Title
Opposing microRNAs Regulate Mouse Embryonic Stem Cell Self-Renewal

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Opposing microRNAs Regulate Mouse Embryonic Stem Cell Self-Renewal

by

Collin Alfred Melton

DISSERTATION

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in

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Dedications and Acknowledgements

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Thank you parents, Lyndel and Marilyn Melton, for your continued love and support. Throughout my life at every level of my education you have encouraged me and pushed me to be the best I can be. When I was in elementary school you helped me do my first science projects. I remember rushing the evening prior to the deadline for one such project. We decided to study condensation forming on a glass of ice water! Every step of the way you were there to help me be the best I could be. Without you I would never have been in a position to write this thesis.

Thank you Archana for all your love and support during the last few years of graduate school. Thank you for listening to me blabber about my miRNAs. Thank you for your help thinking about my experiments and results. Thank you for encouraging me but also for keeping my ego in check! Thank you for calming me when I got mad. Thank you for picking me up when I was sad. Thank you for explaining my research to my parents. Most of all, thank you for making me happy during these past two plus years of graduate school.

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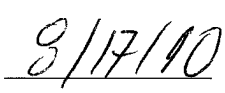
Official business

The text of this thesis Chapter 1 is a reprint with modifications of material as it appears in a chapter in the book *The Cell Biology of Stem Cells*. Chapter 3 is a reprint of material as it appears in *Nature*. The last coauthor listed in these publication, Robert Blelloch, directed and supervised the research that forms the basis for the dissertation/thesis. Robert Judson, coauthor in Chapter 3 contributed to the design and conception of the reprogramming experiments included in the subsection *Inhibition of let-7 promotes de-differentiation.* Experiments performed in Chapter 4 subsection *miR-99b, miR-26a, miR-218, miR-193, and miR-199a-5p regulate self-renewal in Dgcr8 -/- ESCs* was performed in collaboration with Deepa Subramanyam. In Chapter 2, flow cytometry in subsection *A*

subpopulation of Dgcr8 -/- ESCs maintains expression of ESC self-renewal markers was performed in conjunction with fellow graduate student Jan Lui during his rotation. Lastly, Jason Liu helped with qRT-PCR in Chapter 4 subsections *Deregulating the G1/S transition in Dgcr8 -/- ESCs* and *Inhibition of the G1/S transition in wild-type ESCs promotes loss of ESC self-renewal but doesn't sensitize ESCs to miRNA induced silencing of ESC self-renewal.* All other work was conceived and performed solely by Collin Melton under the guidance of Robert Blelloch.

Mentor Signature (Robert Blelloch)



Date

Opposing MicroRNAs Regulate Mouse Embryonic Stem Cell Self-Renewal

Collin Melton

When an embryonic stem cell (ESC) differentiates, it must both silence the ESC self-renewal program as well as activate new tissue-specific programs. In the absence of DGCR8, a protein required for microRNA (miRNA) biogenesis, mouse ESCs are unable to silence the ESC self-renewal program during differentiation. Screening by reintroduction of all known miRNAs one at a time into *Dgcr8* -/- ESCs in differentiation-inducing conditions enabled the identification of numerous miRNAs which silence the ESC self-renewal program. Expression levels of many of these miRNAs are induced during ESC differentiation. Of these miRNAs, most are expressed in specific cell types whereas a single family, the let-7 family, is broadly expressed across differentiated cell types. In various assays of ESC self-renewal, let-7 family miRNAs rescue the inability of *Dgcr8* -/- ESCs to silence self-renewal. However, let-7 miRNAs failed to silence self-renewal in wild-type ESCs, suggesting that ESC-expressed miRNAs inhibit the capacity of let-7 to silence self-renewal. Indeed, introduction of the embryonic stem cell cycle regulating (ESCC) miRNAs blocked the capacity of let-7 to induce silencing of self-renewal in *Dgcr8* -/- ESCs. mRNA profiling and bioinformatic analysis showed that let-7 and ESCC miRNAs function in part through opposite regulation of Myc transcription factors and Lin28. The opposing regulation of these factors contributes to a network, which reinforces the switch from a self-renewing to a differentiated cell state. These results suggested that additional screen positive miRNAs function in similar antagonistic

networks with ESCC miRNAs. Indeed, introduction of the ESCC miRNAs prevented the additional screen positive miRNAs from silencing self-renewal in *Dgcr8* -/- ESCs. mRNA profiling and bioinformatic analyses suggest that screen positive miRNAs and the ESCC miRNAs oppositely regulate multiple molecular pathways including the G1/S cell cycle transition. Inhibition of the G1/S transition in wild-type ESCs promotes loss of markers of ESC self-renewal. These findings suggest that miRNAs through destabilization of the ESC cell-cycle may promote loss of ESC self-renewal during differentiation. These studies show that different classes of miRNAs positioned in the context of complex biological networks function to either promote or antagonize ESC self-renewal.

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Thesis Summary

Embryonic stem cells (ESCs) possess a unique cellular state which allows them to both remain pluripotent (capable of giving rise to all embryonic lineages) and to indefinitely self-renew (self-replicate). These qualities together are referred to as ESC self-renewal. Two hallmarks of the ESC self-renewal program are a self-reinforcing transcriptional network and a specialized cell cycle profile. When an ESC differentiates (transitions to a more specified cell type), it must both silence the ESC self-renewal program as well as activate new tissue-specific programs. These changes during differentiation include a complete alteration in the microRNA (miRNA) expression profile. This thesis investigates the impact of various miRNAs to either reinforce or inhibit the ESC self-renewal program and how this added regulatory layer provides robustness to cell-fate decisions. I focus on murine ESCs and describe miRNA function in self-renewal, differentiation, and de-differentiation.

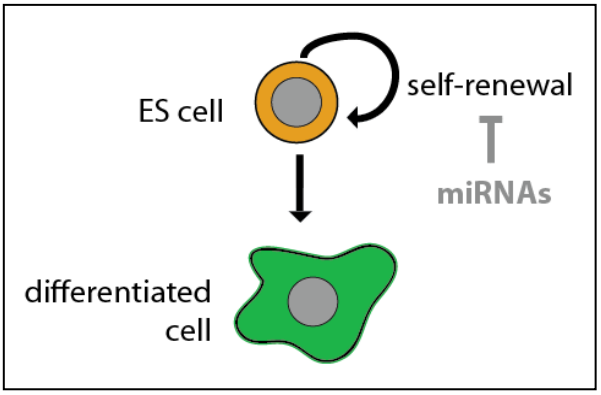


Figure 0-1: miRNAs inhibit the ESC self-renewal program allowing differentiation to occur

MicroRNAs are capable of modulating gene expression of hundreds of mRNA targets at a time. I hypothesized that this ability to act as global regulators of gene expression makes miRNAs well suited to mediate large-scale changes in a cell's proteome during cell fate transitions. I tested this hypothesis by studying differentiation in ESCs that lack canonical microRNAs. In **Chapter 2** of my thesis, I find that in the absence of DGCR8, a protein required for miRNA biogenesis, mouse

ESCs are unable to silence the ESC self-renewal program during differentiation (**Figure 0-1**). This effect was observed in all differentiation conditions tested. To identify the specific miRNAs responsible for this phenotype, I performed a screening assay by adding back miRNA mimics one at a time into *Dgcr8* -/- ESCs in differentiation-inducing conditions. I found a number of miRNAs that strongly induce silencing of self-renewal in *Dgcr8* -/- ESCs. To determine whether these microRNAs are developmentally relevant, I examined whether these miRNAs are normally induced upon differentiation of ESCs. I performed miRNA microarray analysis of wild-type ESCs in two differentiation-inducing conditions: 1) exposure to retinoic acid (RA), a potent inducer of the neural lineage and 2) removal of leukemia inhibitory factor (LIF) from the culture media, which allows spontaneous differentiation down multiple lineages. This data, in addition to published tissue-specific miRNA expression data in both mouse and human, demonstrated that the screen-positive miRNAs fall into two classes: those that are upregulated during differentiation to specific lineages and those that are broadly upregulated during differentiation. In the latter category, I identified a large family of miRNAs known as the let-7 family.

