Title

miR-294/miR-302 Promotes Proliferation, Suppresses G1-S Restriction Point, and Inhibits ESC Differentiation through Separable Mechanisms

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Peer reviewed

MiR-294/-302 promotes proliferation, suppresses G1-S restriction point, and inhibits embryonic stem cell differentiation through separable mechanisms

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Abstract

The miR-294/miR-302 microRNAs promote the abbreviated G1 phase of the embryonic stem cell (ESC) cell cycle and suppress differentiation induced by let-7. Here we evaluated the role of the Retinoblastoma (Rb) family proteins in these settings. Under normal growth conditions miR-294 promoted the rapid G1-S transition independent of the Rb family. In contrast, miR-294 suppressed the further accumulation of cells in G1 in response to nutrient deprivation and cell-cell contact in an Rb dependent fashion. We uncovered five additional miRNAs (miRs-26a, -99b, -193, -199a-5p, and 218) that silenced ESC self-renewal in the absence of other miRNAs, all of which were antagonized by miR-294/302. Four of the six differentiation-inducing miRNAs induced an Rb-dependent G1 accumulation. However, all six still silenced self-renewal in the absence of the Rb proteins. These results show that the miR-294/miR-302 family acts through Rb dependent and independent pathways to regulate the G1 restriction point and the silencing of self-renewal respectively.

Introduction

Embryonic stem cells (ESCs) have rapid and unlimited growth potential while retaining the ability to differentiate into any cell type of the adult (NIH, 2009). In contrast, somatic cells of adult tissues have limited growth and developmental potential. Because of the unique properties of ESCs, there is much promise in their use to study and treat disease, yet the basis of their potential remains incompletely understood.

Somatic cells have an extended G1 phase enabling them to respond to their environment (Blomen and Boonstra, 2007). Somatic cells like fibroblasts arrest in G0/G1 when nutrient starved or when in contact with neighboring cells (contact inhibition). This arrest, which occurs at a checkpoint called the restriction (R) point in the G1 phase, is governed by the Rb family of proteins (Blagosklonny and Pardee, 2002). When Rb proteins are phosphorylated by Cyclin/Cyclin Dependent Kinase (CDK) complexes, cells exit the R point and become committed to completing the cell cycle. The cells will not respond again to the external environment until the next G1 phase. ESCs lack an R point, presumably due to constitutive Cyclin/CDK activity that keeps Rb proteins in a hyperphosphorylated state (Orford and Scadden, 2008; Savatier et al., 1994; Stead et al., 2002; White and Dalton, 2005). As a result, ESCs have a short G1 phase and fail to respond to nutrient deprivation and contact inhibition. However, shortly after the initiation of differentiation, the R point is established (Orford and Scadden, 2008; Savatier et al., 1996; White et al., 2005). It has been proposed that the cell cycle structure of ESCs in part underlies their potential to remain undifferentiated and self-renew indefinitely (Burdon et al., 2002; Neganova and Lako, 2008; Singh and Dalton, 2009). Importantly, many transformed somatic cell lines also lack the R point (Blagosklonny and Pardee, 2002). Therefore, understanding the molecular basis of this unique cell cycle structure is important to both stem cell and cancer biology.

MicroRNAs (miRNAs) play an important role in regulating cell cycle in ESCs (Wang and Blelloch, 2009, 2011). miRNAs are short non-coding RNAs that repress protein translation and mRNA stability (Huntzinger and Izaurralde, 2011). Most mature miRNAs arise following two processing steps: DGCR8/Drosha cleavage of a long pri-miRNA to a hairpin pre-miRNA and Dicer cleavage of the pre-miRNA to the mature miRNA (Kim et al., 2009; Winter et al., 2009). Removal of DGCR8, Drosha, or Dicer results in a loss of miRNAs. Dgcr8 and Dicer null mouse ESCs have a reduced proliferation rate and an altered cell cycle structure with a slight increase in the fraction of cells in the G1 phase of the cell cycle (Murchison et al., 2005; Wang et al., 2008; Wang et al., 2007). Introduction of individual members from a large family of miRNAs highly expressed in pluripotent stem cells can partially rescue the proliferation defect and reverse the accumulation of cells in G1 (Wang et al., 2008). The family shares the seed sequence (AAGUGCU), a sequence near the 5′ end of the miRNA that is thought to largely determine a miRNA's downstream targets (Bartel, 2009). The family has 8 members including miR-294 and miR-302a-d and because of their role in influencing the ESC Cell Cycle have been called the ESCC family of miRNAs (Wang et al., 2008).

The ESCC miRNAs are also promoters of the pluripotent state. When introduced together with the transcription factors Oct4, Sox2, and Klf4 into human or mouse somatic cells, they dramatically enhance the de-differentiation to generate induced pluripotent stem cells (iPSCs) (Judson et al., 2009; Subramanyam et al., 2011). Indeed, it has been proposed that together with just one or two other miRNAs they induce pluripotency in the absence of any exogenously introduced coding genes (Anokye-Danso et al., 2011; Miyoshi et al., 2011). Consistent with this role, the ESCC miRNAs inhibit another family of miRNAs, the let-7 family, from silencing the pluripotency program of ESCs, thereby promoting their self-renewal (Melton et al., 2010). In contrast to the ESCC miRNAs, let-7 is a suppressor of cell cycle progression (Johnson et al., 2007); however, it is unclear whether the cell cycle targets alone can explain the ability of ESCC miRNAs to antagonize the effects of let-7. It also remains unknown whether the ESCC miRNAs can suppress other somatic miRNAs from inducing ESC differentiation.

In this study, we make the surprising finding that G1 accumulation seen in Dgcr8 knockout ESCs under normal growth conditions and reversed by the ESCC miRNAs occurs independent of Rb family proteins and, therefore, is not secondary to the classic G1-S check

or R point. However, under cytostatic conditions (serum starvation and cell confluency), the cells do show evidence of a R point that is absent in wild-type ESCs. In particular, the Dgcr8 knockout ESCs show a dramatic increase in G1, which is reversed by either the addition of miR-294/ miR-302 or the removal of all three Rb family members. Through a miRNA functional screen, we identify five miRNAs that in addition to let-7 can silence the pluripotency program of Dgcr8 knockout ESCs, but not wild-type cells. All these miRNAs are antagonized by the simultaneous addition of miR-294 and four of them induce a Rb-dependent accumulation in G1 suggesting a direct link between the R point and the silencing of self-renewal. Surprisingly though, the simultaneous deletion of the Rb family members along with Dgcr8 did not block the six miRNAs from silencing the pluripotency program. Therefore, the ESCC miRNAs are acting through mechanistically separable pathways to promote passage through G1 under normal growth conditions, suppress the R point under cystostatic conditions, and maintain ESC pluripotency.

Results

ESCC miRNAs promote passage through G1 independent of the G1-S restriction point

We previously hypothesized that miRNA-deficient ESCs accumulate in the G1 phase of the cell cycle due to low-level activation of the G1-S check/restriction point under normal growth conditions (Wang et al., 2008). To test this hypothesis we removed all Rb family members (Rb1, Rbl1, and Rbl2) along with Dgcr8 (Figure S1A-D). The Rb family proteins are obligate regulators of the G1-S R point (Blagosklonny and Pardee, 2002) and, therefore, their loss should remove any accumulation of cells in G1 associated with activation of the R point. Surprisingly, the proliferation rate and the accumulation of cells in G1 was unaffected by the loss of all three Rb family members (Figures S1E and 1A). Furthermore, introduction of the ESCC miRNAs still increased proliferation and decreased the fraction of cells in G1 in the Dgcr8, Rb1, Rbl1, Rbl2 quadruple knockout cells (Figures 1A and S1F). Therefore, the ability of the ESCC miRNAs to promote proliferation and suppress G1 accumulation under normal growth conditions is independent of their action on the G1 R point.

