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Epicardial HDAC3 Promotes Myocardial Growth Through a Novel MicroRNA Pathway

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Abstract

Background: Establishment of the myocardial wall requires proper growth cues from nonmyocardial tissues. During heart development, the epicardium and epicardium-derived cell (EPDC)s instruct myocardial growth by secreting essential factors including fibroblast growth factor 9 (FGF9) and insulin-like growth factor 2 (IGF2). However, it is poorly understood how the epicardial secreted factors are regulated, in particular by chromatin modifications for myocardial formation. The current study is to investigate whether and how histone deacetylase 3 (HDAC3) in the developing epicardium regulates myocardial growth.

Methods: Various cellular and mouse models in conjunction with biochemical and molecular tools were employed to study the role of *HDAC3* in the developing epicardium.

Results: We deleted *Hdac3* in the developing murine epicardium and mutant hearts showed ventricular myocardial wall hypoplasia with reduction of EPDCs. The cultured embryonic cardiomyocytes with supernatants from *Hdac3* knockout (KO) mouse epicardial cells (MECs) also showed decreased proliferation. Genome-wide transcriptomic analysis revealed that *Fgf9* and *Igf2* were significantly downregulated in *Hdac3* KO MECs. We further found that *Fgf9* and *Igf2* expression is dependent on HDAC3 deacetylase activity. The supplementation of FGF9 or IGF2 can rescue the myocardial proliferation defects treated by *Hdac3* KO supernatant. Mechanistically, we identified that microRNA (miR)-322 and miR-503 were upregulated in *Hdac3* KO MECs and *Hdac3* epicardial KO hearts. Overexpression of miR-322 or miR-503 repressed FGF9 and IGF2

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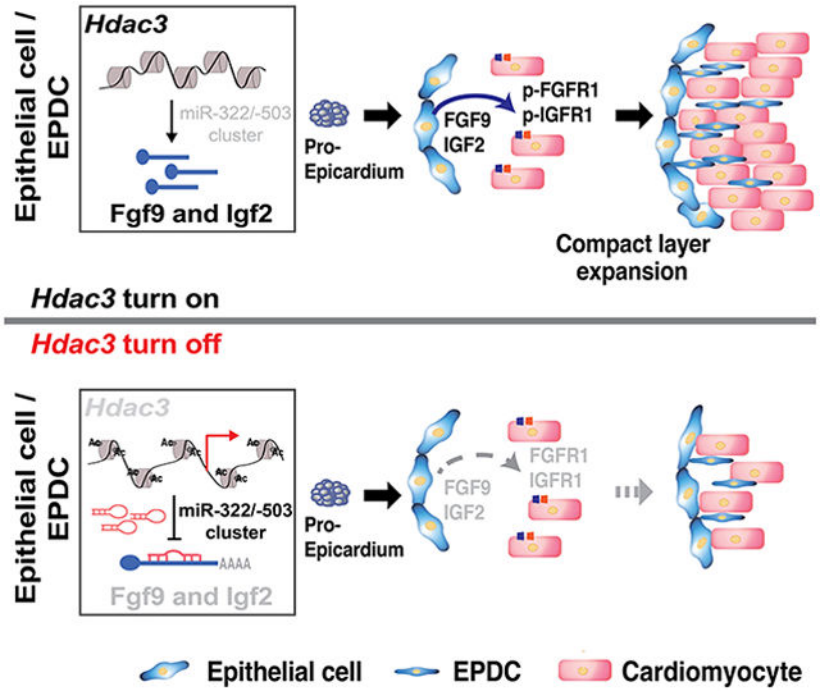
Disclosure

None.

expression, while knockdown of miR-322 or miR-503 restored FGF9 and IGF2 expression in *Hdac3* KO MECs.

Conclusions: Our findings reveal a critical signaling pathway in which epicardial HDAC3 promotes compact myocardial growth by stimulating FGF9 and IGF2 through repressing miR-322/miR-503, providing novel insights in elucidating etiology of congenital heart defects, and conceptual strategies to promote myocardial regeneration.

Graphical Abstract



Keywords

Basic Science Research; Developmental Biology; Mechanisms

Introduction

Congenital heart disease (CHD) is still the most common birth defect worldwide[1]. Myogenic defects are often associated with many forms of CHD. In the past, most research has focused on identifying the intrinsic factors in the myocardium to understand the potential causes, whereas the contributions from nonmyocytes through intercellular communications or regulations between cardiomyocyte (CM)s and nonmyocytes have been largely overlooked[2]. The nonmyocyte compartments such as the epicardium and epicardium-derived cell (EPDC)s are capable of regulating the development of adjacent tissues such as compact myocardium via paracrine signaling crosstalk[3]. Thus, it is possible that the disrupted signaling communications other than myocardium itself account for the myocardial malformations. Further, many of these paracrine signaling pathways are reinvested during heart repair and/or regeneration. For instance, the epicardium acts as a

pivotal hub for mediating heart regeneration[3-6]. Studies in both adult zebrafish hearts and neonatal mouse hearts have revealed that the epicardium plays a critical role for heart regeneration by providing paracrine growth factors, such as fibroblast growth factor (FGF)s and insulin-like growth factor (IGF)s to proliferating CMs[6, 7]. Understanding how these paracrine signals are regulated may provide important insights for both the pathogenesis of CHD and novel strategies for promoting heart regeneration.

The epicardium is composed of a single layer of mesothelial cells. It covers the outermost layer of the heart. The epicardium originates from a cluster of progenitor cells, which are located at the venous pole of the developing heart, known as the proepicardium (PE)[8]. The PE is initiated around embryonic day (E) 8.5 in mice[9]. The EPDCs emancipate from the epicardium through an epithelial-to-mesenchymal transition (EMT) event and give rise to several cardiac cell lineages including interstitial cardiac fibroblasts and coronary smooth muscle cells, which constitute cardiac stroma and provide oxygen and nutrients to heart muscle[10, 11]. During heart development, the epicardium also plays an important role by nurturing the underlying myocardium through secreting paracrine trophic factors such as FGF9 and IGF2[3].

FGF9 belongs to the FGF super family[12]. The binding of FGF9 to FGF receptors (FGFRs) triggers phosphorylation of FGFRs, and then subsequently activates the PI3K/AKT pathway and the MEK/ERK signaling cascade to drive cell proliferation and tissue morphogenesis[12, 13]. Either global deletion of FGF9 or conditional knockout of FGFR1/2 in the myocardium leads to ventricular hypoplasia[14], suggesting that FGF9 and its downstream signaling is important for myocardial growth. IGF2 is another major paracrine growth signal released from epicardial cells and converges to the same downstream AKT and ERK signaling axis[3, 15]. Conditional knockout of either IGF2 in the epicardium or its major receptor, IGFR1 in cardiac progenitors (Nkx2-5+) resulted in reduced CM proliferation and ventricular wall hypoplasia[16-18]. In contrast, conditional deletion of IGF2 in the endocardium or myocardium did not give rise to any apparent cardiac phenotypes[18]. In the developing heart, FGF9 and IGF2 are mainly secreted by the epicardium and its EPDCs, with minor contributions from cardiac endothelial cells[3, 19]. Most previous research has focused on understanding the function and/or the downstream signaling of these growth factors[12, 15]. How the expression of these growth factors in the developing epicardium is regulated is still poorly understood.

Gene transcription can be heavily influenced by chromatin's accessibility, which can be regulated by post-translational modifications of histones, including acetylation/deacetylation, methylation/demethylation, phosphorylation/dephosphorylation and ubiquitination/deubiquitination[20]. By switching between any of those two states, the associated genes can be dynamically programmed to be transcriptionally active or repressed. Histone deacetylase 3 (HDAC3), a member of the class I HDAC family that catalyzes the removal of acetyl groups from lysine residues in histone tails, has been implicated in many biological processes by modulating gene expression[21]. Mesodermal or global knockout of *Hdac3* resulted in myogenic defects and early embryonic lethality[22, 23]. Interestingly, ablation of *Hdac3* specifically in the myocardium does not give rise to cardiac morphological phenotypes during early heart development, but rather compromises cardiac

function at later postnatal stages[23, 24]. These findings suggest that the function of HDAC3 in nonmyocyte compartments may be critical for early myocardial development.