In **Chapter 3**, of my thesis I study the function of the let-7 family in silencing ESC self-renewal. Using various assays of ESC self-renewal, I confirmed that addition of let-7 rescues the inability of *Dgcr8* -/- ESCs to silence self-renewal. Interestingly, let-7 miRNAs failed to induce silencing of self-renewal in wild-type ESCs. This led me to hypothesize that ESC-expressed miRNAs inhibit the capacity of let-7 to silence self-renewal. Indeed, introduction of the members of a family of miRNAs highly expressed in

ESCs—the embryonic stem cell, cell cycle regulating (ESCC) miRNAs—antagonized the capacity of let-7 to silence self-renewal. To understand the molecular basis of this antagonism, I performed mRNA profiling followed by bioinformatic analyses to identify oppositely regulated genes and pathways. Initially, I showed that let-7 and ESCC miRNAs inhibit distinct sets of transcripts with seed sequence matches in their 3' untranslated regions (UTRs) and open reading frames (ORFs). My study is among the first to globally identify miRNA targets with 3' UTR and/or ORF seed sequence matches. Among the genes oppositely regulated by the let-7 and ESCC miRNAs are two with known roles in promoting ESC self-renewal, Lin28 and Sall4. Interestingly, Lin28 is a known negative regulator of let-7 miRNA precursors (**Figure 0-2**).

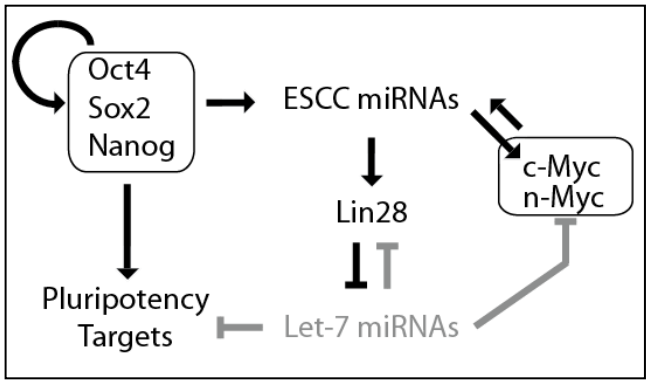


Figure 0-2: Let-7 and ESCC miRNAs oppositely alter expression of critical regulators of ESC self-renewal

To identify pathways oppositely regulated by the let-7 and ESCC miRNAs, I took a novel approach using previously published datasets of genes bound by key ESC transcription factors. I performed analyses to examine whether these transcription factor bound genes were enriched within the transcripts up and/or downregulated by the let-7 and ESCC miRNAs. I found that let-7 directly targets genes in pathways activated by the ESC transcription factors Oct4, Sox2, and Nanog (**Figure 0-2**). I further identified opposing effects of the two miRNA families on Myc transcriptional activity (**Figure 0-2**). I found

and validated that n-Myc is a direct let-7 target while c-Myc is indirectly upregulated by ESCC miRNAs. To follow up on Myc activity as a central regulator of ESC self-renewal, I generated *c-Myc* -/- ESCs. These cells are slightly more prone to let-7 silencing of ESC self-renewal than wild-type ESCs suggesting that Myc is one of multiple factors, which underlie the opposing effects of let-7 and ESCC miRNAs.

In summary, the 3rd chapter of my thesis describes a bistable network of two families of miRNAs and their targets. In the ESC state, the ESCC miRNA family is dominant and via its target interactions functions to support ESC self-renewal and inhibit expression of let-7 miRNAs. In the differentiated state, the let-7 miRNA family is dominant and via its target interactions suppresses the ESC self-renewing state. The opposing regulation of these factors contributes to a network, which reinforces the switch from a self-renewing to a differentiated cell state (**Figure 0-2**). In further support of this concept, inhibition of the let-7 family promotes de-differentiation of somatic cells to self-renewing induced pluripotent stem cells. Together, these findings show how the ESCC and let-7 miRNAs act through common pathways to alternatively stabilize the self-renewing versus differentiated cell fates.

In **Chapter 4**, the final chapter of my thesis, I follow up on the additional

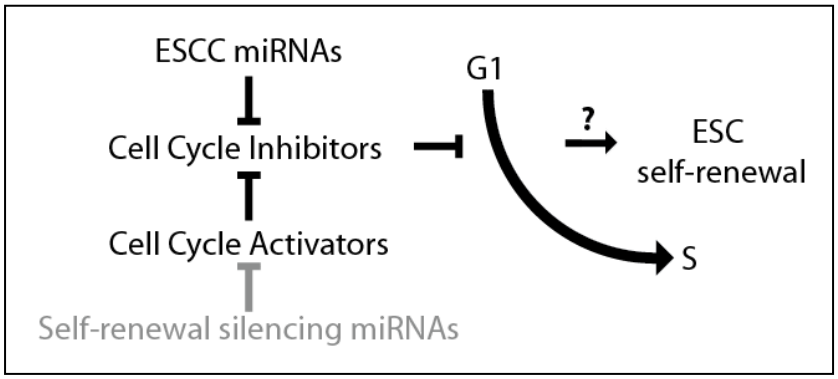


Figure 0-3: ESCC and self-renewal silencing miRNAs oppositely alter G1/S cell cycle regulators to potentially regulate ESC self-renewal

screen positive miRNAs from Chapter 2. These miRNAs, which include miR-99b, miR-26a, miR-218, miR-193, and miR-199a-5p, robustly silence ESC self-renewal when introduced into *Dgcr8 -/-* ESCs. However, like let-7, they are unable to silence ESC self-renewal in wild-type ESCs and their ability to silence self-renewal in *Dgcr8 -/-* ESCs is specifically blocked by the ESCC miRNAs. This finding led me to hypothesize that these miRNAs which silence self-renewal either target common genes or pathways, which are oppositely regulated by the ESCC miRNAs. To address this hypothesis, I have performed microarray profiling and bioinformatic analyses. The ESC self-renewal silencing miRNAs have only a few common mRNA targets, yet via disparate targets regulate multiple molecular pathways including the G1/S cell cycle transition. In contrast, it is well established that ESCC miRNAs have the opposite effect of promoting the G1/S transition. Therefore, I hypothesize that the ESCC miRNA regulation of the cell-cycle protects against ESC differentiation (**Figure 0-3**). I test this hypothesis by blocking the G1/S transition in wild-type ESCs by over-expression of the cell cycle inhibitor P21. I find that p21 over-expression artificially inhibits cell cycle progression and leads to precocious differentiation. This suggests that inhibition of the cell cycle is sufficient to silence the self-renewal program in ESCs. To test whether ESCC regulation of the cell cycle is sufficient to confer resistance to differentiation by ESC self-renewal silencing miRNAs, I attempt to deregulate the G1/S transition in *Dgcr8 -/-* ESCs by removal of the Rb family members p107 and p130. These cells to not have a deregulated cell cycle and are not resistant to miRNA-induced silencing of ESC self-renewal. Likely, the remaining Rb family member pRb is sufficient to maintain a slow cell cycle in *Dgcr8 -/-* ESCs.

Overall, these studies suggest that miRNA regulation of the cell cycle and pluripotency are intrinsically linked processes in ESC differentiation.

In **conclusion**, these studies show that different classes of miRNAs positioned in the context of complex biological networks function to either promote or antagonize ESC self-renewal. These studies in addition to revealing novel aspects of developmental biology have implications for regenerative medicine and cancer. In regenerative medicine, these studies are already contributing to research aimed at more efficiently and safely generating induced pluripotent stem (iPS) cells. Addition of ESCCs or inhibition of let-7 miRNAs promote iPS cell generation. In cancer, ESCC and related miRNAs are oncogenic whereas let-7 miRNAs act as tumor suppressors. Understanding how these miRNAs perform their function will undoubtedly contribute to our understanding of disease and potentially to the generation of more effective therapeutics.

Chapter 1: Introduction

Summary

Stem cell differentiation requires a complex coordination of events to transition from a self-renewing to a differentiated cell fate. Stem cells can be pluripotent (capable of giving rise to all embryonic lineages), multipotent (possessing the potential to give rise to multiple lineages), and unipotent (capable of giving rise to a single cell lineage). Regardless of their potency all stem cells must silence their self-renewal program during differentiation. The self-renewal program can be defined as the integration of external and internal stimuli that enables a cell to proliferate while maintaining its potency. Two hallmarks of the self-renewal program are a self-reinforcing transcriptional network and a specialized cell cycle profile. This thesis investigates the impact of various microRNAs (miRNAs) to either reinforce or inhibit the self-renewal program of stem cells and how this added regulatory layer provides robustness to cell-fate decisions. In this introduction, I focus on embryonic stem cells (ESCs) describing miRNA function in self-renewal, differentiation, and de-differentiation.

