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An Early Developmental Role for miRNAs in the Maintenance of Extraembryonic Stem Cells in the Mouse Embryo

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SUMMARY

The two first cell fate decisions taken in the mammalian embryo generate three distinct cell lineages: one embryonic, the epiblast, and two extraembryonic, the trophoblast and primitive endoderm. miRNAs are essential for early development, but it is not known if they are utilized in the same way in these three lineages. We find that in the pluripotent epiblast they inhibit apoptosis by blocking the expression of the proapoptotic protein Bcl2l11 (*Bim*) but play little role in the initiation of gastrulation. In contrast, in the trophectoderm, miRNAs maintain the trophoblast stem cell compartment by directly inhibiting expression of *Cdkn1a* (p21) and *Cdkn1c* (p57), and in the primitive endoderm, they prevent differentiation by maintaining ERK1/2 phosphorylation through blocking the expression of Mapk inhibitors. Therefore, we show that there are fundamental differences in how stem cells maintain their developmental potential in embryonic and extraembryonic tissues through miRNAs.

INTRODUCTION

At the time of implantation, the mouse embryo is composed of three cell lineages, the epiblast, the trophectoderm, and the primitive endoderm (PE). While the epiblast gives rise to the embryo proper, the trophectoderm and the PE form all the extraembryonic cell types of the placenta and yolk sac. These three cell lineages are thought to be segregated between 2.5 and 3.5 days post coitum (dpc) by a series of reciprocal repressive interactions involving *Cdx2*/*Oct4* for trophectoderm/epiblast segregation and *Nanog*/*Gata6* for epiblast/PE segregation (reviewed by Rossant and Tam, 2009). Insight into how the identity of these early lineages is maintained has been helped by the analysis of three different self-renewing stable progenitor cell types derived from the mouse blastocyst that mimic the behavior of the epiblast, trophectoderm, and PE. These are embryonic stem (ES) cells, trophoblast stem (TS) cells, and extraembryonic endoderm stem (XEN) cells. The undifferentiated ES cell state is dependent on the expression of a combination of transcription factors, most notably *Oct4*, *Sox2*, and *Nanog* and by signaling through Lif and Bmp4. TS cells are derived from the trophectoderm, require expression of *Cdx2*, *Eomesodermin (Eomes)* and other TS-specific transcription factors, and depend on Fgf and Activin/Nodal signaling for self-renewal. XEN cells show morphological similarities to PE derivatives, express lineage specific transcription factors such as *Gata6* and *Gata4*, and require exogenous Fgf signaling for their derivation (reviewed by Rossant, 2008).

miRNAs are endogenous noncoding small RNAs that act as critical posttranscriptional regulators of gene expression in both development and disease. miRNAs are transcribed in the nucleus and sequentially processed by the RNaseIII enzymes Drosha and Dicer into small (~22 nucleotide) RNAs that bind to the 3′ UTR of their target mRNAs. This association inhibits translation and can also lead to message destabilization (reviewed by Valencia-Sanchez et al., 2006). Knockout of *Dicer* leads to severe growth retardation and embryonic death by 7.5 dpc (Bernstein et al., 2003), and similar defects are observed after mutation of *Dgcr8*, a critical component of the Drosha microprocessor complex (Wang et al., 2007). ES cells lacking Dicer activity show proliferation defects and are unable to undergo differentiation (Kanellopoulou et al., 2005; Murchison et al., 2005; Sinkkonen et al., 2008). The differentiation block of *Dicer* mutant ES cells has been proposed to be due to an inability to methylate the *Oct4* promoter upon differentiation because of upregulation of *Rbl2*, a repressor of de novo DNA methyltransferases (*Dnmts*) (Sinkkonen et al., 2008). In addition to this function, miRNAs are also crucial components of the pluripotency regulatory network of ES cells, as core pluripotency factors both regulate the transcription of miRNAs and are targets of differentiation inducing miRNAs (Marson et al., 2008; Xu et al.,

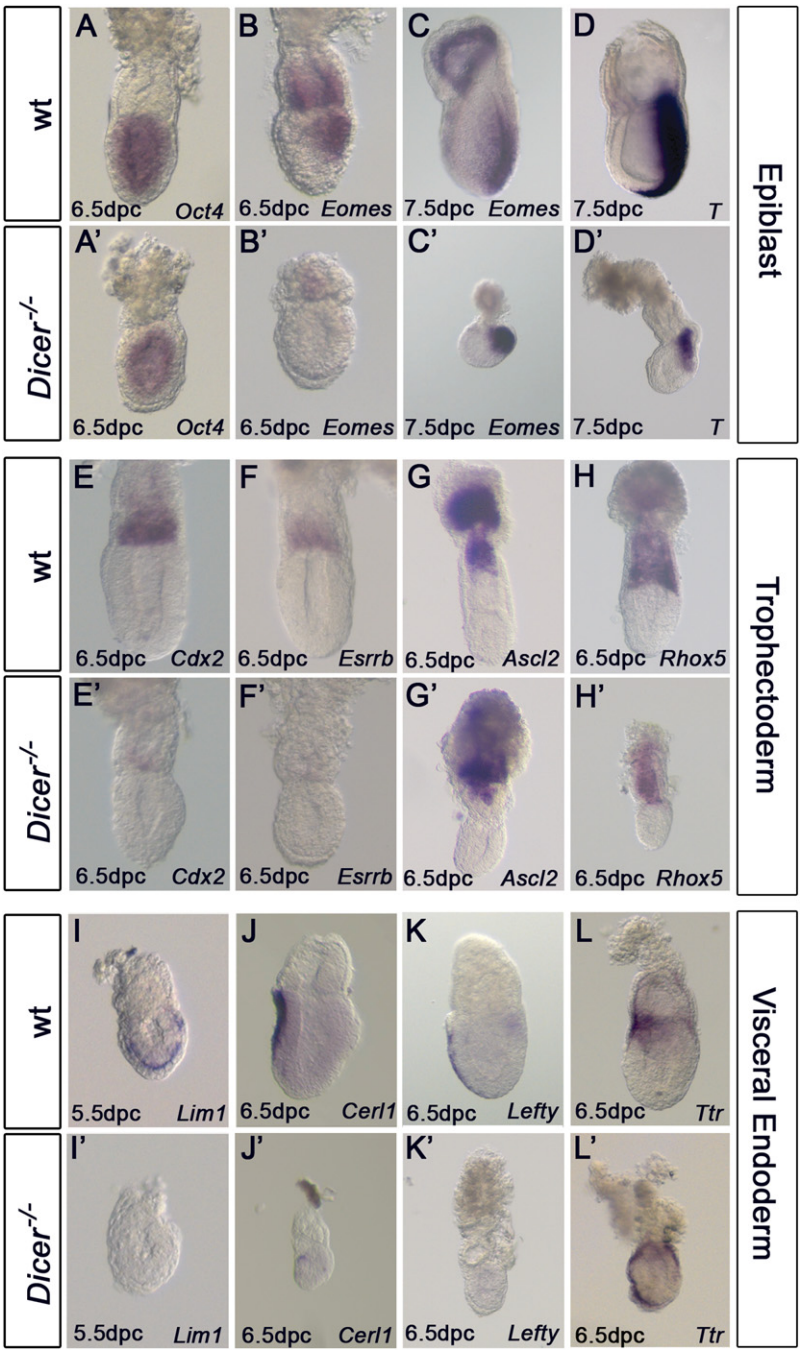

Figure 1. Delayed Initiation of Epiblast Patterning, Loss of the TS Compartment, and Misspecification of the Embryonic Visceral Endoderm in the Absence of *Dicer*

(A–L) Marker gene expression in control and (A′–L′) *Dicer* mutant embryos.