MicroRNAs (miRs) are small non-coding RNAs (about 22 nucleotides in length) that post-transcriptionally regulate gene expression, and are greatly implicated in heart development, disease, and regeneration[25, 26]. MiRs can modulate the expression of a wide range of genes including growth factors. For instance, the expression of FGF2 and IGF2 in neural stem cells, cancer, or skeletal muscle is subject to the regulation by miR-1275, miR-483, and others[27-30]. It is not clear whether and how the expression of FGF9 and IGF2 is epigenetically (e.g., via HDACs or miRs) regulated in the developing epicardium.

In the present study, we investigated the role of HDAC3 in the epicardium during early heart development.

Methods

Data Availability

A detailed description of all experimental procedures and statistical tests can be found in the Supplemental Materials & Methods section in the Supplemental Materials.

Results

Specific inactivation of Hdac3 in the developing epicardium results in ventricular wall hypoplasia

To study the potential role of *Hdac3* in the epicardium during heart development, we specifically ablated *Hdac3* using $Wt1^{CreERT2}$ [10], a tamoxifen inducible epicardial Cre mouse, and *Hdac3* floxed mice[31]. As expected, after tamoxifen induction at E8.5, *Hdac3* was effectively and specifically deleted in the epicardium, whereas its expression in non-epicardial cells, such as CMs, was unaffected (Fig. 1A). We did not observe any leakiness of Cre in the $Wt1^{CreERT2}$ allele (Fig. S1). To evaluate potential impact of *Hdac3* deletion on epicardial development, we performed epicardial lineage tracing in both epicardial *Hdac3* deficient ($Hdac3^{f/f}$, $Wt1^{CreERT2/+}$, $R26^{eGFP/+}$) ($Hdac3^{eko}$) hearts and their littermate control ($Hdac3^{f/+}$, $Wt1^{CreERT2/+}$, $R26^{eGFP/+}$) (CTL) hearts. At E10.5 and E12.5, the epicardium was similarly traced in $Hdac3^{eko}$ and CTL hearts (Fig. S2A and S2B), suggesting proepicardial development and epicardial migration to the heart is not affected by epicardial Hdac3 deletion. From E12.5 to E16.5, EPDCs undergo EMT and migrate into the compact myocardium to become fibroblasts and coronary vascular cells[32, 33]. Interestingly, we found that *Hdac3* deficient EPDCs were significantly fewer in E14.5 $Hdac3^{eko}$ hearts as compared to CTL hearts (Fig. S2A and S2B), although the contributions to each epicardial cell lineage including fibroblasts, smooth muscle cells appeared to be unaffected (Fig. S2C). Furthermore, the percentage of EPDCs that migrate into the compact myocardium was significantly lower in E14.5 $Hdac3^{eko}$ hearts as compared to CTL hearts (Fig. S2D), suggesting a potential EMT/migration defect in *Hdac3* deficient EPDCs. Consistently, epicardial EMT markers such as *Snail2* and *Twist1* were significantly downregulated in $Hdac3^{eko}$ hearts (Fig. S2E). The epicardium is an important source for contributing to embryonic coronary development by both lineage contribution and providing

paracrine patterning cues[3, 34]. Sox17 has been widely used as an early coronary vascular cell marker[35-37]. We found that Sox17+ cells were significantly fewer in *Hdac3*^{eko} hearts as compared to CTL hearts (Fig. S3), suggesting that coronary vascular development is compromised in *Hdac3*^{eko} hearts.

Both epicardium and EPDCs are major contributors to compact myocardial growth through cell-cell crosstalk[3]. Thus, we assessed the myocardia of epicardial *Hdac3* deficient (*Hdac3*^{eko}) hearts at several embryonic stages. The ventricular free wall morphogenesis was similar between *Hdac3*^{eko} and their littermate CTL hearts at E9.5, E11.5 and E12.5 (Fig. S4). However, the compact layer in *Hdac3*^{eko} hearts was significantly thinner than in the littermate CTL hearts at E13.5 and E14.5 (Fig. S4 and Fig. 1B), whereas there was no apparent morphological phenotypic difference in the *Hdac3*^{eko} trabecular myocardia as compared to the littermate CTL hearts.

Next, we investigated whether altered cell proliferation and/or apoptosis contributes to the ventricular wall hypoplastic phenotypes in *Hdac3*^{eko} hearts. At E13.5, we found that the percentage of p-H3+ or BrdU+ myocytes was significantly lower in *Hdac3*^{eko} compact myocardia as compared to the littermate CTL hearts (Fig. 1C). In contrast, there was no significant difference in cell apoptosis between *Hdac3*^{eko} and the littermate CTL hearts (Fig. S5). These findings are consistent with the hypoplastic cardiac phenotypes seen in *Hdac3*^{eko} hearts (Fig. S4 and Fig. 1B).

Reduced expression of FGF9 and IGF2 in Hdac3 deficient epicardial cells contributes to deceased CM proliferation

To further understand the potential molecular mechanisms contributing to the hypoplastic ventricular wall phenotypes in *Hdac3*^{eko} hearts, we effectively deleted *Hdac3* in immortalized MECs using CRISPR/Cas9 technology (Fig. 2A). *Hdac3* KO MECs grow slower than *Hdac3* EV MECs. By performing bulk RNA sequencing and gene ontology (GO) analyses on *Hdac3* KO and EV MECs, we found 1,681 downregulated genes and 1,549 upregulated genes. GO pathway analyses identified that the top 14 out of 20 significantly affected pathways are all involved in cell division (Fig. 2B). In a search for EPDC secreted growth factors in downregulated genes, we identified *Fgf9* and *Igf2* as our top candidates (volcano plot in Fig. 2C). We further validated the decrease of FGF9 and IGF2 at both mRNA and protein level in *Hdac3* KO MECs (Fig. 2D and E). Consistently, FGF9 and IGF2 were also significantly decreased in *Hdac3*^{eko} hearts (Fig. S6). These results are consistent with the cardiac growth defects seen in *Hdac3*^{eko} hearts.

Next, we sought to determine whether reduction of FGF9 and IGF2 accounts for decreased CM proliferation. First, we found that FGF9 and IGF2 were significantly decreased in the supernatants from *Hdac3* KO MECs as compared to that from *Hdac3* EV MECs (Fig. 3A). Then, we treated cultured primary embryonic CMs isolated from E13.5 *Tnnt2*$^{nGFP/+}$[38] hearts with supernatants from either *Hdac3* KO MECs or *Hdac3* EV MECs, respectively. *Hdac3* KO supernatant treatment resulted in significant decrease of the percentage of p-H3+ CMs and the total number of CMs, as compared to *Hdac3* EV supernatant treatment (Fig. 3B and C). Consistently, *Hdac3* KO supernatant attenuated the activation of downstream signaling pathway for CM proliferation, such as p-ERK (Fig. S7A). Lastly, to determine whether

the reduction of FGF9 and IGF2 in *Hdac3* KO supernatants is a major contributor to the decreased CM proliferation, we supplemented *Hdac3* KO supernatants with recombinant mouse FGF9 or IGF2 protein, respectively. Strikingly, supplementation with FGF9 or IGF2 successfully rescued CM proliferation defects by *Hdac3* KO supernatant treatment (Fig. 3B and C). We further confirmed the reactivation of p-FGFR1 or p-IGFR1 by FGF9 or IGF2 supplementation in *Hdac3* KO supernatant treated CMs (Fig. S7B and S7C). Altogether, these results suggest that FGF9 and IGF2 secreted from epicardial cells provide important cues for driving CM proliferation.