The self-renewal program

The stem cell self-renewal program in both embryonic and somatic stem cell populations functions to maintain potency during successive rounds of replication. The degree of potency and proliferative rate vary greatly among stem cell populations in accordance with the evolutionary pressures and biological functions of these populations. ESCs are derived from the inner cell mass of the developing blastocyst and resemble cells of the developing epiblast. The epiblast gives rise to the embryonic endoderm, mesoderm, and

ectoderm, as well as the germ lineage(Surani, Hayashi, & Hajkova, 2007). Epiblast cells initially have a rapid cell cycle and are pluripotent, but eventually differentiate. Like the epiblast cells, ESCs have a rapid cell cycle and are pluripotent. However, unlike epiblast cells, ESCs can self-renew indefinitely in the culture dish.

During embryonic development, the epiblast cells differentiate into specialized fetal stem cell populations that have a more limited potency. These include, among others, the fetal neural stem cells and hematopoietic stem cells. These fetal stem cells retain a high proliferative rate but possess a limited potency(Kriegstein & Alvarez-Buylla, 2009; Mikkola & Orkin, 2006). Eventually, the fetal stem cells are replaced by adult lineage specific stem cells including adult counterparts of the fetal hematopoietic and neural stem cells. These cells also have a limited potency, but unlike their fetal counterparts, typically have a slow proliferative rate. In fact, adult somatic stem cell populations are largely quiescent, and they generate transient populations of progenitor cells, which typically have a rapid proliferative rate more like that of their fetal stem cell counterparts. Quiescence in adult stem cells may have evolved to reduce the chance of harmful mutations, such as those that cause cancer(Arai & Suda, 2008).

Embryonic Stem Cells

The molecular basis of the stem cell self-renewal program has been best studied in ESCs. In these cells the self-renewal program is determined by the interaction of numerous factors at the center of which is a distinct transcriptional network(Jaenisch & Young, 2008). In ESCs, the central transcriptional network includes the transcription factors

Oct4, Sox2, Nanog, Tcf3, and the Myc family of proteins (cMyc and nMyc). The coordinated actions of these transcription factors both directly and indirectly determines an epigenetic state poised to activate or repress upon differentiation the transcription of genes of any lineage of the three germ layers(Jaenisch & Young, 2008). In this way the ESC transcriptional network enables its pluripotency. Additionally the ESC transcriptional network drives expression of factors that enable the cell's high proliferative rate by directly and indirectly maintaining the rapid ESC cell cycle.

During ESC differentiation the many components of the self-renewal program must be shut off and a new differentiated program must be activated. Therefore, this cell fate transition is regulated by factors that both silence self-renewal and induce a lineage specific differentiation program. These factors can be classified broadly as those that influence gene expression at the level of chromatin state, transcription, transcript stability, protein translation, protein stability, or protein function.

In the introduction to this thesis, I will focus on the pro-self-renewal and pro-differentiation functions of miRNAs.

miRNA biogenesis and function

miRNAs are small non-coding RNAs which act to post-transcriptionally silence gene expression through translational inhibition and mRNA destabilization. miRNAs are generated through the sequential processing of RNA transcripts (**Figure 1-1**). miRNAs are first transcribed as long RNA polymerase II transcripts termed primary miRNAs (pri-

miRNAs)(Cai, Hagedorn, & Cullen, 2004; Lee, et al., 2004). These pri-miRNAs can be either non-coding or coding. In the latter case, miRNAs will often reside within the intron of a coding gene(Rodriguez, 2004). In the nucleus, the pri-miRNA is recognized and cleaved by the microprocessor complex, which consists of the RNA binding protein DGCR8 and the RNAse III enzyme DROSHA(Basyuk, Suavet, Doglio, Bordonné, & Bertrand, 2003; Denli, Tops, Plasterk, Ketting, & Hannon, 2004; Gregory, et al., 2004; Han, et al., 2004; Lee, et al., 2003). This complex recognizes a stem loop structure of approximately 33 base pairs in length and posses an enzymatic activity that cleaves the loop 11 base pairs from its base leaving a characteristic 2 nucleotide 3' overhang(Han, et al., 2006). The processed RNA, now termed pre-miRNA, is exported from the nucleus to the cytoplasm by Exportin V where it is recognized by a second complex containing the RNAse III enzyme DICER(Bohnsack, Czaplinski, & Gorlich, 2004; Lund, Güttinger, Calado, Dahlberg, & Kutay, 2004; Yi, Qin, Macara, & Cullen, 2003). This complex recognizes the pre-miRNA hairpin and cleaves it at the base of the hairpin loop again to form a 2 nucleotide 3' overhang to generate an approximately 22 nucleotide mature miRNA duplex(Hammond, 2005). This mature duplex remains double-stranded until it is incorporated into the RNA-induced silencing complex (RISC). Only a single strand of the small RNA duplex is incorporated, typically the strand with the less stable 5' end(Schwarz, et al., 2003).

miRNAs which are loaded into the RISC complex directly interact with their mRNA targets through base pairing to sites in the open reading frame and 3' untranslated region. These interactions depend on base pairing of a 6-8 nucleotide seed sequence of the

Figure 1-1

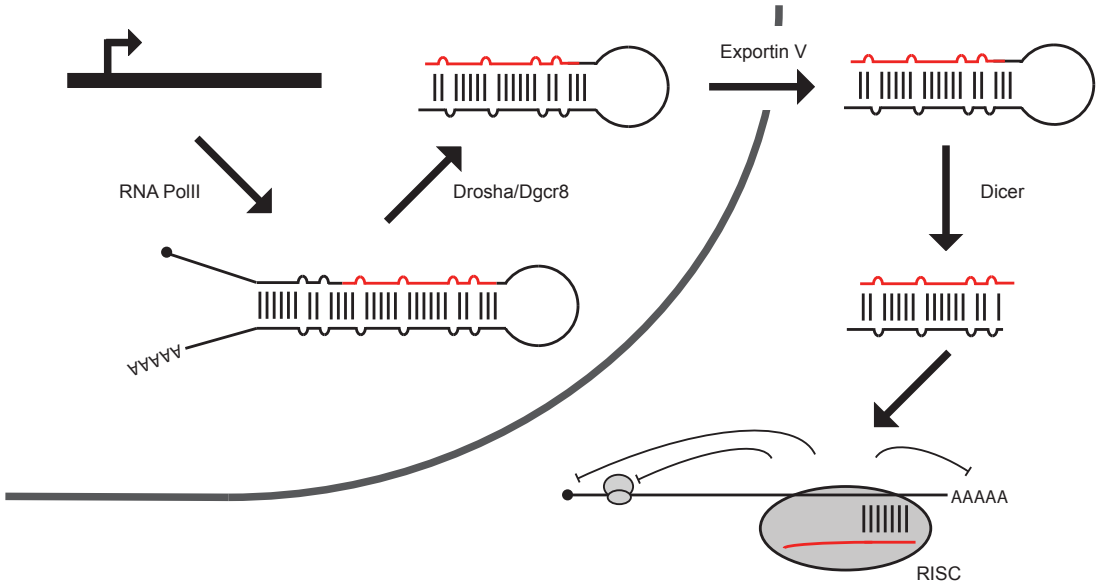


Figure 1-1: miRNA biogenesis. miRNAs are first transcribed as long RNA polymerase II transcripts. The hairpin structure of these transcripts is recognized by the Microprocessor complex composed of DROSHA and DGCR8 and is cleaved to form a smaller pre-miRNA hairpin. The pre-miRNA is exported from the nucleus and subsequently cleaved by DICER to form a mature miRNA duplex. A single strand of this duplex is loaded into the RISC complex. The miRNA loaded complex destabilizes and inhibits translation of its target mRNAs.

miRNA (nucleotides 2-8 on the 5' end) with the mRNA target(Bartel, 2009). The RISC complex which is bound to target mRNAs disrupts protein production through a variety of mechanisms including disruption of ribosome initiation via interacting with the 5' cap, prevention of ribosome elongation, and promotion of RNA degradation by shortening of the polyA tail(Filipowicz, Bhattacharyya, & Sonenberg, 2008).

ESCC miRNAs promote self-renewal

Many miRNAs are co-expressed from a single transcript. One such group is the miR-290 cluster, which consists of 7 miRNAs, and is highly expressed in mouse ESCs. A

subgroup of the miR-290 cluster miRNAs along with a second cluster, the miR-302 cluster, share a common seed sequence and regulate the ESC cell cycle and, therefore, have been coined the ESCC family (ESC cell cycle promoting miRNAs)(Y. Wang, et al., 2008). Related families to the ESCC miRNAs include the mir-17/20/106 family, which has a slightly different seed sequence. The miR-290 cluster is not conserved in human and instead the dominant miRNAs are miR-372, miR-373, and the miR-302 cluster miRNAs, which possess identical seed sequences to their mouse ESCC counterparts(Merav Bar, et al., 2008; M.-R. Suh, et al., 2004).

