Expanded View Figures

Figure EV1. Mouse alleles generated and used in this work, transcriptomic analysis of *mi*iPSCs, and validation of miR-203 mimics and vectors on EBs differentiation.

A Schematic representation of the miR-203-inducible knock-in model generated in this work. In the *ColA1*(miR-203/miR-203); *Rosa26*(rtTA/rtTA) model, the reverse tetracycline transactivator is expressed from the *Rosa26* locus, whereas miR-203 is driven by the tetracycline operator downstream of the *ColA1* locus.

B miR-203 expression, as determined by quantitative PCR, in *ColA1*(miR-203/miR-203); *Rosa26*(rtTA/rtTA) MEFs, iPSCs, and ESCs treated or not with doxycycline (Dox). miR-203 expression is normalized by a control miRNA (miR-142). Data are mean ± SD (n = 3 independent experiments). ***$P < 0.001$ (Student's t-test).

C Time course of miR-203 expression, as determined by qPCR. RNA expression is normalized by a housekeeping miRNA (miR-16) that maintained invariable. Data are mean ± SD (n = 4 independent replicates). $P < 0.001$ (Student's t-test) comparing Dox treatment (green box) versus Dox withdrawal.

D Unbiased clustering of genome-wide RNAseq data (left) and heat map plot showing the comparative expression of 450 genes associated with pluripotency (Chung *et al*, 2012); 2 clones per sample.

E Early expression (RPKM) of the indicated transcripts included in the 2C signature. Data are mean ± SEM (n = 3 independent experiments). *$P < 0.05$; **$P < 0.01$ (Student's t-test).

F Top categories in the Gene Ontology analysis of the genes significantly upregulated in *mi*iPSCs versus un-induced iPSCs (4 independent clones were analyzed; see also Dataset EV1). P-values were calculated by Fisher's exact test.

G (Upper panels) Representative images of EBs derived from either wild-type iPSCs (left) or ESCs (right) transduced with empty pMCSV vector, pMCSV-miR-203, or transfected with control mimics or miR-203 mimics, at day 30 of the differentiation process. Scale bars, 500 μm. (Lower panels) Quantification of EBs with large cavities and beating EBs during the differentiation process. Data are mean ± SEM (n = 3 independent experiments). **$P < 0.01$ (in both iPS and ES cells; Student's t-test).

Source data are available online for this figure.