ESCC miRNAs repress the ability of ESCs to respond to nutrient starvation and cell-cell contact

To more directly evaluate the role of miRNAs in regulating the R point in ESCs, we evaluated the response of Dgcr8 null ESCs to nutrient starvation and cell confluency conditions, well known activators of this checkpoint. The cell cycle profiles of wild-type and Dgcr8 null cells were compared in high and low concentrations of fetal bovine serum (FBS) as well as in increasing density of plated cells. As previously reported (Schratt et al., 2001), wild-type ESCs did not accumulate in G0/G1 in response to low FBS or to increasing cell number (Figures 1B and 1C). In contrast, Dgcr8 null ESCs showed a striking increase in cells in G0/G1 under both conditions (Figures 1B and 1C).

An early event in wild-type ESC differentiation is an accumulation of cells in G1 (Figure S2A) (Savatier et al., 1996; White et al., 2005). To rule out the possibility that nutrient starvation was inducing differentiation of Dgcr8 knockout cells and hence secondarily leading to G1 accumulation, we evaluated multiple markers of pluripotency. Oct4, Nanog, and Klf4 mRNA levels remained high in wild-type and Dgcr8 null ESCs in low serum conditions for 2 days (Figure S2B). Indeed, Oct4 and Nanog mRNA were slightly up in the knockouts as previously described (Wang et al., 2007) likely secondary to the absolute block in differentiation of these cells as well as low level expression of miRNAs in wt ESCs that normally repress these factors to a small degree (Tay et al., 2008a; Tay et al., 2008b). Similarly, OCT4 and NANOG immunohistochemistry as well as alkaline phosphatase activity remained positive in low serum (Figures S2C and S2D). Another alternative

explanation for the accumulation of cells in G0/G1 is selective apoptosis of Dgcr8 null cells in non-G0/G1 phases of the cell cycle. Indeed, both wild-type and Dgcr8 null cells showed increased levels of apoptosis in 1% FBS (Figure S3). To remove apoptosis, we combined Dgcr8 with null alleles of Bax and Bak (Wei et al., 2001). Deletion of both alleles of Bak and one allele of Bax blocked apoptosis in both Dgcr8 heterozygous and Dgcr8 homozygous ESCs (Figures S3A-E). However, the percentage of Dgcr8$^{-/-}$/Bak$^{-/-}$/Bax$^{-/flox}$ ESCs in the G0/G1 phase still showed an increase in response to serum starvation, unlike their Dgcr8$^{-/flox}$/Bak$^{-/-}$/Bax$^{-/flox}$ counterparts (Figure 1D) showing that apoptosis cannot explain the G0/G1 accumulation.

To rule out non-miRNA roles for the Dgcr8 null phenotype, we evaluated Dicer knockout cells. Similar to Dgcr8, Dicer knockout ESCs accumulated in G0/G1 in response to low serum (Figure S4A). Furthermore, acute deletion of Dgcr8 led to a similar increase in the percentage of cells in G0/G1 upon serum starvation, ruling out adaption to Dgcr8 loss as the underlying cause (Figure S4B). Together, these data suggest that miRNAs normally suppress the ability of ESCs to pause in G0/G1 in response to external cues such as nutrient starvation and cell-cell contact.

The ESCC miRNAs act through the Rb pathway to suppress the G1 restriction point in ESCs

The ESCC miRNAs target activators of the G1/S R point including Cdkn1a, Rb1, and Rbl2 (Wang et al., 2008). To directly evaluate whether they can suppress the G1 restriction point, we measured the impact of the ESCC miRNA miR-294 on G0/G1 accumulation under serum starvation and increasing cell density. Introduction of miR-294 mimic into Dgcr8 knockout ESCs blocked the increase in the fraction of cells in G0/G1 under both conditions (Figures 2A and 2B). This block was dependent on the ESCC family seed sequence. Mutation of the seed or the introduction of other ESC expressed miRNAs that have a different seed sequence had no effect on the accumulation of G0/G1 cells (Figure S5A). The effect was stable for the lifespan of the miRNA mimic (Figure S5B). These findings show that the ESCC miRNAs suppress the accumulation of cells in G0/G1 in response to cytostatic growth conditions.

To confirm that the ESCC miRNAs are targeting the Cyclin/Cdk pathway under cystostatic conditions, we evaluated the previously described targets Cdkn1a, Rbl2, and Rb1 (Wang et al., 2008). All three were elevated in Dgcr8 knockout relative to wild-type ESCs under standard culture conditions (Figure 3A, p<0.0002). Upon serum starvation, the three genes remained repressed in wild-type ESCs (p<0.01). In Dgcr8 knockout cells, Rb1 and Rbl2 remained elevated, while Cdkn1a was further elevated (p<0.002). Rbl1 was also elevated in the knockout cells in both culture conditions, but this effect was independent of the 3′UTR as luciferase assays on the 3′UTRs of the three Rb proteins showed that only the 3′UTRs of Rb1 and Rbl2 were suppressed in the wild-type relative to Dgcr8 knockout cells (Figure S6A). We confirmed that protein levels of three Rb genes are higher in Dgcr8 knockout cells under both standard culture and serum starvation conditions (Figure S6B). The re-introduction of miR-294 was able to suppress Cdkn1a, Rbl1, and Rbl2, but not Rb1, under serum starvation conditions, suggesting indirect effects on Rb1 control (Figure 3B). These data show that miR-294 suppresses Cdkn1a and Rbl2 in Dgcr8 knockout ESCs under nutrient starvation conditions.

The above data suggested that the R point is activated in Dgcr8 null cells in cytostatic conditions. To evaluate the role of the R point directly, we compared the Dgcr8 knockout to the Dgcr8, Rb1, Rbl1, Rbl2 quadruple knockout ESCs. Deletion of all three Rb proteins blocked the response of the Dgcr8 knockout cells to serum starvation (Figure 3C). Notably, the presence of even one Rb allele was enough to maintain the R point. Similar results were

obtained under increased cell confluency conditions (Figure 3D). These findings show that unlike the G1 accumulation seen in normal growth conditions, the accumulation seen in cytostatic conditions is Rb-dependent and, therefore, is due to activation of a R point, which is normally suppressed by the ESCC miRNAs in wild-type ESCs.

Multiple miRNAs can induce differentiation and G1 accumulation in miRNA deficient cells

Let-7 is able to silence self-renewal of Dgcr8 knockout cells, but is antagonized by the miR-294/miR-302 family in wild-type embryonic stem cells (Melton et al., 2010). Let-7 levels increase during ESC differentiation coinciding with a decrease in the ESCC miRNAs and an increase in the number of cells in G1 (Melton et al., 2010) (Figure S2A). Therefore, we predicted that let-7 may in part function by lengthening the G1 phase. Indeed, the introduction of let-7 led to an accumulation of Dgcr8 knockout cells in the G1 phase, which was reversed by co-introduction of miR-294 (Figure 4A and 6A). Based on this finding, we hypothesized that there may be additional miRNAs that can induce an accumulation of cells in G1 and silence self-renewal in Dgcr8 knockout cells and whose function is normally antagonized by the ESCC miRNAs in wild-type ESCs.