The common expression of similar miRNAs in pluripotent stem cells in mouse and human suggests an important functional role in ESC self-renewal. Indeed, the first evidence for such a function was uncovered in ESC miRNA knockout models through deletion of either *Dicer* or *Dgcr8*(Kanellopoulou, et al., 2005; Murchison, Partridge, Tam, Cheloufi, & Hannon, 2005; Y. Wang, Medvid, Melton, Jaenisch, & Blelloch, 2007). These ESCs have a slowed proliferation rate and an altered cell cycle profile with an extended G1 phase(Y. Wang, et al., 2007). These findings are particularly interesting as wild-type mouse ESCs are characterized by an atypical cell cycle with a abbreviated G1 phase compared to somatic cells(Savatier, Huang, Szekely, Wiman, & Samarut, 1994). These initial findings suggested that the ESC expressed miRNAs suppress the somatic cell cycle structure.

The abbreviated G1 phase of ESCs promotes their rapid proliferation and is, at least in part, secondary to an alleviation of the G1/S restriction point(Savatier, et al., 1994). In a

typical somatic cell, the G1/S restriction point prevents the initiation of S phase and DNA replication. The G1/S restriction point includes a complex series of signaling events, which must reach a threshold before transitioning into S phase. Key molecular components of this reaction include, but are not limited to, the cyclins, the cyclin dependent kinases (CDKs), cdk inhibitors (CKIs), the Rb family of proteins, and the E2F family of proteins(Planas-Silva & Weinberg, 1997).

D and E type cyclins in complex with CDKs drive phosphorylation of the Rb family of proteins(Giacinti & Giordano, 2006). In mouse ESCs, CyclinE is expressed at high levels independent of cell cycle phase whereas CyclinD is not expressed(Savatier, Lapillonne, van Grunsven, Rudkin, & Samarut, 1996). CyclinE complexes with CDK2, to initiate the phosphorylation and subsequenct inactivation of the Rb family of proteins (pRb, P107, and P130). The Rb family of proteins, when in a hypophosphorylated active state, sequester activating E2Fs (E2F1-3) as well as activate repressive E2F proteins (E2F4 and 5) preventing transcription of S phase genes(Giacinti & Giordano, 2006). When Rb proteins are hyperphosphorylated and inactivated, they no longer activate the repressive E2Fs. Simultaneously, the suppression of the activating E2Fs is relieved, which allows them to drive transcription of S phase genes. Progression to S phase can be blocked by CDK inhibitors, which include members of the CIP and INK families. These inhibitors block activity of CDK/Cyclin complexes(Mittnacht, 1998). INK family inhibitors are non-functional in mouse ESCs as they act through CyclinD, which is not expressed at high levels. CIP family inhibitors, however, are more promiscuous in their inhibitory effects on CDK/Cyclin complexes and are able to bind and inactivate

Figure 1-2

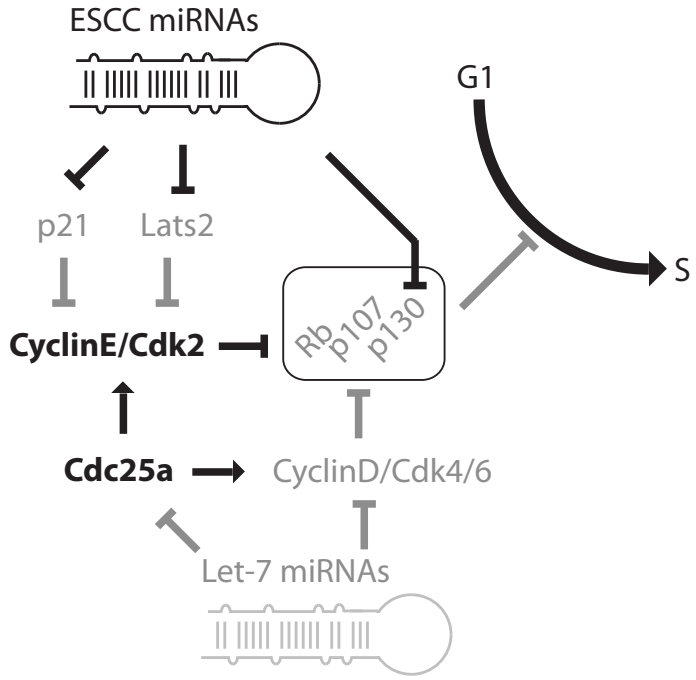


Figure 2: Let-7 and ESCC miRNAs have opposing effects on the G1-S transition. This figure represents a model of the direct inhibitory effects of the ESCC and let-7 miRNAs on factors involved in the ESC G1-S transition. As ESCs transition from a self-renewing to a differentiated state, the ESCC miRNAs are down-regulated and the let-7 miRNAs are upregulated. These changes have direct consequences on the cell-cycle. Dark/bold arrows, lines, and text indicate interactions, miRNAs, and proteins that are up-regulated in the ESC state. Grey arrows, lines, and text indicate interactions, miRNAs, and proteins that are down-regulated in the ESC state. Note the interactions and functional consequences of the let-7 miRNAs on cell-cycle have been tested in various somatic cell populations, but not ESCs.

CDK2/CyclinE complexes(Mittnacht, 1998). In mouse ESCs, CIP family inhibitors are expressed at low levels, as are the Rb proteins(Savatier, et al., 1994; Savatier, et al., 1996).

By screening miRNAs, which enhance proliferation in a *Dgcr8* knockout (-/-) ESC background, the role of ESCC miRNAs in cell cycle control was uncovered. These miRNAs not only increase proliferation of *Dgcr8* -/- ESCs, but also decrease the number

of cells in the G1 phase of the cell cycle. This effect on the G1 phase is in part through direct miRNA targeting of the CIP family CDK inhibitor P21, LATS2, and some of the Rb family of proteins including pRb and P130. Through inhibition of these and other predicted miRNA targets involved in the G1 phase, the ESCC miRNAs promote the ESC cell cycle (**Figure 1-2**)(Y. Wang, et al., 2008).

In **Chapter 3,** I present an in depth analysis of the impact of the ESCC miRNAs on the ESC transcriptome. We discovered that the ESCC miRNAs indirectly activate cMyc expression(Melton, Judson, & Blelloch). Myc is a transcription factor that both promotes proliferation and is required for ESC self-renewal(Cartwright, et al., 2005; Singh & Dalton, 2009). Additionally, in ESCs inhibition of Myc proteins promotes loss of ESC self-renewal, while enforced expression of cMyc prevents loss of self-renewal in the absence of LIF(Cartwright, et al., 2005). Lin *et al*. recently sought to identify the mechanisms by which Myc proteins promote ESC self-renewal. In particular they found that cMyc drives transcription of numerous pro-self-renewal miRNAs including miR-141, miR-200, and miR-429. These miRNAs promote the maintenance of self-renewal in the absence of LIF although the biological mechanisms underlying these effects remain unknown(C.-H. Lin, Jackson, Guo, Linsley, & Eisenman, 2009). Furthermore, cMyc regulates expression of the ESCC miRNAs forming a positive feedback loop as described below.

