Supplementary methods

Reagents

Anti-ErbB4 antibody (111B2), Anti-pAkt antibody(#9271), anti-Akt antibody(#9272), anti-cleaved caspase3 antibody (#9661) , Anti-p53 antibody (1C12), Anti-GAPDH antibody (14C10) were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-ErbB2 (Neu) antibody (sc-284), anti-EGFR (sc-03) antibody and anti-Neuregulin (NRG) 1β1 antibody (sc-347) were obtained from Santa Cruz Biotechnology (CA, USA). Anti-bcl-2 antibody (83-8B) was obtained from MBL (Nagoya, Japan). Recombinant NRG1-β1 (396-HB) was obtained from R&D Systems (MN, USA). Tetramethylrhodamine, ethylester, perchlorate (TMRE) was obtained from Molecular Probes (OR, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Z-Leu-Leu-Leu-CHO (MG-132) were obtained from Sigma Aldrich (Saint Louis, MI, USA). Doxorubicin was obtained from WAKO (Osaka, Japan).

siRNA-mediated knock down of rat ErbB4

The oligonucleotides used for siRNA of ErbB4 were the following:

ErbB4 siRNA1: sense, (GCCCUCAACCAGUUUCGUU) and antisense, (AACGAAACUGGUUGAGGGC).

ErbB4 siRNA2: sense, (GGACCUGACAACUGUACAA) and antisense, (UUGUACAGUUGUCAGGUCC).

Randomly shuffled form of ErbB4 siRNA1 were used as control. Every siRNA construct was made by the use of pSINsi-mU6 vector (Takara Bio Inc.) and the siRNA producing constructs were introduced to lenti-virus vector plasmid and transduced to

cardiac myocytes. Two or Three days after the transduction, cells were used for analysis.

RNA extraction and quantitative real-time PCR for mRNA

Total RNA was isolated using TRIzol® reagent (Invitrogen) and cDNA was synthesized by using SuperScript II reverse transcriptase (Invitrogen). For real-time PCR, the reaction was performed with a SYBR Green PCR master mix (Applied Biosystems), and the products were analyzed using a thermal cycler (ABI Prism 7900HT sequence detection system). The levels of GAPDH transcripts were used to normalize cDNA levels. Gene-specific primers were shown as follows.

rat ErbB4 sense: AACCAGCACCATACCAGAGG

rat ErbB4 antisense: TTCATCCAGTTCTGCTCGTG

rat NRG1β sense: CAGAAGAAGCCAGGGAAGTC

rat NRG1β antisense: TGGCAACGATCACCAGTAAA

rat GAPDH sense; TTGCCATCAACGACCCCTTC

rat GAPDH antisense; TTGTCATGGATGACCTTGGC

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Fig.1

Representative western blotting results for ErbB4 in the hearts of mice at the indicated time points after intra-peritoneal injection of Dox (20 mg/g). Because the antibody against ErbB4 recognizes the C-terminus of ErbB4, full length of ErbB4 is recognized at 180 kD and cleaved-ErbB4 is recognized at 80 kD. Note that cleaved-ErbB4 was not seen at 80 kD. GAPDH was used as a loading control.