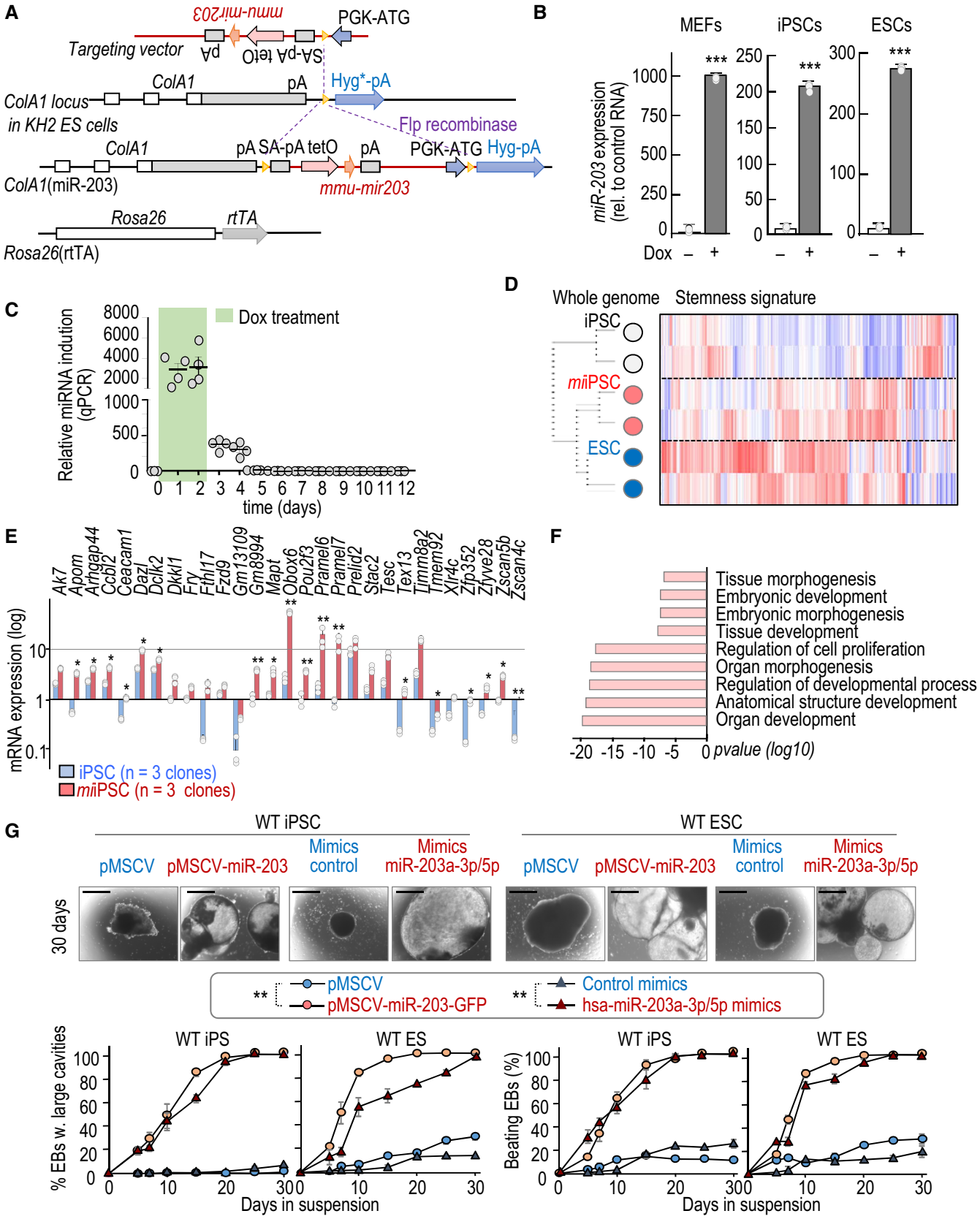


Figure EV1.

Figure EV2. Histopathological analysis of teratomas generated from *mii*PSCs.

A Representative images of teratomas 20–25 days after subcutaneous injection of wild-type iPSCs or *mii*PSCs expressing GFP, as indicated in Fig 2A. Images on the right show an example of a *mii*PSC-derived embryo-like structure, analyzed 20 days after intraperitoneal (i.p.) injection of *mii*PSCs expressing GFP. Scale bar, 5 mm (two left panels) and 1 mm (two right panels).

B Representative example of a highly differentiated teratoma generated from *mii*PSCs after i.p. injection in nude mice. Most of these complex teratomas were detected in the proximity of the uterus or as ovarian cysts in the host mice. The panel shows higher magnifications (H&E staining) of several differentiated tissues and cells observed in the teratoma. Scale bars, 1 mm (central image) or 50 μm (insets).

C Histopathological examples (H&E staining) of specific tissues found in *mii*PSC-derived teratomas. Scale bars, 100 μm. A magnification of a trophoblast giant cell stained against placental lactogen-1 (PL-1) is also shown (scale bar, 50 μm).