RESULTS

Patterning of the Epiblast Is Initiated but Delayed in *Dicer* Mutant Embryos

To address whether the epiblast is correctly specified in *Dicer* mutant embryos (Cobb et al., 2005), we studied the expression of the pluripotency marker *Oct4* and the early epiblast marker *Cripto*. Both these genes were strongly expressed in control and *Dicer* mutant embryos (Figures 1A, 1A′, and S1, available online), suggesting a normal maintenance of pluripotency and initial specification of the epiblast in the absence of *Dicer*. Gastrulation begins with the formation of mesoderm in the posterior epiblast at 6.5 dpc. To see if this process is correctly initiated in the absence of *Dicer*, we examined expression of *Eomes*. At 6.5 dpc, we found it not to be expressed in the majority of $Dicer^{-/-}$ embryos (Figures 1B and 1B′). In contrast to this, by 7.5 dpc, a stage at which *Dicer* mutant embryos are morphologically abnormal (Figures 1C′ and 1D′), *Eomes* and other markers of the primitive streak such as *T* and *Nodal* were expressed robustly and appropriately restricted to the posterior in the majority of $Dicer^{-/-}$ embryos (Figures 1C–1D′ and S1). Although the expression of these markers was restricted to the posterior side, in no case did we observe them to reach the distal tip of the embryo, indicating that there is a failure to elongate the primitive streak in *Dicer* null embryos. In addition to this, markers of the definitive endoderm such as *Hex* and *Cerl1* were drastically reduced or lost in the majority of mutant embryos at this stage (Figure S1), suggesting defects in definitive endoderm specification. These observations indicate that in the absence of miRNAs, initiation of gastrulation and mesoderm formation does take place, although with a delay with respect to wild-type embryos. However, the failure to elongate the primitive streak, the abnormal morphology of the $Dicer^{-/-}$ embryos, and the definitive endoderm patterning defects shows that progression of patterning is abnormal in mutant embryos.

miRNAs Are Required for Maintaining Trophectoderm Stem Cells and Correct Patterning of the Visceral Endoderm in the Embryo

To study how the trophectoderm derivatives are patterned in the absence of *Dicer*, we analyzed the expression of the trophoblast

2009). In contrast, little is known about the role of miRNAs in extraembryonic development or in TS and XEN cells. In order to bridge this gap, we have analyzed the developmental defects of extraembryonic structures in *Dicer* mutant embryos and the role of miRNAs in regulating the identity of extraembryonic stem cells. We have found that contrary to what occurs in the epiblast where miRNAs modulate cell survival and in ES cells where they are required for differentiation to occur, in the trophectoderm, PE, and the stem cells derived from these tissues, miRNAs act to maintain multipotency and self-renewal.

stem (TS) cell markers *Eomes*, *Cdx2*, and *Esrrb*. The expression of all these genes was severely downregulated or lost at 6.5 and 7.5 dpc in *Dicer*$^{-/-}$ embryos (Figures 1B–1C′, 1E–1F′, and S1; data not shown). However, the expression of markers of differentiated trophectodermal cell types such as *Ascl2* and *Rhox5* was still found in *Dicer* mutant embryos (Figures 1G–1H′ and S1). Loss of the TS stem cell population was not due to a disruption of the signaling interactions between the epiblast and extraembryonic ectoderm as the expression of *Fgf4* and *Nodal* in the epiblast as well as *Fgfr2, Spc1*, and *Spc4* in the extraembryonic ectoderm was still observed in *Dicer* mutant embryos at 6.5–7.5 dpc (Figure S1; data not shown). In addition, we observed correct phosphorylation of ERK1/2 in the extraembryonic ectoderm of these embryos (Figure S1). These results point to a specific requirement for miRNAs in maintaining the trophoblast stem cell compartment in the mouse embryo.

To determine how patterning of the visceral endoderm is affected in *Dicer* mutant embryos, we analyzed the expression of embryonic and extraembryonic visceral endoderm markers. We found a loss or severe downregulation in the expression of the embryonic visceral endoderm marker *Lim1* in 50% of mutant embryos analyzed at 5.5 dpc (Figures 1I and 1I′). At 6.5 dpc, expression of the anterior visceral endoderm marker *Cerl1* was downregulated in two-thirds of *Dicer*$^{-/-}$ embryos (Figures 1J and 1J′) and expression of *Lefty1* was lost in all mutant embryos analyzed (Figures 1K and K′). At 7.5 dpc, *Cerl1* and *Hex* expression was downregulated in 78% of *Dicer* null embryos analyzed Figure S1). In contrast to this, we observed that the expression of the extraembryonic visceral endoderm marker *Ttr* was expanded into the embryonic visceral endoderm of three-quarters of mutant embryos analyzed at 6.5 dpc (Figures 1L and 1L′). Therefore, in the absence of miRNAs, we find that the visceral endoderm is not correctly patterned.

The phenotype displayed by *Dicer* mutant embryos raises the possibility that the first three lineages to form in the embryo, the epiblast, the trophectoderm, and the primitive endoderm, can be correctly specified in the absence of miRNAs. However, processed miRNAs may still be present in *Dicer* mutant embryos at preimplantation stages due to perdurance of maternal Dicer. To address this, we examined in *Dicer*$^{-/-}$ morulas/blastocysts the expression of three processed members of the miR-290 miRNA cluster, which is highly expressed in preimplantation embryos (Tang et al., 2007). We observed that these miRNAs (miR-291-3p, miR-295, and miR291-5p) were expressed in a similar way in control and mutant embryos (Figures S1M–S1O′). This indicates that at the stage when the epiblast, trophectoderm, and primitive endoderm are specified, processed miRNAs are still present. For this reason, in this study, we were unable to determine if miRNAs have a role in the specification of the first lineages of the blastocyst.

miRNA Loss Leads to Different Effects on Proliferation and Apoptosis in Embryonic and Extraembryonic Tissues

Given the decreased size of *Dicer*$^{-/-}$ embryos compared with wild-types, we analyzed the rate of cell division and found that there was a significant decrease in the proportion of mitotic cells (PH3-positive) in both the trophectoderm (9.90% ± 3.33% versus 24.52 ± 1.75 in controls; Figures 2A, 2A′, and 2C) and visceral endoderm (3.21% ± 0.94% versus 15.54% ± 1.78% in controls; Figure 2C) of *Dicer*$^{-/-}$ embryos. In contrast to this, we saw no significant change in the proliferation rate in the epiblast of *Dicer* mutant embryos (35 ± 3.33 versus 29.61% ± 1.75% in controls; Figures 2B and 2C). Therefore, while proliferation defects are likely to contribute to the phenotypes observed in the extraembryonic structures, they are not the cause of the developmental delay in the epiblast.

This observation prompted us to analyze the patterns of cell death in *Dicer* mutant embryos. When we studied the distribution of apoptotic cells, we found a drastic increase in TUNEL positive nuclei in the epiblast but no apoptosis in either the trophectoderm or the visceral endoderm of *Dicer* null embryos at 5.5 and 6.5 dpc (Figures 2D–2E′). The specific increase in apoptosis found in the epiblast could be intrinsic to this tissue or secondary to the defects seen in extraembryonic tissues. To distinguish between these possibilities, we specifically deleted *Dicer* from the epiblast using the *Sox2Cre* line (Hayashi et al., 2002). *Dicer* deletion from the epiblast led to embryos that survived until 9.5 dpc (data not shown). In these embryos, as expected, the visceral endoderm and the trophoblast stem cell compartment were unaffected as indicated by the normal expression of *Cerl1* and *Cdx2* at 6.5 dpc (Figure S2). Similarly, a number of genes involved in anterior-posterior patterning such as *Six3*, *Cerl1*, *Hnf3b*, *Nodal*, or *Lim1* were expressed normally at 7.5–8.0 dpc in *Dicer*$^{fx/-}$*;Sox2Cre* embryos (Figure S2), showing correct establishment of the anterior-posterior axis. In contrast to this, we observed a dramatic increase in the levels of TUNEL staining at 9.0 dpc in *Dicer*$^{fx/-}$*;Sox2Cre* embryos (Figures 2F and 2F′), indicating that miRNAs are required specifically in the epiblast to inhibit apoptosis. The increased survival of the *Dicer*$^{fx/-}$*;Sox2Cre* embryos also suggests that the lack of correct development of the extraembryonic tissues is an important factor contributing to the primitive streak elongation failure, the abnormal morphology, and the definitive endoderm patterning defects seen in the null mutants.

Association of miRNAs with Argonaute (Ago) proteins is essential for miRNA induced gene silencing (Hutvagner and Simard, 2008). ES cells deficient for Ago1-4 show increased apoptosis due to the upregulation of the proapototic protein Bcl2l11 (Bim) (Su et al., 2009). When we analyzed *Dicer*$^{fx/-}$*;Sox2Cre* embryos, we found Bim was significantly upregulated when compared with controls (Figure 2G), indicating that this is the likely cause for the increased apoptosis observed in the epiblast in the absence of *Dicer*. miRNAs of the miR-17 to 92 and miR-106b to 25 clusters have been shown to directly target *Bim* (Fontana et al., 2008; Inomata et al., 2009; Kan et al., 2009; Ventura et al., 2008). For this reason, we analyzed by qPCR the expression of miR-17-5p, miR-19a, miR-92, and miR-25 that belong to these clusters, in 6.5 and 8.5 dpc embryos. At both stages, we detected high expression of these four miRNAs, with them showing expression levels comparable to those of the small nucleolar RNAs (snoRNAs) which are used as ''housekeeping genes'' for normalization of miRNA expression levels. Given that these are the stages at which we see increased apoptosis in the *Dicer* null and *Dicer*$^{fx/-}$*;Sox2Cre* embryos, these miRNAs are likely to be regulating Bim protein expression in the early mouse embryo.