Supplementary Fig.2

A. Three potential binding sites of miR-146a in the 3'UTR of ErbB4. (Hsa: human, Ptr: Chimpanzee, Mmu: mouse, Rno: rat).
B. The expression of miR-146a in NRCMs and cardiac fibroblasts. Values are the means ± S.E. of 3 independent experiments. The mean values of miR-146a, normalized with U6 was set at 10.0.
C. The expression of miR-133a in NRCMs and cardiac fibroblasts. Values are the means ± S.E. of 3 independent experiments. The mean values of miR-133a, normalized with U6 was set at 10.0.
D. MicroRNAs were transduced into NRCMs using lentivirus vectors. The transduction efficiency, which is shown using GFP in the right-hand panel, was always over 90%.
E. Representative western blotting results for ErbB4 in cardiac myocytes and 293T cells, into which miR-control or miR-146a had been introduced. GAPDH was used as a control.
F. Human ErbB4 3'UTR firefly-luciferase (F-luc) activity normalized using renilla reniformis luciferase (R-luc). About 500 bp fragments were amplified from human cDNA and inserted into a pMIR-REPORT vector. Reporters (0.1 μg) were co-transfected with miR-control or miR-146a (0.5 μg) into 293T cell. The mean values of miR-control were set at 100%. Values are the means ± S.E. of 8 independent experiments (#$p<0.05$, +$p=0.06$).
G. Rat ErbB4 3'UTR F-luc activity normalized using R-luc. About 500 bp fragments were amplified from rat cDNA and inserted into a pMIR-REPORT vector. Reporters (0.1 μg) were co-transfected with miR-control or miR-146a (0.5 μg) into 293T cells. The mean values of miR-control were set at 100%. Values are the means ± S.E. of 8 independent experiments (#$p<0.05$, ##$p<0.01$).
H. Wild-type and mutant sequence of miR-146a binding site 3 of ErbB4 3'UTR.

Supplementary Fig.3

A. Microscopy images of NRCMs transduced with miR-control or miR-146a with or without 1

μM Dox for 24 h.

B. Fluorescence microscopy images of NRCMs treated with or without 1 μM Dox for 24 h and stained with TMRE dye, as described in the materials and methods.

Supplementary Fig.4

A. Flow cytometric analysis of TMRE in miR-control or miR-146a over-expressing NRCMs infected with either lacZ or ErbB4 with or without Dox treatment for 24 h.
B. The ratio of TMRE intensity with Dox treatment compared with without Dox for each group. Values are the means ± S.E. of 3-4 independent experiments (#$p<0.05$).

