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DICER1 Is Essential for Self-Renewal of Human Embryonic Stem Cells

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SUMMARY

MicroRNAs (miRNAs) are the effectors of a conserved gene-silencing system with broad roles in post-transcriptional regulation. Due to functional overlaps, assigning specific functions to individual miRNAs has been challenging. DICER1 cleaves pre-miRNA hairpins into mature miRNAs, and previously *Dicer1* knockout mouse embryonic stem cells have been generated to study miRNA function in early mouse development. Here we report an essential requirement of DICER1 for the self-renewal of human embryonic stem cells (hESCs). Utilizing a conditional knockout approach, we found that *DICER1* deletion led to increased death receptor-mediated apoptosis and failure of hESC self-renewal. We further devised a targeted miRNA screening strategy and uncovered essential pro-survival roles of members of the *mir-302-367* and *mir-371-373* clusters that bear the seed sequence AAGUGC. This platform is uniquely suitable for dissecting the roles of individual miRNAs in hESC self-renewal and differentiation, which may help us better understand the early development of human embryos.

INTRODUCTION

MicroRNAs (miRNAs) are ~20- to 24-nucleotide long non-coding RNAs that bind target mRNAs through partial complementarity with their 3′ UTRs to promote target mRNA decay and/or inhibit translation (Ebert and Sharp, 2012). The RNase III enzyme Dicer1 is required for the processing of precursor miRNA hairpins into their mature functional products (Yang and Lai, 2011). *Dicer1* knockout mouse embryos exhibit post-implantation morphological abnormalities at around embryonic day 7.5 (E7.5) (Bernstein et al., 2003; Spruce et al., 2010), emphasizing the importance of miRNAs for early mammalian development. Due to overlapping miRNA requirements, no individual miRNA mouse knockouts have recapitulated the *Dicer1* knockout phenotype (Kuhnert et al., 2008; Liu et al., 2008; Park et al., 2010; Ventura et al., 2008; Zhao et al., 2007). Mouse embryonic stem cells (mESCs) have been used to gain further understanding of miRNA functions in the pre-implantation period. mESCs lacking either Dicer1 or Dgcr8, factors essential for miRNA biogenesis, can self-renew but show reduced proliferation and a relative accumulation of cells in the G_1 phase of the cell cycle (Kanellopoulou et al., 2005; Murchison et al., 2005; Wang et al., 2007, 2008). miRNA rescue experiments in *Dgcr8* knockout mESCs have shown that the rapid cell cycle of mESCs is regulated by members of the *mir-290-295* (homologous to human *mir-371-373*), *mir-302-367*, and *mir-17-92b* clusters, known collectively as the ESC-specific cell-cycle-regulating (ESCC) family of miRNAs (Wang et al., 2008).

Thus far the exact requirements for *DICER1* in human embryonic stem cells (hESCs) have not been established. One study used a knockdown approach in hESCs but *DICER1* mRNA was only reduced about 5-fold (Qi et al., 2009). Compared with mESCs, hESCs reflect a later developmental stage (Brons et al., 2007; Tesar et al., 2007), and there could be human-specific DICER1 and miRNA requirements. Here we report the generation and characterization of *DICER1*-deficient hESCs. We show that in hESCs, unlike in mESCs, DICER1 is essential for self-renewal and inhibition of death receptor-mediated apoptosis. Furthermore, ESCC seed sequence-containing members of the *mir-302-367* and *mir-371-373* clusters could reverse the apoptotic phenotype. Our study provides a valuable platform for the discovery of individual miRNA functions during early development of human embryos and hESC differentiation.

RESULTS

Generation of *DICER1* Conditional Knockout hESCs

To investigate the role of *DICER1* in human pluripotency, we attempted to generate *DICER1* knockout hESC lines. Despite using the highly efficient iCRISPR platform (Gonzalez et al., 2014), we were unable to generate homozygous null mutant hESC lines after screening 464 lines generated with nine guide RNAs (gRNAs) (Figures S1A–S1C). We