HDAC3 induces FGF9 and IGF2 expression dependent on its deacetylase enzymatic activities

To determine whether the deacetylase activities are required for HDAC3 to induce the expression of FGF9 and IGF2, we treated MECs with RGFP966, a selective HDAC3 deacetylase inhibitor[39]. Interestingly, RGFP966 treatment significantly decreased FGF9 and IGF2 (Fig. 4A and B). To further confirm that HDAC3 regulates FGF9 and IGF2 dependent on its enzymatic activities, we performed a set of genetic rescue experiments. As expected, re-expression of lentiviral wildtype *Hdac3* in *Hdac3* KO MECs successfully restored the expression of *Fgf9* and *Igf2* (Fig. 4C). In contrast, re-expression of lentiviral *Hdac3* Y298H, a deacetylase dead mutant *Hdac3*[40], failed to rescue the expression of *Fgf9* and *Igf2* (Fig. 4C).

HDAC3 induces the expression of FGF9 and IGF2 through repressing miR-322 and miR-503

The expression of FGFs and IGFs can be modulated by miRs[41, 42], which can be downstream targets of HDACs in certain biological contexts[43]. To identify the potential HDAC3 downstream miR targets, we performed miR sequencing in *Hdac3* KO and EV MECs. Through differential expression analyses, 42 miRs were significantly upregulated in *Hdac3* KO MECs (Fig. 5A). Among the top 20 hits (fold change equal to or greater than 1.5 and $P<0.01$), we identified 11 miRs that have putative binding sites on either *Fgf9* or *Igf2* using the “DIANA MicroT-CDS” analyses tool[44]. We treated MECs with these 11 miR mimics and found that the treatment of miR-322 mimics or miR-503 mimics significantly inhibited the expression of both *Fgf9* and *Igf2* (Fig. 5B). miR-322 and miR-503 are encoded as one cluster by H19X, which is located in chromosome X, and they share the same “AGCAGC” sequences within the seed region at the 5’ end. The 3’ UTRs of both *Fgf9* and *Igf2* harbor putative binding sites for miR-322 and miR-503 (Fig. 5C). We further validated the significant inhibitory effects of miR-322 mimics or miR-503 mimics treatment on the expression of FGF9 and IGF2 by western blot (Fig. 5D). These results indicate that miR-322/miR-503 represses the expression of *Fgf9* and *Igf2*. Further, we treated cultured E13.5 CMs with either miR-322 or miR-503 mimics and found that these treatments significantly decreased the percentage of p-H3+ CMs (Fig. 5E), suggesting that miR-322 and miR-503 inhibit CM proliferation.

We further validated the upregulation of miR-322 and miR-503 in *Hdac3* KO MECs (Fig. 5F). Interestingly, miR-322 and miR-503 were similarly significantly upregulated when the deacetylase activity of HDAC3 is inhibited by RGFP966 treatment (Fig. 5F). The significant

upregulation of miR-322 and miR-503 was also observed in E13.5 *Hdac3*^{eko} hearts as compared to the littermate CTL hearts (Fig. 5F).

To determine whether the upregulation of miR-322 and miR-503 has causal effects on the reduction of FGF9 and IGF2 when *Hdac3* is knocked out or inhibited, we knocked down the expression of miR-322 or miR-503 by miRZip lentivirus in *Hdac3* KO MECs (Fig. 6A). Remarkably, knockdown of miR-322 or miR-503 significantly restored the expression of FGF9 and IGF2 in *Hdac3* KO MECs (Fig. 6B). These results suggest that HDAC3 promotes the expression of FGF9 and IGF2 through repressing miR-322 and miR-503.

HDAC3 represses miR-322/miR-503 promoter activity

H3K27Ac is a marker for active promoters and a direct downstream target of HDAC3[21]. As expected, *Hdac3* deletion resulted in significant increase of H3K27Ac in MECs (Fig. 7A). Identified by the ENCODE project, the promoter region of miR-322/miR-503 is subject to epigenetic regulations such as H3K27Ac (Fig. 7B). To determine whether *Hdac3* deletion would affect the chromatin accessibility of the miR-322/miR-503 promoter, we surveyed H3K27Ac binding affinity in *Hdac3* KO MECs by ChIP-qPCR. We found that *Hdac3* deletion significantly increased the binding of H3K27Ac to the miR-322/miR-503 promoter (Fig. 7C), suggesting that *Hdac3* deletion renders the miR-322/miR-503 promoter more accessible to transcriptional factors. As expected, HDAC3 binds to this promoter region, as determined by HDAC3 ChIP-qPCR (Fig. 7D). To further test whether this increased chromatin accessibility would affect the miR-322/miR-503 promoter activities, we performed an miR-322/miR-503 promoter luciferase reporter assay. We found the luciferase activity was significantly increased in *Hdac3* KO MECs as compared to *Hdac3* EV MECs (Fig. 7E). Strikingly, inhibition of HDAC3 by RGFP966 replicated this result (Fig. 7E), suggesting that the regulation of HDAC3 on the miR-322/miR-503 promoter is dependent on its deacetylase activities.

Discussion

Early elegant studies in avian embryos have revealed that delay or blockade of the epicardium formation leads to decreased CM proliferation in the ventricular free wall without affecting trabecular myocardial development, which is more dependent on the support from the endocardium[45, 46]. Subsequent studies found that epicardium stimulates ventricular wall expansion by providing mitogens to CMs such as retinoic acid, FGFs, and IGFs[16, 47, 48]. However, it is unclear how these mitogens are initially induced in the epicardium. Epigenetics has been increasingly recognized as an important regulator of gene transcription in a variety of physiological/pathological processes including cardiac development and congenital diseases[49]. Our current study suggests that HDAC3 induces the expression of FGF9 and IGF2 in the epicardium, and thus stimulates ventricular myocardial wall expansion through paracrine signaling. Our study provides strong evidence that the epigenetics in the epicardium regulates ventricular wall morphogenesis, a new perspective on the mechanisms of cardiac development.

HDAC1, HDAC2 and HDAC3 are categorized in the same family (Class I HDACs), and global knockout in each case is lethal although their cardiovascular phenotypes are all

dinstinct[50], suggesting that they all have unique functions. HDAC1 and HDAC2 are almost identical and they are often found in same repressive complexes such as Sin3 and NuRD[51], while HDAC3 usually partners with Nuclear receptor co-repressor 1 (NCoR1 or NCoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT)[52]. The difference in these biochemical characteristics may underlie their distinct functions. Knockout of *Hdac3* alone in the developing epicardium resulted in cardiac defects, indicating that other HDACs are not functionally redundant with HDAC3/unable to compensate for the loss of HDAC3. Meanwhile, it awaits investigation whether deletion of other *Hdacs* in the developing epicardium will elicit cardiac phenotypes.

The molecular action of HDAC3 is complex. NCoR and SMRT are both nuclear receptor co-repressors. The NCoR/SMRT complex stoichiometrically recruits HDAC3 and activates its intrinsic deacetylase activity[21]. HDAC3 mainly deacetylates H3K9Ac and H3K27Ac. Deacetylation of these two sites renders chromatin structure unfavorable for the recruitment of transcriptional factors, and thus dampens gene transcription. This is the mechanism by which HDAC3 epigenetically suppresses gene transcription in regulation of skeletal muscle metabolism[53, 54]. However, when HDAC3 determines CM fate, it acts as a recruiter to tether genes to the nuclear periphery for silencing and its deacetylase enzymatic activity is dispensable[55]. Interestingly, several recent studies highlight a critical function for HDAC3 as a gene activator, rather than a gene repressor[55-58]. Whether HDAC3 acts as a repressor or an activator might depend on the nature of its interacting partners and cell context. Our current study demonstrates that in the developing epicardium, HDAC3 works as a gene repressor to suppress the expression of miR-322/miR-503 through its deacetylase activities.