Our data show that miRNAs are required to maintain the self-renewal and multipotency of the cells within the extraembryonic

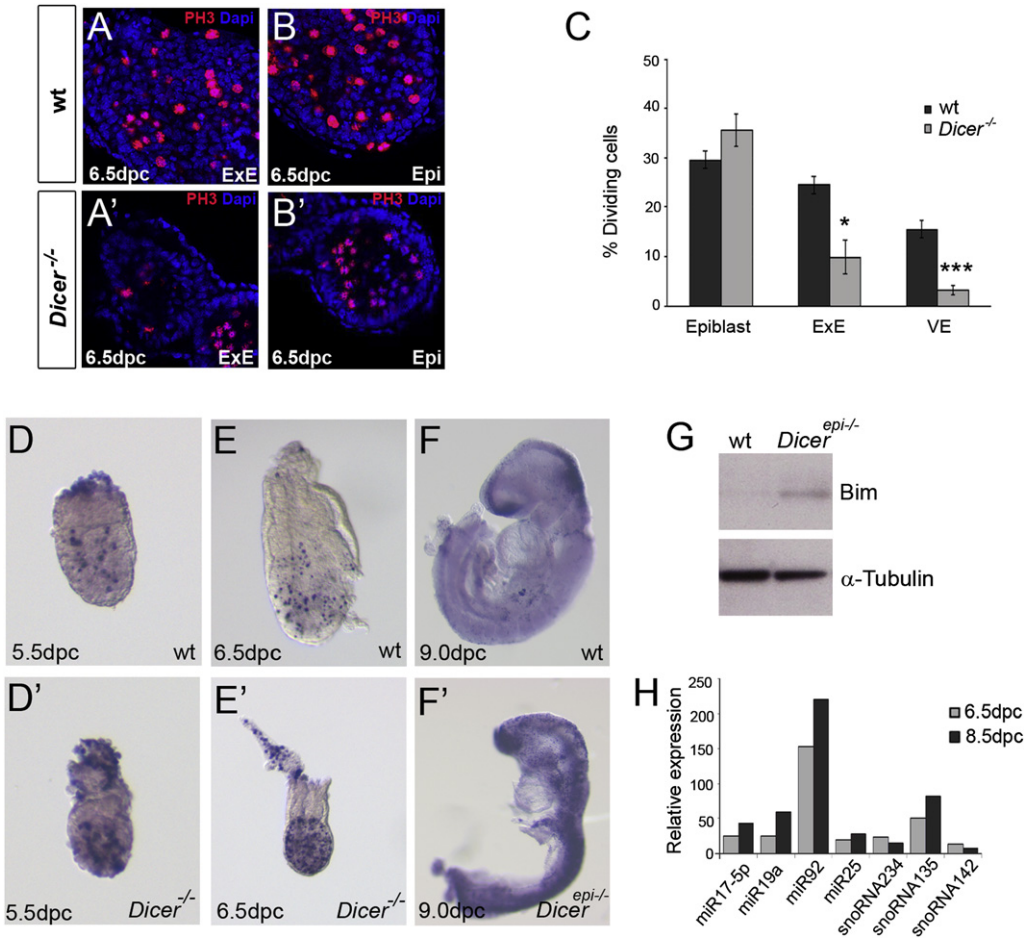

Figure 2. Dicer Is Required to Maintain Cell Proliferation in Extraembryonic Tissues and to Inhibit Apoptosis in the Epiblast

(A and B) Phospho-Histone H3 (PH3) expression in control and (A′ and B′) *Dicer* mutant embryos.

(C) Quantification of dividing cells in embryonic and extraembryonic tissues of *Dicer*$^{-/-}$ and control embryos. The average percentage of PH3-positive nuclei from nine wild-type and six *Dicer*$^{-/-}$ (±SEM) is shown.

(D–F′) TUNEL staining of wild-type, *Dicer* null, and epiblast-specific *Dicer*-deleted embryos.

(G) Expression levels of Bim in protein extracts from 9.5 dpc wild-type and epiblast-specific *Dicer*-deleted embryos.

(H) Relative expression levels of miRNAs regulating *Bim* and small nucleolar RNAs (snoRNAs) in pools of E6.5 and E8.5 wild-type embryos.

Student's t test. *$p < 0.05$ and ***$p < 0.001$ as indicated in the text.

tissues of the trophectoderm and visceral endoderm. In contrast, in the epiblast miRNAs are involved in controlling cell survival. Here, *Dicer* loss leads to increased Bim expression, apoptosis, and developmental delay but has no major effects on the initiation of gastrulation.

Maintenance of the Multipotency and Self-Renewal Capacity of TS and XEN Cells by miRNAs

To study in greater detail the roles of miRNAs in extraembryonic development, we isolated TS and XEN cell lines from embryos carrying a conditional mutation in *Dicer* (Cobb et al., 2005). *Dicer* deletion was carried out by infecting *Dicer*$^{fx/fx}$ TS cells with AdenoCre-GFP and sorting for GFP positive cells 48 hr post-infection. Genotyping of this sorted population confirmed the Cre-mediated deletion of *Dicer* (data not shown). As a control, *Dicer*$^{fx/fx}$ TS cells were infected with Adeno-GFP and sorted in parallel. Four days after infection, we observed a 300-fold reduction in the levels of *Dicer* mRNA and the appearance of the first signs of morphological differentiation (data not shown). By 7 days after AdenoCre-GFP infection, deleted TS cells presented a morphology characteristic of differentiated trophoblast giant cells, with a significantly larger cell size than controls, an enlarged nucleus (Figures 3A and 3A′) and an increase in the proportion of polyploid cells (data not shown). A severe proliferation block was also observed in these cells (Figure 3B), and for this reason we could not establish a stable *Dicer*-deleted TS cell line. When we analyzed changes in gene expression 7 days after AdenoCre-GFP infection by qPCR, we observed a downregulation in the expression of TS cell markers such as *Cdx2*, *Esrrb*, *Eomes*, and *Fgfr2*. This was accompanied by a strong increase in the expression of the trophoblast giant cell markers *Pl-1* and *Pai1*, and a smaller, but significant, increase in the expression of the trophoblast giant cell differentiation determinants *Stra13* and *Hand1* (Figure 3C). Therefore, after *Dicer* deletion we saw

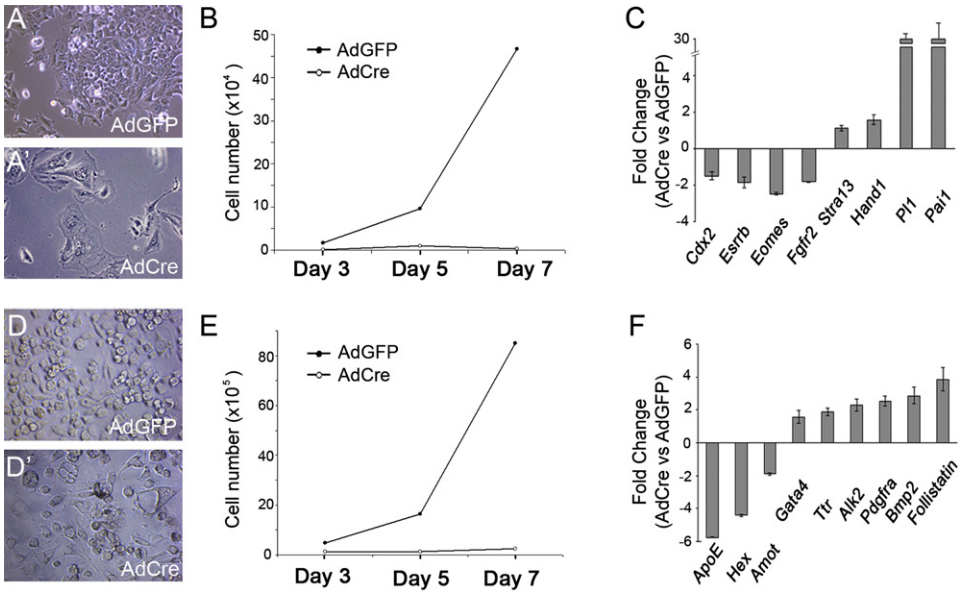

Figure 3. *Dicer* Loss Leads to Growth Arrest and Differentiation in TS and XEN Cells

(A) Morphology of Ad-Cre (A′) and Ad-GFP *Dicer*$^{fx/fx}$ TS cells 7 days postinfection.

(B) Growth rates of Ad-Cre and Ad-GFP-treated *Dicer*$^{fx/fx}$ TS cells.

(C) qRT-PCR analysis of differentiation and multipotency markers in Ad-Cre and Ad-GFP-treated *Dicer*$^{fx/fx}$ TS cells 7 days postinfection. The average fold change from two independent experiments (±SEM) is shown.

(D) Morphology of Ad-Cre and (D′) Ad-GFP-treated *Dicer*$^{fx/fx}$ XEN cells 7 days postinfection.

(E) Growth rates of Ad-Cre and Ad-GFP-treated *Dicer*$^{fx/fx}$ XEN cells.

(F) qRT-PCR analysis of differentiation and multipotency markers in Ad-Cre and Ad-GFP-treated *Dicer*$^{fx/fx}$ XEN cells 7 days postinfection. The average fold change from three independent experiments (±SEM) is shown.

clear morphological and molecular signs of differentiation of TS cells into trophoblast giant cells, accompanied by a decrease in proliferation.