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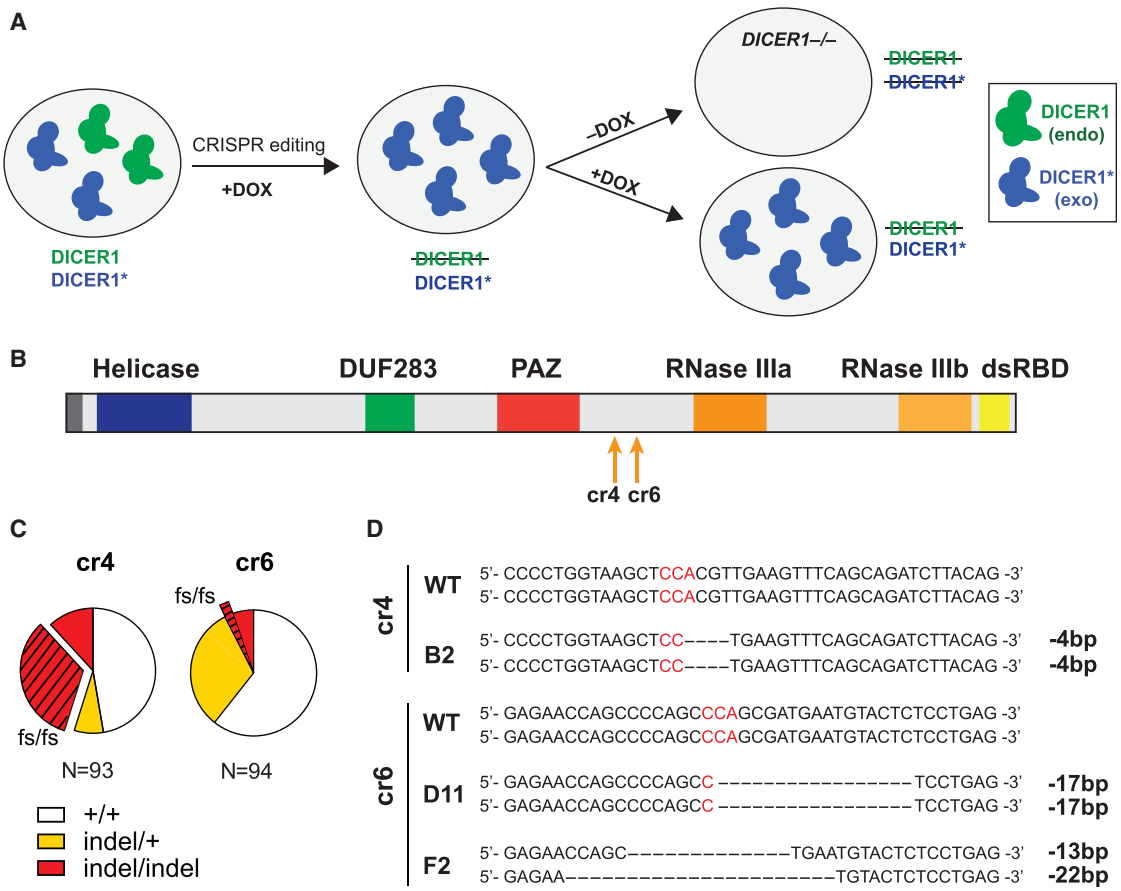

Figure 1. Generation of *DICER1* Conditional Knockout hESC Lines

(A) Schematic of the procedure to generate conditional *DICER1* knockout hESCs. CRISPR/Cas9 was used to generate *DICER1* knockout mutations in cells infected with a gRNA-immune doxycycline (DOX)-inducible *TRE-DICER1** transgene. Cas9 and DICER1* protein expression was induced by doxycycline treatment. *DICER1* knockout mutants were maintained in doxycycline until the start of experiments. An asterisk is used to distinguish the exogenous modified *DICER1** from endogenous *DICER1*.

(B) Schematic representation of DICER1 protein with domains. Orange arrows represent gRNA-targeting loci upstream of the RNase IIIa functional domain. cr, CRISPR; DUF, domain of unknown function; dsRBD, double-stranded RNA-binding domain.

(C) CRISPR/Cas9 targeting results of conditional *DICER1* hESC lines. White, wild-type lines; yellow, monoallelic mutant lines; red, biallelic mutant lines. Stripes indicate *DICER1* knockout lines with frameshift mutations in both alleles. fs, frameshift allele.

(D) Representative endogenous *DICER1* sequences of three selected conditional *DICER1* knockout clones with PAM sequences labeled in red. WT, wild-type.

Unless otherwise indicated, scale bars represent 100 μm in all figures. See also Figure S1.

retargeted a line already carrying a heterozygous *DICER1* frameshift mutation, but still failed to generate a frameshift mutation in the second allele (Figure S1D). These results suggest that *DICER1*-deficient hESCs have self-renewing defects. To overcome the apparent cell lethality issues, we designed a conditional knockout strategy. We first selected two CRISPRs (cr4 and cr6) based on their ability to efficiently generate indel (insertion/deletion) mutations (Figure S1C). We then generated two Tet-On transgenic lines expressing *DICER1* transgenes that have a silent mutation in the PAM sequence and thus are immune to cr4 or cr6 gRNA targeting (referred to as *TRE-DICER1** lines) (Figures S1E and S1F). We next used CRISPR/Cas9 to knock out endogenous *DICER1* while maintaining expression of exogenous DICER1* by doxycycline treatment (Figure 1A). This approach enabled us to create 32 conditional biallelic frameshift knockout lines out of 187 clonal lines analyzed (Figure 1C). We focused further analysis on three conditional knockout lines: B2 (generated using cr4), and D11 and F2 (generated using cr6) (Figures 1B and 1D). When maintained with doxycycline treatment, these conditional *DICER1* knockout hESC lines were indistinguishable from wild-type hESCs and exhibited normal karyotypes (Figure S1G).