To identify the potential targets of HDAC3 in the epicardium, we narrowed down to FGF9 and IGF2 by both unbiased screen (RNA-Seq) and candidate (known growth factors) approaches. It is possible that other unaccounted dysregulated genes caused by *Hdac3* deletion might contribute to the hypoplastic ventricular wall phenotype. We will explore those possibilities in future studies.

miR-322 (ortholog of human miR-424) and miR-503 belong to the miR-15/107 family. They are encoded as one cluster by H19X. miR-322 and miR-503 regulate many fundamental processes such as cell proliferation, death, differentiation, metabolism, and stress response. Thus, they are widely implicated in many disorders such as cardiovascular disease, neural disease, and cancer[59, 60]. During heart development, miR-322/miR-503 has been shown to drive early cardiac progenitor cells toward CM lineage, although the underlying mechanism is undetermined[61]. Our current study demonstrates that HDAC3 represses miR-322 and miR-503 expression to induce the expression of several major growth ligands including FGF9 and IGF2 for ventricular wall CM proliferation (Fig. 7F). This is the first line of study to demonstrate that the myocardial growth cues from the epicardium are tightly controlled by double inhibitory epigenetic regulation with HDACs and microRNAs. HDAC3 modulates the accessibility of miR-322/miR-503 promoter (Fig. 7C). Using transcription factor search bioinformatic tools, we identified over 300 putative transcription factors that bind to the 1.5kb miR-322/miR-503 promoter region. These transcription factors include C/EBPß, RXRα, Smad, ETS-1 and LEF-1/TCF-1, which have been shown to regulate

epicardial development[62-66]. Some of these transcription factors may work with HDAC3 to regulate miR-322/503 expression. We will explore it more in future studies.

Many forms of CHD, such as hypoplastic left heart syndrome, are associated with defective cardiac myogenesis, which further compromises cardiac contractile function[67]. Proper cardiac myogenesis requires precise coordination among multiple cell types in the developing heart, rather than just requiring CMs. Nonmyocytes including EPDCs provide key growth signals to the adjacent compact myocardium in a paracrine fashion[2]. Our findings suggest that disrupted epicardial signaling (FGF9 and IGF2) significantly compromises the compact wall expansion in *Hdac3*^{eko} hearts. Epicardium also plays important roles during heart regeneration and repair, such as providing paracrine signals and stimulating neovascularization[3-6, 68]. It's very common that some growth signaling pathways employed during heart development are reactivated upon myocardial injuries/stresses and contribute to heart regeneration/repair. In future studies, it will be interesting to determine whether this HDAC3—miR-322/miR-503—FGF9/IGF2 axis is reutilized during regenerative responses and manipulation of this pathway affects the outcome of neonatal/adult heart repair after injuries.

During heart development, EPDCs mainly give rise to interstitial cardiac fibroblasts and coronary smooth muscle cells[11], maybe to CMs and endothelial cells with controversial results[10, 69-71]. We found that the total number of EPDCs is significantly decreased in *Hdac3*^{eko} hearts, whereas the percentage of contributions to each cell type was not affected (Fig. S2). The reduction of EPDC population in *Hdac3*^{eko} hearts likely also contributes to the ventricular hypoplasia phenotypes since the total amount of FGF9 and IGF2 will be decreased as a result of EPDC population reduction. Further, *Hdac3* deletion appeared to specifically affect EPDC invasion to the compact myocardium (Fig. S2). It is not well understood how the lineage contribution by EPDCs is regulated. Growth factors (e.g., FGF10) can promote this process by either inducing EMT or stimulating the proliferation of epicardium-derived terminal cells[48, 72]. It is possible that the reduction of FGF9 and IGF2 in *Hdac3*^{eko} hearts accounts for decreased derivation of epicardial lineages.

In summary, we demonstrated that epicardial HDAC3 orchestrates ventricular wall expansion by inducing FGF9 and IGF2 expression through repressing miR-322/miR-503. Our study provides strong evidence that epigenetic factors such as HDAC3 play pivotal roles for the expression of these paracrine growth signals in the epicardium. Our findings strengthen the importance of epicardial paracrine signaling for myocardial development, which is implicated in the pathogenesis of CHDs and adult heart regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CHD	Congenital Heart Disease
EPDC	Epicardium-Derived Cell
CM	Cardiomyocyte
EMT	Epithelial-to-Mesenchymal Transition
HDAC3	Histone Deacetylase 3
FGF9	Fibroblast Growth Factor 9
IGF2	Insulin-like Growth Factor 2
miR	MicroRNA
BrdU	5-bromo-2'-deoxyuridine

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Novelty and Significance

WHAT IS KNOWN?

- Paracrine growth signals including fibroblast growth factors (FGFs) and insulin-like growth factors (IGFs) from the developing epicardium and epicardium-derived cells (EPDCs) promote the expansion of compact myocardium. However, it is unclear how these signals are transcriptionally regulated.
- Histone deacetylase 3 (HDAC3) is essential for heart development in general, but its function in the developing epicardium has not been studied.
- microRNA (miR)-322 and miR-503 are important for cardiac progenitor cell proliferation and differentiation. Their potential roles in other cardiac cells including EPDCs have been elusive.

WHAT NEW INFORMATION DOES THIS ARTICLE CONTRIBUTE?

- The expression of *Hdac3* in the developing epicardium is critical for compact myocardial growth.
- HDAC3 is important for EPDC derivation and migration.
- miR-322 and miR-503 suppress the transcription of Fgf9 and *Igf2*.
- HDAC3 promotes the transcription of *Fgf9* and *Igf2* by repressing the expression of miR-322 and miR-503.

In the present study, we discovered that deletion of *Hdac3* in epicardial cells compromises developing CM proliferation both *in vitro* and *in vivo*. Through lineage tracing at several developmental stages, we found that deletion of *Hdac3* in the epicardium resulted in reduced EPDC derivation and migration. *Hdac3* deletion led to downregulation of FGF9 and IGF2 both *in vitro* and *in vivo*. Further, we showed that HDAC3 induces the expression of FGF9 and IGF2 dependent on its deacetylase activity. We found that miR-322 and miR-503 were significantly upregulated in *Hdac3* deficient epicardial cells both *in vitro* and *in vivo*, whereas ectopic expression of these miRs in epicardial cells resulted in the reduced expression of FGF9 and IGF2, and subsequently led to decreased CM proliferation. Importantly, inhibition of miR-322 or miR-503 restored the expression of FGF9 and IGF2 in Hdac3 deleted epicardial cells. Finally, we demonstrated that HDAC3 physically binds to the promoter of miR-322/miR-503 and suppresses their expression. Overall, we conclude that epicardial HDAC3 regulates compact myocardial growth by inducing FGF9 and IGF2 expression through repressing miR-322 and miR-503. Our study exemplifies how the expression of growth factors is regulated by a dual epigenetic mechanism (histone modifiers and microRNAs), and dysregulation of such signaling axis among distinct cell types may elicit congenital heart defects. On the other hand, this mechanism may well be invested to induce cardiac regeneration and repair.