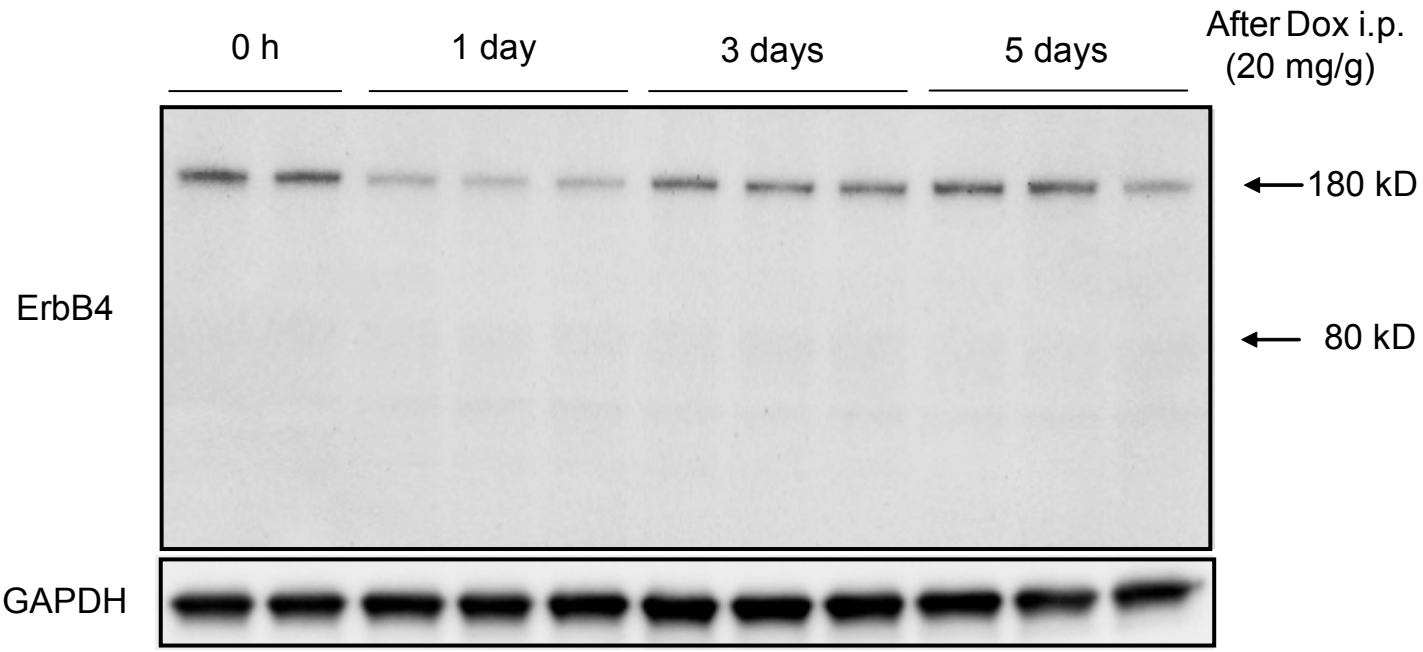
Supplementary Fig.5

A. Scheme of the luciferase 'decoy' gene. 3'UTR of firefly luciferase was modified to include 3 or 6 tandem sequences complementary to miR-146a separated by 3 nucleotides spacers
B. Control-decoy (0.1 μg) was co-transfected with miR-control and/or miR-146a or miR-133a expression vectors (total 0.5 μg) into 293T cells, and F-luc activity was measured and normalized using R-luc. The mean values of miR-control were set at 100%. Values are the means ± S.E. of 4 independent experiments.
C. Decoy-miR-146a×3 (0.1 μg) was co-transfected with miR-control and/or miR-146a or miR-133a expression vectors (total 0.5 μg) into 293T cells, and F-luc activity was measured and normalized using R-luc. The mean values of miR-control were set at 100%. Values are the means ± S.E. of 4 independent experiments (#$p<0.05$ vs miR-control).
D. Decoy-miR-146a×6 (0.1 μg) was co-transfected with miR-control and/or miR-146a or miR-133a expression vectors (total 0.5 μg) into 293T cells, and F-luc activity was measured and normalized using R-luc. The mean values of miR-control were set at 100%. Values are the means ± S.E. of 4 independent experiments (#$p<0.05$ vs miR-control).
E. The same amounts (0.5 μg) of plasmids (control-decoy, decoy-miR-146a×3, or decoy-miR-146a×6) were transfected into NRCMs, and F-luc activity was measured and normalized using R-luc. The reduction in luciferase activity was considered as the effect of the 'decoy' gene. The mean level of luciferase activity in the control-decoy was set at 100%. Values are the means ± S.E. of 4 independent experiments (#$p<0.05$ vs control-decoy).
F. Scheme of the GFP 'decoy' gene. The 3'UTR of GFP was modified to include 3 or 6 tandem sequences complementary to miR-146a separated by 3 nucleotides spacers.
G. Flow cytometric measurement of FITC intensity in NRCMs, infected with GFP-control-decoy, GFP-decoy-miR-146a×3, or GFP-decoy-miR-146a×6 using lentivirus vectors. The reduction in FITC intensity was considered as the effect of the 'decoy' gene.
H. The sequence of anti-miR-146a used in constructing 'decoy' genes.