To determine if additional miRNAs are able to silence self-renewal, we performed a screen reintroducing 256 different miRNAs individually into the knockout cells (Figure 4B, Table S1). To follow the silencing of self-renewal, we qualitatively scored the degree of loss of alkaline phosphatase (AP) activity on a scale from 1 to 8 with 8 being complete loss of staining (Figure 4C). We uncovered 32 miRNAs that decreased the number of cells showing AP activity by approximately 75 percent or greater (score   6). To narrow down the miRNAs followed up from the screen, we performed microarray analysis of miRNAs under two differentiation conditions (minus LIF and retinoic acid) (Figure 4D, Table S2). 14 miRNAs with a differentiation score   6 were upregulated under both differentiation conditions. A subset of these miRNAs was confirmed by reverse transcriptase-quantitative PCR (RT-qPCR) (Figure S7). To focus on a small number, we combined our profiling data with published profiling data for human ESC embryoid body differentiation, mouse embryonic fibroblasts, and mouse neural progenitor cells (Figure 4E) (Bar et al., 2008; Marson et al., 2008). We selected five miRNAs in addition to let-7 that had a differentiation score   6 and were up in 2 or more differentiation conditions. These were miR-26a, miR-99b, miR-193, miR-199a-5p, and miR-218.

To further evaluate the role of these miRNAs in silencing self-renewal, we tested re-synthesized mimics for a representative let-7 family member (let-7c) and five of the newly identified miRNAs. miR-134 was also tested as it had been previously described in inducing differentiation of wild-type mouse ESCs (Tay et al., 2008b). All six miRNAs identified in our screen robustly silenced AP activity, as did miR-134 (Figure 5A, panel i). RT-qPCR for three additional markers of the pluripotency program, Oct4, Sox2, and Nanog also showed robust silencing with the screen-positive miRNAs and miR-134 (Figure 5B, panel i). Together these experiments show that multiple miRNAs in addition to let-7, which are upregulated upon ESC differentiation, can silence the self-renewal program in Dgcr8 knockout ESCs.

Similar to let-7, none of the newly uncovered miRNAs were able to silence the pluripotency program in wild-type cells (Figures 5A and 5B, panel ii). miR-134 was able to suppress AP activity and to a small degree Oct4, Sox2 and Nanog levels in wild-type cells. Let-7 and the screen positive miRNAs were all antagonized by miR-294 and miR-302b. Specifically, the co-introduction of miR-294 or miR-302b, but not mutant miR-294, blocked the ability of miRs-26a, 99b, 193, 199a-5p and 218 to down-regulate AP activity or Oct4, Sox2, and Nanog mRNA levels (Figures 5A and 5B, panel i). While miR-294 and miR-302b were unable to suppress miR-134's ability to silence AP activity, it did inhibit, albeit to a smaller

degree, the downregulation of Oct4, Sox2, and Nanog, consistent with the findings when miR-134 was introduced into wild-type cells. These findings suggest that the ESCC miRNAs are general inhibitors of miRNA-induced differentiation rather than being a specific antagonist of let-7.

Next, we asked whether the screen-positive miRNAs, like let-7, were able to induce the accumulation of cells in G1. Indeed, four of the five newly uncovered differentiation-inducing miRNAs increased the fraction of cells in G1 when introduced into Dgcr8 knockout cells (Figure 6A, $p < 0.02$ except miR-199a-5p, $p=0.7$). Similar to let-7, the effects of these miRNAs on G1 accumulation were reversed by the introduction of the ESCC miRNA, miR-294 (Figure 6A). Therefore, differentiation-associated miRNAs both silence the pluripotency program and induce accumulation of cells in G1, both of which are antagonized by the ESCC miRNAs.

The ESCC miRNAs act independently of the Rb pathway to antagonize other miRNAs from silencing self-renewal

Based on the above findings, we hypothesized that wild-type ESCs were resistant to the effects of the differentiation-inducing miRNAs because of the capacity of miR-294/miR-302 family to suppress the R point. To test this hypothesis, we introduced the differentiation-inducing miRNAs into Dgcr8, Rb1, Rbl1, Rbl2 quadruple knockout ESCs and evaluated their effects on both the accumulation of cells in G1 and the silencing of self-renewal. The loss of all three Rb proteins blocked the ability of the differentiation-inducing miRNAs to cause an accumulation of cells in G1 (Figure 6B), consistent with these miRNAs activating the R point. In striking contrast, the loss of all three Rb proteins did not inhibit the ability of these miRNAs to silence self-renewal. In particular, the six differentiation inducing miRNAs were equally effective at suppressing Oct4, Nanog, and Sox2 levels in the quadruple knockouts as the Dgcr8 alone knockout ESCs (Figures 5B and 6C). These findings demonstrate that the ability of the differentiation-inducing miRNAs to silence self-renewal is independent of their capacity to induce the R point.

Discussion

ESCs have a unique cell cycle structure lacking a G1 restriction point, which has been hypothesized to play an important role in the maintenance of pluripotency (Burdon et al., 2002; Neganova and Lako, 2008; Orford and Scadden, 2008; Singh and Dalton, 2009). Here, we show that a family of miRNAs, the ESCC miRNAs, suppresses the R point as measured by the response of ESCs to cytostatic conditions (serum starvation and increased cell confluency). Furthermore, using genetic tools, we show that the miRNAs are acting upstream of the Rb family of proteins. Surprisingly, the previously reported G1 accumulation seen in Dgcr8 knockout cells grown under normal growth conditions, occurs independent of the G1-S restriction point. Similarly, the ability of the ESCC miRNAs to antagonize other miRNAs from silencing ESC self-renewal occurs independently from their ability to suppress the R point. Therefore, the abilities of the ESCC miRNAs to promote the G1-S transition under normal growth conditions, suppress the R point under cystostatic conditions, and block ESC differentiation are mechanistically separable (Figure 7).

The role of Rb family of proteins in the regulation of the G1/S transition has been well studied, but remains complex. Knockout of the three Rb genes removes the R point in mouse embryonic fibroblasts in response to serum starvation and cellular confluency (Sage et al., 2000). However, their loss has little effect on ESCs (Wirt et al., 2010), presumably because the R point is already suppressed by the ESCC miRNAs (this study). Interestingly, loss of the Rb family members does not block the accumulation of many cell types in G1 during ESC differentiation (Wirt et al., 2010). That is, cells can still exit the cell cycle and early

organogenesis proceed largely undisturbed following injection of triple knockout ESCs into early embryos. Therefore, there must be multiple mechanisms associated with G1 accumulation seen during ESC differentiation. In particular, considering their differing dependencies on the Rb family, the mechanism that controls the R point that responds to serum starvation or cellular confluency must be different from the mechanisms that mediate G1 accumulation in normal growth conditions and during ESC differentiation. It will be important in future studies to determine if the accumulation seen in Dgcr8 knockout cells under normal growth conditions share common mechanisms with the Rb-independent pathways seen with differentiation.

Previously, we had found that the ESCC and let-7 miRNAs antagonize one another in the switch between ESC self-renewal and differentiation. Here, we describe 5 additional miRNAs that promote the silencing of ESC self-renewal and are antagonized by the ESCC miRNAs. Interestingly, each of these miRNAs has a distinct seed sequence from let-7 with very different predicted or experimentally determined downstream targets (Targetscan and Melton et al, unpublished data). Furthermore, unlike let-7, these additional miRNAs have more restricted expression patterns, some being expressed in a small number of tissues (Landgraf et al., 2007). Therefore, their functional targets in silencing self-renewal are likely quite different from one another, which will be the focus of future studies. The ability of the ESCC miRNAs to suppress these alternative mechanisms would suggest a more global role for the ESCC miRNA in promoting the pluripotent state. Such a conclusion fits well with the ability of the ESCC miRNAs to dramatically enhance the de-differentiation of somatic cells to induced pluripotent stem cells (Judson et al., 2009).