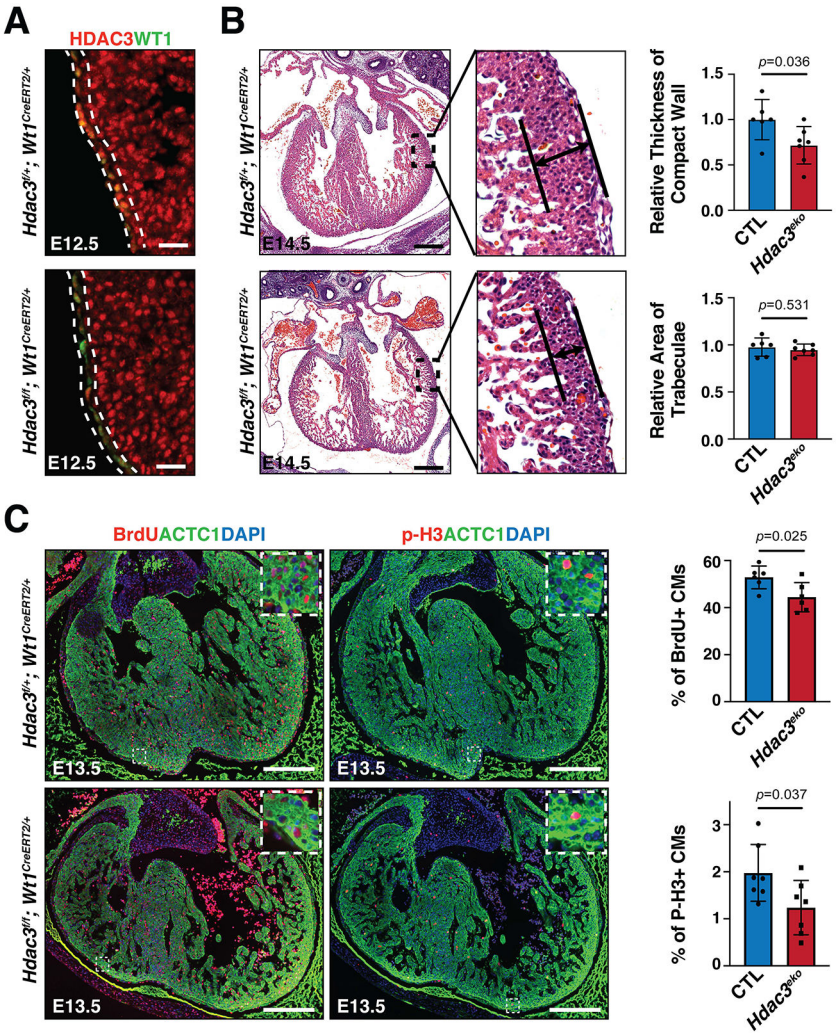

Figure 1. Epicardial deletion of Hdac3 resulted in hypoplasia of ventricular compact wall.

(A) Representative immunofluorescence staining of HDAC3 and WT1 in *Hdac3*^{eko} (*Hdac3*$^{f/f}$; *Wt1*$^{CreERT2/+}$) hearts and control (CTL; *Hdac3*$^{f/+}$; *Wt1*$^{CreERT2/+}$) hearts. Tamoxifen was given to dams intraperitoneally (150 mg/kg body weight) at E8.5. Scale bars: 25 μm. **(B)** Representative H&E staining of *Hdac3*^{eko} and CTL hearts. Scale bars: 250 μm. Quantification is shown on the right. CTL: n=6, *Hdac3*^{eko}: n=7. The relative thickness of compact wall and the relative area of trabeculae for each sample was derived by dividing its actual measured thickness or trabecular area by the mean measured thickness or area value of the CTL group. **(C)** Representative immunofluorescence staining of BrdU and p-H3 in *Hdac3*^{eko} hearts and CTL hearts. Scale bars: 250 μm. Quantification is shown on the right. For BrdU, CTL: n=6, *Hdac3*^{eko}: n=6; For P-H3, CTL: n=7, *Hdac3*^{eko}: n=7. *P*-values were determined by unpaired two tailed Student's *t*-test for **(B)** and **(C)**.

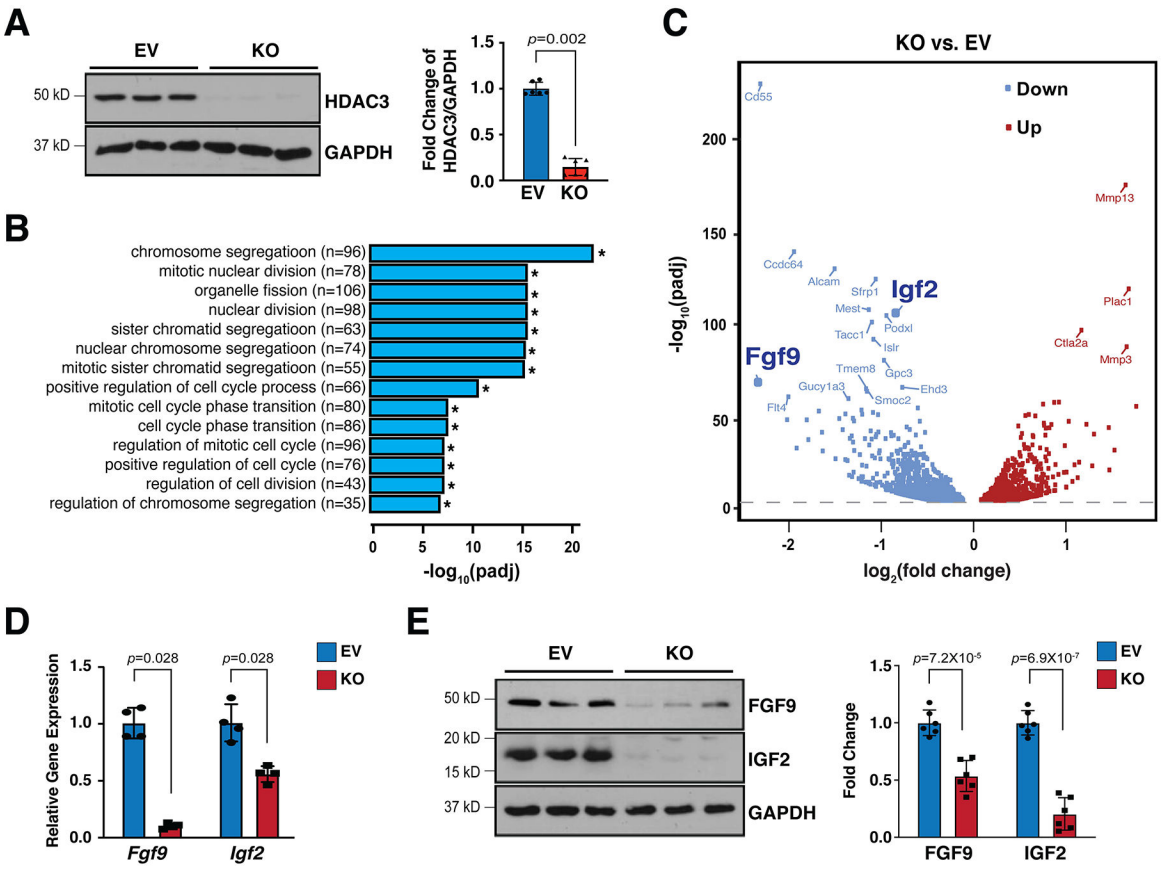

Figure 2. *Hdac3* deletion resulted in downregulation of FGF9 and IGF2.

(A) Generation of *Hdac3* knockout (KO) and empty vector control (EV) MECs by CRISPR/Cas9. Deletion of *Hdac3* was verified by western blot. n=4 in each group. **(B)** Gene ontology (GO) pathway analyses and **(C)** Volcano plot of RNA Sequencing in *Hdac3* KO and EV MECs. n=3 in each group. Log_2 fold changes were calculated by RPKM (Reads per kilo base of transcript per million mapped reads) per gene in the KO group divided by the mean RPKM per gene in the EV group then followed by calculation of Log (fold change, 2). Significantly downregulated genes are shown in light blue, and significantly up-regulated genes are shown in red. Cut-off criteria: adjusted *P*-value<0.01. **(D)** Quantification of *Fgf9* and *Igf2* in *Hdac3* KO MECs by qRT-PCR. *Gapdh* was used as cDNA loading control. n=4 in each group. **(E)** Quantification of FGF9 and IGF2 in *Hdac3* KO and EV MECs by western blot. GAPDH was used as protein loading control. n=6 in each group. *P*-values were determined by the Mann-Whitney U test for **(A), (D)** and unpaired two tailed Student's *t*-test for **(E)**.

