**SUPPLEMENTAL MATERIAL**

**DETAILED METHODS**

**Cardiac Fibroblasts Isolation and Transfection**

Cardiac fibroblasts were isolated from 1-day old C57BL/6 mouse neonates and isolated as described[1]. Briefly, harvested hearts were minced and digested for 30 minutes at 37°C using 100 U/mL of Collagenase II in HBSS. The cell mixture was filtered using a 100 µm cell strainer and centrifuged at 400g for 5 min. To isolate the cardiac fibroblasts and eliminate any cardiomyocytes, the cell pellet was resuspended in growth media (DMEM, 15% FBS, Pen/Strep) and subjected to a Percoll gradient centrifugation. Isolated fibroblasts were plated on 0.2% gelatin-coated 25cm$^2$ flasks and cultured in growth media at 37°C with 5% $CO_2$. Cells were passaged twice before being used for transfection.

One day prior to transfection, 24-wells plates were seeded with cardiac fibroblasts at a density of 5500 cells per cm$^2$. Transient transfections were performed using Dharmafect I reagent (Dharmacon) in antibiotics-free media according to manufacturer's instructions. Synthetic mimic of pre-miRs (Ambion/Applied Biosystems) and siRNAs (FlexiTube siRNA, Qiagen) were used at a final concentration of 50 nmol/L. A list of the siRNAs used is provided in the table below. After 24 hours, transfection media was replaced with growth media and cells were cultured for 3 or 14 days. For the DZNep treatment, growth media containing 2 µmol/L of DZNep (EMD Millipore) was added 24 hours after transfection, changed daily until day 14.

| **microRNAs Information** | | |
|---|---|---|
| **miR Name** | **Full name and mature miR sequence** | **ID** |
| Negative Control (Ambion) | | Ctrl_Control_1 |
| miR-1 (Ambion) | hsa-miR-1 UGGAAUGUAAAGAAGUAUGUA | AM17100 / PM10617 |
| miR-133a (Ambion) | hsa-miR-133a-3p UUUGGUCCCCUUCAACCAGCUG | AM17100 / PM10413 |
| miR-208a (Ambion) | hsa-miR-208a-3p AUAAGACGAGCAAAAAGCUUGU | AM17100/PM10677 |
| miR-499 (Ambion) | hsa-miR-499a-5p UUAAGACUUGCAGUGAUGUUU | AM17100/PM11352 |
| Let-7c (Ambion) | hsa-let-7c-3p CUGUACAACCUUCUAGCUUUCC | AM17100/PM12580 |
| **siRNAs Information** | | |
| **Gene Name** | **siRNA Target Sequence** | **ID** |
| Negative Control (Qiagen) | AATTCTCCGAACGTGTCACGT | Ctrl_Control_1 |
| Eed (Qiagen) | ATGGAGGATGATATAGATAAA<br>AGGCATTATAAGAATAATTAA<br>AAGCAACAGAGTAACCTTATA<br>CAGGCCATTTATTTCCCAGAA | Mm_Eed_1<br>Mm_Eed_2<br>Mm_Eed_3<br>Mm_Eed_4 |
| Ezh1 (Qiagen) | CTCTTATCTAATAAAGTGTTA<br>CTGGTCATGAGGAATGCTTTA<br>CAGGGATACCCTCCAGCCTAA<br>CGGAAGCGCCATGCTATCGAA | Mm_Ezh1_4<br>Mm_LOC100044129_1<br>Mm_LOC100044129_2<br>Mm_LOC100044129_3 |
| Ezh2 (Qiagen) | CAGGATGGCACTTTCATTGAA<br>CTGCAGAAAGATACAACTGAA<br>TTGGTCGCCCTTACAACAGAA | Mm_Ezh2_3<br>Mm_Ezh2_4<br>Mm_Ezh2_5 |

| | | |
|---|---|---|
| | AAAGATCTAGAGGATAATCGA | Mm_Ezh2_6 |
| Kdm6A (Dharmacon) | AGTTAGCAGTGGAACGTTA<br>GGACTTGCAGCACGAATTA<br>GGTACGGCCTACTGGAATT<br>CCACGTTGGTCATACTATA | J-042844-09<br>J-042844-10<br>J-042844-11<br>J-042844-12 |
| Kdm6B (Qiagen) | CTCACTTAATTTATTAAGAAA<br>CAGCATCTATTTGGAGAGCAA<br>TTGTGTGAGAATATTATTAAA<br>CACCGTGCAGCTATACATGAA | Mm_Jmjd3_1<br>Mm_Jmjd3_2<br>Mm_Jmjd3_3<br>Mm_Jmjd3_4 |

**RNA isolation and Quantitative Real-Time PCR**

Total RNAs were isolated using an RNeasy miniprep kit (Qiagen) and following manufacturer's instructions. RNA concentrations and purity were quantified by Nanodrop (Applied Biosystems). Reverse Transcription was carried out using a High-Capacity cDNA Synthesis Kit (Thermo Fisher) according to manufacturer's instructions. Quantitative expression of each gene was assessed using Taqman Gene Expression Assays on a StepOnePlus Real-Time PCR System (Applied Biosystems) with a minimum of 40 ng of cDNA per reaction. A list of the primers used in this study is provided in the table below.

| **Taqman Primers Information** | | |
|---|---|---|
| **Gene Name** | **Gene Category/Function** | **Taqman Primer ID** |
| Gapdh | Housekeeping Gene | Mm99999915_m1 |
| Gata4 | Cardiac Transcription Factor | Mm00484689_m1 |
| Hand2 | Cardiac Transcription Factor | Mm00439247_m1 |
| Mef2C | Cardiac Transcription Factor | Mm01340482_m1 |
| Tbx5 | Cardiac Transcription Factor | Mm00803518_m1 |
| Actn2 | Mature Cardiomyocyte Marker | Mm00473657_m1 |
| Cacna1c | Mature Cardiomyocyte Marker | Mm00437917_m1 |
| Kcnj2 | Mature Cardiomyocyte Marker | Mm00434616_m1 |
| Myh6 | Mature Cardiomyocyte Marker | Mm00440359_m1 |
| Scn5a | Mature Cardiomyocyte Marker | Mm01342518_m1 |
| Tnni3 | Mature Cardiomyocyte Marker | Mm00437164_m1 |
| Ezh1 | H3K27 methyltransferase | Mm00468440_m1 |
| Ezh2 | H3K27 methyltransferase | Mm00468464_m1 |
| Eed | PRC2 Member | Mm00469659_m1 |
| Kdm6A | H3K27 Demethylase | Mm01283053_m1 |
| Kdm6B | H3K27 Demethylase | Mm01332668_g1 |