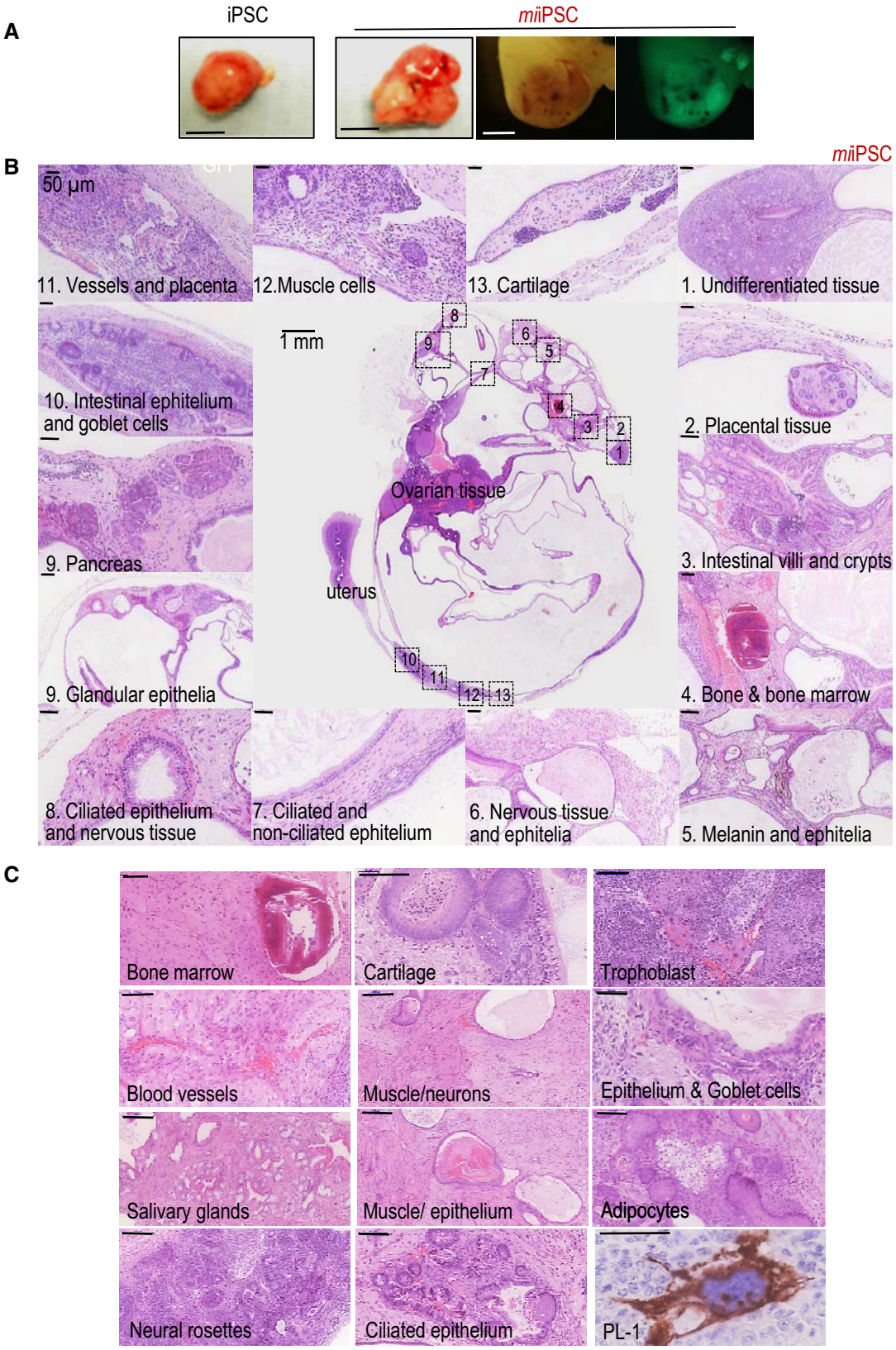


Figure EV2.

Figure EV3. miR-203-exposed human iPSCs efficiently contribute to human–mouse interspecies chimeras.

A Summary of interspecies chimera assays in which one single Td-Tomato fluorescent-labeled human iPSC, either control or transiently transfected with miR-203-expressing vectors (*mi*iPSCs), was injected into 8C-stage mouse embryos. These embryos were cultured *in vitro* for 48 h to reach the blastocyst stage and then transfected to 2.5 days post-coitum pseudo-pregnant females. The post-implantation mouse conceptuses were dissected at the E9.5 developmental stage and analyzed by immunofluorescence.