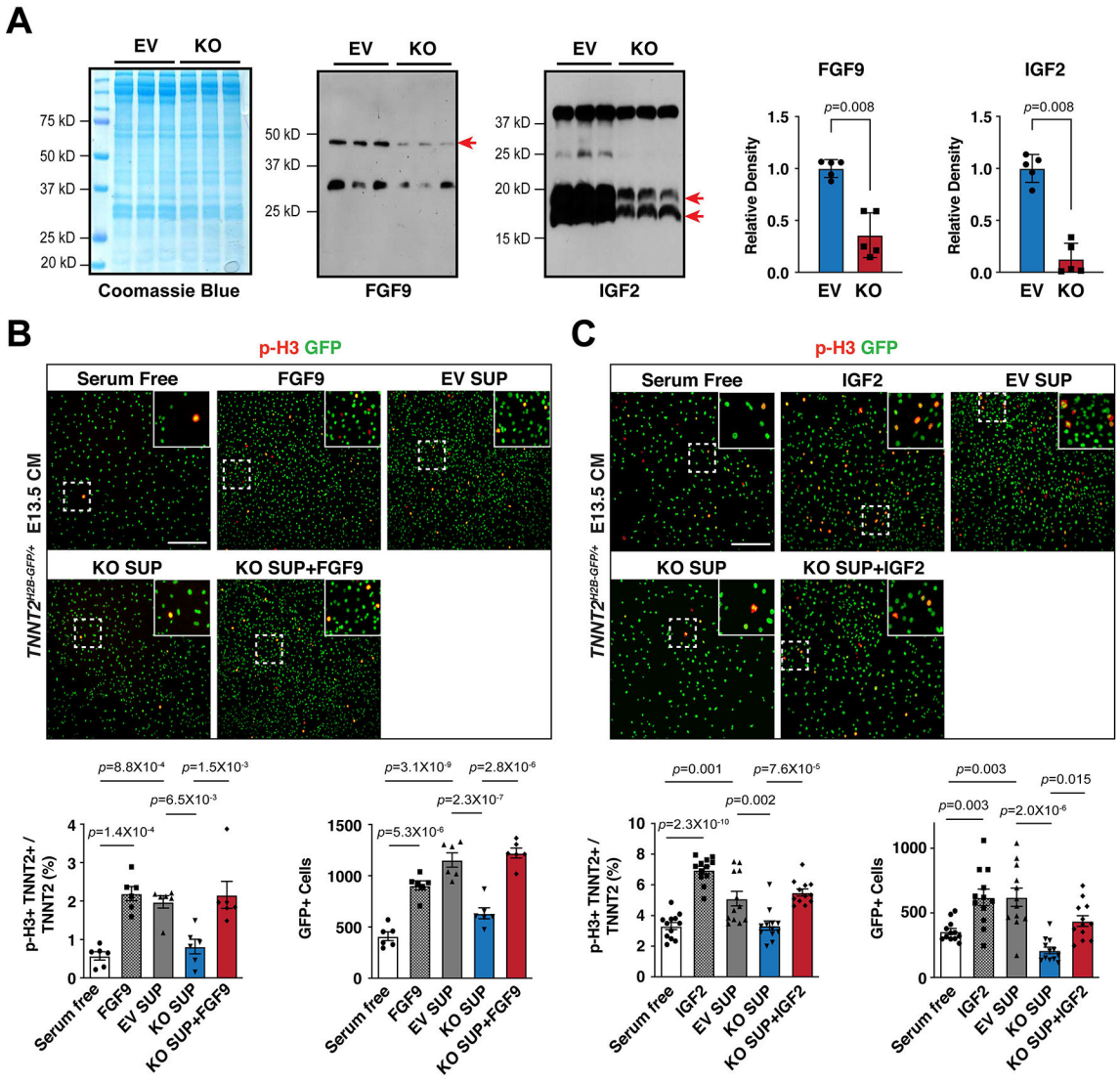

Figure 3. Supplementation of FGF9 or IGF2 rescues CM proliferation defects.

(A) Secretion of FGF9 and IGF2 from *Hdac3* KO and EV MECs. Coomassie brilliant blue staining of total extracted proteins from supernatants served as protein loading controls. FGF9 and IGF2 in the MEC supernatants were detected by western blot. Arrows point to the target bands. Quantifications are shown on the right. n=5 in each group. **(B and C)** The effects of MEC supernatants and/or recombinant FGF9 or IGF2 on E13.5 *Tnnt2*$^{nGFP/+}$ CM proliferation. Representative immunofluorescence micrographs are shown. Scale bars: 275 μm. Percentage of p-H3+ CMs and total number of CMs were quantitated. Independent samples: FGF9, n=6 in each group; IGF2, n=12 in each group. *P*-values were determined by the Mann-Whitney U test for **(A)** and One-way ANOVA followed by the Tukey post hoc test for **(B)** and **(C)**.

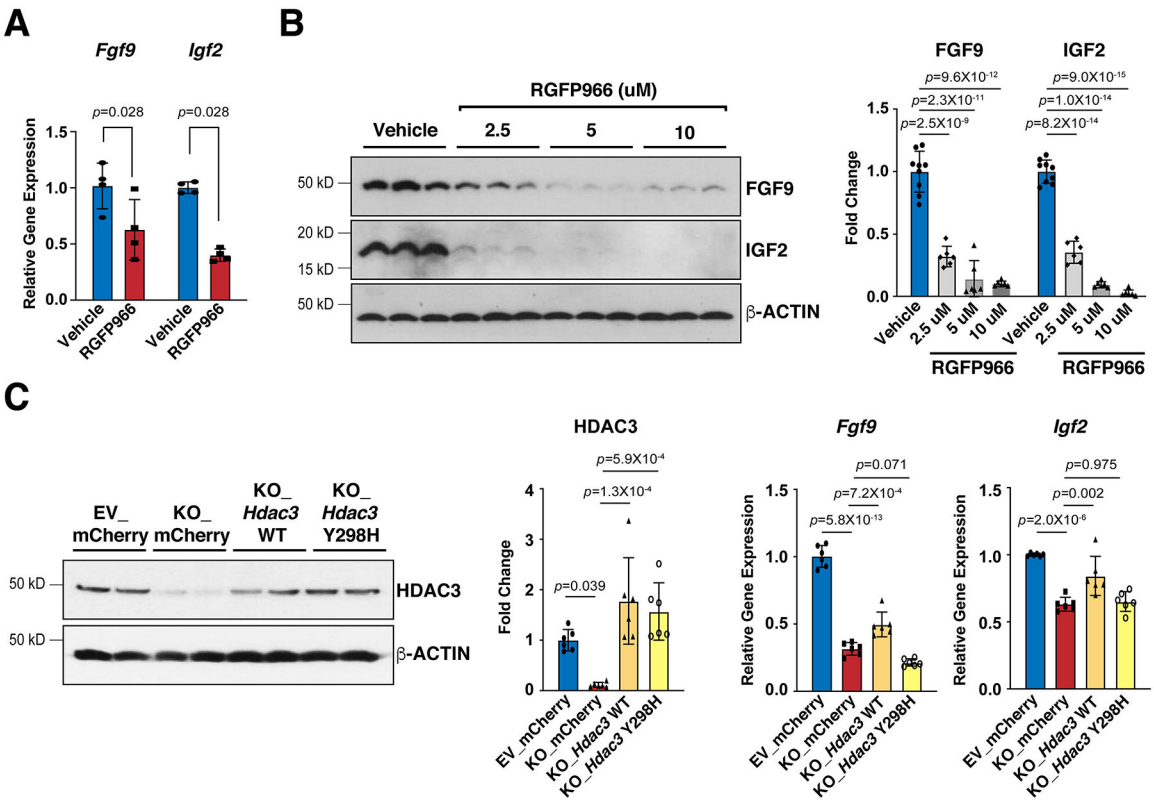

Figure 4. HDAC3 induces the expression of FGF9 and IGF2 dependent on its deacetylase activity.