A number of other factors have been identified as indirectly upregulated by the ESCC miRNAs including the DNA methyl transferases (DNMT3a & b)(Benetti, et al., 2008;

Sinkkonen, et al., 2008). The increase in expression of these DNA methyl transferases is required to maintain appropriate DNA methylation in sub-telomeric regions, which in turn is required to prevent abnormal telomere elongation(Benetti, et al., 2008). The regulation of DNMT3a & b occurs via ESCC targeting of P130—a negative regulator of DNMT3a & b transcription)(Benetti, et al., 2008; Sinkkonen, et al., 2008). In addition to the DNA methyl transferases, a number of other pluripotency-associated transcripts are indirectly upregulated by the ESCC miRNAs. These include Lin28, Trim71, and Sall4(Melton, et al.). Together these numerous molecular changes induced by the ESCC miRNAs have a profound effect on promoting the cell cycle and preserving faithful maintenance of telomeres to ensure proper ESC self-renewal and maintenance of pluripotency.

miRNAs induced during ESC differentiation suppress the self-renewal program

As miRNAs are suited to stabilizing the self-renewing state, so are they well situated to promote the transition from self-renewal to differentiation. Mouse ESCs globally deficient in miRNAs are unable to silence the ESC self-renewal program when exposed to differentiation inducing conditions(Kanellopoulou, et al., 2005; Murchison, et al., 2005; Y. Wang, et al., 2007). This suggested to us that microRNAs are required for the silencing of the ESC self-renewal program. Indeed, we and others find microRNAs that silence ESC self-renewal. These microRNAs can be categorized by their targets and by their expression patterns. A small number of miRNAs have been found to directly target components of the central ESC transcriptional network(Tay, Zhang, Thomson, Lim, & Rigoutsos, 2008; Y. M. S. Tay, et al., 2008; N. Xu, Papagiannakopoulos, Pan, Thomson,

& Kosik, 2009). These same miRNAs are induced rapidly during ESC differentiation down specific lineages. A second class of microRNAs is induced during differentiation down a broad set of lineages and broadly suppress ESC associated genes but not the central ESC transcription factors themselves(Melton, et al., 2010). They also promote a somatic cell cycle(Johnson, et al., 2007; M. S. Kumar, et al., 2008; Schultz, Lorenz, Gross, Ibrahim, & Kunz). These two classes of pro-differentiation miRNAs likely play distinct roles in the differentiation process. The first class of microRNAs directly suppress ESC self-renewal state, while the second class of microRNAs predominantly stabilize the differentiated state—much like the ESC microRNAs stabilize the ESC state.

MiRNAs miR-134, miR-296, and miR-470 have been discovered to directly suppress Nanog, Pou5f1 (also known as Oct4), and Sox2 in mouse ESCs(Y. Tay, et al., 2008; Y. M. S. Tay, et al., 2008). These miRNA-target interactions occur predominantly through interactions in the open reading frame. These miRNAs are highly upregulated during retinoic acid (RA) induced differentiation, which induces predominantly neural differentiation suggesting that these miRNAs may be involved in lineage specific silencing of ESC self-renewal. In human ESCs, miR-145 was found to directly suppress ESC self-renewal via targeting Oct4, Sox2, and Klf4(N. Xu, et al., 2009). Understanding the biological functions and relative *in vivo* contributions of various direct miRNA suppressors of self-renewal will be an important area of future pursuit.

In contrast to the miRNAs which directly suppress ESC self-renewal, in **Chapter 3**, I find that the let-7 family of miRNAs are stabilizers of the differentiated cell fate(Melton,

et al., 2010). Mutations in let-7 were first discovered in C. elegans in a mutagenesis screen for genes that prevented terminal differentiation of seam cells in the hypodermis(Reinhart, et al., 2000). Since the discovery of let-7 in C. elegans, homologues of let-7 have been found in all metazoans studied(Pasquinelli, et al., 2000). In mouse and human there are 9 distinct let-7 family members with varied tissues specific expression patterns(Griffiths-Jones, 2004; Griffiths-Jones, Grocock, van Dongen, Bateman, & Enright, 2006; Griffiths-Jones, Saini, van Dongen, & Enright, 2008; Landgraf, et al., 2007). In ESCs an elegant mechanism exists which allows for the post-transcriptional silencing of let-7 transcripts(Hagan, Piskounova, & Gregory, 2009; Heo, et al., 2008; Heo, et al., 2009; Rybak, et al., 2008; Thomson, et al., 2006; Viswanathan, Daley, & Gregory, 2008). A complex of the RNA binding protein, Lin28, and the terminal uridyl-transferase, TUT4, binds to and induces the degradation of pre-let-7 transcripts. Lin28 expression is quickly lost during ESC differentiation(L. Wu & Belasco, 2005; Yang & Moss, 2003), which allows for the rapid increase in let-7 expression(Thomson, et al., 2006).

In **Chapter 3** of this thesis I will present data indicating that let-7 family members induce silencing of self-renewal in the miRNA deficient *Dgcr8 -/-* ESCs but not in wild-type ESCs(Melton, et al., 2010). This observation suggested that miRNAs expressed in ESCs normally prevent let-7 from silencing ESC self-renewal. Indeed, the ESCC miRNAs that predominate in ESCs, are able to prevent loss of self-renewal induced by the let-7 miRNAs. Let-7 preferentially targets transcripts that are enriched in ESCs, including many transcripts that are regulated by the pluripotency transcription factors Oct4, Sox2,

Nanog, and Tcf3. Additionally, a number of direct targets of let-7 are indirectly upregulated by the ESCC miRNAs, which can explain how the ESCCs antagonize let-7. Among the targets with opposing regulation by let-7 and the ESCCs are the Myc proteins, Sall4, Lin28, and Trim71(Melton, et al., 2010).

The antagonism observed between the ESCC and let-7 miRNAs, and the targets which are regulated in opposing fashion by these miRNAs, suggest a network in which ESCC miRNAs and let-7 miRNAs have mutually exclusive expression and function (**Figure 1-3**). In ESCs, the ESCC miRNAs lead to upregulation of Lin28, which directly suppresses let-7 maturation. Additionally, ESCCs indirectly upregulate cMyc and other direct let-7 targets that promote ESC self-renewal. By these mechanisms ESCC miRNAs counteract the effects of let-7. ESCC miRNA expression is promoted by Oct4, Sox2, and Nanog(Marson, et al., 2008). As ESCs differentiate, Oct4, Sox2, and Nanog expression decrease resulting in a corresponding decrease in ESCC expression. In the absence of ESCCs, Lin28 levels also decrease. In this differentiated state, let-7 is no longer inhibited and feeds back to directly target Lin28 thereby reinforcing its own expression. Furthermore, let-7 now stabilizes the differentiated state by limiting expression of factors required for the ESC fate including transcripts that were previously activated by the pluripotency transcription factors Nanog, Oct4, and Sox2.

The let-7 miRNAs in addition to suppressing the ESC transcriptional program also promote the somatic cell cycle (**Figure 1-3**). Let-7 miRNAs target both directly and indirectly multiple activators of the G1-S transition including cdc25a, cdk6, cyclinD1,

Figure 1-3

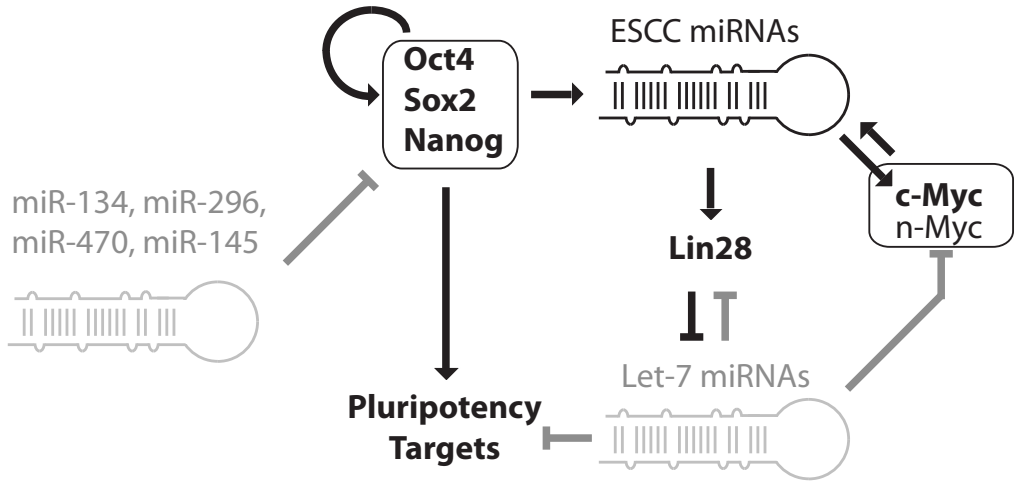


Figure 3: miRNA interactions in the ESC self-renewal network. This figure represents a model of the direct inhibitory and indirect activating effects of the ESCC, let-7, and miR-134, miR-296, miR-470, and miR-145 miRNAs. Dark/bold arrows, lines, and text indicate interactions, miRNAs, and proteins that are up-regulated in the ESC state. Grey arrows, lines, and text indicate interactions, miRNAs, and proteins that are down-regulated in the ESC state. As ESCs differentiate, the miR-134, miR-296, miR-470, and miR-145 miRNAs destabilize the Oct4/Sox2/Nanog transcriptional network to promote differentiation, whereas the let-7 miRNAs inhibit Myc and downstream targets of the Oct4/Sox2/Nanog network to stabilize the differentiated state.

and cyclinD2(Johnson, et al., 2007; Schultz, et al.). These interactions and others contribute to the overall effect of the let-7 miRNAs on increasing the number of cells in the G1 phase of the cell cycle(Johnson, et al., 2007; M. S. Kumar, et al., 2008; Schultz, et al.). It remains unclear how or if the cell cycle directly influences ESC self-renewal. It has been postulated that in the G1 phase cells are most susceptible to pro-differentiation signaling cascades including MAPK signaling(Burdon, Smith, & Savatier, 2002). It will be important to understand in more detail the interactions between the cell cycle and the ESC transcriptional network and to understand the impact of miRNAs on these interactions.