How the ESCC miRNAs are able to maintain self-renewal in the presence of differentiation-inducing miRNAs or promote the de-differentiation of somatic cells remains an open question. Several genomic studies have shown that these miRNAs target hundreds of mRNAs (Hanina et al., 2010; Leung et al., 2011; Melton and Blelloch, 2010; Sinkkonen et al., 2008). Functional analysis of a small number of targets chosen based on known roles for the encoded proteins has begun to give some insight into their impact on reprogramming to iPSCs (Liao et al., 2011; Subramanyam et al., 2011). However, knockdown of individual members thus far has failed to recapitulate the full effects of the ESCC miRNAs (Subramanyam et al., 2011). Further, our attempts to recapitulate ESCC function by knocking down a small number of individual targets failed to block differentiation by let-7 and the other miRNAs (Melton and Blelloch, data not shown). A more systematic dissection of the ESCC miRNA targets including combinatorial suppression of multiple targets will be required to understand how the ESCC miRNAs can have such a powerful impact on stabilizing and promoting the pluripotent state.

Materials and Methods

Tissue culture, cell cycle, apoptosis and luciferase reporter analysis

Mouse ESCs were grown on gelatin-coated plates or irradiated mouse embryonic fibroblast feeders as previously described (Wang et al., 2007). For serum starvation experiments, 150,000 or 300,000 cells for wild type and Dgcr8 knockout ESCs were plated in a well of a 6-well plate. After growing for ~24 hours in standard culture media (15% FBS), for standard culture control, cells were then fixed for cell cycle analysis as previously described (Wang et al., 2008; Wang et al., 2007); for serum-starved samples, media was replaced with 1% FBS culture media and cells were grown for another 24 hours or 48 hours before fixation for cell cycle analysis. For serum starvation of miRNA transfected cells, media were replaced with low serum media at ~24 hours after transfection. For contact inhibition experiments, 200,000 to 1,600,000 cells were plated in a well of a 24-well plate and grown in standard culture media for ~24 hours before fixation for cell cycle analysis. For contact inhibition of

miRNA transfected cells, cells were trypsinized and plated at increasing densities at ~24 hours after transfection. Notably, all cell cycle analysis were internally controlled as there was slight variability between cell cycle distribution under differing conditions including day of experiment, location of experiment, and specifics of experiment. The sequence for the control mimic in this study is UUCUCCGAACGUGUCACGUTT, which is obtained from Shanghai Genepharma and predicted to have no homology with mouse genes. For apoptosis analysis, cells were labeled with propidium iodide and FITC-Annexin V and analyzed by flow-cytometry. Fraction of PI negative and Annexin V positive cells in total population was calculated to track early stage of apoptosis. miRNA transfection and luciferase reporter assay were performed essentially the same as previously described (Wang et al., 2008). For luciferase assay, 4,000 cells for wild type and 8,000 cells for Dgcr8 knockout ESCs were plated in 96-well plate either in the standard media or 1% FBS media. After growing for ~16 hours, reporter constructs were transfected and cells were grown in respective media for ~36 hours before lysis. For alkaline phosphatase staining and qPCR of differentiation markers, 6,000 wild-type or 20,000 Dgcr8 knockout cells were plated in a 24 well plate, transfected the next day, and fixed or lysed in Trizol on the fourth day. For cell cycle experiments with differentiation inducing miRNAs, 50,000 Dgcr8 knockout or quadruple knockout cells were plated in a 12 well plate, the next day they were transfected, and on the second day harvested for cell cycle analysis.

RNA extraction, qPCR and miRNA RT-PCR

Total RNA was extracted according to standard Trizol protocol (Invitrogen). Samples were centrifuged at >12,000 g during washing steps to preserve small RNAs. qPCR and miRNA RT-PCR were performed using Sybr Green mix (Applied Biosystems) as previously described. Sequences for qPCR primers were previously reported (Melton et al., 2010; Wang et al., 2008). miRNA qPCR was performed by polyadenylating the miRNAs and then using a modified oligodT reverse transcription primer as described previously (Shi et al 2005).

Alkaline phosphatase staining and immunofluorescence (IF) analysis

Cells were fixed in 4% paraformaldehyde for 10-15 minutes. Alkaline phosphatase staining was performed using kit from Vector labs according to the manufacture's protocol. For IF analysis, primary antibodies for Oct4 (Santacruz, sc-9081) and Nanog (CalBiochem, sc-1000) were diluted 200-400 fold. Secondary antibodies were Alex Fluor conjugated antibodies from Invitrogen.

miRNA screening

On day 0, 4000 Dgcr8 knockout ESCs were plated per well of a 96 well plate in media without LIF. On day 1, miRNA mimics were transfected one per well at a concentration of 100nM final volume 100 uL. On day 4, cells were fixed and stained for alkaline phosphatase activity using Vector Red substrate (Vector Labs). The screen was repeated in triplicate and scoring was done manually and blind to the layout of the screening plate.

miRNA microarray analysis

400, 000 cells were plated in a 6cm plate and differentiated either in media without LIF or equivalent media with 1uM all-trans retinoic acid (Sigma). RNA was isolated using Trizol (Invitrogen). 2ug total RNA was labeled with an Exiqon miRCURY LNA microRNA Power Labeling Kit (Exiqon) following the manufacturer's protocol for manual hybridization. All hybridizations were dual labeled using day0 undifferentiated ES cell RNA as a reference. One array in each case for (−) LIF and RA differentiation was repeated in reverse color. Arrays were scanned and data were extracted using a GenePix Scanner (Molecular Devices)

and associated software. Data were discarded for spots where at least one color was not 2 fold above background. Background was subtracted for individual spots and quadruplicate spots from each array were averaged. Data at this point was manually filtered to remove data for inconsistent values within quadruplicate spots. Arrays were median centered based on the median of probes with meaningful data on all three arrays (i.e. commonly unfiltered probes). The average of data for all probes passing analysis is given in Figure 4 for probes present both on the RA and (−) LIF arrays.

miRNA expression meta analysis

Human ES and EB deep sequencing data were downloaded from the supplementary materials of (Bar et al., 2008). Mouse NPC, MEF, and ESC deep sequencing data were downloaded from the supplementary material of (Marson et al., 2008). Data were manipulated in Microsoft Excel, clustered in Cluster (http://rana.lbl.gov/EisenSoftware.htm), and visualized in Java Treeview (http://jtreeview.sourceforge.net/).

Dgcr8 and Rb targeting in Rbl1$^{-/-}$, Rbl2$^{-/-}$ ESCs

Rbl1−/−, Rbl2−/− mouse ESCs (Sage et al., 2000) were kindly provided by Julien Sage (Stanford University). These ESCs were targeted with R26CreER and Dgcr8 as previously described (Wang et al., 2007). The Rb floxed puromycin targeting construct (Sage et al., 2000) also kindly provided by Julian Sage was targeted once, the Puromycin selection cassette was removed by CreER activation, and then the second allele was targeted to generate the final line. qPCR primers were designed to detect the expression of exon3 in Rb1, exon1 in Rbl1 and exon2 in Rbl2. The sequences for these primers are: Rb-F, TCATCCGTGGATGGAATCCTG; Rb-R, GATCAACTGCTGCGATAAAGATGC; Rbl1-F, CCGAAGCCCTGGATGACTT; Rbl1-R, ATGCCAGCCAGTGTATAACTTCTCC; Rbl2-F, GGACCGCTGAAGGAAACTATGTA; Rbl2-R, CTTCCCACTTCTTCATCTTGTTAAAA. Western analysis for Rb1, Rbl1, and Rbl2 was done as previously described (Melton et al., 2010) using Rb D20 antibody from Cell Signaling, the p130 C-20 sc-317 antibody from Santa Cruz Biotech, and the p107 C-18: sc-318 antibody from Santa Cruz Biotech.