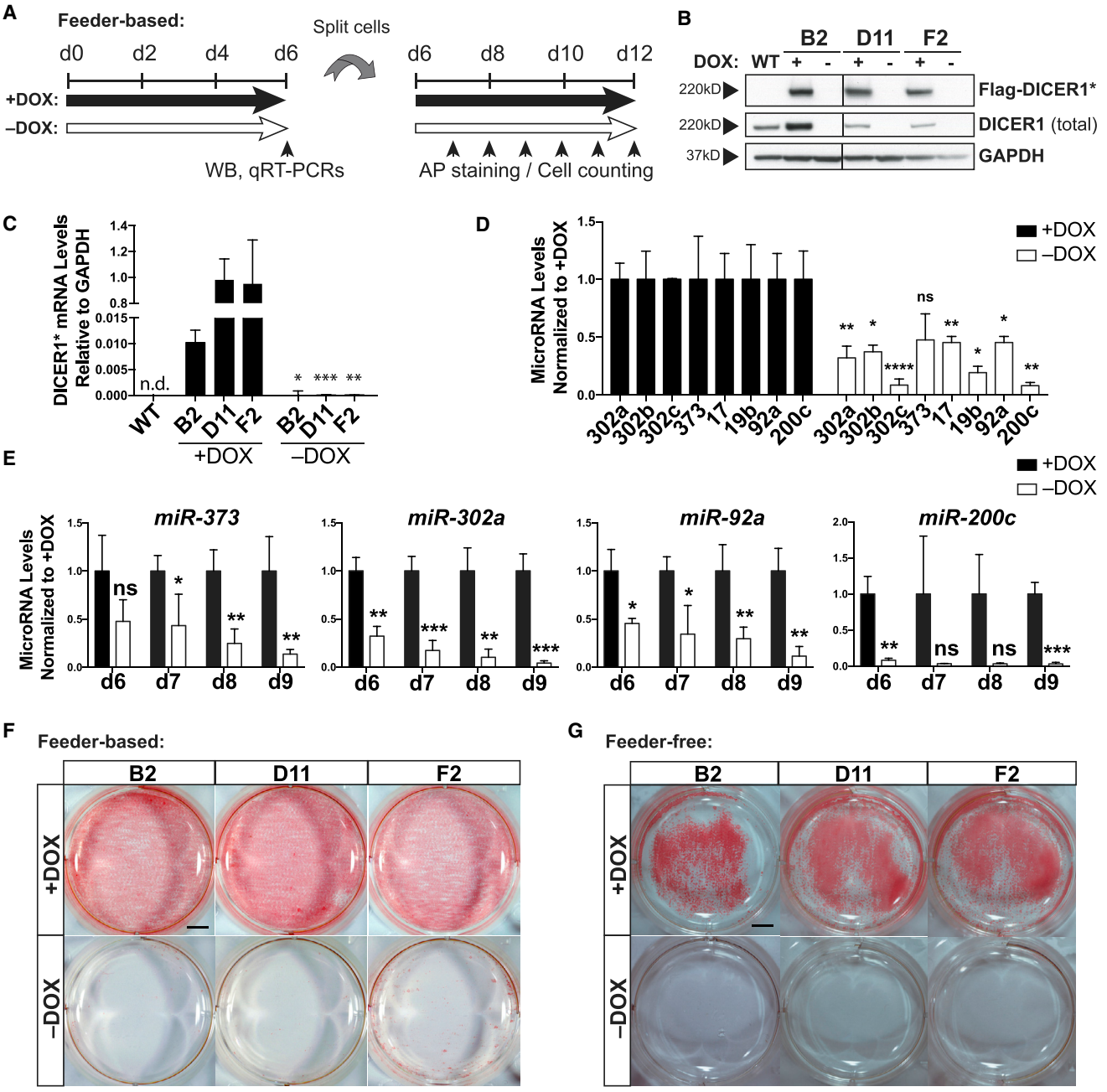

Figure 2. DICER1 Is Essential for hESC Self-Renewal

(A) Schematic representation of experimental procedure in feeder-based condition. Cells were split at 28,000 cells/cm^2. DOX, doxycycline; WB, western blot; AP, alkaline phosphatase.

(B) Western blot of FLAG-DICER1* and total DICER1 levels of three conditional *DICER1* knockout lines (B2, D11, F2) in feeder-based condition with and without DOX treatment for 6 days. WT, wild-type.

(C) qRT-PCR analysis of *DICER1** transgene expression in three conditional *DICER1* knockout lines in feeder-based condition with and without doxycycline treatment for 6 days. Levels relative to GAPDH. Statistical analysis was performed in comparison with +DOX samples. n = 3 independent experiments. n.d., not detected.

(D) Mature miRNA qRT-PCR analysis of eight hESC-expressed miRNAs in *DICER1* conditional knockout line B2 after 6 days of DOX withdrawal in feeder-based condition. Levels are normalized to +DOX. Statistical analysis was performed in comparison with +DOX samples. n = 3 independent experiments.

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DICER1 Is Essential for hESC Self-Renewal

We removed doxycycline to investigate the effects of *DICER1* deletion in conditional *DICER1* knockout hESCs (Figure 2A). Six days after doxycycline withdrawal, no DICER1* mRNA or protein expression was detected, nor did we observe significant expression of the endogenous DICER1 protein (Figures 2B and 2C). The *DICER1*-depleted hESCs survived and maintained comparable protein levels of pluripotency markers OCT4 (POU5F1), NANOG, and SOX2 based on immunofluorescence staining, although some reduction of mRNA levels was observed by qRT-PCR (Figures S2A and S2B). We also tested the levels of several mature miRNAs that are abundant in wild-type hESCs including *miR-373* and *miR-302a*. All miRNAs examined were significantly reduced in *DICER1*-depleted hESCs by qRT-PCR and northern blot, and there was a concomitant increase in precursor miRNAs (Figures 2D and S2C). These results confirmed that miRNA processing was compromised in *DICER1* null hESCs. However, miRNAs were not fully depleted after 6 days, which is consistent with the notion that miRNAs are highly stable molecules (Guo et al., 2015).