A small number of miRNAs suppress ESC self-renewal in a similar fashion to the let-7 family. These miRNAs, like the let-7 family, only suppress ESC self-renewal in the absence of the ESCC miRNAs. Many of these miRNAs similar to the let-7 family may suppress progression though the G1 phase of the cell cycle. These data are discussed further in **Chapter 4**.

Regulatory networks controlling miRNA expression

In ESCs, ESCC miRNA expression from the miR-290 cluster is controlled by the pluripotency transcription factors Nanog, Oct4, Sox2, and Tcf3 as well as by the Myc transcription factors nMyc and cMyc(Judson, Babiarz, Venere, & Blelloch, 2009; Marson, et al., 2008). ESCC miRNAs indirectly upregulate cMyc to form a positive feedback loop which likely reinforces their own expression. When ESCs differentiate, pluripotency transcription factors are downregulated and in turn so are the ESCC miRNAs(Marson, et al., 2008).

Transcriptional control of expression of direct miRNA suppressors of ESC self-renewal remains an open area of research; however, high-throughput sequencing of chromatin immuno-precipated factors (ChIP seq) data in ESCs give us some insight into their regulation. In ESCs the miR-296 promoter is bound by Oct4, Sox2, Nanog, and Tcf3; however, it is also marked by repressive H3K27 methylation and is bound by the polycomb group protein Suz12(Marson, et al., 2008). These data suggest a mechanism by which miR-296 is poised to be activated in ESCs. If upon differentiation the repressive H3K27 histone mark is rapidly lost prior to loss of Oct4, Sox2, and Nanog,

these transcription factors could transiently drive transcription of miR-296. This regulation would form a negative feedback loop leading to more robust loss of ESC self-renewal. One way in which H3K27 methylation could be rapidly lost would be through the post-translational inactivation of the transcriptional repressor responsible for recruiting the polycomb complex. The binding data of these factors at the miR-134 and miR-470 promoters has yet to be determined.

Likewise transcriptional control of let-7 expression remains relatively unclear. Different let-7 transcripts are expressed in the various differentiated tissues and thus likely diverse transcription factors are able to induce let-7 expression(Griffiths-Jones, 2004; Griffiths-Jones, et al., 2006; Griffiths-Jones, et al., 2008; Landgraf, et al., 2007). In ESCs, Oct4, Sox2, and Nanog drive expression of the let-7g primary transcript(Marson, et al., 2008). The primary transcripts are processed to pre-miRNAs in ESCs where they are degraded by the Lin28/Tut4 complex(Hagan, et al., 2009; Heo, et al., 2008; Heo, et al., 2009; Rybak, et al., 2008; Thomson, et al., 2006; Viswanathan, et al., 2008). As ESCs differentiate, suppression by Lin28/Tut4 is lost and mature let-7 is produced(Thomson, et al., 2006; L. Wu & Belasco, 2005; Yang & Moss, 2003). Additional miRNAs are regulated in this way in ESCs(Heo, et al., 2009).

Recently, a new class of regulatory RNA binding proteins, the Trim-NHL proteins, has been discovered. In neural stem cells, Schwamborn *et al.* showed that expression of Trim32 potentiates let-7 inhibition of targets and is associated with the differentiation of NSCs(Schwamborn, Berezikov, & Knoblich, 2009). In ESCs, the ESCC miRNAs

promote expression of Trim71 (also known as Mlin-41). Trim71 is a let-7 target essential for mouse development(Maller Schulman, et al., 2008). Rybak *et al.* demonstrated that Trim71 acts as an ESC expressed E3 ubiquitin ligase that functions to degrade Ago2 protein, a component of the RISC complex(Rybak, et al., 2009). Both Trim32 and Trim71 are members of a larger family of Trim-NHL proteins, which also include the Drosophila proteins Brat and Mei-P26. These Drosophila proteins also function to modulate the miRNA pathway through interactions with Ago1(Neumuller, et al., 2008). It will be important to understand if Trim71 simply functions to modulate activity of the entire miRNA pathway via degradation of Ago2 or if like Trim32 it can associate and increase the activity of specific miRNA subtypes.

Conclusion

The data summarized in this introduction and presented in this thesis support an important role for various miRNA species in either stabilizing the self-renewing state of stem cells or in promoting their differentiation. These miRNAs are similar to other global regulators of gene expression as different subclasses of these miRNAs can either promote or inhibit stem cell self-renewal. These impacts on self-renewal occur both through regulation of the cell cycle and through regulation of the stem cell transcriptional program. As we learn more about the miRNAs that influence stem cell self-renewal it is becoming clear these miRNAs are tightly regulated in complex biological networks. This regulation can occur at various levels both transcriptional and post-transcriptional. Furthermore, different classes of miRNAs can inhibit or activate each other's expression.

Understanding the extent and function of these networks in development will greatly enhance our knowledge of both developmental and disease states.

Chapter 2: MicroRNAs are required for silencing mouse ESC self-renewal

Summary

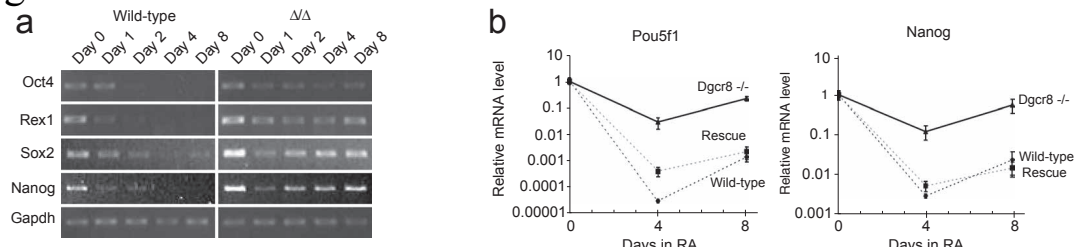
MicroRNAs are capable of modulating gene expression of 100s of mRNA targets at a time. I hypothesized that this ability to act as a global regulator of gene expression makes them well suited to mediate large-scale changes in a cells proteome during cell fate transitions. I tested this hypothesis by studying differentiation in ESCs that lack canonical microRNAs. I found that in the absence of DGCR8, a protein required for microRNA (miRNA) biogenesis, mouse ESCs are unable to silence the ESC self-renewal program during differentiation. This effect, observed in all differentiation conditions tested, was demonstrated using multiple assays. To identify the specific miRNAs responsible for this phenotype, I performed a screening assay adding back miRNA mimics one at a time into *Dgcr8* -/- ESCs in differentiation-inducing conditions. I found a number of miRNAs that strongly induce silencing of self-renewal in *Dgcr8* -/- ESCs. To examine whether these miRNAs are normally induced upon differentiation of ESCs, I performed miRNA microarray analysis of wild-type ESCs in two differentiation-inducing conditions: 1) exposure to retinoic acid (RA), a potent inducer of the neural lineage and 2) removal of leukemia inhibitory factor (LIF) from the culture media, which allows spontaneous differentiation down multiple lineages. This data, in addition to published tissue-specific miRNA expression data, demonstrated that the screen-positive miRNAs fall into two classes: those that are upregulated during differentiation to specific lineages and those that are broadly upregulated during differentiation.