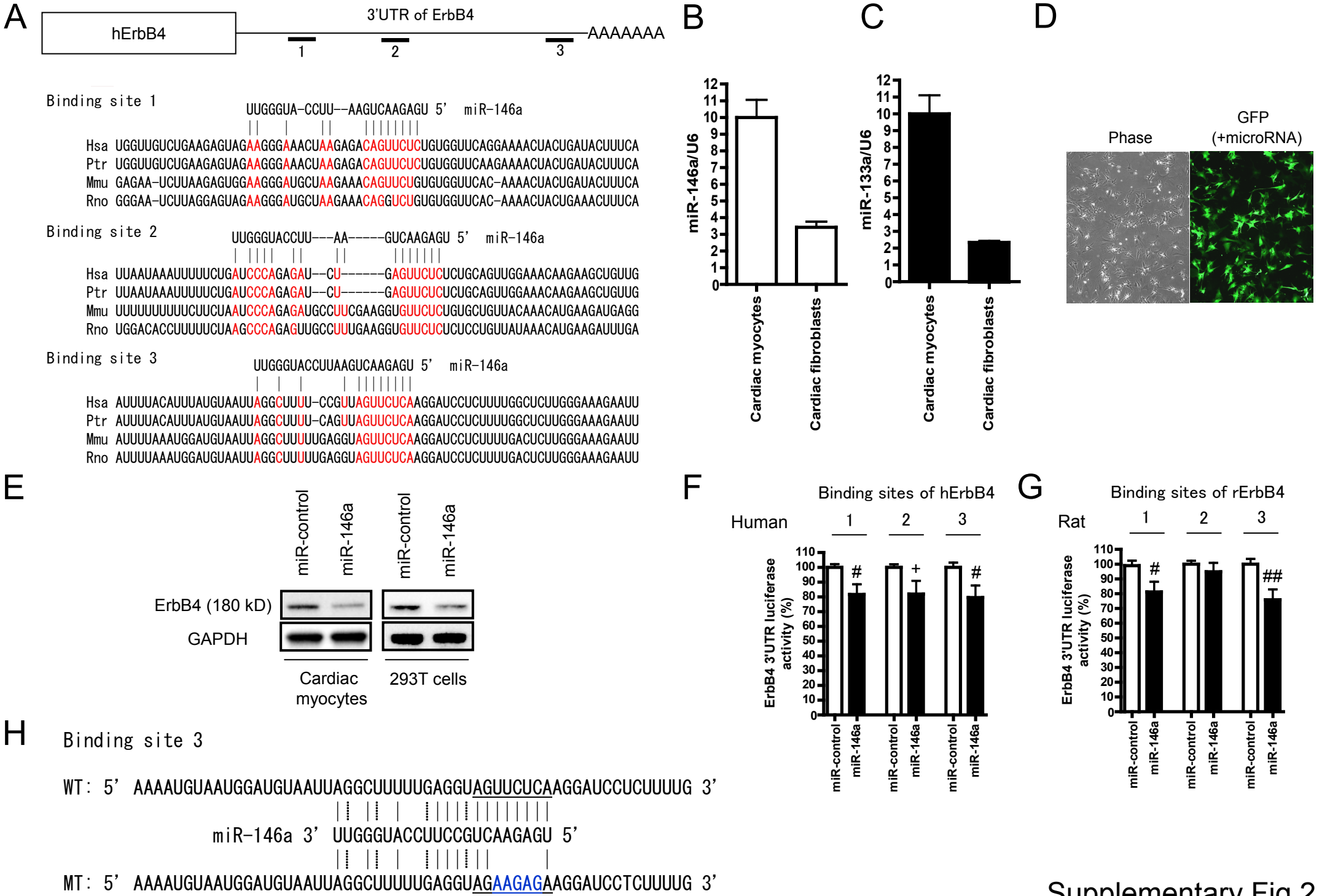
I. Flow cytometric analysis of TMRE in NRCMs infected with control or decoy gene with or without Dox treatment for 24 h.
J. The ratio of TMRE intensity with Dox treatment compared with without Dox for each group. Values are the means ± S.E. of 6 independent experiments (#$p<0.05$).

Supplementary Fig.6

A. Western blotting results for NRG1β in NRCMs stimulated with 1 μM Dox for the indicated time periods. Full means full length, and CTF means C terminus fraction. GAPDH was used as a loading control.
B. Quantitative real-time PCR analysis for NRG1β in miR-control or miR-146a over-expressing NRCMs. The mean values of miR-control normalized using GAPDH, were set at 100%.
C. Western blotting results for NRG1β in miR-control or miR-146a over-expressing NRCMs stimulated with 1 μM Dox for the indicated time periods. GAPDH was used as a loading control.
D. Quantitative real-time PCR analysis for NRG1β in NRCMs transduced with control-siRNA or ErbB4-siRNAs. The mean values of control-siRNA normalized using GAPDH were set at 100%.
E. Western blotting results for NRG1β in NRCMs transduced with control-siRNA of ErbB4-siRNAs stimulated with 1 μM Dox for the indicated time periods. GAPDH was used as a loading control.