(A) Decease of *Fgf9* and *Igf2* mRNAs after RGFP966 (a selective *Hdac3* inhibitor) treatment. MECs were treated with 10 uM RGFP966 or vehicle for 24 hours. mRNA levels were quantified by qRT-PCR. *Gapdh* was used as a cDNA loading control. *Fgf9*, n=4 in each group; *Igf2*, n=5 in each group. **(B)** Decease of FGF9 and IGF2 protein levels after RGFP966 treatment. MECs were treated with 2.5 uM (n=6), 5 uM (n=6) or 10 uM (n=6) RGFP966 or vehicle (n=9) for 24 hours. FGF9 and IGF2 were quantified by western blot. GAPDH was used as a protein loading control. **(C)** mRNA levels of *Fgf9* and *Igf2* in *Hdac3* KO and EV MECs after 24 hours treatment with *Hdac3* WT (wild type), Y298H mutant, or mCherry (CTL) lentivirus. The expression of HDAC3 was quantified by western blot. n=6 in each group. The mRNA levels of *Fgf9* and *Igf2* were quantified by qRT-PCR. n=6 in each group. *P*-values were determined by the Mann-Whitney U test for **(A)** and One-way ANOVA followed by the Tukey post hoc test for **(B)** and **(C)**.

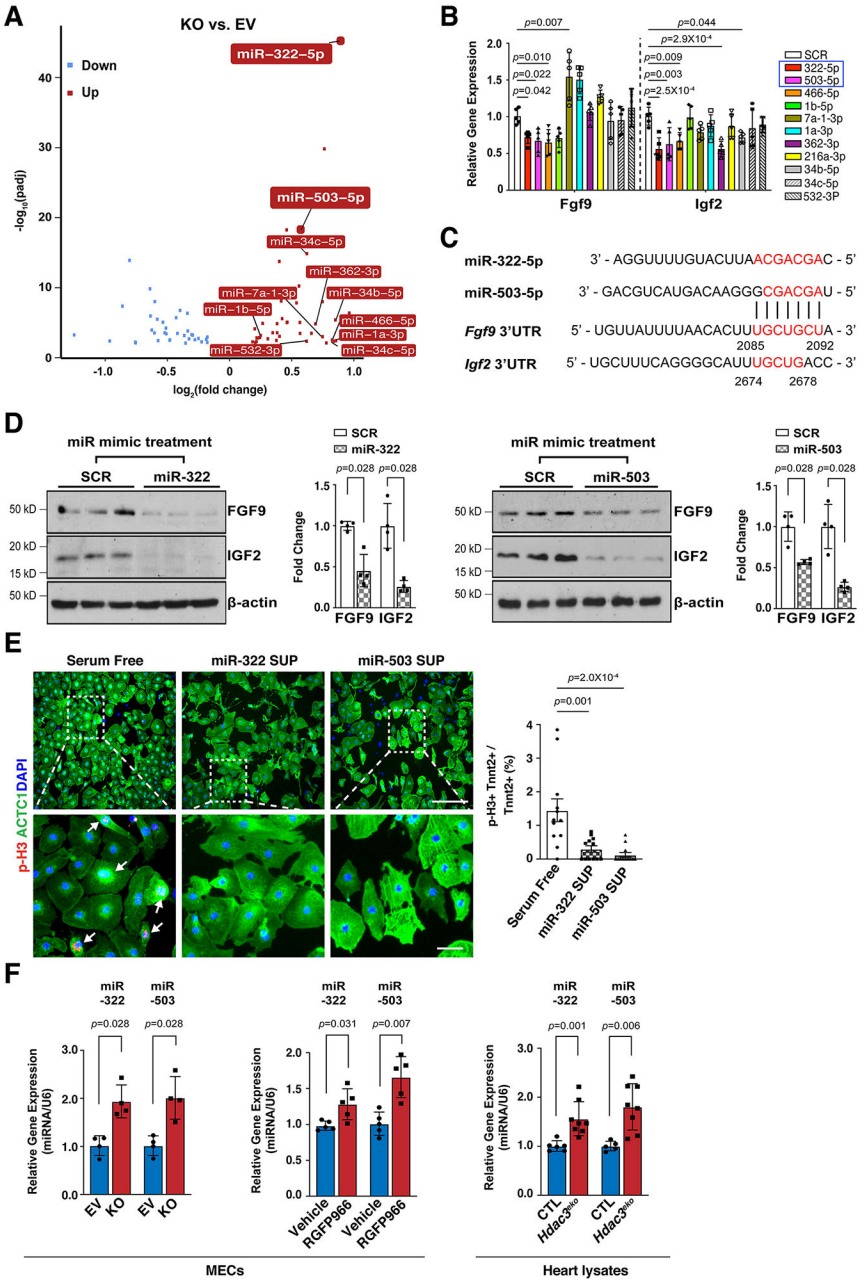

Figure 5. miR-322 and miR-503 repress the expression of FGF9 and IGF2 and CM proliferation.

(A) Volcano plot of miR sequencing of *Hdac3* KO and EV MECs. n=3 in each group. MiRNAs with reads less than 100 were discarded and miRNA expression levels were normalized by TPM (transcript per million) values (TPM = (miRNA total reads/total clean reads) × 10^6). Log_2 fold changes were calculated by TPM per miR in the KO group divided by the mean TPM per miR in the EV group then followed by calculation of Log (fold change, 2). Significantly downregulated miRs are shown in light blue, and significantly up-regulated miRs are shown in red. Cut-off criteria: adjusted *P*-value<0.01. **(B)** Quantification of *Fgf9* and *Igf2* expression in MECs after miR mimics treatment (final concentration: 10 nM) by qRT-PCR. *Gapdh* was used as a cDNA loading control. *Fgf9*,

n=7 in each group; *Igf2*, n=6 in each group. Fold changes were compared to the miR scrambles (SCR) group. **(C)** miR-322 and miR-503 share high similarity of their seed binding motifs to 3'UTRs of *Fgf9* and *Igf2*. Binding motifs or complementary bases are in red. **(D)** Quantification of the expression of FGF9 and IGF2 after miR-322 or miR-503 mimics treatment by western blot. n=4 in each group. **(E)** The effects of supernatants from miR-322 and miR-503 mimics treated MECs on E13.5 CM proliferation. Representative immunofluorescence micrographs are presented. Scale bars: 275 um (upper images); 50 um (lower images). Percentage of p-H3+ CMs were quantified. Independent samples: n=12 in each group. **(F)** Quantification of the expression of miR-322 and miR-503 in *Hdac3* KO MECs, RGFP966-treated MECs (10 uM), or E13.5 $Hdac3^{eko}$ hearts by qRT-PCR. U6 snRNA was used for normalization. miR-322 or miR-503 expression in EV or KO MECs: n=4 in each group; miR-322 expression after vehicle or RGFP966 treatment: n=5 in each group; miR-503 expression after vehicle or RGFP966 treatment: n=4 in each group; miR-322 expression in E13.5 hearts: CTL (n=6), $Hdac3^{eko}$ (n=8); miR-503 expression in E13.5 hearts: CTL (n=5), $Hdac3^{eko}$ (n=8). *P*-values were determined by One-way ANOVA followed by the Dunnett post hoc test for **(B)**, One-way ANOVA followed by the Tukey post hoc test for **(E)**, and the Mann-Whitney U test for **(D)** and **(F)**.