After passaging these *DICER1*-depleted cells and maintaining them in the absence of doxycycline for another 6 days (Figure 2A), we observed further reduction in the accumulation of mature miRNAs (Figure 2E). Coinciding with this reduction in miRNA levels, although the *DICER1*-depleted cells were properly attached to the dish after replating, they began to show compromised growth 10 days after doxycycline withdrawal as determined by staining for alkaline phosphatase activity and live cell counting (Figures S2D and S2E). By day 12 of doxycycline withdrawal there were barely any cells detected in all three conditional *DICER1* knockout lines (Figures 2F and S2F). The few remaining *DICER1*-depleted cells could not be propagated without doxycycline treatment.

In addition to the original feeder-based condition, we also tested the requirements for DICER1 in the Essential 8 (E8) chemically defined feeder-free culture condition (Chen et al., 2011). Upon doxycycline withdrawal, *DICER1*-depleted cells could not be propagated in the E8 condition either, and almost no cells were detected by alkaline phosphatase staining after 8 days of doxycycline withdrawal (Figure 2G). Therefore, DICER1 is required for self-renewal of hESCs cultured in feeder-based and feeder-free conditions. *DICER1*-depleted cells survived longer in the feeder-based condition presumably due to pro-survival effects of the feeder cells. In comparison, *Dicer1*-depleted mESCs are capable of self-renewal although they proliferate at a slower rate than wild-type mESCs (Bernstein et al., 2003; Kanellopoulou et al., 2005; Murchison et al., 2005; Wang et al., 2007, 2008).

Loss of DICER1 in hESCs Causes Apoptosis

Unlike *Dicer1*-deficient mESCs, we did not observe an apparent mitotic defect based on flow-cytometry analysis of the mitotic marker phosphohistone H3 (Figures S3A and S3B), nor defects in cell-cycle progression based on cell-cycle analysis by propidium iodide staining (Figure S3C). Instead, we detected significantly increased numbers of cleaved caspase-3 (C-CSP3)-positive cells by flow cytometry starting from day 8 after doxycycline removal and peaking at day 10 in both *DICER1* conditional knockout lines (B2 and D11) cultured in the feeder-based condition (Figures 3A, 3B, S3D, and S3E). The increased apoptotic rate coincided with the compromised cell growth that first became apparent around days 9–10 (Figures S2D and S2E). C-CSP3-positive cells lost OCT4 expression and showed nuclear condensation and fragmentation typical of apoptotic cells when examined by immunofluorescence staining at days 9 and 10 (Figure 3C). Similarly, *DICER1* conditional knockout cells in the E8 condition also showed significantly increased numbers of apoptotic cells by flow cytometry for C-CSP3 expression and annexin-V staining (another apoptosis assay) after doxycycline removal (Figures 3E–3G and S3F). Therefore, unlike its pro-proliferation role in mESCs, DICER1's main function in hESCs is pro-survival.

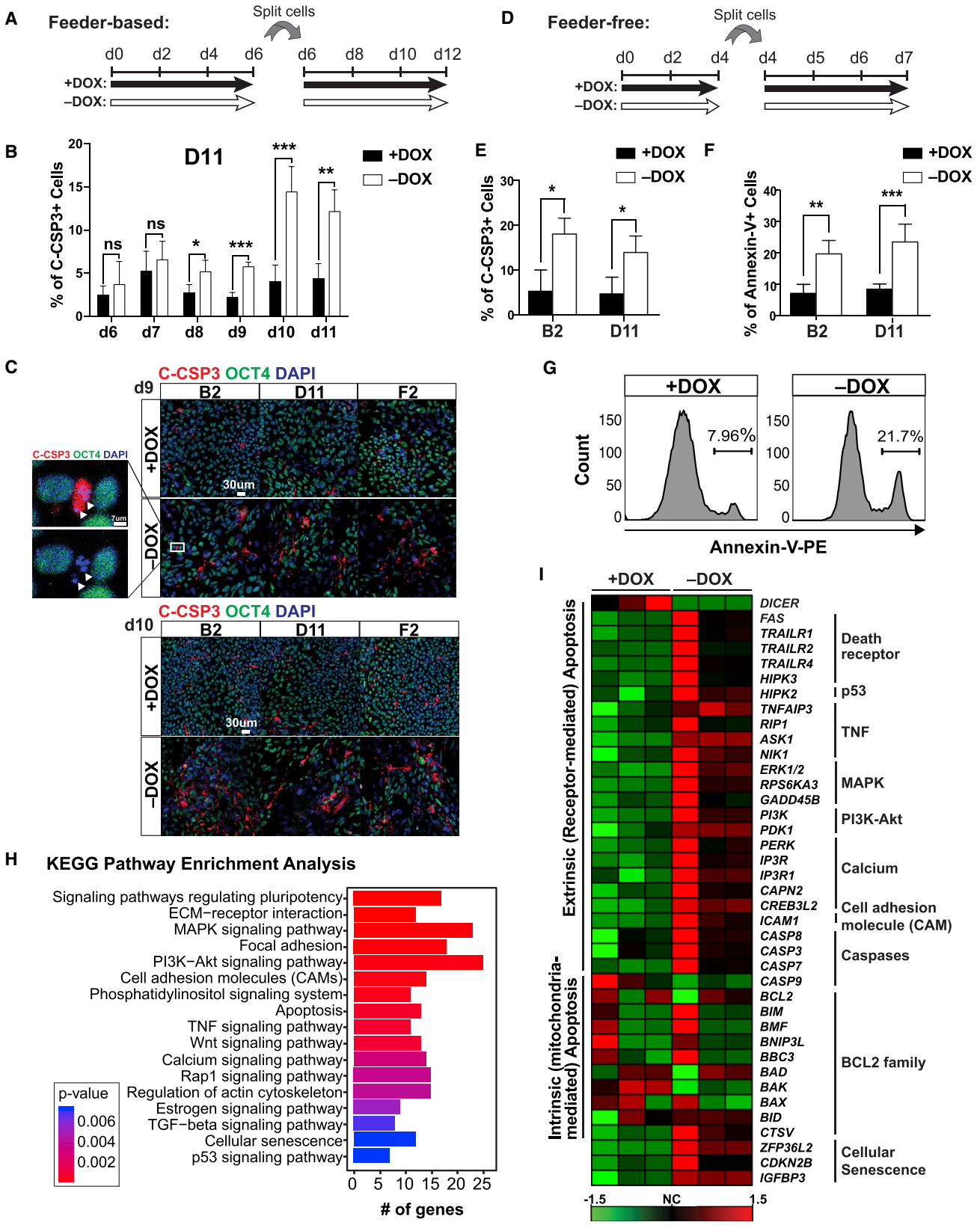
RNA sequencing (RNA-seq) analyses identified 692 significantly upregulated and 277 significantly downregulated genes in *DICER1*-depleted hESCs on day 9 of doxycycline withdrawal in the feeder-based condition compared with the doxycycline-treated control (Figure S3G). As expected, *DICER1* was the most significantly downregulated