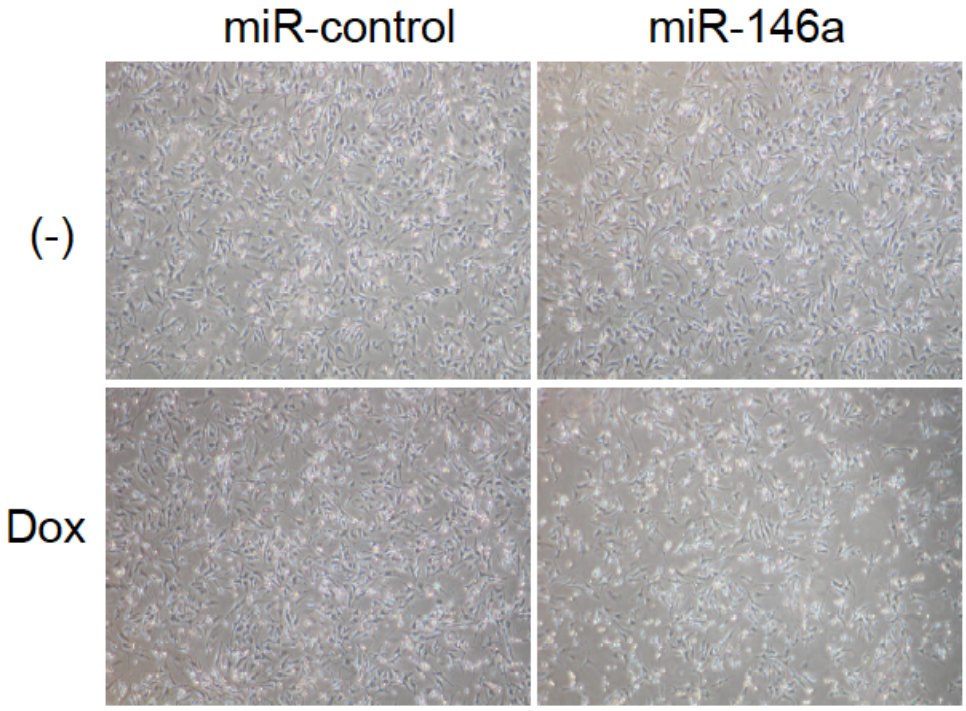


Supplementary Fig.1



Supplementary Fig.2

A



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