**Immunoblotting**

Histone extracts were isolated from harvested cells using the EpiQuick Total Histone Extraction Kit (Epigentek) according to the manufacturer's instructions. Nuclear extracts were isolated from harvested cells by standard nuclear extraction protocol (Abcam). Protein concentrations were determined by the Lowry protein assay and 2 µg of histone extract or 5 µg of nuclear extract were loaded per well. Samples were run on 4-12% Bis-Tris gels (NuPAGE, Thermo-Fisher) in MES-SDS running buffer (NuPAGE, Thermo-Fisher) for histone extracts, or in MOPS-SDS (NuPage, Thermo-Fisher) for nuclear extracts. After 90min of transfer at 30mV, nitrocellulose membranes were stained overnight at 4ºC with anti-H3K27me3, anti-H3K4me3, anti-H3K9me3, anti-H4K20me3 anti-H3, anti-Mef2c, anti-Tbx5 and anti-TBPdiluted in blocking buffer (TBS, 0.1% Tween, 5% BSA). A list of the antibodies used and their working dilution is provided in the table below. Protein detection was performed

using HRP-coupled antibodies (Cell Signaling) and the ECL Prime detection reagent (GE Healthcare). Membranes were imaged and quantified using the G:BOX Chemi XT4 and associated software (Syngene).

**Fluorescence-activated cell sorting**

Neonatal cardiac fibroblasts were washed with PBS and allowed to detach by incubation with 0.05% trypsin for 5 minutes at 37ºC. Cells were pelleted at 800g for 5 min at 4ºC and fixed with 4% paraformaldehyde for 15 minutes at 4ºC. After fixation, FACS buffer (1xPBS, 5% BSA, 0.1% Tween-20) was added and cells were pelleted by centrifugation at 800g for 5 minutes at 4ºC. Cells were blocked for 1 hour in FACS buffer at 4ºC with constant rotation. Cells were then incubated with FACS buffer containing 1/20 dilution of anti-Myh6-APC (Miltenyi Biotech) and anti-Tnnt2-PE (BD Biosciences); or 1/100 dilution of anti-Gata4 or anti-Mef2cfor 1hour at 4 ºC with constant rotation. For Gata4 and Mef2c, we incubated cells for 1hour at 4 ºC with the secondary antibody Alexa 488 anti-rabbit diluted 1/100 in FACS buffer. After two washes in FACS buffer, cells were re-suspended in 250µl of FACS buffer. FACS was performed using a BD Biosciences FACSCanto. Single stained samples and IgG controls were used for compensation. FloJo version 10 was used for compensation and data analysis.

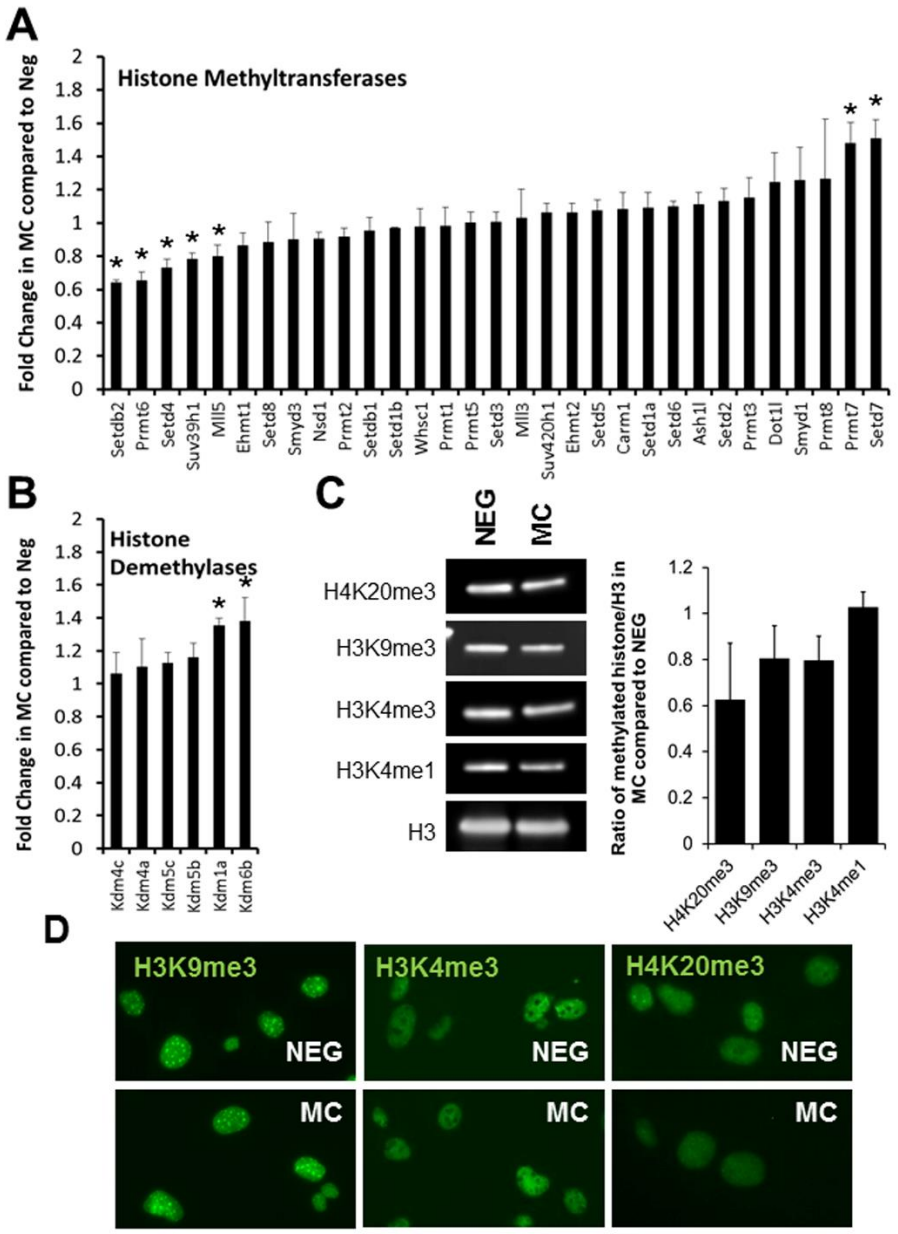
**Immunocytochemistry**

Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 10 minutes at room temperature and washed 3 times in PBS-Tween (PBS 1X, 0.1% Tween). After blocking in PBS-Tween containing 5% BSA (Sigma), cells were stained using primary antibodies for H3K27me3, H3K9me3, H3K4me3, H4K20me3, Tnni3 (cTnI) and Myh6 (α MHC) overnight at 4°C using the dilution indicated in the table below. Cells were then incubated for 2 hours at room temperature with secondary anti-mouse or anti-rabbit antibodies