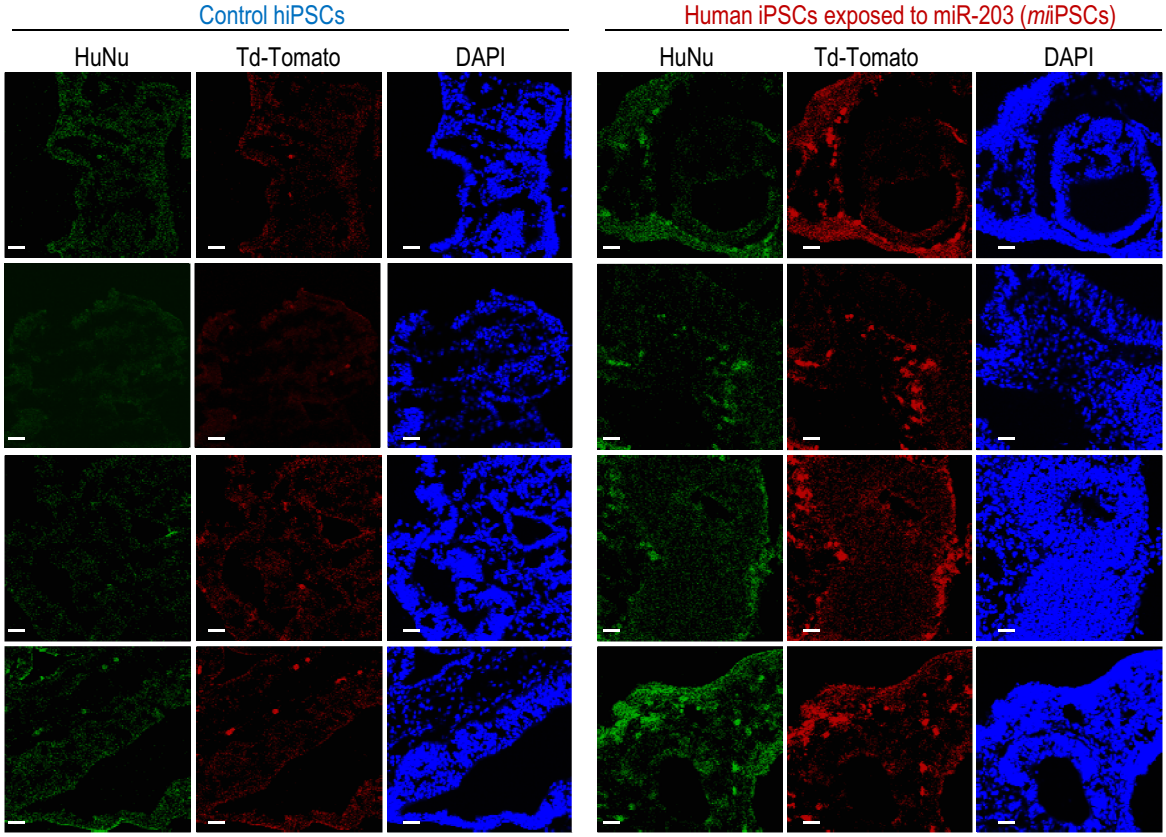
B Representative immunofluorescence images from these E9.5 embryos summarized in panel (A). Anti-human HuNu antibody was co-stained with Td-Tomato direct fluorescence to detect human cells in E9.5 mouse embryos. Scale bars, 20 μm.

C Representative immunofluorescence images from these E9.5 embryos summarized in panel (A), co-stained with human HuNu antibody and Gata4, Sox2, or hSOX17 antibodies showing differentiation and proper integration of human cells into the mouse embryo. Scale bars, 20 μm.

A

	Injected embryos	Tranferred embryos	Recovered embryos	Implantation sites
Control hiPSCs P29	20	14	3	9
Human *mii*PSCs P29+0	21	16	8	4
Human *mii*PSCs P36+2	21	16	9	5

B



C

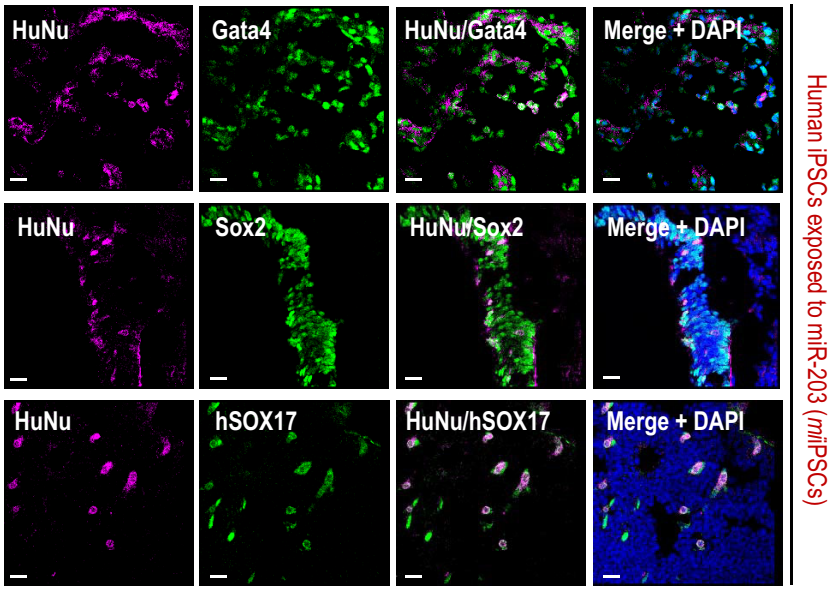


Figure EV3.

Figure EV4. miR-203 targets *Dnmt3a/b* 3′-UTR sequences.