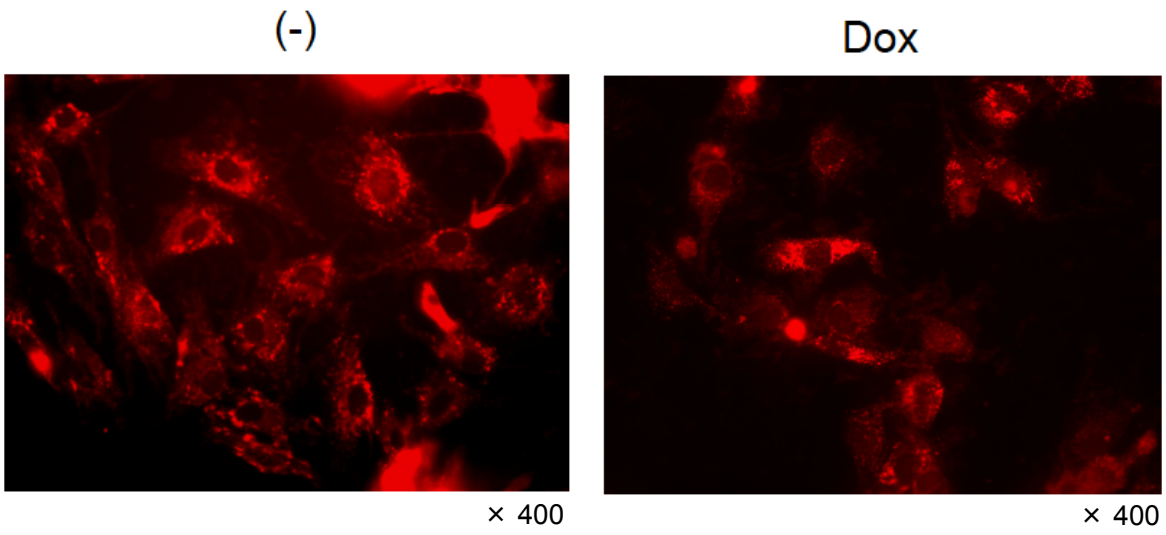
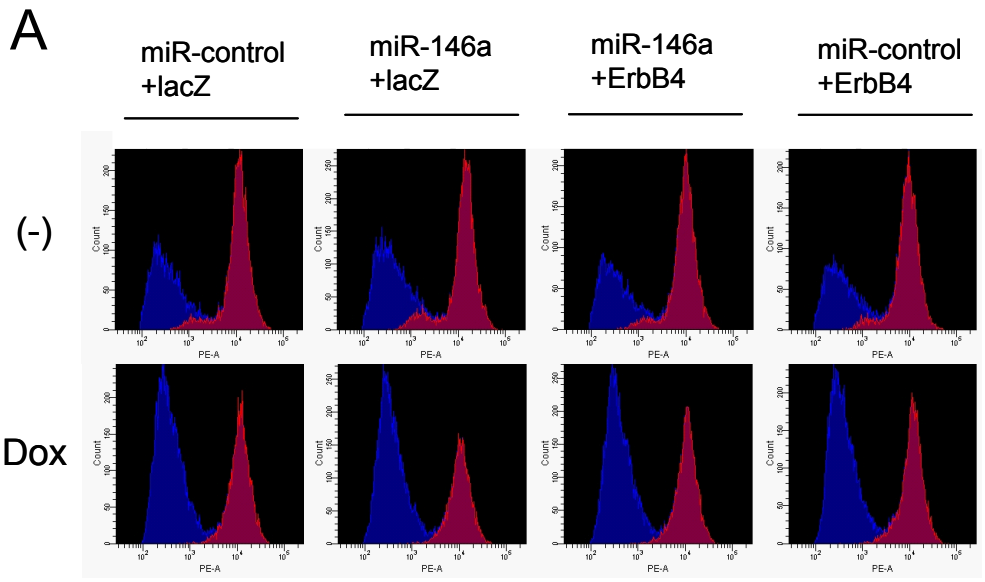
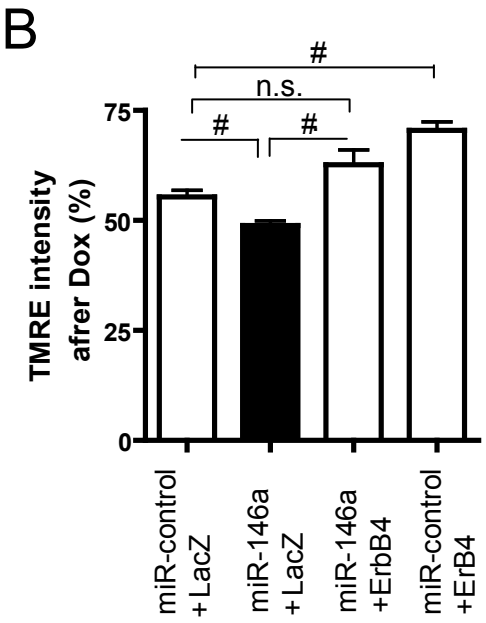