Dgcr8 and R26 targeting in Bak$^{-/-}$, Bax$^{flox/flox}$ ESCs

Derivation of Bak$^{-/-}$,Bax$^{flox/flox}$ ESCs will be described elsewhere (Eric Wang, Nichole Reyes, CM, RB, and Scott Oakes, manuscript in preparation). These cells were targeted with R26CreER and Dgcr8 as has been previously described (Y. Wang, et al., 2007). Following the targeting of the first Dgcr8 allele to generate Dgcr8$^{flox/+}$, cells were treated with 1 uM Tamoxifen for 1 hour to remove the HygroTK selection cassette. This resulted in Dgcr8$^{-/+}$ allele that was then targeted again to generate Dgcr8$^{-/flox}$. This line (Bak$^{-/-}$, Bax$^{-/flox}$, Dgcr8$^{-/flox}$) was used as control. The line was treated with tamoxifen and subcloned to generate the experimental lines (Bak$^{-/-}$, Bax$^{-/flox}$, Dgcr8$^{-/-}$). Tamoxifen treatment did not result in complete loopout of the Bax allele (Figure S2).

Animal use

All animal experiments described in this study were approved by UCSF's Institutional Animal Care and Use Committee.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- miR-294/miR-302 promote G1-S in ESCs through Rb dependent and independent mechanisms

- Multiple miRNAs silence the pluripotency program of Dgcr8 null, but not wt ESCs

- A subset of the diff-inducing miRNAs activate the Rb dependent restriction point