| **Antibody Information** | | |
|---|---|---|
| **Name** | **Application/Dilution** | **Company/Catalog Number** |
| H3 | Immunoblot / 1:5000 | Active Motif / 61475 |
| H3K27me3 | Immunocytochemistry / 1:500<br>Immunoblot / 1:250 | Active Motif / 39155<br>Cell Signaling / 9733 |
| H3K4me3 | Immunocytochemistry / 1:500<br>Immunoblot / 1:500 | Active Motif / 39155<br>Active Motif / 39155 |
| H3K9me3 | Immunocytochemistry / 1:500<br>Immunoblot / 1:1000 | Abcam / ab8898<br>Cell Signaling / 5327 |
| H4K20me3 | Immunocytochemistry / 1:500<br>Immunoblot / 1:1000 | Active Motif / 39671<br>Abcam / ab9053 |
| Mef2c | Immunoblot/ 1:400 | Cell Signaling / 5030 |
| Tbx5 | Immunoblot / 1:400 | R&D / AF5918 |
| Anti-mouse-HRP | Immunoblot / 1:10000 | Cell Signaling / 7076 |
| Anti-rabbit-HRP | Immunoblot / 1:2000 | Cell Signaling / 7074 |
| Anti-sheep-HRP | Immunoblot / 1:1000 | R&D / HAF016 |
| Tnni3 | Immunocytochemistry / 1:200 | Abcam / ab47003 |
| Myh6 | Immunocytochemistry / 1:200 | Abcam / ab207926 |
| Tnnt2-PE | FACS / 1:20 | BD Biosciences / 564767 |
| Myh6-APC | FACS / 1:20 | Miltenyi Biotech / 130-106-215 |
| Gata4 | FACS / 1:100 | Cell Signaling / 36966 |
| Mef2c | FACS / 1:100 | Cell Signaling / 5030 |
| Anti-rabbit-Alexa 488 | FACS / 1:100 | Invitrogen / A21026 |