Background

To date three global miRNA knockout models have been generated in ESCs: a *Dicer* conditional knockout, a *Dgcr8* conditional knockout, and an *Ago*1-4 knockout(Kanellopoulou, et al., 2005; Murchison, et al., 2005; Su, Trombly, Chen, & Wang, 2009; Y. Wang, et al., 2007). Work by others in the Blelloch laboratory demonstrated that *Dgcr8* -/- ESCs cells are globally deficient in canonical miRNA biogenesis(Joshua E. Babiarz, Ruby, Wang, Bartel, & Blelloch, 2008; Y. Wang, et al., 2007). This was shown by loss of specific precursor and mature miRNA transcripts as assayed by Northern blot analysis and globally by miRNA microarray expression arrays and high-throughput sequencing analysis(Joshua E. Babiarz, et al., 2008; Y. Wang, et al., 2007). Like DGCR8, DICER protein is essential for miRNA biogenesis; however, DICER, unlike DGCR8, is known to be required for the processing of double stranded RNA to generate siRNAs. Likewise, Argonautes are required for siRNA function and their loss in ESCs leads to apoptosis(Su, et al., 2009). Both loss of *Dicer* and *Ago1-4* in ESCs leads to increased cell death whereas *Dgcr8 -/-* ESCs are viable. Thus, the *Dgcr8 -/-* miRNA knockout ESC model is not only the most specific to canonical miRNAs but also is not prone to increased apoptosis thus making it the best choice for studying the specific role of miRNAs in ESCs.

Prior to the generation of *Dgcr8 -/-* ESCs, limited evidence suggested a role for miRNAs in ESC differentiation. The strongest such evidence comes from differentiation assays of *Dicer -/-* ESCs. In embryoid body differentiation assays, *Dicer -/-* ESCs are deficient in

Figure 2-1 a-b



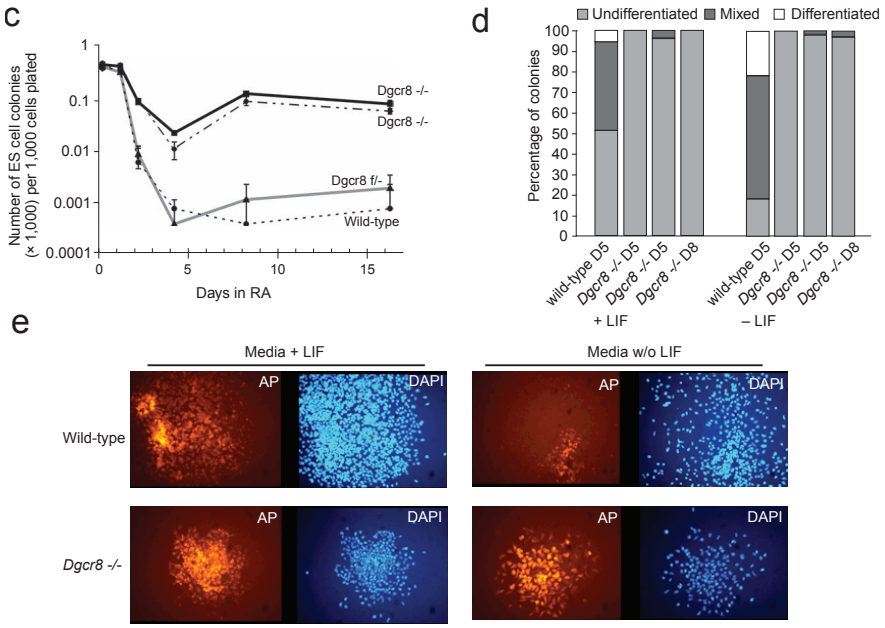
Dgcr8 -/- ESCs are resistant to differentiation. (a) RT-PCR analysis of pluripotency markers. Wild-type and *Dgcr8* -/- ESCs were plated as a monolayer and treated with retinoic acid (RA) in the absence of LIF to induce differentiation. Gapdh was used as a loading control. (b) Quantitative PCR analysis of the pluripotency markers Oct4 and Nanog (n=3). The beta-actin gene was used as a reference. For each sample, data were normalized to the mRNA level at day 0.

expression of the markers of various differentiated lineages and do not significantly down-regulate expression of Oct3/4, a key marker of undifferentiated ESCs(Kanellopoulou, et al., 2005). Furthermore, prior to my work, preliminary data showed that *Dgcr8* -/- embryoid bodies maintain a higher expression of markers of ESC self-renewal(Y. Wang, et al., 2007).

***Dgcr8* -/- ESCs exposed to retinoic acid fail to down-regulate ESC self-renewal markers**

To interrogate the role of miRNAs in the silencing of self-renewal during ESC differentiation, I first tested the ability of *Dgcr8* -/- ESCs to differentiate in a retinoic acid (RA) induced differentiation protocol. RA rapidly induces near complete differentiation of wild-type ESCs to a neural fate(Walker, et al., 2007). In this assay, ESCs are plated as a monolayer on gelatin coated plates in the absence of MEFs and LIF and in the presence of 100nM all trans retinoic acid. Strikingly, *Dgcr8* -/- ESCs failed to fully down-regulate expression of the key pluripotency associated genes Nanog and Oct3/4, retaining

Figure 2-1 c-e



Dgcr8 -/- ESCs are resistant to differentiation. (c) ESC colony formation of differentiated cells. After varying durations of differentiation, cells were returned to ESC culture conditions and assayed for their ability to form alkaline phosphatase–positive colonies. Error bars indicate the range of measurements (n=3). (d) Clonal analysis of ESC differentiation in the absence of MEF feeders and in the presence or absence of LIF, as indicated. Shown are percentages of undifferentiated, mixed and differentiated colonies under the indicated conditions. 'D5' indicates 5 d; 'D8' indicates 8 d. (e) Representative examples of clonal analysis from d.

approximately 100 fold greater expression of these markers compared to both wild-type ESCs and a rescue cell line (*Dgcr8 -/-* ESCs retargeted to *Dgcr8 f/-*) (**Figure 2-1a & b**).

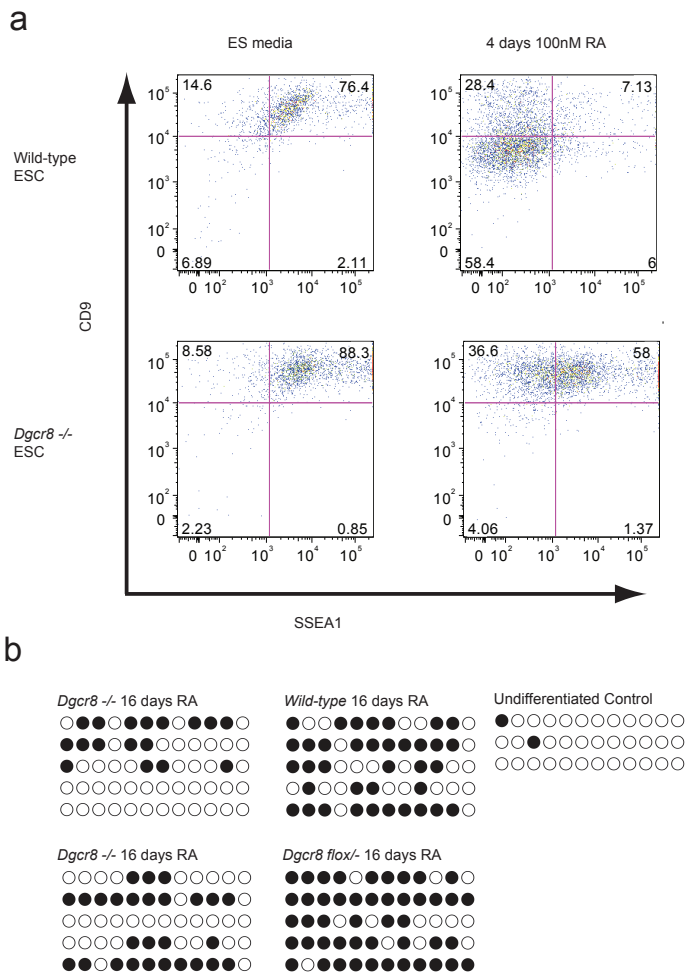
To test the functional capacity of the RA differentiated cultures to reform ESC colonies, I disassociated differentiated cultures and plated a defined number of cells at colony forming density in ESC conditions (media + LIF and MEFs). After 5 days of culture, colonies grew out from these individually plated cells. These colonies were fixed, stained for alkaline phosphatase (AP) activity (an established marker of ESCs), and counted. The results of this experiment match those of the expression data (**Figure 2-1c**).

Approximately 10% of the *Dgcr8 -/-* cells after even up to 16 days of exposure to RA are able to reform ESC colonies as opposed to roughly 0.1% for the control cells.