Next, we tested the effects of *Dicer* deletion in XEN cells by infecting *Dicer*$^{fx/fx}$ cells with AdenoCre-GFP and sorting for GFP 24 hr postinfection, as described for TS cells. Similar to what occurred in TS cells, 4 days after infection we observed a 200-fold reduction in the levels of *Dicer* mRNA expression and clear changes is cell morphology (data not shown). By 7 days postinfection, *Dicer*-deleted XEN cells were significantly larger than controls, had big vacuoles, had extended large protrusions (Figures 3D and 3D′), and presented a block in proliferation (Figure 3E). At the molecular level, these cells presented a downregulation in the expression of embryonic visceral endoderm markers such as *Hex*, *ApoE*, and *Amot* and showed an increase in the expression of extraembryonic visceral endoderm markers such as *Gata4*, *Ttr*, *Alk2*, and *Bmp2* as well as an increase in the expression of parietal endoderm markers such as *Pdgfr*α and *Follistatin* (Figure 3F). These results parallel what we observed in *Dicer* mutant embryos, where the loss of embryonic visceral endoderm markers was accompanied by the expansion of extraembryonic visceral endoderm markers into the embryonic region (Figures 1J–1L′). Therefore, in XEN cells *Dicer* deletion leads to differentiation and a block in proliferation.

Together these data show that miRNA depletion in both TS and XEN cells recapitulates the defects we see in *Dicer*-deficient embryos. This indicates that the defects present in the trophectoderm and primitive endoderm are autonomous to these tissues. Therefore, both in vivo and in vitro, we see that depletion of miRNAs leads to a loss of multipotency and self-renewal in the extraembryonic lineages of the early embryo.

Cell Cycle Control by miRNAs in Extraembryonic Stem Cells

Given the proliferation defects we observed in TS and XEN cells, we investigated whether inhibitors of cell cycle progression were upregulated upon *Dicer* deletion. In ES cells, upregulation of *Cdkn1a* (*p21*), *Rbl2*, and *Lats2*, which are a set of G1/S regulators targeted by miRNAs, contributes to the proliferation defects observed in these cells after loss of mature miRNAs (Wang et al., 2008). In both extraembryonic stem cell types, concomitant with the growth defects we also observed an upregulation of these genes when *Dicer* was deleted (Figures 4A and 4B). This suggests that this aspect of cell cycle control is shared between the different stem cells derived from the blastocyst.

In ES cells, these genes are regulated by members of the miR-290 cluster of miRNAs which is claimed to be ES cell specific (Houbaviy et al., 2003; Wang et al., 2008). However, when we analyzed the expression of three members of the miR-290 miRNA cluster in ES, TS, XEN, and MEF cells, we found that they were highly expressed in all stem cells but not in MEFs (Figure 4C). This suggests that the regulation of G1/S transition by miRNAs is shared between all three early embryonic stem cell types and furthermore shows that the miR-290 cluster is not ES cell specific as previously reported.

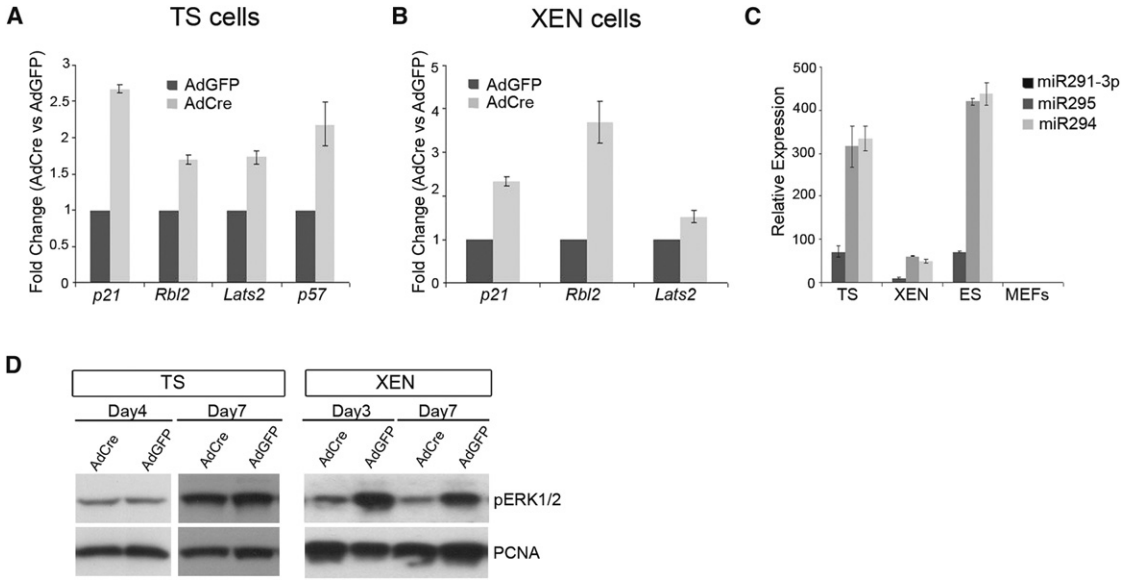

Figure 4. Dicer Regulates the Expression of Cell Cycle Inhibitors in TS and XEN Cells and Maintains ERK1/2 Signaling Levels in XEN Cells
(A) qRT-PCR analysis of the expression levels of cell cycle regulators in Ad-Cre and Ad- GFP-treated *Dicer*$^{fx/fx}$ TS and (B) *Dicer*$^{fx/fx}$ XEN cells 4 days postinfection. The average fold change from two independent experiments (±SEM) is shown. (C) Expression levels of miR-291-3p, miR-294, and miR-295 in XEN, TS, ES, and MEF cells. The data shown for each cell type represent the average expression of two cell lines (±SEM). (D) Western blot analysis of phospho-ERK1/2 expression levels in Ad-Cre and Ad-GFP-treated *Dicer*$^{fx/fx}$ TS and *Dicer*$^{fx/fx}$ XEN cells at day 3 postinfection.

Entry into the endoreduplication cycle is a critical step for differentiation into trophoblast giant cells and is driven by the upregulation of a regulatory network of genes that ensures mitotic arrest but continued progression through S phase. In TS cells, the upregulation of the Cdk inhibitors Cdkn1c (p57) and Cdkn1a (p21) block entry into mitosis and promotes their differentiation into trophoblast giant cells (Ullah et al., 2008). Given that *Dicer*-deficient TS cells show growth arrest and differentiate into trophoblast giant cells (Figures 3A, 3A′, and 3C), we investigated if, together with *p21*, *p57* is also affected by loss of miRNAs. We found that after *Dicer* deletion in TS cells, an upregulation of *p57* could be observed, something not seen in XEN cells (Figure 4A; data not shown). This indicates that miRNAs have an additional role in cell cycle control in the trophectoderm, where by regulating *p57* expression they block entry into the endoreduplication cycle and thus differentiation into trophoblast giant cells.

Regulation of ERK1/2 Signaling by miRNAs in XEN Cells

In the embryo, the Fgf signaling pathway is required for the maintenance of TS cells and the formation of the primitive endoderm (Chazaud et al., 2006; Nichols et al., 2009; Tanaka et al., 1998; Yamanaka et al., 2010). In the absence of *Dicer*, we see a loss of TS cell identity and XEN cells exhibit growth arrest and loss of multipotency. For these reasons, we examined the status of activation of the Fgf signaling pathway after *Dicer* deletion. When this was done, we did not observe any significant change in the levels of ERK1/2 phosphorylation in the trophectoderm or in TS cells (Figures S1 and 4D). On the contrary, XEN cells suffer a drastic decrease in the levels of phosphorylated ERK1/2 as early as 3 days after AdenoCre-GFP infection (Figure 4D), which precedes any overt changes in XEN cell morphology.

To address if a decrease in ERK1/2 signaling could be the cause for the phenotypes observed in XEN cells, we blocked signaling using specific inhibitors for the Fgf receptors (SU5403 and PD17307) or for Mek (U0126), the kinase that phosphorylates ERK1/2. Interestingly we found that while Fgf receptor inhibitors had no effect on XEN cells, Mek inhibition led to growth defects and downregulation of embryonic visceral endoderm marker gene expression (Figure S3). Therefore, in XEN cells inhibition of ERK1/2 signaling recapitulates some of the phenotypes caused by miRNA depletion. We next asked if excess Fgf4 or Pdgf, ligands that can stimulate ERK1/2 phosphorylation (Figure 5A), could rescue the phenotypes caused by loss of Dicer. We found that treatment of *Dicer*-deleted XEN cells with either of these two factors rescued the morphological changes associated with miRNA loss and prevented the downregulation in the expression of the embryonic visceral endoderm markers *ApoE*, *Amot*, and *Hex*, markers associated with the multipotent state of XEN cells (Figures 5B and 5C). However, neither excess Fgf4 or Pdgf could block the upregulation of parietal or extraembryonic visceral endoderm markers and could only partially rescue the proliferation defects of *Dicer*-deleted XEN cells (Figure 5D; data not shown). Therefore, miRNA-mediated mechanisms independent of ERK1/2 signaling are likely to be contributing to these specific phenotypes. Together, these results indicate that in XEN cells miRNAs are involved in the maintenance of early markers of the undifferentiated visceral endoderm by regulating the Mapk signaling pathway.