**SUPPLEMENTAL FIGURES AND FIGURE LEGENDS**

**Online Figure I**

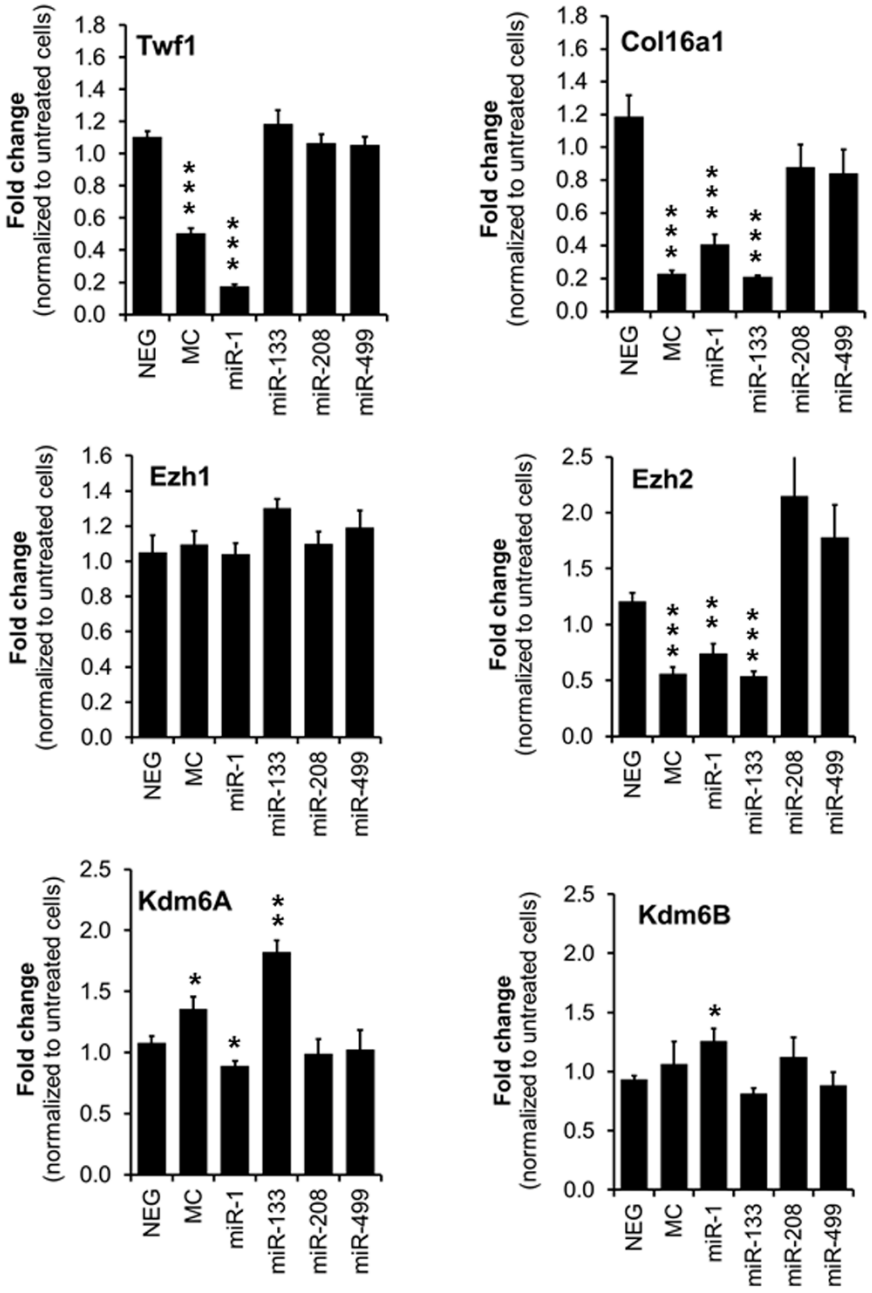



**Online Figure I: Role of Histone Methylation in direct cardiac reprogramming by miR combo.**

(**A** and **B**) Selected results of the qPCR array (RT² Profiler™ PCR Array Mouse Epigenetic Chromatin Modification Enzymes) performed on mRNAs isolated from neonatal cardiac fibroblasts transfected with negmiR (NEG) or miR combo (MC) and cultured for 3 days. Expression levels of various histone methyltransferases (A) and demethylases (B) in MC-treated neonatal cardiac fibroblasts is presented as a fold change compared to NEG control cells. (**C**) Immunoblot analysis of various histone methylations in histone extracts isolated from NEG and MC-treated neonatal cardiac fibroblasts 3 days after transfection (H3K4me1: N=6; H3K4me3: N=6; H3K9me3: N=5; H4K20me3: N=4). (**D**) Immunocytochemistry for H3K9me3, H3K4me3 and H4K20me3 in corresponding transfected neonatal cardiac fibroblasts. Cells were co-labeled with the nuclear stain DAPI (N=2).

Statistical difference was determined by standardized T-test between NEG- and MC-transfected cells (*P≤0.05).

## Online Figure II

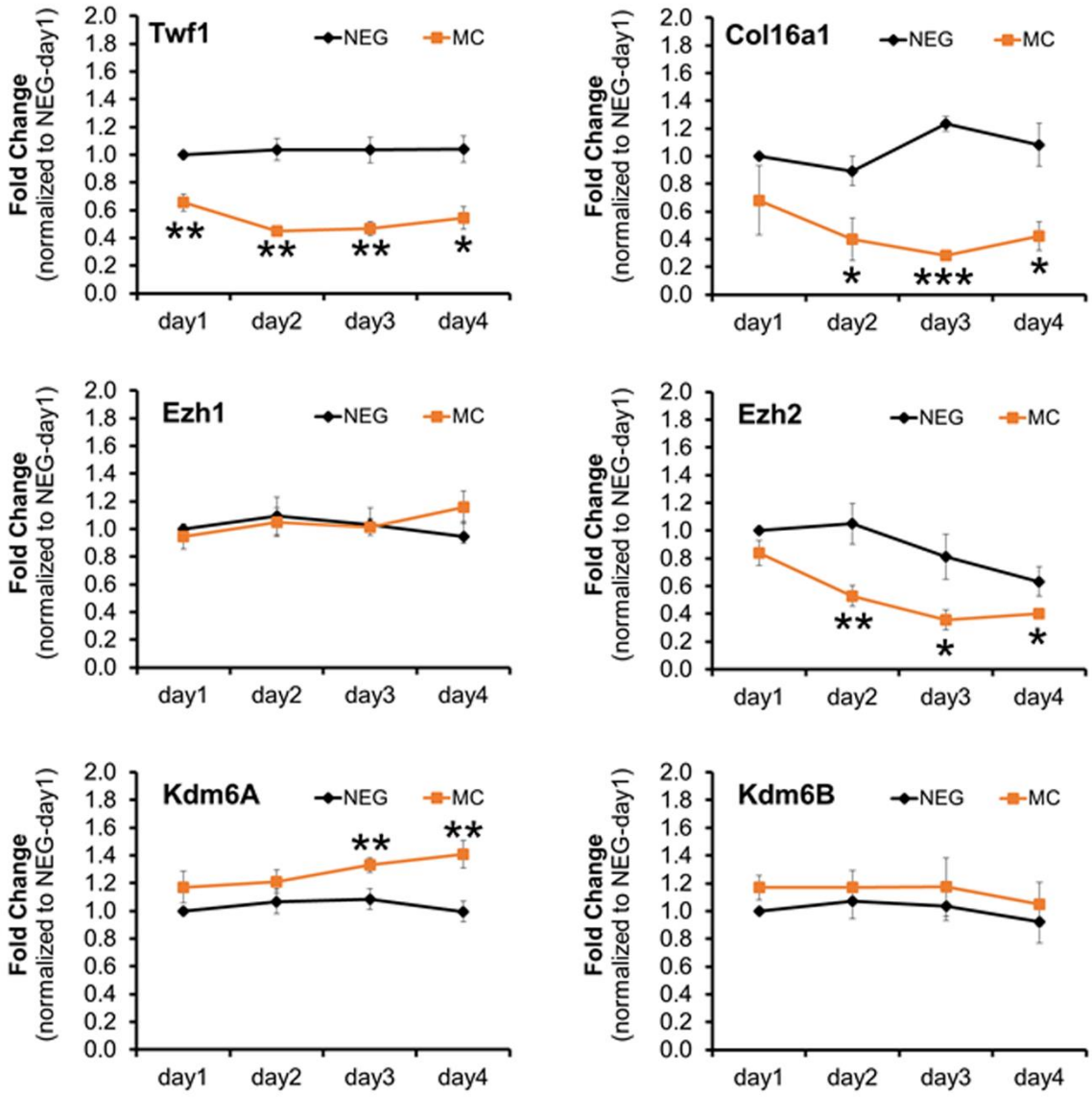



**Online Figure II: Regulation of the expression of H3K27 modifiers by individual microRNA**

Analysis of Twf1 (miR-1 target), Col16a1 (miR-1/133 target), Ezh1, Ezh2, Kdm6A and Kdm6B mRNA levels in neonatal cardiac fibroblasts transfected with NEG, MC or each individual microRNA (miR-1, miR-133, miR-208, miR-499). Expression values were determined by qPCR, 3 days after transfection, and normalized to NEG controls (N=3-6).

Asterisks indicate statistical significance between NEG and MC-treated cells determined by standardized T-test (*P ≤0.05, **P ≤0.005 or ***P≤0.0005).