A *Dnmt3a* and *Dnmt3b* 3′-UTR alignment in several representative species. The seed region of the miR-203 target site contained in these 3′-UTRs is highlighted in blue and aligned with the corresponding miR-203 seed sequence.

B Schematic representation of the luciferase reporter, carrying the wild-type *Dnmt3a* (left) or *Dnmt3b* (right) complete 3′-UTRs or the corresponding mutated versions, downstream of the luciferase gene. The seed region of the miR-203 target site contained in these 3′-UTRs is written in blue and aligned with the corresponding miR-203 seed sequence. The mutated residues are indicated in red.

C Major pathways from the Gene Ontology database upregulated in wild-type iPSCs treated with specific siRNAs against *Dnmt3a* and *Dnmt3b* versus scrambled sequences (*siC*). P-values were calculated by Fisher's exact test.

D Representative images of embryoid bodies (EBs) generated from wild-type iPSCs treated with specific siRNAs either against *Dnmt3a* or *Dnmt3b* (left panel) or transfected with a combination of miR-203-resistant Dnmt3a/b cDNAs (right panel) at the indicated time points during the differentiation process. Scale bars, 500 μm.

E Detection of Dnmt3a (117 KDa) and Dnmt3b (96 KDa) protein levels by Western blot, in 5 independent clones of mouse un-induced iPSCs or *mi*iPSCs and one human cell line, transfected with mimics control (hiPSCs) or miR-203 mimics (h*mi*iPSCs). Protein extracts were evaluated immediately after miR-203 exposure, to detect the modulation of Dnmt3a/b protein levels. Vinculin levels are included as loading control.