(E) qRT-PCR analysis of four hESC-abundant miRNAs (*miR-373*, *miR-302a*, *miR-92a*, and *miR-200c*) at days 6 through 9 of DOX withdrawal. miRNA levels in the +DOX samples are used for comparison. Analysis was done on the B2 *DICER1* conditional knockout line in feeder-based condition. For each time point, statistical analysis was performed in comparison with +DOX samples. Day-6 data are reused from (D) for comparison purposes. n = 3 independent experiments.

(F) AP staining of three conditional *DICER1* knockout lines in feeder-based conditions with and without DOX treatment for 12 days. n = 6 independent experiments. Scale bar, 5 mm.

(G) AP staining of three conditional *DICER1* knockout lines in feeder-free condition with and without doxycycline treatment for 8 days. n = 3 independent experiments. Scale bar, 5 mm.

Error bars indicate SD, and significance is indicated as *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001; ns, not significant ($p \geq 0.05$). See also Figure S2.



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gene (Figure S3G). KEGG pathway enrichment analysis of these differentially expressed genes identified pathways involved in apoptosis, pluripotency, and cell adhesion (Figure 3H), while gene ontology analysis revealed mostly development-related terms (Figure S3I). In particular, *DICER1* deletion led to increased expression of pro-apoptotic genes of the extrinsic (receptor-mediated) pathway, notably *FAS*, *NIK* (*MAP3K14*), and *TRAILR4* (*TNFRSF10D*) as verified by qRT-PCR analyses (Figures 3I and S3H). Key genes involved in the intrinsic (mitochondria-mediated) apoptotic pathway were generally not upregulated in our RNA-seq analysis or qRT-PCR validation, except for *BIM* (Figures 3I and S3H). *DICER1*-depleted hESCs did not show increased expression of *BNIP3L*, an intrinsic pathway pro-apoptotic factor upregulated upon *miR-302/367* knockdown in hESCs (Figures 3I and S3H) (Zhang et al., 2015). This difference could be due to perturbing global versus specific miRNAs, or the knockdown versus the knockout approach. Together, these results demonstrate a previously unappreciated role for DICER1 in preventing receptor-mediated apoptosis in hESCs.

miRNA Rescue of *DICER1* Deficiency

To further understand the *DICER1*-dependent hESC survival, we attempted to rescue the *DICER1* knockout phenotype by transfecting *DICER1*-depleted hESCs with selected miRNA families (Figure 4A). We tested the ESCC-family of miRNAs (*mir-302-367*, *mir-371-373*, and *mir-17-92*) since they have been previously implicated in mESC proliferation and they are among the most abundant miRNAs in both mouse and human ESCs (Greve et al., 2013; Wang et al., 2008). The most striking rescues were observed for members of the *mir-302-367* and *mir-371-373* clusters based on alkaline phosphatase staining and cell number counts (Figures 4B and S4B). The transfected cells expressed pluripotency markers OCT4 and NANOG (Figure S4A). Although members of the *mir-17-92* cluster were sufficient to rescue the proliferation defect in miRNA-depleted mESCs (Wang et al., 2008), their effects were relatively mild in hESCs (Figures 4B and S4B), suggesting differences between mouse and human ESCs in miRNA requirements. Interestingly, not all members of the *mir-302-367* and *mir-371-373* clusters rescued the *DICER1* knockout hESC phenotype. In the *mir-302-367* cluster, *miR-302a*, *miR-302b*, *miR-302c*, and *miR-302d* showed significant rescue but miR-367 did not (Figures 4B and S4B). Likewise, in the *mir-371-373* cluster, *miR-372* and *miR-373* but not *miR-371* showed significant rescue (Figures 4B and S4B). Notably, the miRNAs with significant rescue effects all shared the ESCC seed sequence "AAGUGC" (Figure 4C).