- miR-294/miR-302 antagonize the diff-inducing miRNAs through a Rb independent mechanism
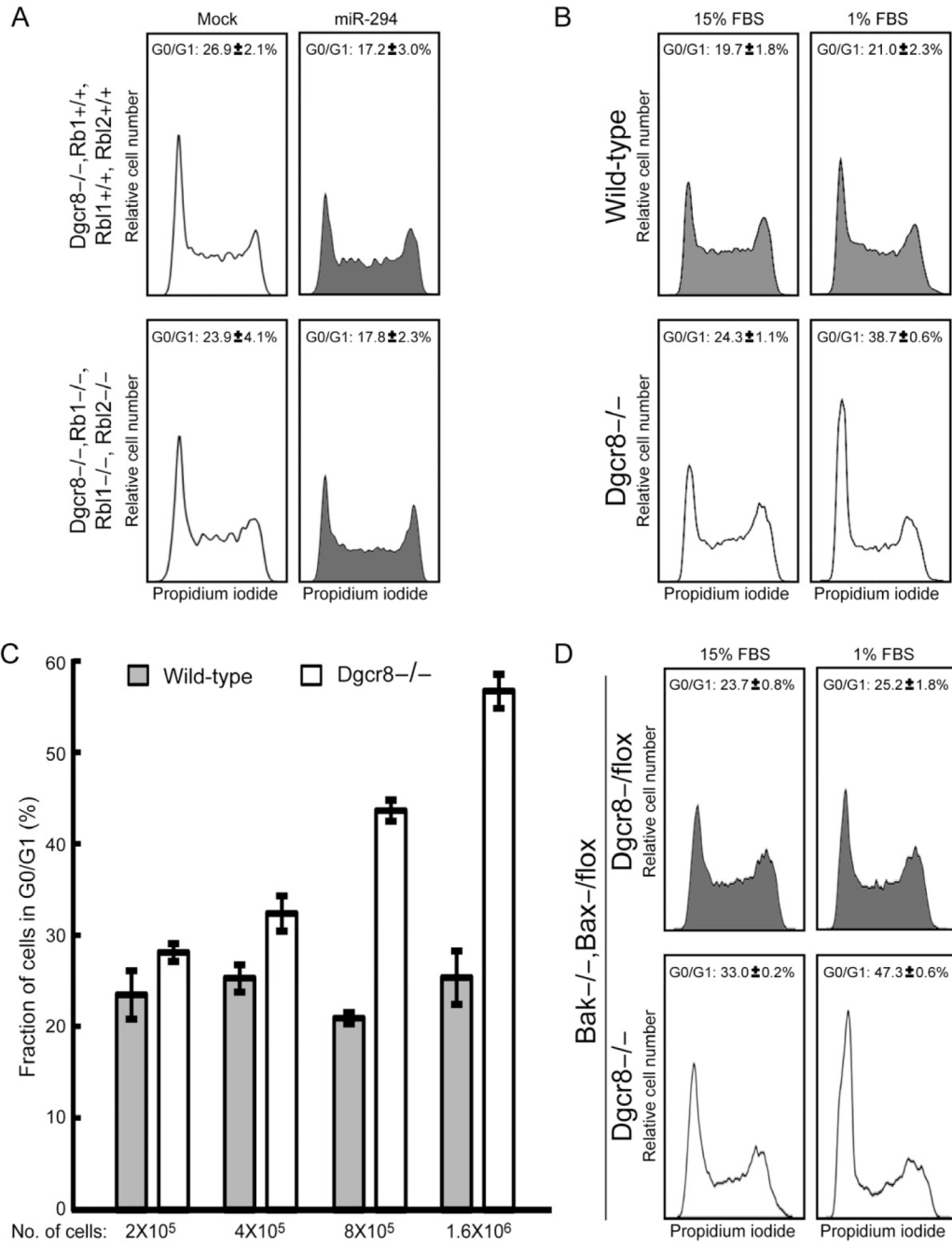
Figure 1.
miRNAs suppress the G1 restriction point in mouse ESCs. (A) Cell cycle profile of mock or miR-294 transfected Dgcr8 knockout and triple Rb, Dgcr8 knockout ES cells. Shown is flow cytometry analysis of propidium iodide stained cells. (B) Cell cycle profile of wild type and Dgcr8 knockout ESCs before and after serum starvation. (C) Fraction of cells in the G0/G1 phase for wild type and Dgcr8 knockout ESCs at increasing densities. (D) Cell cycle profile of Bak$^{-/-}$/Bax$^{-/flox}$ ESCs before and after serum starvation. Representative experiments are shown here. All results shown as mean±s.d., n=3. See also Figures S1-S4.
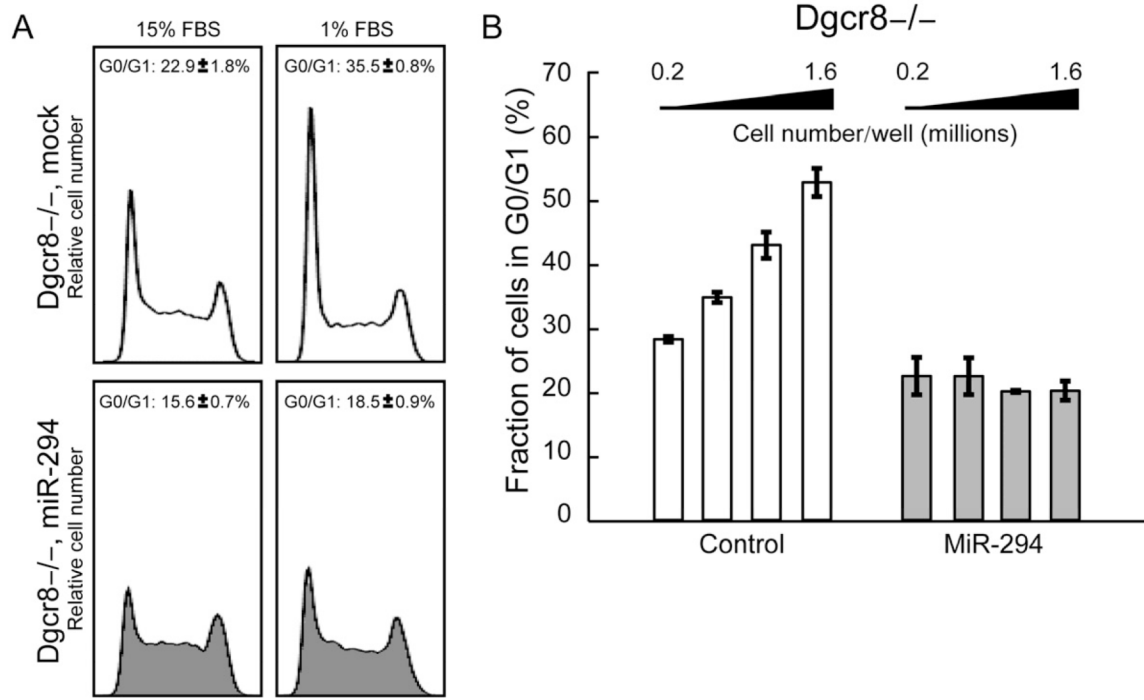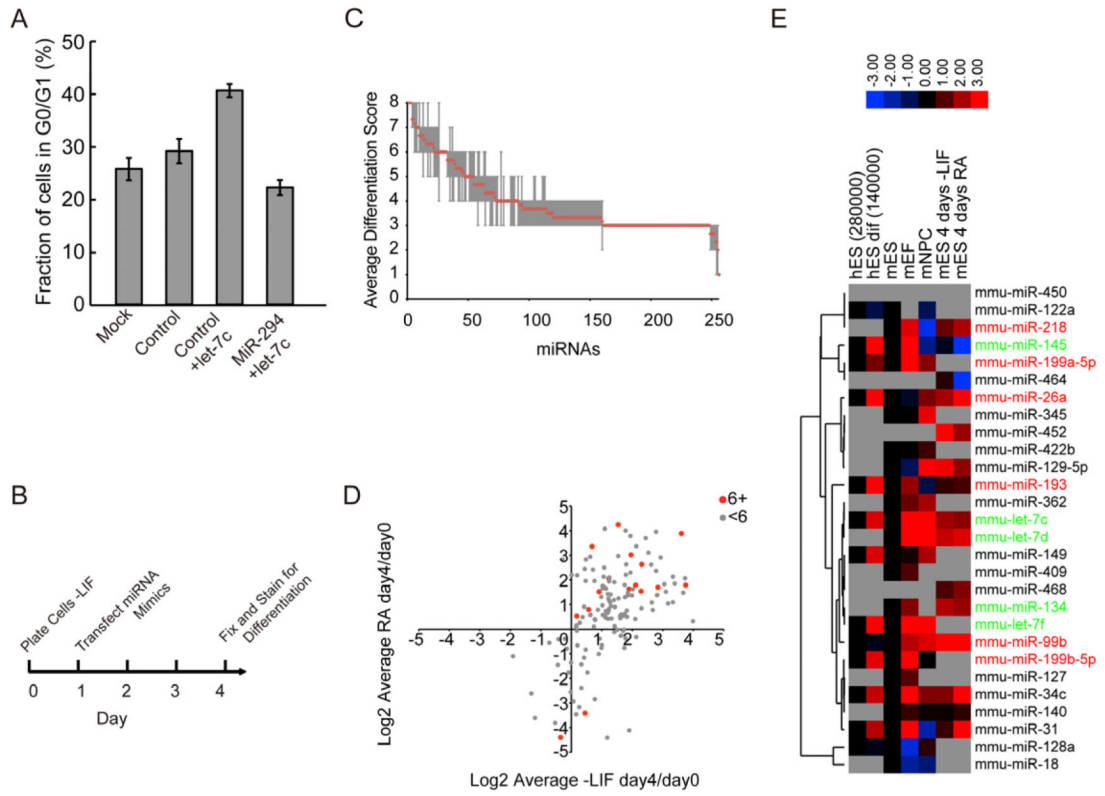
Figure 2.
ESCC miRNAs suppress the G1 restriction point in ESCs. (A) Cell cycle profile of mock and miR-294 transfected Dgcr8 knockout ESCs before and after serum starvation. (B) Fraction of cells in the G0/G1 phase for Dgcr8 knockout ESCs transfected with control mimics or miR-294 at increasing densities. Representative experiments are shown here. All results shown as mean±s.d., n=3. See also Figure S2.
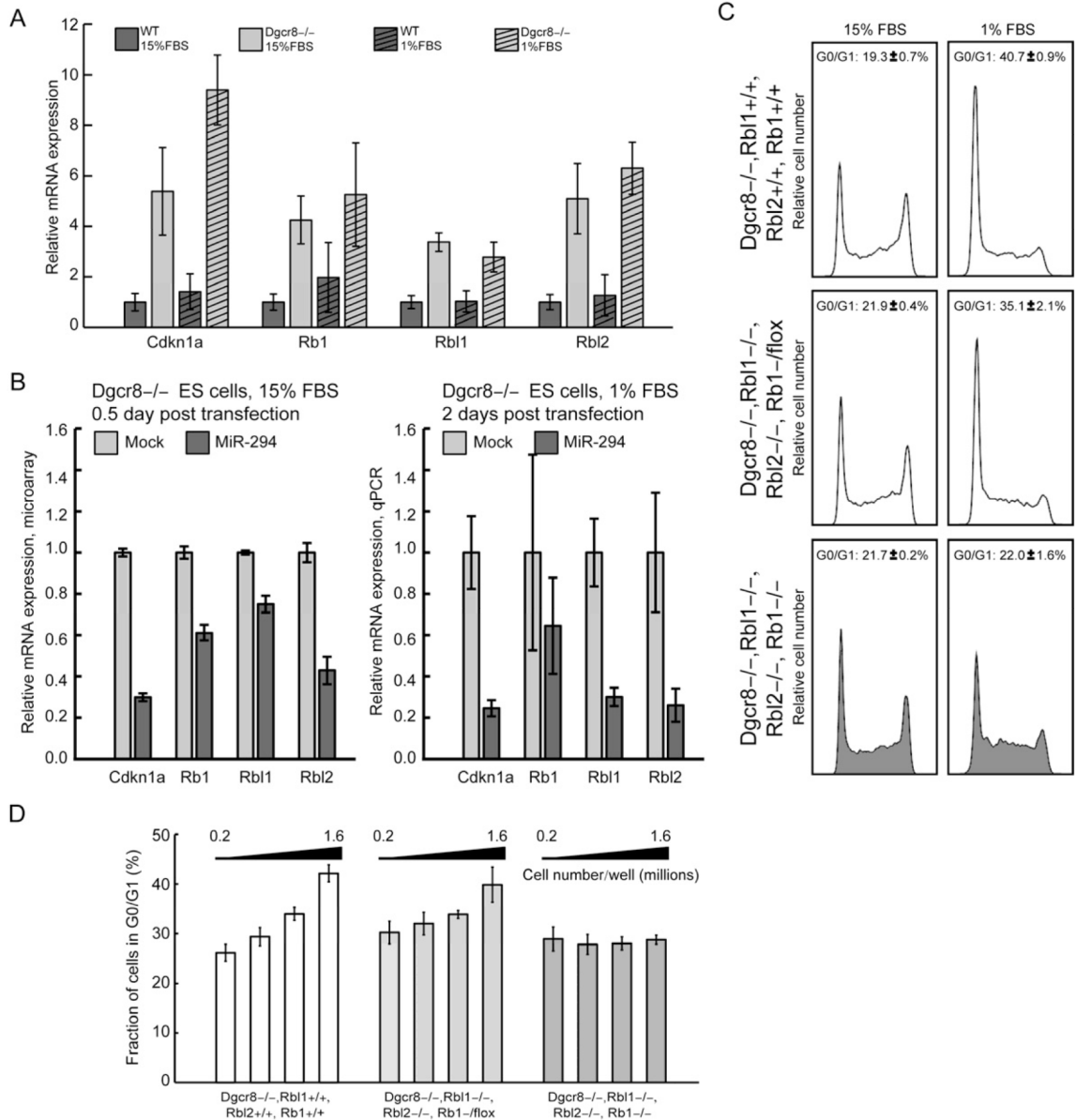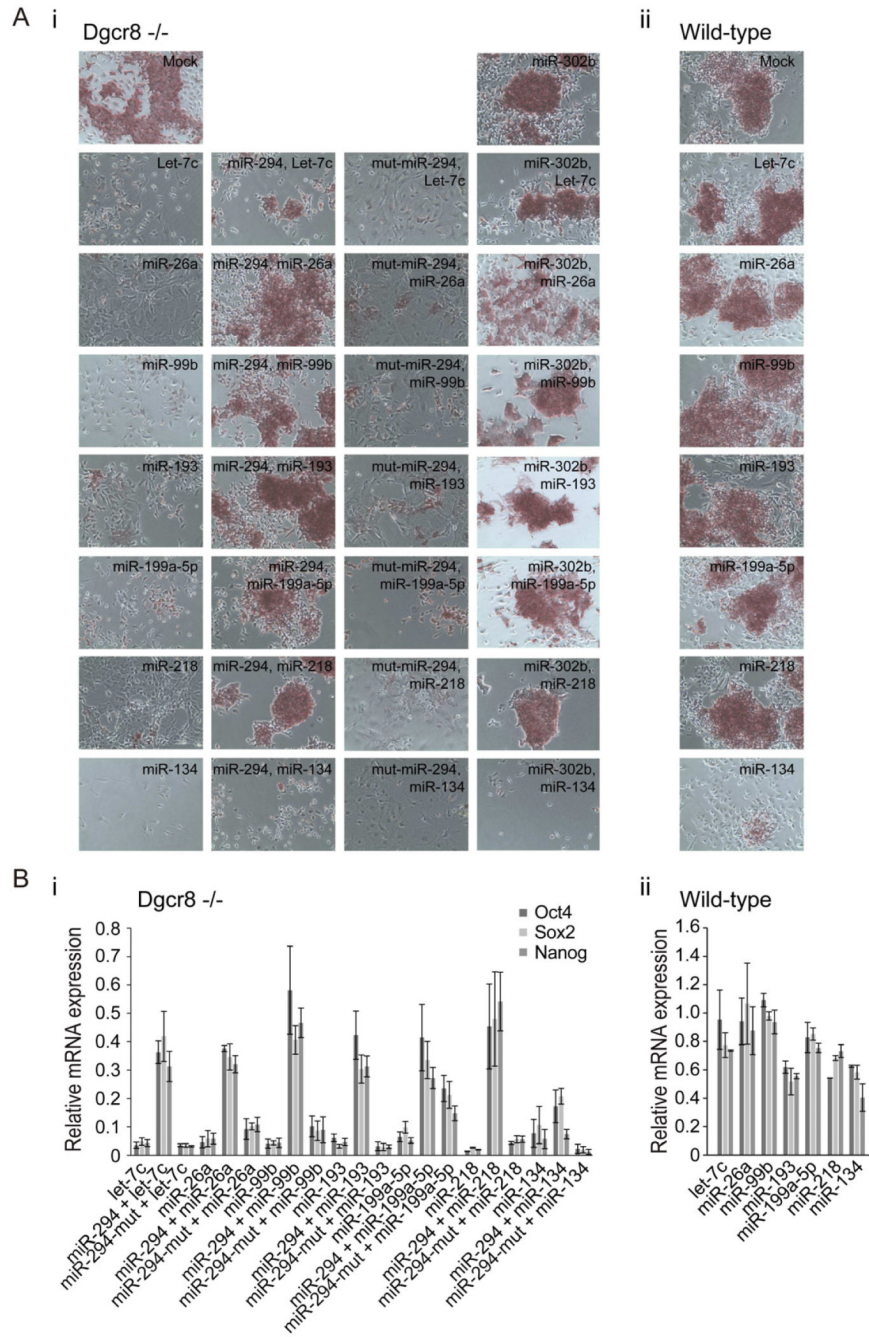
Figure 3.
The ESCC miRNAs act through the Rb pathway to suppress G1 restriction point in ESCs.
(A) Quantatitive PCR analysis of Rb family genes in wild type and Dgcr8 knockout ESCs before and after serum starvation. Rpl7 gene was used as loading control. mRNA expression was normalized to wild type ESCs grown at standard culture conditions. Error bars indicate s.d. n=6. (B) mRNA expression of Rb family genes in mock and miR-294 transfected Dgcr8 knockout ESCs. Left panel shows the microarray result of cells in standard culture conditions (p<0.001). Right panel shows qPCR results in serum starved cells(Cdkn1a, Rbl1, and Rbl2, p<0.02; Rb1, p=0.31). Error bars indicate s.d. n=3. (C) Cell cycle profile of Rb family knockout ESCs before and after serum starvation. (D) Fraction of cells in the G0/G1 phase for Rb family knockout ESCs and controls at increasing densities. Representative experiments are shown here. All results shown as mean±s.d., n=3. See also Figure S6.