We next screened for negative regulators of the pathway whose expression is upregulated in the absence of *Dicer* and therefore would result in decreased ERK1/2 activity. We found an increase in the expression of *Sulf2*, *Rasa2*, and *Dusp1* (Kupzig et al., 2006; Lamanna et al., 2008; Owens and Keyse, 2007) in

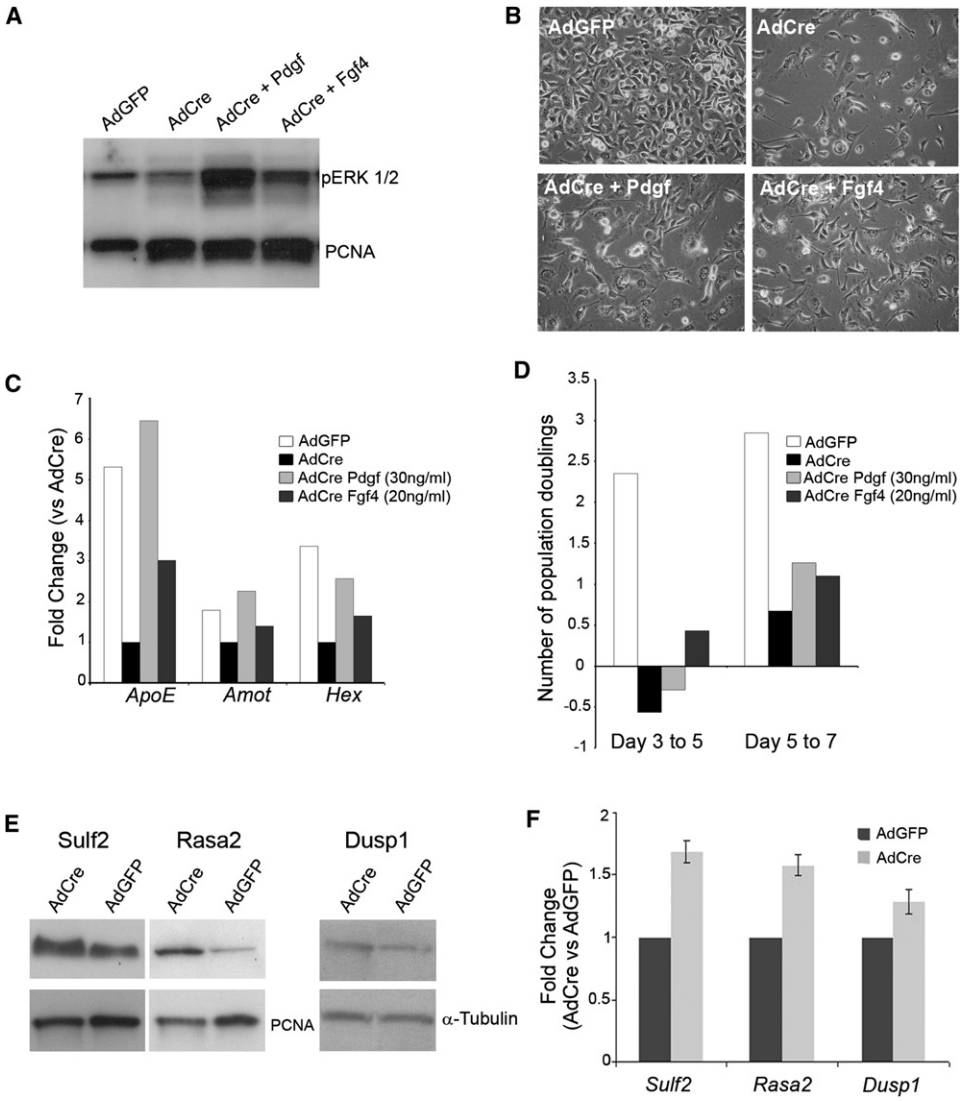

Figure 5. Loss of Multipotency in *Dicer*$^{-/-}$ XEN Cells Is Due to Decreased ERK1/2 Signaling

(A) Western blot analysis of phospho-ERK1/2 levels in Ad-GFP-treated *Dicer*$^{fx/fx}$ XEN cells and Ad-Cre-treated *Dicer*$^{fx/fx}$ XEN cells cultured in normal media or in the presence of Fgf4 or Pdgf at 3 days postinfection. (B) Morphology of Ad-GFP and AdCre *Dicer*$^{fx/fx}$ XEN cells left untreated or treated with the indicated growth factors at 7 days postinfection. (C) qPCR analysis of embryonic visceral endoderm markers and (D) cell division rate calculated as number of cell divisions per 2 days of Ad-GFP or AdCre *Dicer*$^{fx/fx}$ XEN cells treated with the indicated growth factors at 7 days postinfection. A representative experiment is shown. (E) Western blot and (F) qPCR analysis of the expression levels of *Sulf2*, *Rasa2*, and *Dusp1* in Ad-Cre and Ad-GFP-treated *Dicer*$^{fx/fx}$ XEN cells 4 days postinfection. The average fold change from three independent experiments (±SEM) is shown.

XEN cells upon *Dicer* deletion both at the mRNA and protein levels (Figures 5E and 5F). This suggests that in XEN cells, miRNAs maintain multipotency through ERK1/2 activation, by decreasing the levels of Mapk pathway inhibitors.

Identification of miRNAs Highly Expressed in TS and XEN Cells

To identify specific miRNAs highly expressed in the trophectoderm and primitive endoderm that could be responsible for maintaining the multipotent state, we carried out an expression screen by qPCR of 312 miRNAs in TS and XEN. As a point of comparison, we also analyzed the expression of these miRNAs in ES cells and mouse embryonic fibroblasts (MEFs). The first conclusion that can be drawn from this study is that the profiles of miRNA expression in XEN and TS cells are very similar and cluster together when compared to those of ES cells and MEFs (Figure 6A). miRNAs can be grouped into families according to a common seed site and therefore to a common set of target genes (Grimson et al., 2007) (from now on we will use a representative member to refer to the whole miRNA family; the other members of the family can be found in the database of TargetScan, www.targetscan.org). The fact that all 22 of the highly expressed miRNA families in XEN cells are also highly expressed in TS cells (Figure 6B) illustrates the degree of similarity between the XEN and TS miRNA expression profiles. These miRNA expression profiles of TS and XEN cells are in turn closer