Supplementary Fig.3





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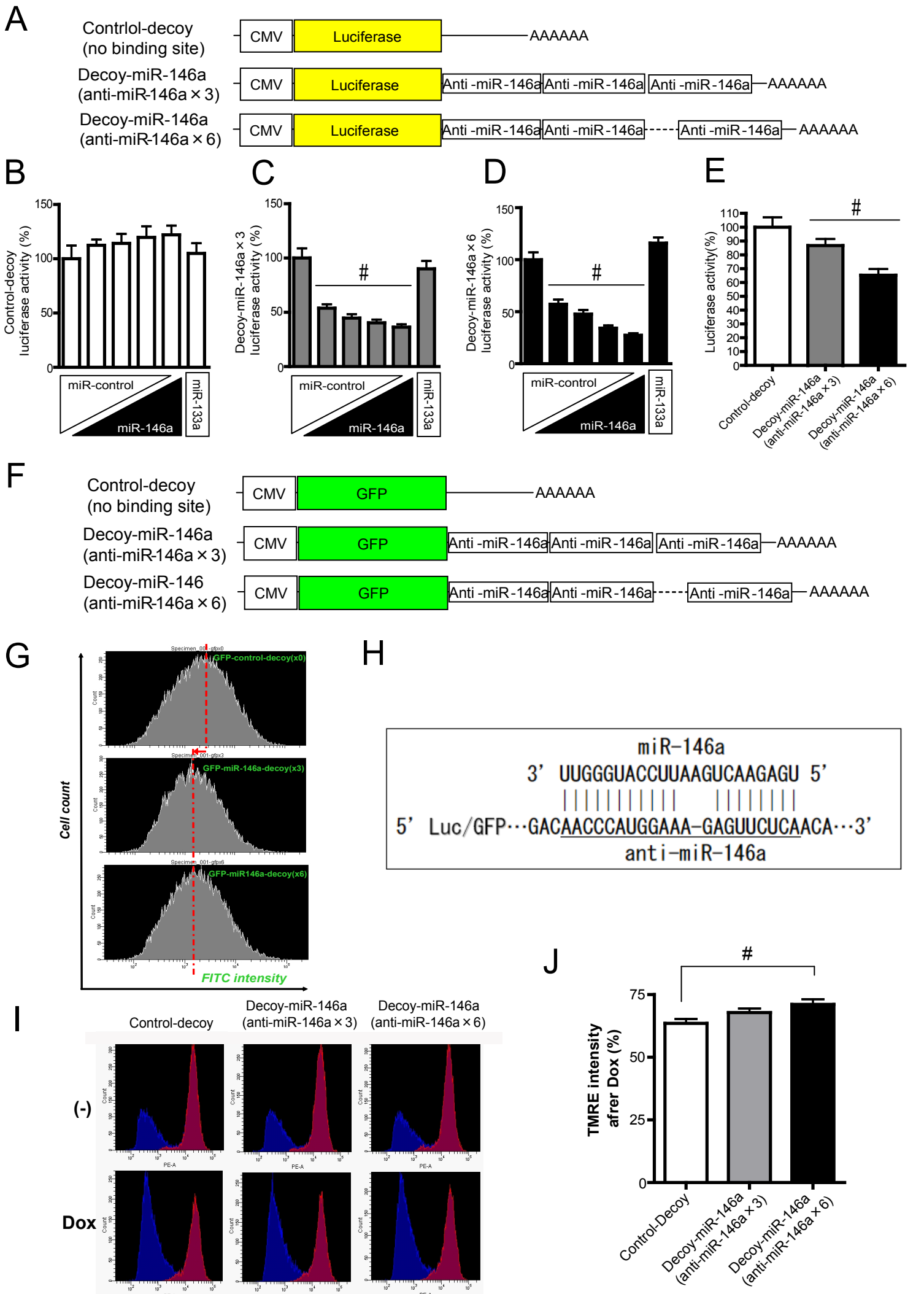