We also observed similar rescue of cell survival in the E8 feeder-free condition, and annexin-V staining showed a significant reduction of apoptotic rates in *DICER1*-deficient hESCs transfected with *miR-372*, *miR-373*, *miR-302b*, and *miR-302d* (Figures 4D, 4E, and S4C). *In silico* analysis using TargetScan and MirTarget (Agarwal et al., 2015; Wong and Wang, 2015) showed that *FAS*, *NIK*, *TRAILR4*, and *BIM* genes, significantly upregulated in *DICER1*-depleted hESCs, were predicted targets of the ESCC seed sequence-bearing miRNAs (Table S1). Therefore, we examined whether their upregulation could be reversed in hESCs transfected with *miR-372* or *miR-302b*. By qRT-PCR

Figure 3. DICER1 Depletion in hESCs Causes Caspase-3-Mediated Apoptosis

(A) Schematic representation of the experimental design in feeder-based condition. Cells were split at 28,000 cells/cm^2. DOX, doxycycline.

(B) Flow cytometry of cleaved caspase-3 (C-CSP3) from days 6 through 11 in the D11 conditional *DICER1* knockout line with and without DOX in feeder-based condition. n = 3 independent experiments.

(C) Representative immunofluorescence pictures of C-CSP3 and OCT4 in the D11 conditional *DICER1* knockout line maintained with and without DOX at days 9 and 10 in feeder-based condition. Inset represents a close-up view showing fragmentation (arrowheads) of nuclei and loss of OCT4 in a C-CSP3-positive cell. Scale bars, 30 μm (main images) and 7 μm (close-up). n = 3 independent experiments.

(D) Schematic representation of the experimental design in feeder-free condition. Cells were split at a density of 14,000 cells/cm^2.

(E) Flow-cytometry detection of C-CSP3 expression in B2 and D11 conditional *DICER1* knockout lines in feeder-free condition maintained with and without DOX for 7 days. n = 4 independent experiments.

(F) Flow cytometry of annexin-V in B2 and D11 conditional *DICER1* knockouts cultured in feeder-free condition maintained with or without DOX for 7 days. n = 4 independent experiments for B2, and n = 5 independent experiments for D11.

(G) Representative histograms of annexin-V flow cytometry on D11 conditional *DICER1* knockout line maintained with or without DOX in feeder-free condition. n = 5 independent experiments.

(H) KEGG pathway enrichment analysis was performed in R (v3.4.2) with clusterProfiler (v3.6.0) on RNA-seq data of B2 conditional *DICER1* knockout line cultured in feeder-based condition with or without DOX for 9 days. n = 3 independent experiments.

(I) Heatmap of apoptosis-related genes. Data are derived from RNA-seq of B2 conditional *DICER1* knockout hESCs with and without DOX for 9 days in feeder-based condition. The heatmap was generated by cluster analysis using the online analysis tool CARMAweb (https://carmaweb.genome.tugraz.at/genesis) with the methods normalized gene. The variation between the biological repeats could be due to the rapid nature of apoptosis. n = 3 independent experiments.

Error bars indicate SD, and significance is indicated as *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant ($p \geq 0.05$). See also Figure S3.