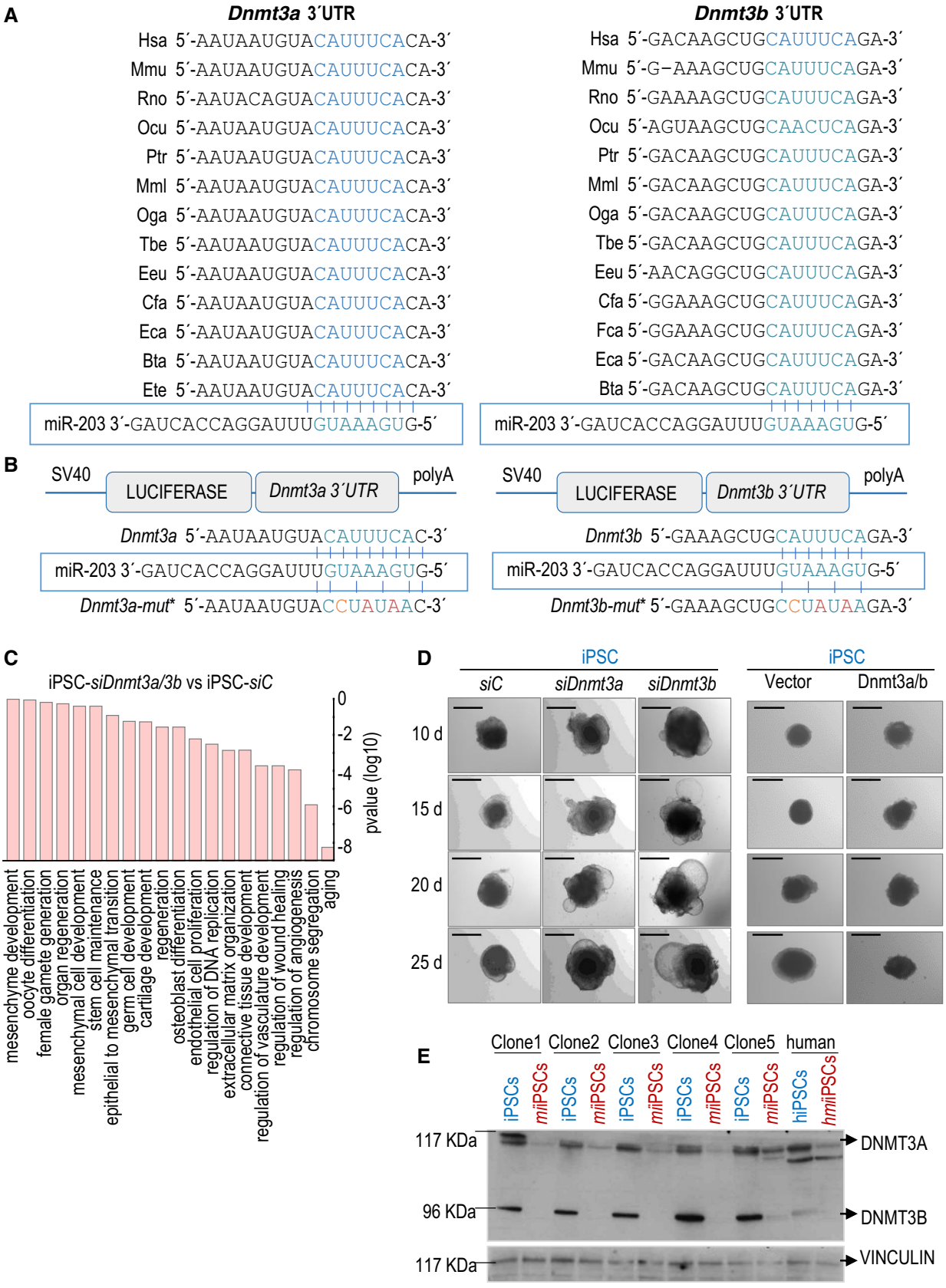


Figure EV4.

Figure EV5. Genome-wide methylation of iPSCs or embryoid bodies after transient exposure to miR-203.

A Expression levels, as determined by quantitative PCR, of *miR-203*, and transcripts for the DNA methyltransferases *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Dnmt3a2*, and *Dnmt3l*, or pluripotency (*Dazl*) and differentiation (*Gata6*) markers. *ColA1*(miR-203/miR-203); *Rosa26*(rtTA/rtTA) iPSCs treated or not with doxycycline (Dox) and simultaneously transduced with Dnmt3a/b cDNAs or empty vector were used as shown in the schematic representation of the experimental design. The pink shadow indicates the time lapse in which the cells were treated or not with Dox and transduced or not with Dnmt3a/b cDNAs. The green shadow indicates the differentiation process to embryoid bodies. Data are represented as mean of three technical replicates per experiment (n = 2 independent experiments).

B Experimental design used for the validation of methylation data in the indicated *Elf5* differentially methylated region (DMR).

C DNA was isolated as indicated and sequenced after bisulfite modification. Eight to ten independent clones were sequenced per condition. Histograms show the percentage of DNA methylation at the *Elf5* DMR in the different conditions.

D Experimental protocol followed to test DNA methylation rescue by miR-203-resistant Dnmt3a/b cDNAs.

E The specific differentially methylated regions (DMRs) at the *Elf5* locus were analyzed by PCR amplification and sequencing of bisulfite-modified DNA. The quantification of methylated versus unmethylated CpGs is shown in the histogram.

Source data are available online for this figure.