## Online Figure III

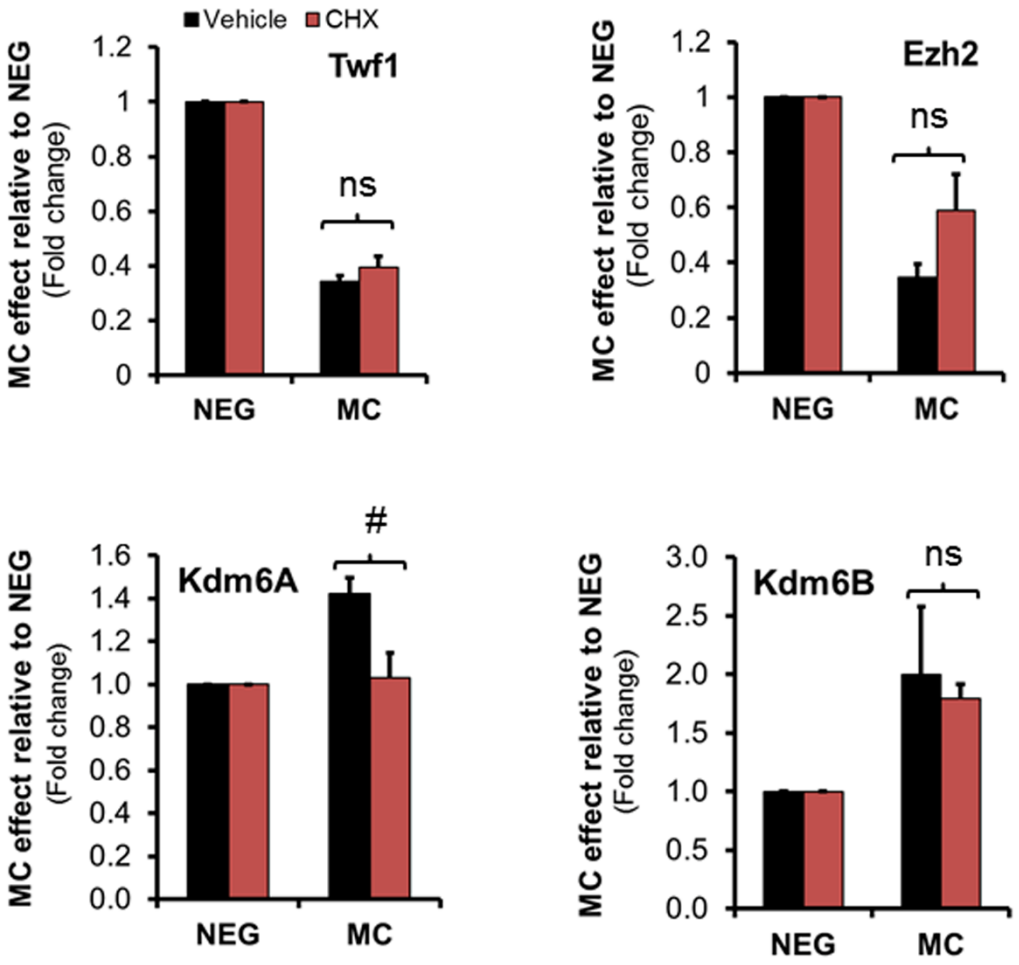



**Online Figure III: Time-course analysis of the expression of H3K27 modifiers.**

Analysis of the expression of Twf1 (miR-1 target), Col16a1 (miR-1/133 target), Ezh1, Ezh2, Kdm6A and Kdm6B in neonatal cardiac fibroblasts transfected with NEG, MC over the course of 4 days. RNA were isolated at 1, 2, 3 and 4 days after transfection and analyzed by qPCR. Expression values were normalized to NEG controls at day1 (N=3-6).

Asterisks indicate statistical significance between NEG and MC-treated cells determined by standardized T-test (*P ≤0.05, **P ≤0.01 or ***P≤0.005).