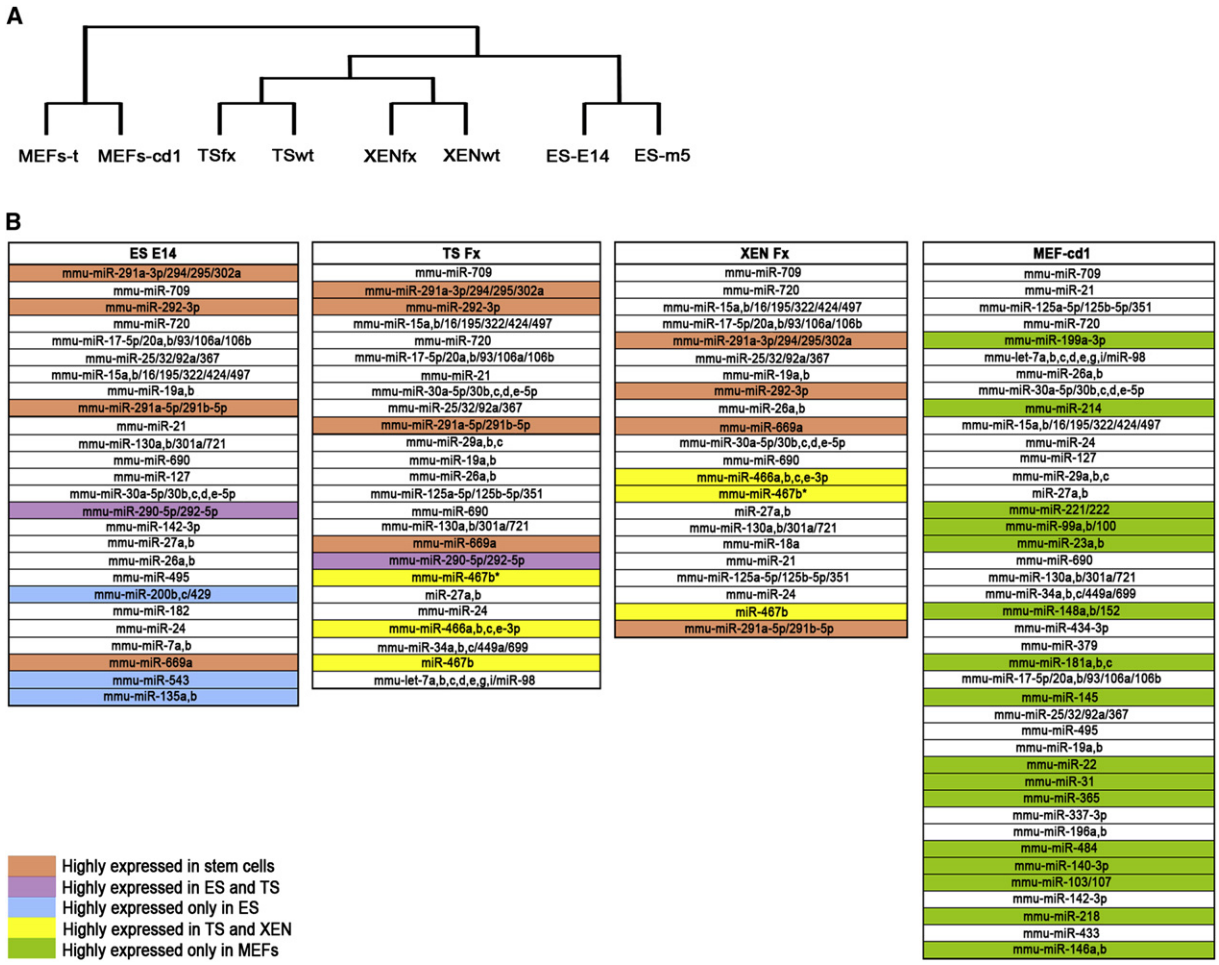

Figure 6. miRNA Expression Profiling of Blastocyst-Derived Stem Cells

(A) Hierarchical clustering of the miRNA expression profiles of TS, XEN, ES, and MEF cells.

(B) Tables showing all miRNA families with a relative expression greater than 10 in the cell lines shown. All miRNA family members found in the array are listed.

to the profile of ES cells than to those of MEFs, correlating with the stem cell nature and early embryonic origin of TS and XEN cells (Figure 5A). Interestingly (Figure 6B), we found three families of miRNAs that were specifically enriched in extraembryonic stem cells (miR-466a-3p, miR-467b and miR-467b*), four that were enriched in all three stem cell types (miR-291a-3p, miR-292-3p, miR-291a-5p, and miR-669a), one that was enriched in ES and TS cells (miR-290-5p) and three miRNA families that were enriched in ES cells only (miR-200b, miR-543, and miR-135a).

This study allowed us to identify 26 highly expressed families of miRNAs in TS cells and 22 highly expressed families of miRNAs in XEN cells (Figure 6B). These miRNAs made up 95% and 94% of the total quantity of miRNA examined in the two cell lines respectively and are likely to account for the majority of miRNA induced silencing. We used the miRNA target prediction program TargetScan to determine which of these miRNAs could be regulating the genes that we found to be responsible for the phenotypes observed in the trophectoderm and primitive endoderm (Figure 7A). Of the 26 highly expressed miRNA families identified in TS cells, we found two that were predicted to target *p57*, the miR-25 family and the miR-466a-3p family. A number of members of the miR-25 family have already been shown to directly inhibit p57, miR-92b has been shown to directly target *p57* in human ES cells (Sengupta et al., 2009) and miR-25 has been shown to target *p57* in gastric cancer cells (Kim et al., 2009). It is therefore likely that this family of miRNAs is regulating *p57* expression in the trophectoderm.

In XEN cells, we found a number of highly expressed miRNAs predicted to target *Rasa2*, *Dusp1*, and *Sulf2* (Figure 7A). Given that none of these miRNA families have been reported to target these genes, we tested if representative miRNAs could regulate the expression of a luciferase reporter attached to the 3′ UTR of *Rasa2*, *Dusp1*, or *Sulf2*. In the case of *Rasa2*, miR-20a, a member of the miR-17-5p family, miR-30b, a member of the miR-30 family, and miR-466a-3p, a member of the miR-466a-3p family, could all significantly decrease activity of the 3′ UTR-luciferase reporter of this gene in HEK293T cells, while miR-295 did not affect reporter expression (Figure 7B). Mutation of the predicted binding sites for miR-20a made the *Rasa2* 3′ UTR-luciferase reporter insensitive to the overexpression of this miRNA, indicating that it is through this site that miR-20a controls *Rasa2*

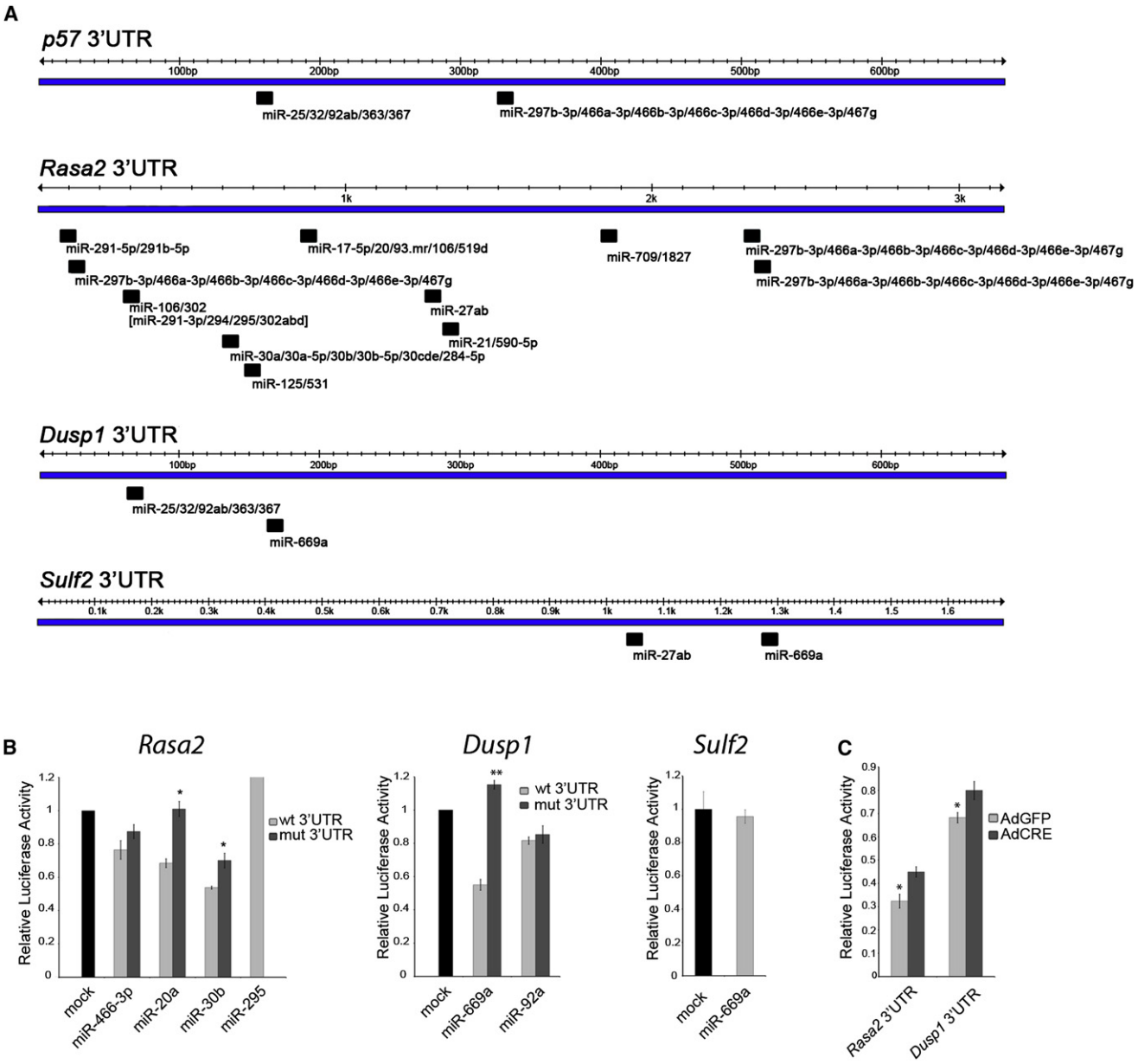

Figure 7. miRNAs that Are Highly Expressed in TS and XEN Cells Directly Target Cell Cycle Regulators and ERK Signaling Modulators

(A) Predicted target sites for miRNAs highly expressed in XEN or TS cells in the 3′ UTR of *Rasa2*, *Dusp1*, *Sulf2*, and *p57*.

(B) Validation of *Rasa2*, *Dusp1*, and *Sulf2* target sites by dual-Luciferase assay in HEK293T cells cotransfected with the stated miRNA. In the mutant versions of each 3′ UTR, four nucleotides from the seed site of the miRNA have been substituted.