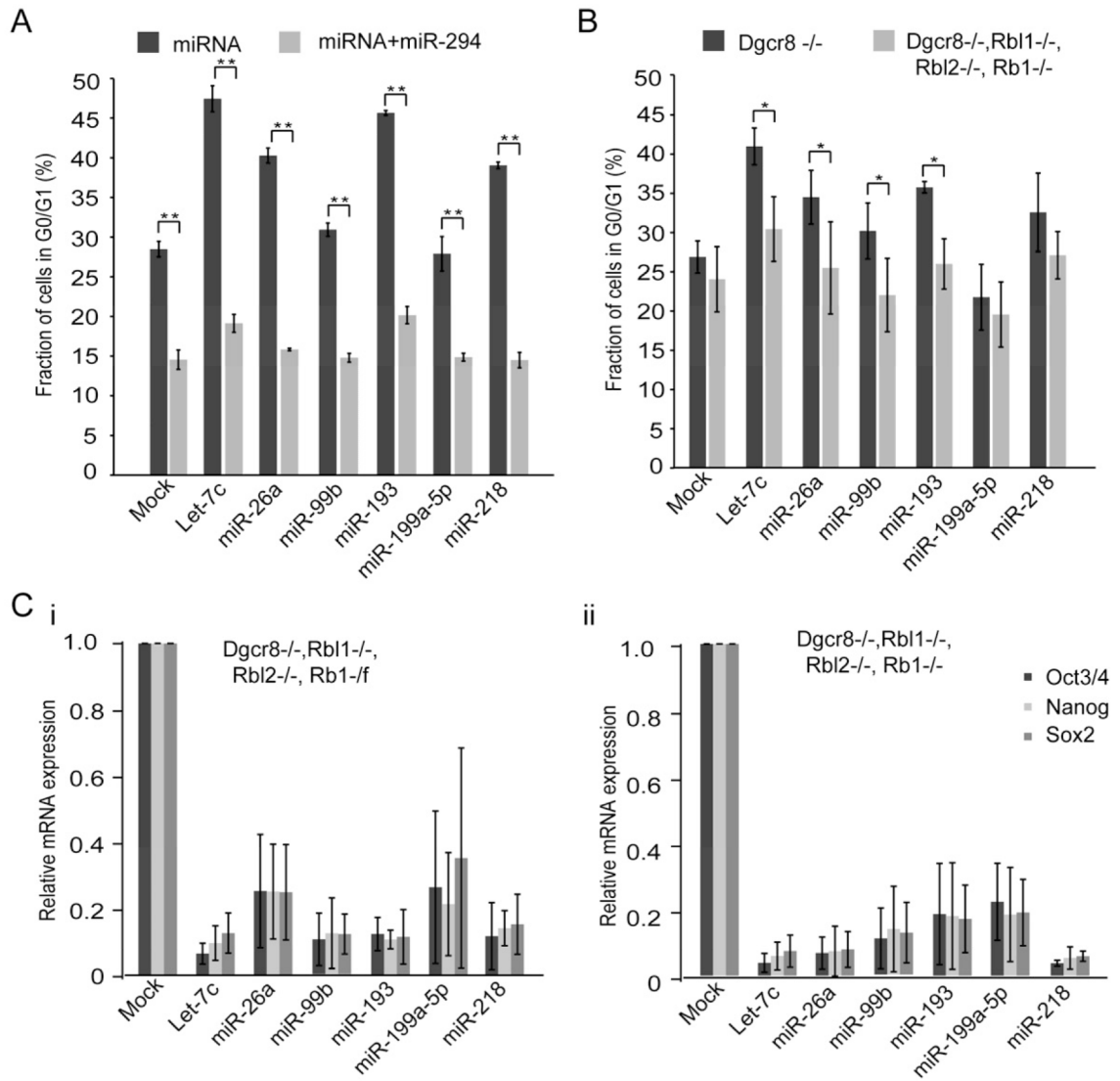
Figure 4.
Screening identifies multiple miRNAs that silence ESC self-renewal in Dgcr8 knockout ESCs. (**A**) A bar graph depicting fraction of cells in G0/G1 for Dgcr8 knockout ESCs mock transfected, transfected with control mimics, or with let-7c in combination with control mimics or miR-294. Results shown as mean±s.d., n=3. (**B**) A schematic of the screening strategy. (**C**) miRNA screen data plotted for individual miRNAs with the error representing the range of scores for n = 3. (**D**) A scatterplot depicting the results for individual miRNAs based on miRNA array data in mouse ESCs 4 days after LIF withdrawal or in 1 micromolar all-trans retinoic acid (n = 3 for each condition). Red dots show miRNAs with a screen score greater than or equal to 6. (**E**) A heatmap depicting miRNA expression changes in mouse NPC and MEF relative to mouse ESC, human EB differentiation, and mouse –LIF and RA differentiation. MiRNAs labeled in green were previously implicated in ESC differentiation while those in red were chosen for further investigation in this study. See also Figure S7 and Tables S1 and S2.
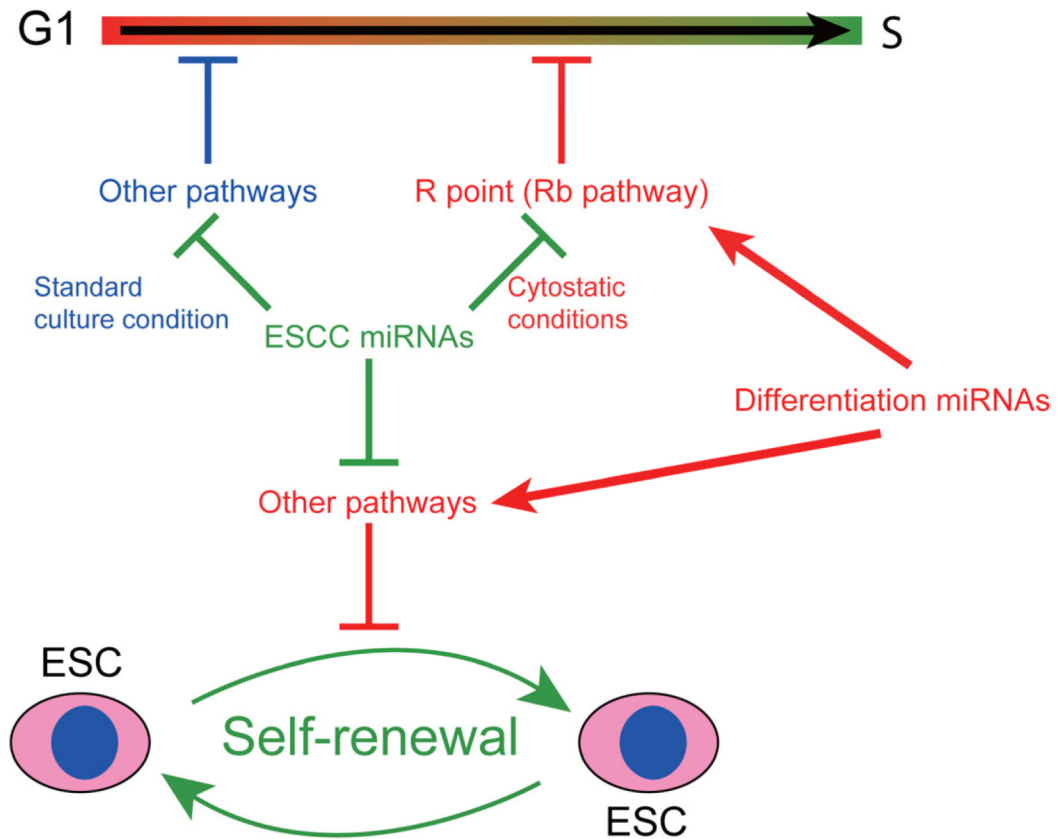
Figure 5.
ESCC miRNAs antagonize differentiation-inducing miRNAs from silencing ESC self-renewal. **(A)** Representative alkaline phosphatase staining in Dgcr8 knockout **(i)** and wild type **(ii)** ESCs after transfection with let-7c, miR-26a, miR-99b, miR-193, miR-199a-5p, and miR-218 alone or in combination with miR-294, mutant-miR-294 or miR-302b. **(B)** qRT-PCR for Pou5f1/Oct4, Sox2, and Nanog normalized first to beta-actin then to mock transfection after miRNA introduction as in **A**. Results shown as mean±s.d., n=2.