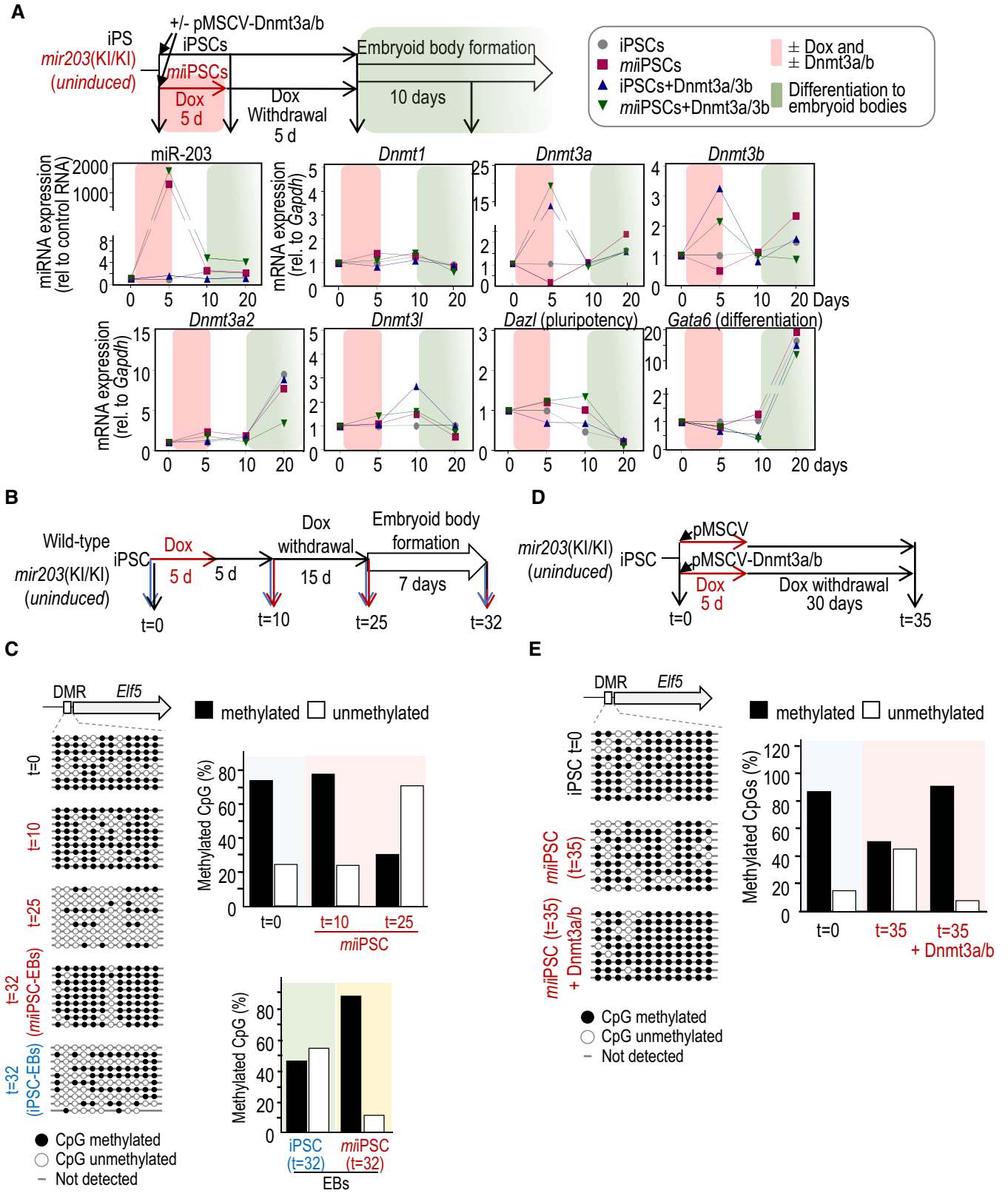


Figure EV5.

Figure EV6. Improved cardiomyocyte differentiation and maturation after transient expression of miR-203.

A Representative immunofluorescence showing EdU (green) and nuclei (Hoechst, blue) staining of primary cardiomyocytes extracted at post-natal day 1 and transiently transfected with control or miR-203 mimics 24 h after extraction. Pictures were taken 3 days after transfection. Scale bar, 60 μm. The histogram shows the percentage of EdU-positive cells at different days post-transfection. Data are mean ± SD (total number of cells counted is indicated in the figure; the dots represented in the bars correspond to $n = 2$ independent experiments, with 5 and 4 different replicates, respectively.

B RNA expression as determined by quantitative PCR of miR-203 (24 h after transfection) and *Ccnb1*, *Myh6*, and *Myh7* transcripts (5 days after transfection). The *Myh6/Myh7* ratio is calculated as an indicator of cardiomyocyte maturation. Data are mean ± SD (the dots represented in the bars correspond to $n = 3$ independent experiments, with 2 replicates each).

C Representative immunofluorescence showing phospho-histone 3 (Ser-10; pH3) in green, cardiac Troponin T (cTnT) in red, and Hoechst for nuclei staining in blue, in primary cardiomyocytes extracted at post-natal day 1 and transiently transfected with control or miR-203 mimics 24 h after extraction. Images were taken 3 days after transfection. Scale bars, 64 μm. White arrows point to cardiomyocytes positive for pH3. Yellow arrows point to cells positive for pH3 but not properly differentiated to cardiomyocytes, according to cardiac Troponin T staining. Middle histogram shows the proliferation rate measured as the percentage of pH3-positive cells respect to the total number of cTnT-positive cells at day 3 post-transfection. Data are mean ± SD Total number of cells counted is indicated in the figure; the dots represented in the bars correspond to $n = 2$ independent experiments, with 8 different replicates each. The plot on the right shows the total number of cells at day 3 post-transfection. Data are mean ± SD. The dots represented in the bars correspond to $n = 2$ independent experiments with 5 replicates each.