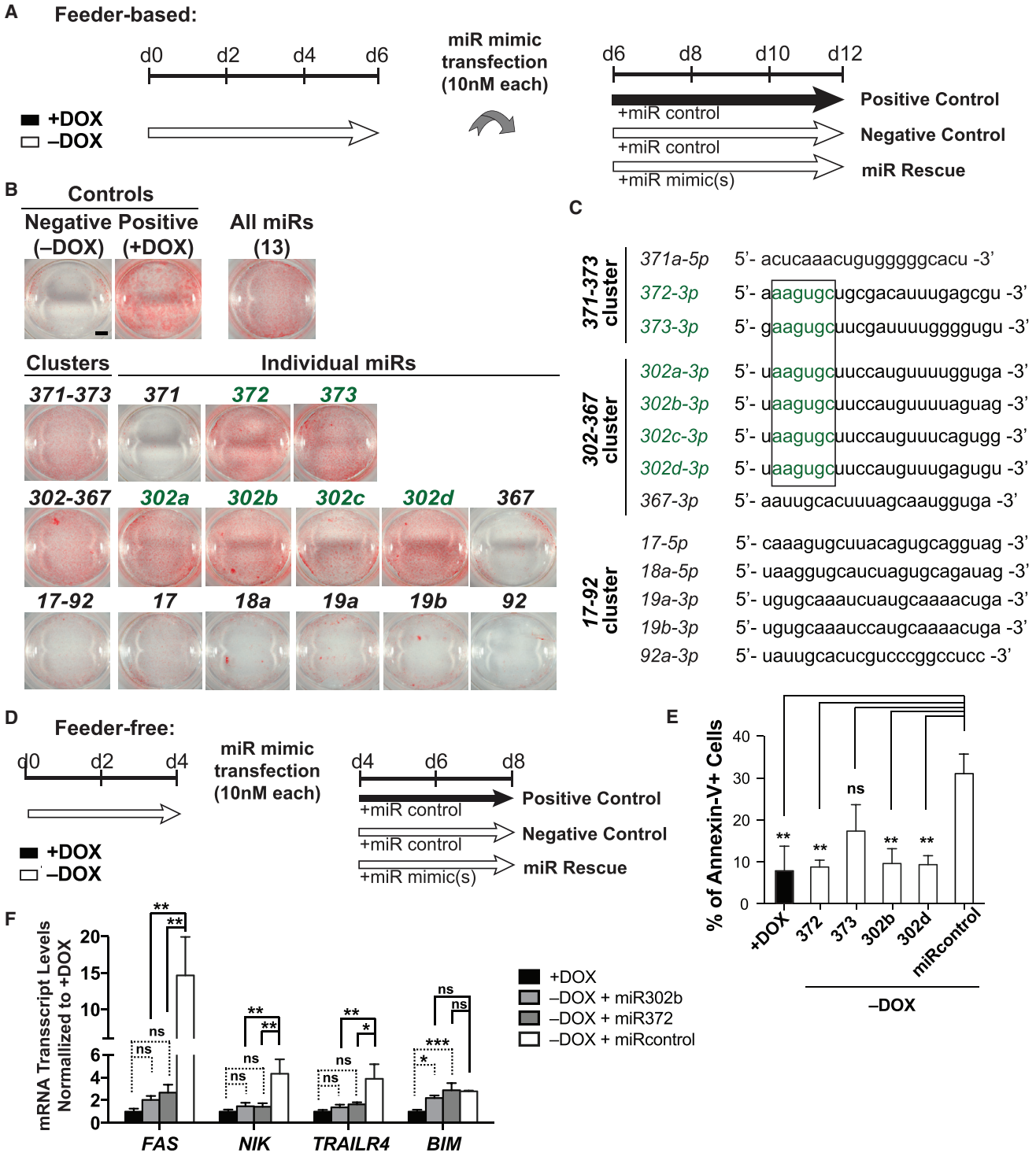

Figure 4. ESCC Seed Sequence-Containing miRNAs Rescue *DICER1* Knockout Defects

(A) Schematic representation of miRNA mimic transfection procedure in feeder-based condition. DOX, doxycycline.

(B) Representative AP-stained D11 conditional *DICER1* knockout hESCs with or without transfection of miRNAs in feeder-based condition. In green are the six miRNAs that partially rescue the *DICER1* knockout phenotype. n = 5 independent experiments. Scale bar, 5 mm.

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analysis, the expression levels of *FAS*, *NIK*, and *TRAILR4*, but not *BIM*, were significantly rescued to levels close to those of doxycycline-treated control hESCs expressing *DICER1* (Figure 4F). These results suggest that the ESCC seed sequence-bearing members of *mir-302-367* and *mir-371-373* clusters are responsible for preventing apoptosis in *DICER1* knockout hESCs primarily through the regulation of death receptor-mediated apoptosis pathway genes such as *FAS*.

DISCUSSION

Overlapping functions of miRNAs pose a technical challenge to fully understanding their exact roles during early embryonic development (Greve et al., 2013). Our conditional *DICER1* knockout hESCs provide an excellent platform to investigate the roles of individual miRNAs (Park et al., 2010). For instance, we find that reintroduction of ESCC seed sequence-containing miRNAs of the *mir-302-367* and *mir-371-373* clusters is sufficient to promote cell survival in *DICER1* knockout hESCs, whereas *mir-17-92* does not play a significant role in hESCs in this context despite its prominent role in mESC cell-cycle progression (Wang et al., 2008). Our platform is a valuable tool for the discovery of novel miRNA requirements for both hESC pluripotency maintenance and stem cell differentiation.

In contrast to the essential requirement for DICER in hESCs, Dicer1 is not required for mESC survival. This could be due to the different developmental stages thought to be represented by mouse and human ESCs. Supporting this idea, recent studies have uncovered essential requirements for *EZH2* and *DNMT1* in hESCs, which are not observed in mESCs (Collinson et al., 2016; Liao et al., 2015; Shen et al., 2008; Tsumura et al., 2006). In this regard, it may be more appropriate to compare *DICER1* knockout phenotypes in hESCs with those observed in mouse epiblast stem cells (mEpiSCs). In mEpiSCs, *Dicer1* deletion causes increased *Bim* expression, leading to activation of the intrinsic apoptotic pathway (the mitochondrial pathway); these effects are mitigated by reintroduction of *miR-20*, *miR-92*, and *miR-302* family members into *Dicer1*-depleted cells (Pernaute et al., 2014). Although we also observed some upregulation of *BIM* expression in *DICER1*-depleted hESCs, there was no clear rescue of *BIM* expression upon reintroduction of *miR-302b* or *miR-372*. Instead, *DICER1*-depleted hESCs upregulate a cohort of extrinsic apoptotic pathway genes. Reintroduction of *miR-302b* or *miR-372* in *DICER1* knockout cells rescued the cell-survival defect and reversed the aberrant upregulation of extrinsic pathway genes *FAS*, *NIK*, and *TRAILR4*. It is of interest to note that the human, but not the mouse, *FAS* and *TRAILR4* 3′ UTR sequences were predicted by *in silico* analyses to contain the ESCC targets (Table S1). These differences may explain the differing roles for DICER1 in hESCs compared with mESCs or mEpiSCs.