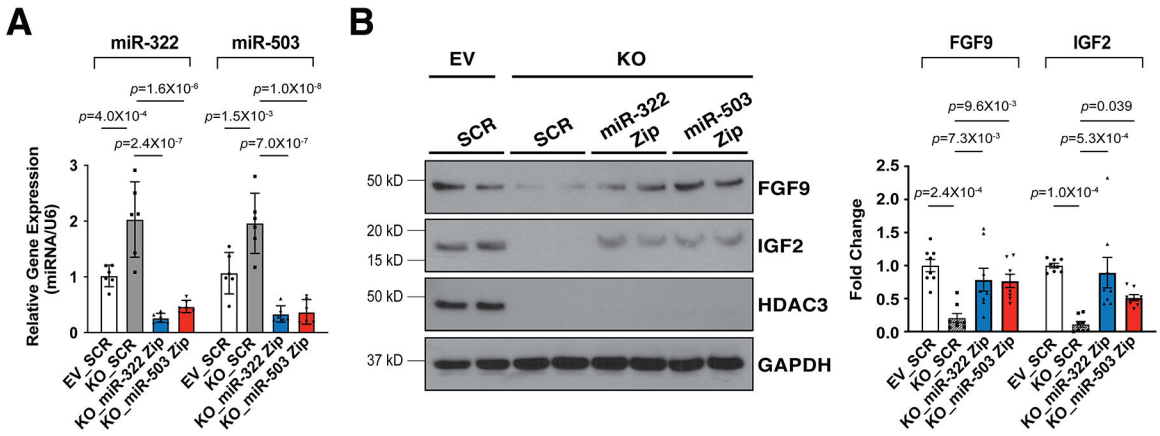

Figure 6. Knockdown of miR-322 or miR-503 restores the expression of FGF9 and IGF2.

(A) Quantification of miR-322 and miR-503 after *Hdac3* KO or EV MECs were infected with LentimiRa-GFP-miRZip (miR-322 or miR-503) or pGreenPuro Scramble Hairpin control lentivirus (SCR) respectively. miR levels were quantified by qRT-PCR. n=6 in each group. **(B)** Quantification of the expression of FGF9 and IGF2 after miRZip lentiviral treatment by western blot. n=8 in each group. *P*-values were determined by One-way ANOVA followed by the Tukey post hoc test for **(A)** and **(B)**.

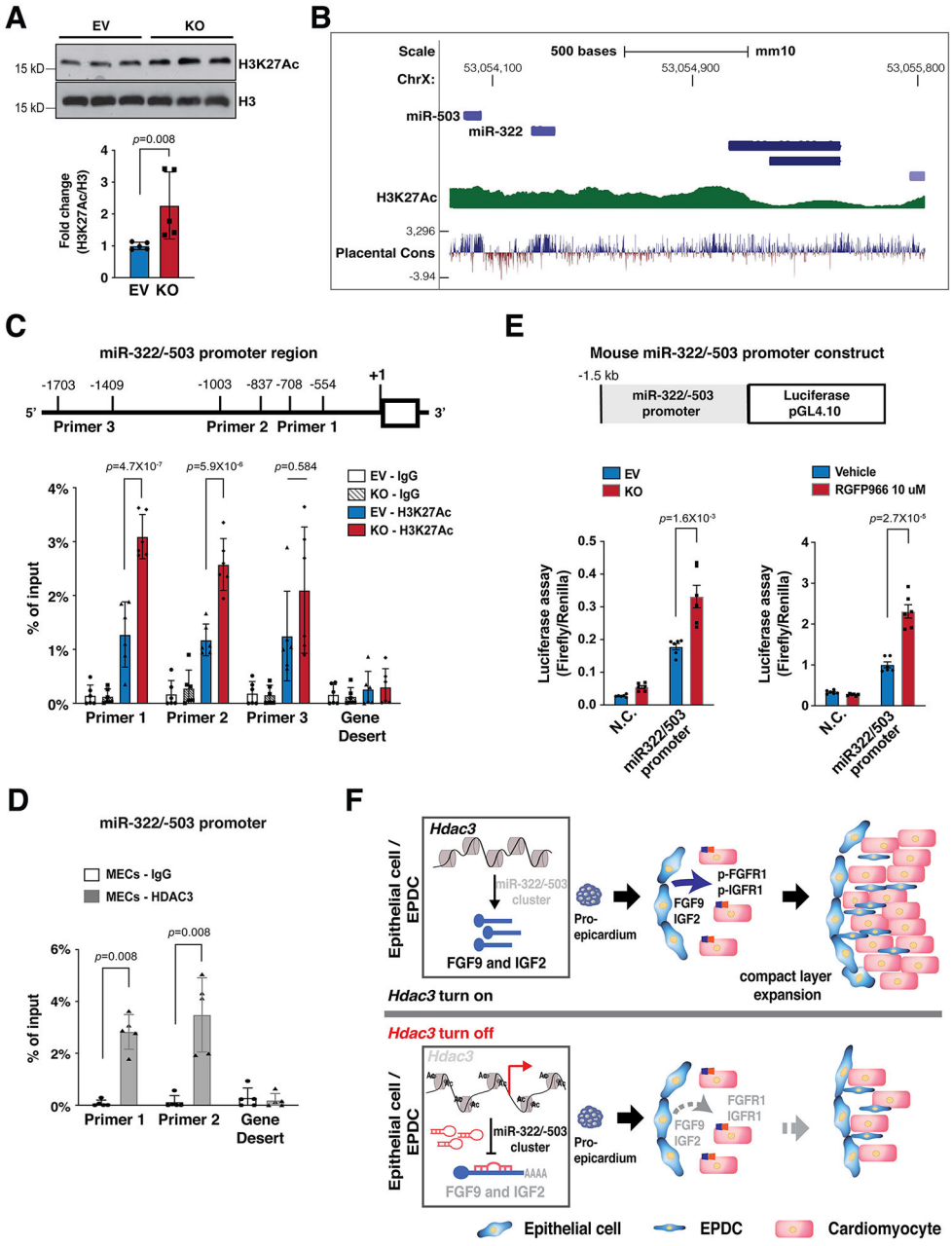

Figure 7. HDAC3 represses miR-322/miR-503 promoter activity.

(A) Quantification of H3K27Ac in *Hdac3* EV and KO MECs by western blot. n=5 in each group. **(B)** Schematic diagram of the miR-322/miR-503 locus from the UCSC Genome Browser. In the upstream regulatory regions as well as gene bodies, active epigenetic marker H3K27Ac was identified by the ENCODE project. **(C)** Quantification of H3K27Ac binding affinity in the miR-322/miR-503 promoter region in *Hdac3* KO and EV MECs by ChIP-qPCR. Primers targeting a gene desert region were used as a negative control. n=6 in each group. **(D)** Quantification of binding of HDAC3 to the miR-322/miR-503 promoter region by ChIP-qPCR in MECs. n=5 in each group. (E) Dual luciferase reporter assays on the −1.5 kb miR-322/miR-503 promoter when *Hdac3* is either knocked out or inhibited

by RGFP966 (10 uM) treatment. The ratio of firefly:Renilla luciferase light units (RLU) was determined 48 hours after transfection. n=6 in each group. **(F)** The schematics of the working model. In the developing epicardium, HDAC3 represses the expression of miR-322/miR-503 to release their suppression on the expression of FGF9 and IGF2. When *Hdac3* is deleted, the expression of miR-322/miR-503 is increased, which subsequently suppresses the expression of FGF9 and IGF2 to a stronger extent, and the decrease of FGF9 and IGF2 leads to ventricular wall hypoplasia. *P*-values were determined by the Mann-Whitney U test for **(A)** and **(D),** the one-way ANOVA followed by the Tukey post hoc test for **(C)** and unpaired two tailed Student's *t*-test for **(E)**.