Figure 6.
ESCC miRNAs act independently of the Rb pathway to antagonize other miRNAs from silencing ESC self-renewal. **(A)** A bar graph depicting fraction of cells in the G0/G1 phase after transfection of Dgcr8 knockout ESCs with let-7c, miR-26a, miR-99b, miR-193, miR-199a-5p, and miR-218 alone or in combination with miR-294. Mean±s.d. for n = 2-5. **, $p < 0.001$. **(B)** A bar graph depicting fraction of cells in the G0/G1 phase after transfection of Dgcr8 knockout and Rb family knockout and Dgcr8 knockout ESCs with let-7c, miR-26a, miR-99b, miR-193, miR-199a-5p, miR-218, and miR-294. Mean±s.d. for n = 4. *, $p < 0.05$. **(C)** qRT-PCR for Pou5f1/Oct4, Sox2, and Nanog normalized first to beta-actin then to mock transfection after miRNA introduction in **(i)** near Rb family and Dgcr8 knockout and **(ii)** Rb family and Dgcr8 knockout ESCs. n ≥ 3

Figure 7.
ESCC miRNAs suppress the R point and silencing of self-renewal through different mechanisms. ESCC miRNAs suppress the R point and G1 accumulation induced by differentiation-inducing miRNAs through targeting the Rb pathway. However, knocking out Rb family proteins is not sufficient to prevent silencing of self-renewal by differentiation-inducing miRNAs. Therefore, other pathways must be regulated by ESCC miRNAs to antagonize silencing of self-renewal by differentiation-inducing miRNAs. Additionally, ESCC miRNAs regulate Rb-independent pathways to promote G1/S transition and proliferation at standard culture conditions, since triple knockout of Rb family proteins neither prevent G1 accumulation nor promote proliferation rate in Dgcr8 knockout ES cells.