(C) miRNA regulation of *Rasa2* and *Dusp1* 3′ UTR in control and *Dicer*-deleted XEN cells. Three independent experiments were conducted. The graphs displayed show the average, ±SEM. Student's t test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ of the effects of a miRNA binding site mutation.

expression. In contrast to this, mutation of the miR-30b site only partially rescued the 3′ UTR-luciferase reporter activity and mutation of the miR-466a-3p sites did not significantly affect the reporter's activity, suggesting that these miRNAs may be acting through other sites (Figure 7B). We also observed that the 3′ UTR-luciferase reporter of *Rasa2* shows significantly higher activity in *Dicer*-deficient XEN cells than in control XEN cells (Figure 7C). Therefore, together these findings support a direct role for miRNAs in regulating *Rasa2* expression.

With *Dusp1*, miR-669a and miR-92a, a member of the miR-25 family, decreased 3′ UTR-luciferase reporter activity but only mutation of the miR-669a predicted binding site restored activity, indicating that it is likely that other sites for miR-92a exist within the *Dusp1* 3′ UTR (Figure 7B). Again, the higher activity of the *Dusp1* 3′ UTR-luciferase reporter observed in *Dicer*-deficient XEN cells than in controls points to the direct regulation of *Dusp1* by miRNAs (Figure 7C). miR-669a, which is highly expressed in XEN cells and predicted to target *Sulf2*, did not affect expression

of this gene (Figure 7B). This suggests that either other miRNAs are regulating *Sulf2* expression in the primitive endoderm or that increased *Sulf2* expression is not a direct effect of miRNA depletion. Our studies have therefore identified that miRNAs belonging to the miR-17-5p, miR-25, miR-30, miR-466a-3p, and miR-669a families are repressing the expression of inhibitors of the Mapk pathway in the primitive endoderm and therefore are likely to be regulating the multipotency of this tissue.

DISCUSSION

Analysis of *Dicer*-deficient embryos and extraembryonic stem cell lines has allowed us to address what roles miRNAs play in the early embryo. In the epiblast of *Dicer*$^{-/-}$ embryos, gastrulation is initiated although a day later than in controls. This delay is likely due to the smaller size of the embryos as a critical cell number is necessary for the initiation of gastrulation (Tam and Behringer, 1997). The smaller size of *Dicer* mutant embryos is unlikely to be caused by proliferation defects as we see little change in the proliferation rates of epiblast cells from *Dicer*$^{-/-}$ embryos compared to controls. In contrast to this, we observe an increase in the levels of apoptosis in this tissue, and it is likely that this accounts for the reduced size and developmental delay observed in *Dicer* null embryos. This increase in apoptosis is also seen when *Dicer* is specifically deleted from the epiblast, although in this case it is only detected at a later developmental stage (7.5–8.5 dpc). *Dicer*$^{fx/-}$*;Sox2Cre* embryos present a much less severe phenotype than that seen in the full knockout, with embryos surviving to 9.5 dpc and displaying normal establishment of the embryonic axes. Given the essential role that extraembryonic tissues have on the patterning and nutrition of the epiblast, it is likely that the less severe phenotype observed in *Dicer*$^{fx/-}$*;Sox2Cre* embryos is at least in part due to the extraembryonic tissues being normal in these embryos. However, differences in the timing of the loss of mature miRNAs could also account for the delay in the appearance of the phenotype in the *Dicer*$^{fx/-}$*;Sox2Cre* embryos. In these embryos *Dicer* deletion would only occur at around 4.5–5.5 dpc (Hayashi et al., 2002), and from then there needs to be a loss of any residual Dicer protein and a subsequent loss of processed miRNAs. Therefore, mature miRNAs are likely to be lost later in *Dicer*$^{fx/-}$*;Sox2Cre* embryos than in *Dicer*$^{-/-}$ embryos. However, in both mutants the increase in apoptosis precedes any obvious morphological defects, supporting the hypothesis that elevated cell death is responsible for the majority of the phenotypes observed in embryonic tissues upon *Dicer* deletion.

In *Dicer*$^{fx/-}$*;Sox2Cre* embryos elevated apoptosis was accompanied by increased Bim expression. Bim is a proapoptotic protein (O'Connor et al., 1998) that can trigger apoptosis by neutralizing prosurvival Bcl2-like molecules and/or by activating the Bcl2 inhibitor Bax (Pinon et al., 2008). Indeed, when the miRNA RISC complex components *Argonaute1-4* are removed from ES cells, increased apoptosis is observed as a consequence of raised *Bim* expression (Su et al., 2009). Our observation that the members of the miR-17 to 92 and miR-106b to 25 clusters miR-17-5p, miR-19a, miR-92, and miR-25, previously shown to directly target *Bim* (Ventura et al., 2008), are highly expressed at stages when apoptosis is severely increased in *Dicer*$^{-/-}$ and *Dicer*$^{fx/-}$*;Sox2Cre* embryos, suggests that these miRNAs may be directly regulating apoptosis in the early mouse embryo.

In a previous analysis of the *Dicer*$^{-/-}$ mutation, the expression of *Oct4* and *T* were found to be much reduced in mutant embryos (Bernstein et al., 2003). It is possible that differences in the genetic background of the embryos analyzed could account for the increased severity of the previously published study. However, the extended analysis we have carried out both in the null mutant embryos as well as in embryos where *Dicer* has been removed specifically from the epiblast, strongly supports our view that miRNAs are not directly involved in the initiation of gastrulation and germ layer specification in the mouse embryo. Our findings also contrast with those made in ES cells where *Dicer*$^{-/-}$ cells cannot initiate the differentiation program (Kanellopoulou et al., 2005; Murchison et al., 2005; Sinkkonen et al., 2008) because of an inability to methylate and silence the *Oct4* promoter (Sinkkonen et al., 2008). Furthermore, *Dicer* and *Dcgr8*-deficient ES cells present growth defects attributed to a lengthening of the cell cycle (Kanellopoulou et al., 2005; Murchison et al., 2005; Sinkkonen et al., 2008; Wang et al., 2008). Previously maternal Dicer has been demonstrated to allow for correct processing of miRNAs during early preimplantation development (Murchison et al., 2007; Tang et al., 2007), and in this study we show that expression of processed forms of miRNAs of the miR-290 cluster persist at the blastocyst stage. Given that we only see increased apoptosis from 5.5 dpc and ES cells are derived from 3.5 dpc preimplantation embryos, it is likely that the dissimilar phenotypes seen in the *Dicer* null epiblasts and ES cells reflect different requirements for miRNAs during development rather than different roles of miRNAs in vivo and in vitro.

Very little is known about the roles of miRNAs in extraembryonic tissues. We find that in stark contrast to what happens in the epiblast, extraembryonic cell types require miRNAs both in vivo and in vitro to prevent differentiation and maintain the self-renewal capacity of progenitor cell types. This is most clearly seen in trophectoderm of *Dicer* null embryos where there is a loss of expression of genes that mark the TS cell compartment such as *Cdx2*, *Eomes*, or *Esrrb*, but not of genes found in trophoblast derivatives. This phenotype is recapitulated in vitro, with *Dicer* deletion in TS cells leading to differentiation and cell cycle arrest. These results argue that in *Dicer* null embryos, the TS cell population is correctly established but as development proceeds and differentiation progresses, this pool is quickly depleted due to an inability to maintain multipotency and self-renewal.

Two main signaling pathways have been shown to prevent the differentiation of TS cells and thus maintain their stem cell condition; Fgf signaling via ERK1/2 activation (Tanaka et al., 1998) and Activin signaling via Smad2/3 phosphorylation (Erlebacher et al., 2004; Natale et al., 2009). However, we see no change in the levels of ERK1/2 or Smad2/3 phosphorylation after *Dicer* deletion in TS cells (this study and data not shown). Similarly the expression of components of these pathways, as well as ERK1/2 phosphorylation appears normal in *Dicer* null embryos. Little is known regarding other mechanisms controlling TS cell multipotency. The overexpression of *Hand1* and *Stra13* has been previously shown to cause the differentiation of TS cells into trophoblast giant cells independently of ERK1/2

signaling (Hughes et al., 2004). However, 4 days after *Dicer* deletion in TS cells, when we first observe signs of morphological differentiation, neither of these genes is significantly upregulated (data not shown). More recently it has been demonstrated that inhibition of Cdk1 in TS cells causes entry into the endoreduplication cycle and differentiation into trophoblast giant cells (Ullah et al., 2008). It has also been shown that the Cdk1 inhibitor p57 is required for differentiation into trophoblast giant cells to occur (Ullah et al., 2008). Given that we observe an upregulation of the Cdk inhibitors *p57* and *p21* shortly after *Dicer* deletion in TS cells, it is likely that this is what drives their differentiation into trophoblast giant cells.