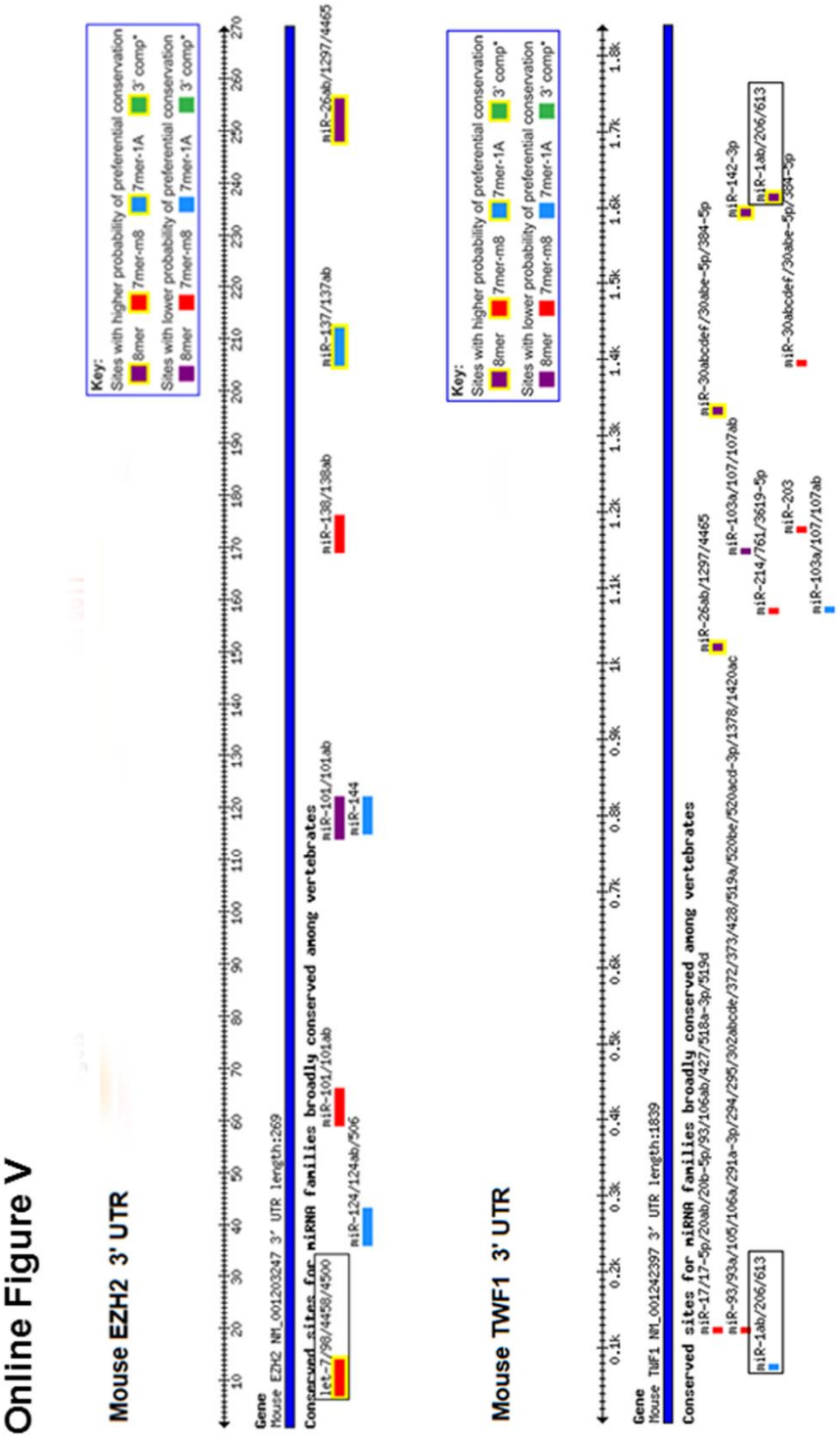
## Online Figure IV




**Online Figure IV: The regulation of the expression of H3K27 modifiers by miR combo is unaffected by CHX treatment.**

Analysis of the expression of Twf1 (miR-1 target), Ezh2, Kdm6A and Kdm6B by qPCR in neonatal cardiac fibroblasts transfected with negmiR (NEG), miR combo (MC) and treated for 12 hours with vehicle or 50µg/ml of cycloheximide (CHX). For each treatment (vehicle or CHX) the expression values for MC were normalized to their respective NEG control (N=4-6).

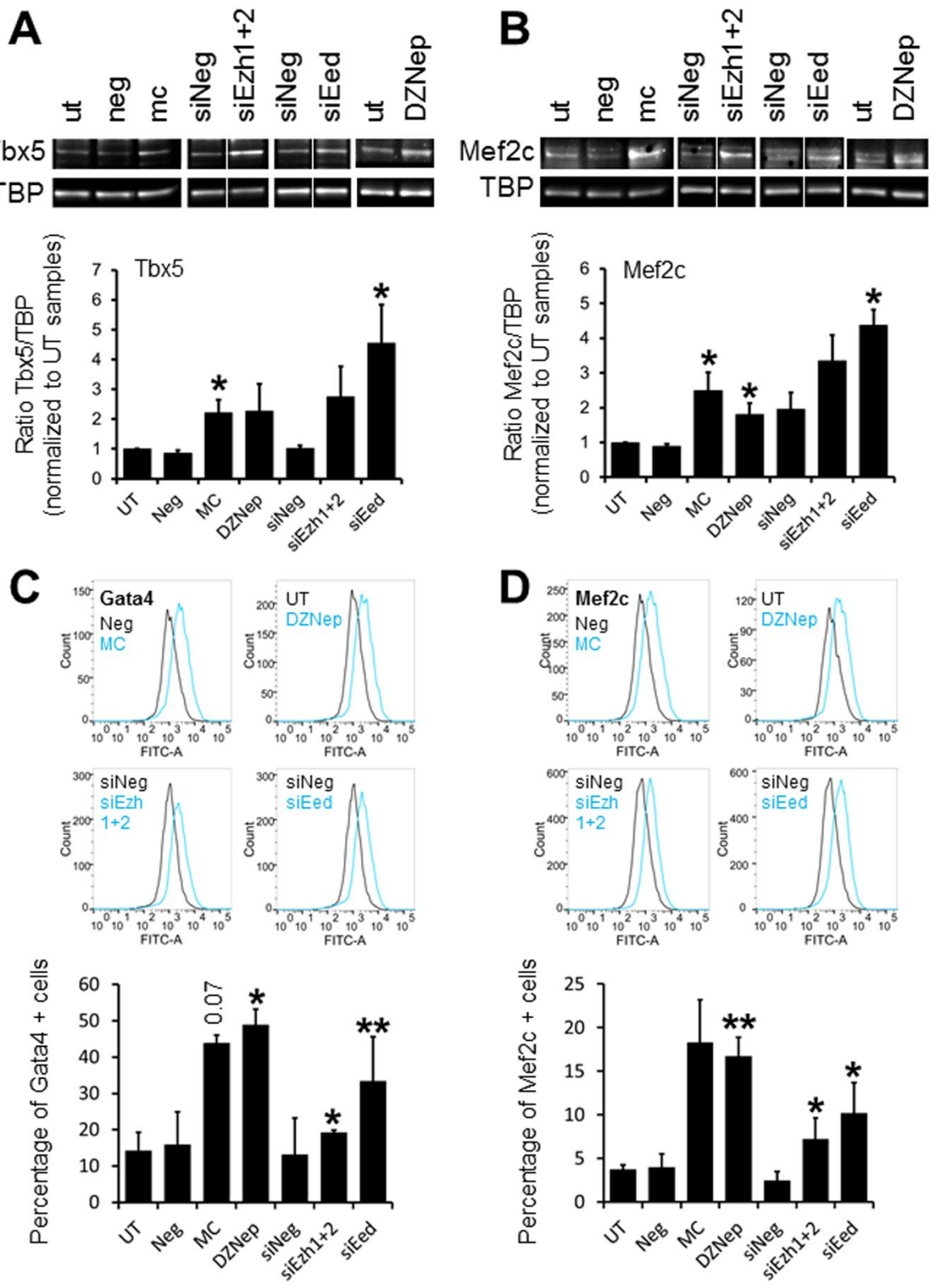
T-test comparing the MC effect relative to NEG between vehicle and CHX treated groups (#: $p \leq 0.05$; ns: not significant)




**Online Figure V: Putative microRNA binding sites in mouse Ezh2 3'UTR and Twf1 3'UTR**

Schematic representation of the 3'UTR sequences for Ezh2 (top) and Twf1 (bottom) indicating the location of conserved binding sites for microRNA families broadly conserved among vertebrates as determined by TargetScan version 6.2.