D mRNA levels as determined by quantitative PCR of the indicated transcripts at different time points before and during cardiomyocyte differentiation. iPSCs were transfected either with control mimics or with miR-203 mimics, maintained during 15 days in culture, and then differentiated *in vitro*. Data are mean ± SD The dots represented in the bars correspond to $n = 2$ independent experiments, with 3 replicates each.

E mRNA levels of the indicated transcripts at different time points during cardiomyocyte differentiation. Data are mean ± SD The dots represented in the bars correspond to $n = 2$ independent experiments, with 3 replicates each.

Data information: In (A–E) data, $*P < 0.05$, $**P < 0.01$ (Student's *t*-test).
Source data are available online for this figure.

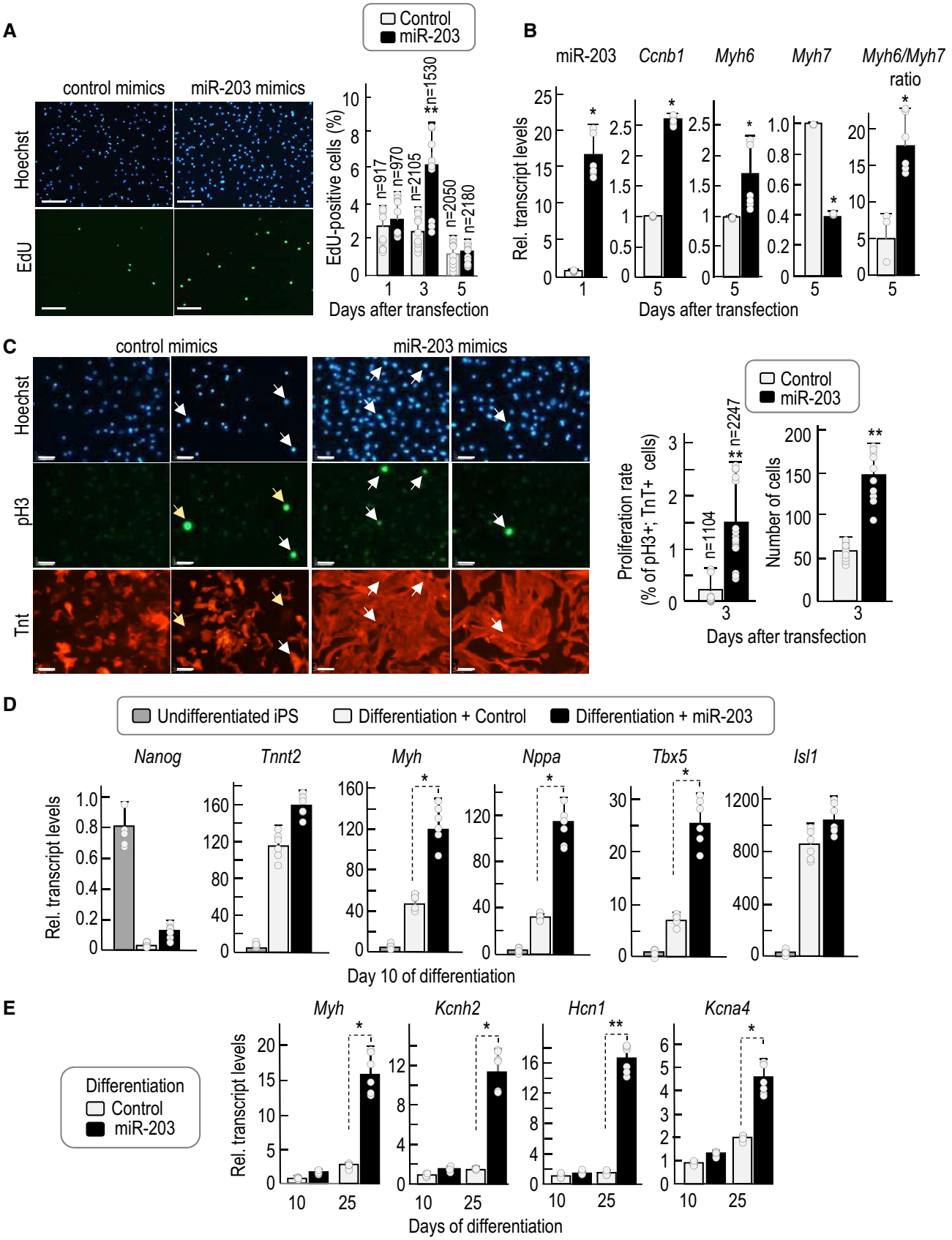


Figure EV6.