In the visceral endoderm and in XEN cells, we find that miRNAs are required to maintain multipotency. The ERK1/2 signaling pathway is essential for the specification of the primitive endoderm, formation of the visceral endoderm, and XEN cell maintenance (Liu et al., 2009; Nichols et al., 2009; Yamanaka et al., 2010; this study). Our work indicates that miRNAs maintain the embryonic visceral endoderm by modulating ERK1/2 signaling, via the inhibition of negative regulators of this pathway such as *Sulf2*, *Rasa2*, and *Dusp1*. Surprisingly, although Fgf signaling via ERK1/2 is required for TS cell multipotency (Tanaka et al., 1998), we have found no effect of miRNAs in modulating this pathway in TS cells, indicating the miRNAs contribute to the maintenance of multipotency of TS and XEN cells in different ways.

qPCR-based analysis of the miRNA expression profiles of TS and XEN cells has allowed us to precisely identify miRNAs that are highly expressed in these cell types, and that presumable account for most of the phenotypes observed after *Dicer* deletion. Interestingly, we find that the miRNA families that are highly expressed in XEN cells are also highly expressed in TS cells. This overlap is reflected when we analyze the miRNA families that are likely to regulate *p57* in the trophectoderm and that regulate the Mapk inhibitors in the primitive endoderm. For example, miRNAs of the miR-25 family have been shown to target *p57* (Kim et al., 2009; Sengupta et al., 2009), and we find that they also target *Dusp1* in XEN cells. Also, members of the miR-466a-3p family are predicted to target *p57* and we have shown that they target *Rasa2* in XEN cells. Therefore, it seems that a common set of miRNA families are important for maintaining multipotency in both extraembryonic tissues, although through the regulation of different genes.

Of particular interest are the miRNAs that we find to be enriched in TS and XEN cells with respect to ES cells and MEFs. These miRNAs, of the miR-466a-3p, miR-467b and miR-467b* families, cluster together and map to the intronic region of the *Sfbmt2* gene. *Sfmbt2* is a polycomb gene (Klymenko et al., 2006) that is imprinted (Kuzmin et al., 2008) and its expression is restricted to extraembryonic tissues (Frankenberg et al., 2007; Kuzmin et al., 2008). This suggests that the expression in TS and XEN cells of this cluster of miRNAs is likely to be due to coregulation with *Sfmbt2*. miRNA families within this cluster are predicted to target *p57*, and we have shown that they target *Dusp1* and *Rasa2* in XEN cells and therefore are likely to play important roles in the maintenance of both the trophectoderm and primitive endoderm.

We also found that, as occurs in ES cells (Wang et al., 2008), the expression of inhibitors of the G1/S transition, such as *p21*, *Rbl2*, and *Lats2*, is increased upon miRNA depletion in TS and XEN cells. Furthermore, we found that miRNAs of the miR-290 cluster, that regulate the cell cycle by blocking the expression of these inhibitors in ES cells and have previously been assumed to be ES cells specific (Houbaviy et al., 2003; Wang et al., 2008), are also highly expressed in extraembryonic stem cells. This argues that, in addition to the tissue-specific factors that control the cell cycle in TS cells (p57) and in XEN cells (ERK signaling), there is an additional level of regulation of cell cycle progression by miRNAs that is conserved in all three preimplantation lineages of the mouse embryo and is provided by miRNAs of the miR-290 cluster. The implication of miRNAs in the control of G1/S transition via regulation of *p21* has been described in stem cells from other organisms such as the germline stem cells of *Drosophila* (Hatfield et al., 2005; Yu et al., 2009). This suggests that this role for miRNAs is evolutionarily conserved and could be present in multiple different progenitor cell types of the embryo.

EXPERIMENTAL PROCEDURES

Mouse Lines

Dicer$^{fx/fx}$, *Dicer*$^{+/-}$ (Cobb et al., 2005), and *Sox2Cre*$^{+/-}$ mice (Hayashi et al., 2002) were maintained on a mixed background. Embryos were genotyped as described (Martinez Barbera et al., 2000) using published conditions.

Whole-Mount In Situ Hybridization, TUNEL Staining, and Immunohistochemistry

Whole-mount in situ hybridization (WISH) was carried following standard procedures (Thomas and Beddington, 1996). WISH for microRNA detection was carried out using 3′ DIG-labeled LNA probes (Exiqon) as described (Kloosterman et al., 2006) with the following modifications. Preimplantation embryos were dehydrated in serial dilutions of Ethanol (25%, 50%, and 70%) in saline (150 mM) and stored in 70% ethanol/saline at −20°C. When used, blastocysts were rehydrated in serial dilutions of ethanol/saline before being permeabilized for 10 min in fresh RIPA buffer and postfixed for 20 min in 0.2% glutaraldehyde /4% paraformaldehyde in PBS. Whole-mount TUNEL staining was carried out using the ApopTag Plus Peroxidase In Situ Apoptosis Kit (Chemicon International). Staining for Phospho-ERK (Cell Signaling) was performed as described (Corson et al., 2003) and for PH3 as described (Rivera-Perez et al., 2003) with the following modifications: embryos were cultured for 1 hr in N2B27 (Invitrogen) containing 200 nm nocodazole and after fixation embryos were washed in Triton-X100 (0.5% in PBS). Ten percent goat serum was used in the blocking solution. Anti-PH3 (1:500) was diluted in blocking solution diluted 1 in 10 in PBS-Triton-X100 (0.5%). Cell counting was performed on four mutant and six wild-type embryos using the ImageJ program (Abramoff et al., 2004). Three confocal sections were counted for each embryo. PH3-stained cells were expressed as a percentage of total cells and averaged to give overall percentage for wild-types and mutants. Statistical significance was measured using a two-tailed Student's t test ($p < 0.05$).

Cell Culture

TS and XEN cell lines were derived from *Dicer*$^{fx/fx}$ intercrosses and cultured as described (Kunath et al., 2005; Tanaka et al., 1998). Cells were infected with either Ad-GFP or AdCre-GFP adenovirus (University of Iowa Gene Transfer vector Core). GFP-positive cells were purified by FACS either 24 hr (for XEN cells) or 48 hr (for TS cells) after infection. *Dicer*$^{fx/fx}$ XEN cells were treated with Fgf4 (20 ng/ml), Pdgf (30 ng/ml) (R&D Systems), PD17307 (100 ng/ml), SU5043 (10 μM), U0126 (25 μM), or DMSO (1:1000) or left untreated for up to 7 days. For growth curves cumulative population doublings per passage were calculated as log2 (number of cells at time of subculture divided by number of cells plated) and plotted against total time in culture.

Quantitative PCR and Western Blot Analysis

mRNA was extracted using the RNeasy MiniKit (QIAGEN). cDNA was prepared from 0.75 μg total RNA using Superscript III reverse transcriptase (Invitrogen)

and random nonamer primers (Invitrogen). qPCR was performed using SYBR Green I (QIAGEN). Primers and conditions for PCR reactions are detailed in the Supplemental Experimental Procedures.

For qPCR analysis of miRNA expression RNA was extracted using the miRVanamiRNA Isolation Kit (Ambion). PCRs were performed by Geneservice using the TaqMan Mouse microRNA Assay Set v1.0 (Applied Biosystems). For embryos, miRNA was extracted from pools of embryos and expression normalized to sno202 expression. For cells, miRNA expression analysis was conducted on ES-E14, ES-m5, TS-*Dicer*$^{fx/fx}$, TS-B1, XEN-*Dicer*$^{fx/fx}$, XEN- IM8A1, MEF-cd1, and MEF-t cells and data were normalized to sno135 expression.

The hierarchical clustering of the miRNA expression profiles in the different cell lines was carried out with the program MultiExperimental Viewer Version 4.1 (MeV v4.1) using the complete linkage method and the correlation coefficient as the similarity measure.

For identification of highly expressed miRNA families, miRNAs were grouped by seed sequence as occurs on the TargetScan website. The total relative expression for each family was calculated as the sum of the relative expression of each member of that family present in our array.

The antibodies used for western blot analysis are listed in the Supplemental Experimental Procedures.

Luciferase Assays

The complete 3′ UTR regions of the mouse genes *Rasa2*, *Dusp1*, *Sulf2*, and *Cdkn1c* (sequences obtained from the Ensembl Genome Browser, www.ensembl.org) were amplified from mouse genomic DNA and cloned into the NotI and XhoI sites of the psiCHECK-2 vector (Promega), the primers used for cloning the 3′ UTR as well as the point mutations introduced in the mutated versions are detailed in the Supplemental Experimental Procedures. HEK293T cells were cotransfected in triplicate with 400 ng of 3′ UTR/psiCHECK construct and 100 nM of each microRNA mimic (Dharmacon) using Lipofectamine 2000 (Invitrogen) according to manufacturers' instructions. In the mock experiment, only the 3′ UTR/psiCHECK2 vector was transfected. XEN cells were infected with AdCre-GFP or Ad-GFP FACS sorted as previously described. Three days after infection, cells were transfected in triplicate with 800 ng of 3′ UTR/psiCHECK2 construct or the empty psiCHECK2 vector using jet-PRIME transfection reagent (Polyplus-Transfection). The luciferase and renilla activities were measured 24 to 36 hr after transfection using the Dual Luciferase Reporter Assay System (Promega). The relative luciferase activity in transfected XEN cells was normalized against the activity of the empty vector.

Three independent experiments were conducted. A Student's t test was used to verify significance, $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at doi:10.1016/j.devcel.2010.07.014.

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