In addition to the human-mouse species difference, the pro-survival requirement for *DICER1* also depends on the cellular context. Mouse studies have shown that Dicer1 is essential for the survival of a number of developmental cell types such as limb mesoderm, αβ T cells, and melanocyte stem cells and differentiated melanocytes (Cobb et al., 2005; Harfe et al., 2005; Levy et al., 2010). On the other hand, Dicer1 is not required for survival of the γδ T cells (Cobb et al., 2005), and it is dispensable in the specification and survival of the fetal adrenal cortex (Krill et al., 2013). In HCT116 human colorectal cancer cells, DICER1 is also not essential for survival (Kim et al., 2016). Combined with directed differentiation, our *DICER1* conditional knockout hESC lines can be used in the future to determine cell-context-dependent DICER1 and miRNA functions, and inform us about human-specific aspects of post-implantation development.

EXPERIMENTAL PROCEDURES

hESC Culture

All experiments were performed on HUES8 hESCs (NIHhESC-09-0021). HUES8 iCas9 cells were generated as previously described (Gonzalez et al., 2014). For experiments conducted in the feeder-based condition, irradiated mouse embryonic fibroblasts were used as feeder cells, and hESCs were cultured in DMEM/F12

(C) List of mature miRNAs (3p or 5p arms) transfected in D11 conditional *DICER1* knockout line. In green are the mature miRNAs that significantly rescued the *DICER1* knockout phenotype. The box encloses the seed sequence shared among those mature miRNAs that rescue the *DICER1* knockout phenotype.

(D) Schematic representation of miRNA mimic transfection procedure in feeder-free condition.

(E) Flow cytometry of annexin-V in D11 conditional *DICER1* knockout line cultured in feeder-free conditions for 8 days and transfected with mature miRNAs or control miRNA as shown in (D). For comparison between multiple experimental groups in (E) and (F), one-way ANOVA was used followed by multiple comparisons with Tukey correction. n = 3 independent experiments.

(F) qRT-PCR analysis of representative pro-apoptotic genes belonging to the extrinsic apoptotic pathway (*FAS*, *NIK*, *TRAILR4*) as well as intrinsic marker *BIM* in feeder-free condition with and without doxycycline treatment for 8 days and transfected with *miR-302b*, *miR-372*, or *miR* control as shown in (D). n = 3 independent experiments.

Error bars indicate SD, and significance is indicated as *p < 0.05, **p < 0.01, and ***p < 0.001; ns, not significant ($p \geq 0.05$). See also Figure S4.

(without HEPES) supplemented with 20% KnockOut Serum Replacement, 1× nonessential amino acids, 1× GlutaMAX, 100 U/mL penicillin/100 μg/mL streptomycin (Gemini), 0.055 mM 2-mercaptoethanol, and 10 ng/mL recombinant human basic fibroblast growth factor. Cultures were passaged at a 1:12 split ratio every 4–6 days using TrypLE to dissociate the cells. Rho-associated protein kinase inhibitor Y-27632 (Selleck Chemicals, S1049) (5 μM) was added to the culture medium when passaging cells.

For experiments conducted in the feeder-free condition, hESCs were cultured in the complete E8 medium (Thermo Fisher Scientific, A1517001) on vitronectin (VTN-N; Thermo Fisher Scientific, A14700) pre-coated plates. Cells were replated at 125,000 cells/mL in 6-well plates and passaged every 4 days after cell dissociation using 0.5 mM EDTA (KD Medical, RGE-3130). The hESC culture work was conducted as per NIH guidelines and approved by the Tri-SCI Embryonic Stem Cell Research Oversight Committee.

miRNA Mimics Rescue Assay

For the miRNA mimics rescue experiments, *DICER1* knockout hESCs (B2 and D11 lines) were cultured with or without doxycycline for 6 days (feeder-based condition) or 4 days (feeder-free condition). They were replated at 125,000 cells/mL in 24-well plates in feeder-based conditions and at 125,000 cells/mL in 12-well plates in feeder-free conditions, and transfected while in suspension with miRNA mimics (*mir*Vana miRNA mimics; Thermo Fisher, 4464066) using Lipofectamine RNAiMAX (Thermo Fisher Scientific, 13778150) as per manufacturer's instructions. For the positive control, doxycycline was added back. For the negative control, a control miRNA mimic that contains a nonspecific sequence that does not bind to any mRNAs was transfected. The final concentration of each miRNA mimic was 10 nM, as previously described (Wang et al., 2008). The transfected cells were allowed to grow without doxycycline for 6 days (feeder-based condition) or 4 days (feeder-free condition), at which point the cells were harvested for analyses.

Statistical Analysis

All values are shown as mean ± SD. Data were analyzed using unpaired two-tailed Student's t test in GraphPad Prism unless otherwise indicated. p values of ≥ 0.05 were considered not significant (ns) and p values of <0.05 (*), <0.01 (**), <0.001 (***), and <0.0001 (****) were considered significant.

ACCESSION NUMBERS

All sequencing datasets are available from the Gene Expression Omnibus under accession number GEO: GSE113375.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.07.013.

AUTHOR CONTRIBUTIONS

V.T. led, designed, and performed most experiments; D.Y. performed most of the revision experiments and assisted with flow cytometry; S.M. performed northern blots; F.G. cloned the *DICER1** vectors; R.K. and C.X. analyzed the RNA-seq data; R.W.R. and N.V. assisted with additional experiments; D.H. and E.C.L. supervised the study; V.T. and D.H. wrote the manuscript; all other authors provided editorial advice.

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