## Online Figure VI

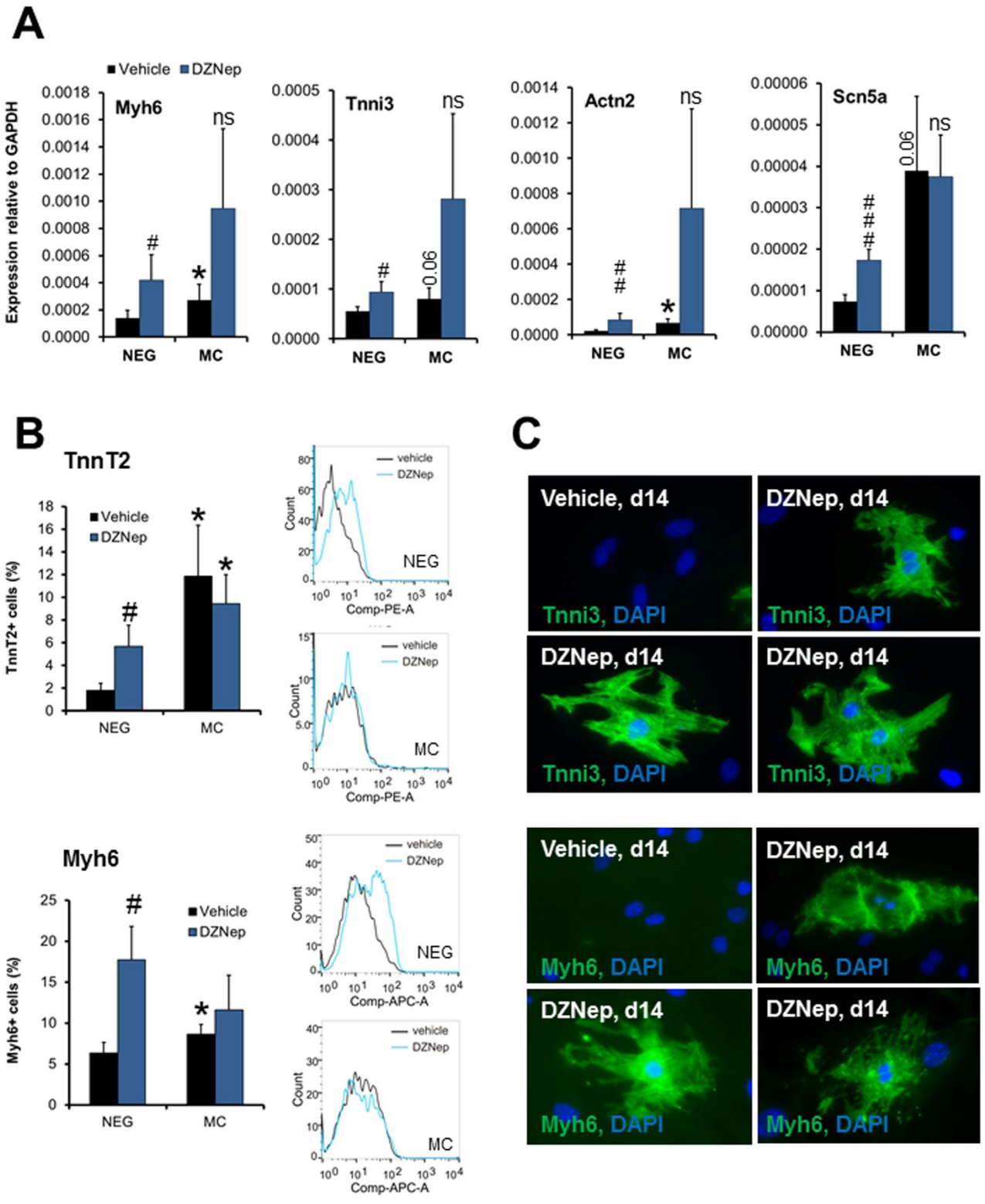


**Online Figure VI: Inhibition of H3K27 methyltransferase activity increases cardiogenic factors at the protein level.**

(**A-B**) Analysis of Tbx5 (A) and Mef2c (B) protein level by immunoblot in nuclear extracts isolated from neonatal cardiac fibroblasts transfected with negmiR (NEG) or miR combo (MC), negative siRNA (siNeg), Ezh1+Ezh2 siRNAs (siEzh1+2), Eed siRNA (siEed) or treated with 2 μM of DZNep for 3 days (N=4-5 for MC, N=3-6 for DZNep, N=3-6 for si-Ezh1+2 and N=3-5 for si-Eed). (**C-D**) FACS analysis of Gata4+ (C) and Mef2c+ (D) cells present in neonatal cardiac fibroblasts transfected with negmiR (NEG) or miR combo (MC), negative siRNA (siNeg), Ezh1+Ezh2 siRNAs (siEzh1+2), Eed siRNA (siEed) or treated with 2 μM of DZNep for 7 days (N=2 for MC, N=4 for DZNep and N=3 for si-Ezh1+2 and si-Eed). Bar graphs present the average percentage of Gata4+ and Mef2c+ for each condition. Representative FACS traces are presented on top of each panel.

Statistical differences between MC/NEG, DZNep/UT, siEzh1+2/siNeg and siEed/siNeg were determined by standardized T-test (*$P \leq 0.05$, **$P \leq 0.01$).