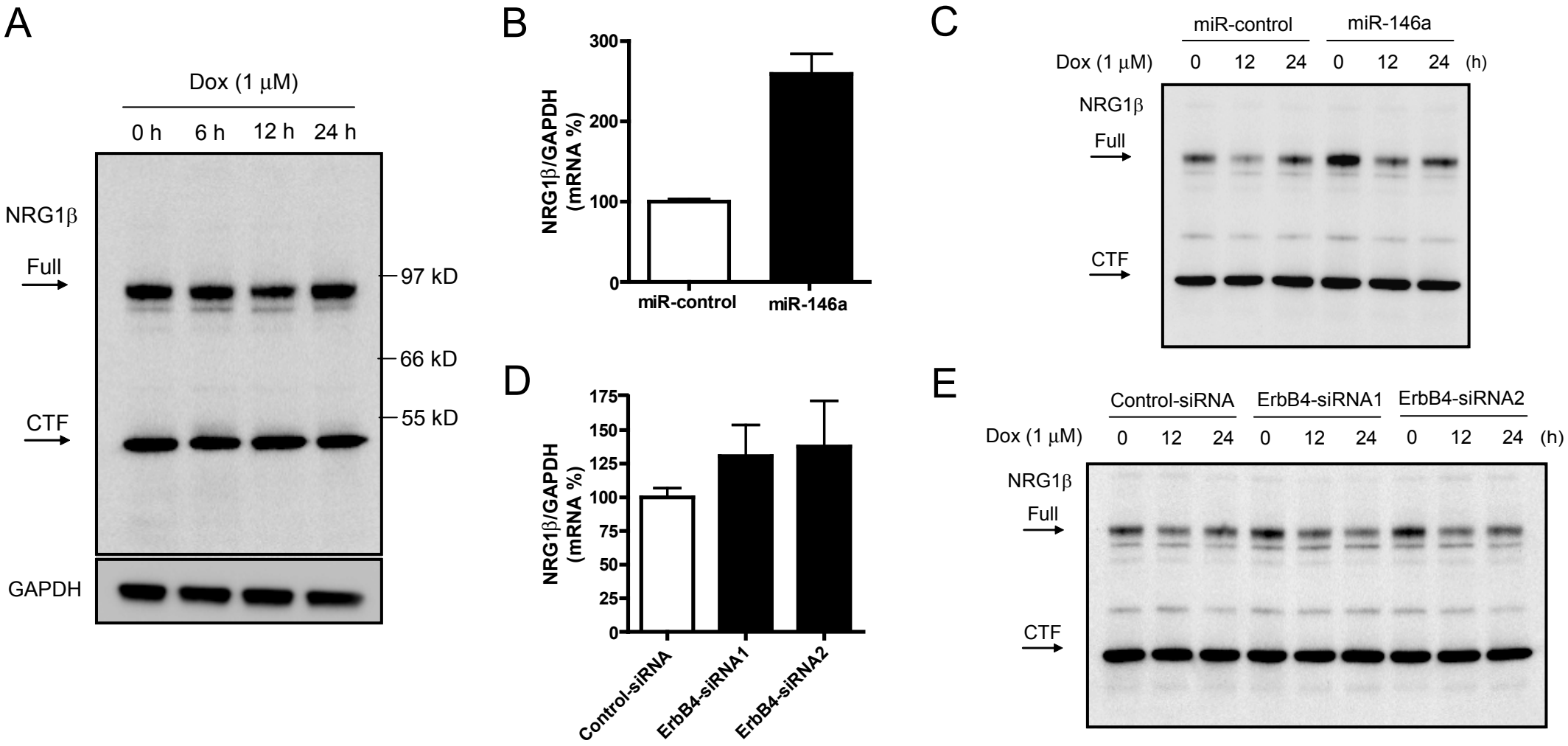


Supplementary Fig.5



Supplementary Fig.6