proliferation-promoting proteins of neural cells in the zebrafish retina [172,173].

Histone methyltransferases such as G9a/GLP, Suv39h1/2 di, and tri methylate H3K9 are essential for suppressing cell proliferation and enabling cell cycle exit [4,168,175]. Dimethylated H3K9 is present in euchromatin and heterochromatin loci, while trimethylated H3K9 is found in the heterochromatin region, indicating their different roles in gene silencing [4]. Trimethylated H3K9 along with retinoblastoma protein and p130 can result

in heterochromatin formation in cardiomyocytes. HP1 family proteins (HP1α, -β, and -γ) are recruited by Rb proteins and play an important role in gene silencing by maintaining heterochromatin [168,176–179]. These proteins are expressed in adult cardiomyocytes. However, their role in the heart largely remains unknown, and their subnuclei localize at different sites. HP1γ stably represses adult cardiomyocyte-gene promoters by binding to G2/M and cytokinesis gene promoters, and the other members of the HP1 family, HP1α and -β, cannot compensate for the loss of function in HP1γ [4]. When Rb/p130 is deleted, HP1γ disassociates from the G2/M and cytokinesis gene promoters. Although trimethylated H3K9 remains intact, it leads to the disruption of heterochromatin and cell cycle re-entry by re-expression of G2/M and cytokinesis. However, the role of trimethylated H3K27 in differentiation termination in adult cardiomyocytes is unclear, but it plays a role in the suppression of E2F-dependent genes [180]. Moreover, trimethylation of H3K27 may play a role during the early stages of heterochromatin formation.

5. Conclusions

Here, we summarize the results of the studies that have been performed on epigenetic regulation in cardiac differentiation and development. Several reports on gene manipulation techniques and inhibitors have revealed the importance of epigenetic factors in cardiac development. However, specific target genes and histone modification mechanisms as well as the role of related enzymes in cardiac development, require further investigation.

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