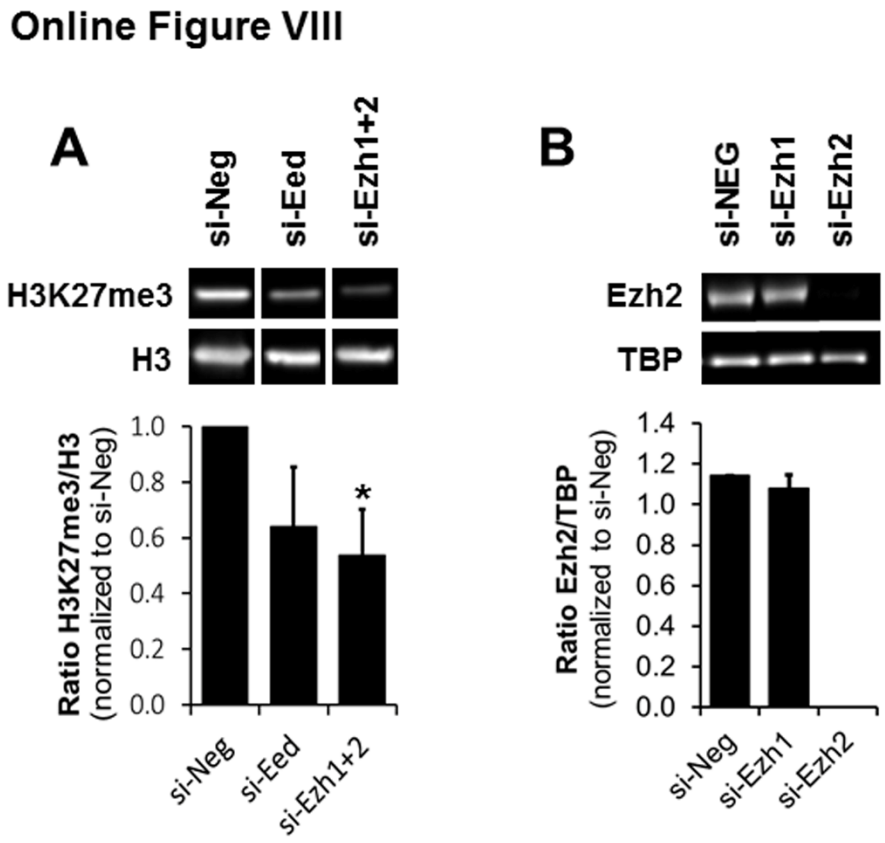
# Online Figure VII



**Online Figure VII: DZNep treatment increases the number of cardiomyocyte-like cells.**

(**A**) mRNA levels of various cardiomyocyte markers was determined by qPCR in neonatal cardiac fibroblasts transfected with negmiR (NEG) or miR combo (MC) and cultured for 14 days with 2 μM of DZNep or vehicle (N=6-7). (B) FACS analysis showing the percentage of Tnnt2+ (top) and Myh6+ (bottom) cells present in NEG and MC-transfected fibroblasts treated with vehicle or 2μM of DZNep and cultured for 14 days (N=4 for Tnnt2; N=3 for Myh6). Representative FACS traces are presented on the right. (**C**) Immunocytochemistry for Tnni3 and Myh6 in neonatal cardiac fibroblasts after 2 weeks of treatment with 2μM of DZNep or vehicle (N=2).

Statistical difference was determined by standardized T-test between DZNep and vehicle treated cells (# P≤0.05, ## P≤0.01, ###P≤0.0005) or between NEG- and MC-transfected cells (*P≤0.05).

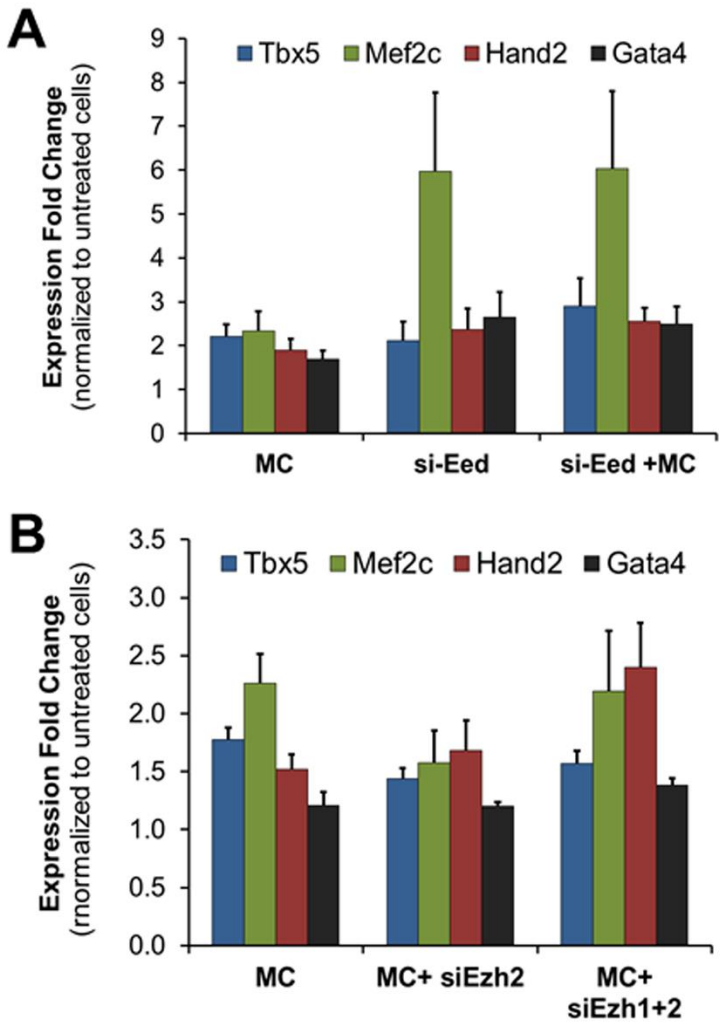



**Online Figure VIII: Analysis of H3K27me3 and Ezh2 levels in siRNA transfected fibroblasts.**

(**A**) Immunoblot analysis of H3K27me3 levels in histone extracts isolated from si-Neg, si-Eed and siEzh1+2 – transfected neonatal cardiac fibroblasts (N=2-3). H3 was used as loading control. (**B**) Immunoblot analysis of Ezh2 protein levels in nuclear extract isolated from neonatal cardiac fibroblasts transfected with negative siRNA (si-Neg) or siRNA targeting Ezh1 (si-Ezh1) or Ezh2 (si-Ezh2). TBP was used as loading control.

Statistical difference was determined by standardized T-test between targeting siRNA and si-Neg (*P≤0.05).

## Online Figure IX




**Online Figure IX: Knock-down of PRC2 members does not increase the effect of miR combo.**

(**A**) Expression levels of Tbx5, Mef2c, Hand2 and Gata4 in neonatal cardiac fibroblasts transfected with miR combo (MC), siRNA against Eed (si-Eed) or the combination of MC and si-Eed. mRNAs were isolated and analyzed by qPCR 3 days after transfection (N=3-5). (**B**) Similar experiment using neonatal cardiac fibroblasts transfected with MC, the combination of MC and siRNA against Ezh2 (MC+si-Ezh2) (N=3) or the combination of MC and siRNAs against Ezh1 and Ezh2 (MC+ si-Ezh1+2) (N=5).

Standardized T-test showed no significant difference between MC+si-Eed versus si-Eed, MC+si-Ezh2 versus MC, or MC+Ezh1+2 versus MC.

**SUPPLEMENTAL REFERENCE**

1. Jayawardena T, Mirotsou M and Dzau VJ. Direct reprogramming of cardiac fibroblasts to cardiomyocytes using microRNAs. *Methods Mol Biol*. 2014;1150:263-72.