Dgcr8 -/- ESCs fail to down-regulate ESC self-renewal markers in the absence of LIF

It was possible that the observed defect in the differentiation of *Dgcr8 -/-* ESCs is specific to retinoic acid induced differentiation but not other differentiation protocols. To investigate the generality of the observed defect in the silencing of ESC self-renewal during differentiation, I used a clonal differentiation assay. In this assay, wild-type and *Dgcr8 -/-* ESCs were plated at colony forming density on gelatin either in the presence or absence of LIF. Both conditions, plus and minus LIF, are differentiating with the absence of LIF being an even stronger differentiating condition. After five and eight days of colony formation, the colonies were fixed and stained for AP activity. Cultures were scored as undifferentiated (all cells stained AP positive), mixed (typically an inner core of cells remained AP positive with a surrounding differentiated ring), or differentiated (no detectable AP positive cells). The results as seen in **Figure 2-1d & e** demonstrate a striking resistance to differentiation in the *Dgcr8 -/-* ESCs. 100% and > 95% of *Dgcr8 -/-* colonies remained totally undifferentiated in the presence and absence of LIF respectively compared to approximately 50% and < 20% for wild-type controls. These experiments demonstrate that *Dgcr8 -/-* ESCs are globally resistant to silencing of ESC self-renewal in conditions that promote differentiation to diverse lineages.

Figure 2-2



Assaying silencing of ESC self-renewal in *Dgcr8 -/-* ESCs at the single cell level. (a) Flow cytometric analysis of wild-type or *Dgcr8 -/-* ESCs untreated or treated for 4 days in 100nM RA. Staining was performed with SSEA1 and CD9 antibodies. (b) bisulfite sequencing of the Oct3/4 promoter was performed on *Dgcr8 -/-*, *Dgcr8 flox/-*, and wild-type ESCs treated for 16 days with 100 nM RA. Undifferentiated ESC DNA was used as a control.

A subpopulation of Dgcr8 -/- ESCs maintains expression of ESC self-renewal markers

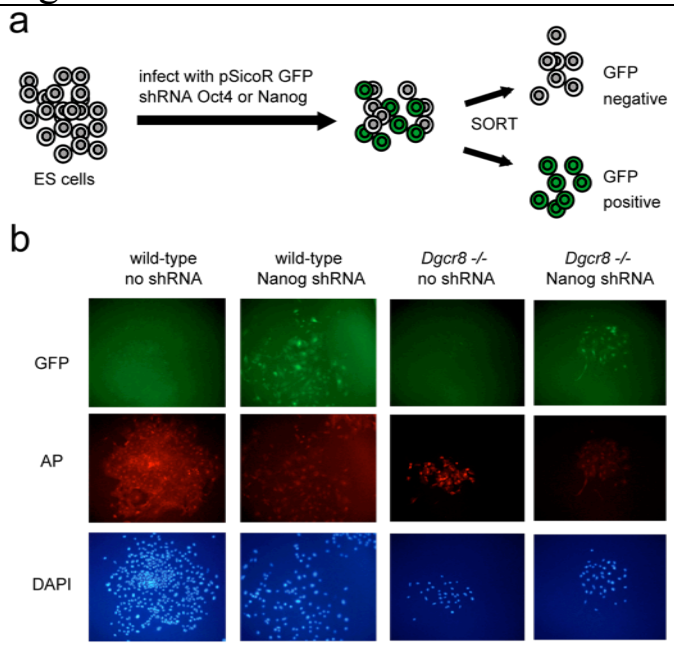
The results thus far, testing the ability of *Dgcr8 -/-* ESCs to silence self-renewal during differentiation, are based on experiments assaying differentiation at the population level. These results do not address the possibility of an incomplete block in differentiation (i.e.

that some small population of *Dgcr8 -/-* ESCs are able to differentiate). If this were true and enough undifferentiated cells continued to self-renew, an equilibrium could be reached in which a constant fraction of cells mantain the ESC state. Alternatively, it is also possible that *Dgcr8 -/-* ESCs fail to lock in a differentiated state and are in equilibrium between the ESC state and some intermediate stage of differentiation. To begin to test these hypotheses, I used flow cytometric analysis to analyze differentiation at the single cell level.

I chose to pursue this approach with antibodies against CD9 (BD pharmigen) and SSEA1 (University of Iowa Developmental Studies Hybridoma bank). These markers were previously shown to be expressed highly in ESCs and are rapidly down-regulated upon differentiation(Cui, et al., 2004). Our results confirm that these two surfaces markers are expressed highly in undifferentiated wild-type ES cells and are significantly down-regulated upon 4 days of exposure to retinoic acid in the absence of LIF. Similar analyses in *Dgcr8 -/-* ESCs reveals a small population, on the order of 10% of cells which retain expression of both CD9 and SSEA1 after 4 days of RA differentiation (**Figure 2-2a**).

As an alternative method to measure differentiation status on a single cell level, I performed bisulfite sequencing of the Oct3/4 promoter of RA treated *Dgcr8 -/-* ESCs. This promoter is unmethylated in ESCs and highly methylated upon differentiation. I performed sequencing on 16 day RA treated *Dgcr8 -/-*, wild-type, and heterozygous cells in addition to an undifferentiated wild-type ESC control. 3 of 10 *Dgcr8 -/-* clones

Figure 2-3 a&b

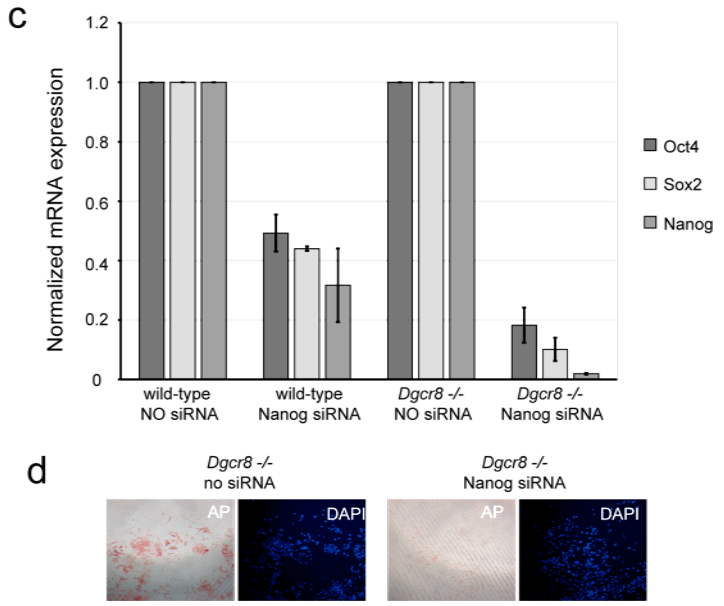


Depletion of Nanog in *Dgcr8* -/- ESCs by RNAi silences ESC self-renewal. (a) A schematic of the shRNA infection and sorting strategy used in b. (b) Colonies isolated from infected (GFP +) and uninfected (GFP -) *Dgcr8* -/- and wild-type ESCs. Images of GFP mark infected cells, alkaline phophatase (AP) marks self-renewing ESCs, and DAPI marks the nuclei.

compared to 0 of 10 wild-type and heterozygous clones sequenced remained completely unmethylated at the Oct3/4 promoter (**Figure 2-2b**).

These data suggest that at any given moment on the order of 10-30% of *Dgcr8* -/- ESCs express markers of ESC self-renewal and remain undifferentiated. It remains a possibility that the remaining 90% of cells are capable of reverting back to the undifferentiated state. However, this seems unlikely considering that only approximately 10% of cells are able to reform colonies at these same time points after exposure to RA. These data suggest that miRNAs are required for the efficient silencing of ESC self-renewal during ESC differentiation.

Figure 2-3 c&d



Depletion of Nanog in *Dgcr8 -/-* ESCs by RNAi silences ESC self-renewal. (c) Nanog siRNA was transfected into wild-type and *Dgcr8 -/-* ESCs in the absece of LIF and qRT-PCR was performed for Oct4, Sox2, and Nanog (n=2). (d) As in (c) Nanog siRNA was transfected into *Dgcr8 -/-* ESCs. Images of alkaline phophatase stain mark undifferentiated ESCs and DAPI marks the nuclei.

Knockdown of Nanog silences self-renewal in *Dgcr8 -/-* ESCs

To understand in further mechanistic detail the resistance of *Dgcr8 -/-* ESCs to differentiation, I tested the effects of shRNA mediated knock-down of Oct4 and Nanog. I used shRNA vectors generated and validated by Gaspar-Maia *et al.*(Gaspar-Maia, et al., 2009) Wild-type and *Dgcr8 -/-* ESCs were infected with pSicoR GFP shRNA vectors and subsequently sorted for GFP positive and negative cells. The sorted cells were plated at colony forming density on gelatin-coated plates in the presence of LIF (**schematic in Figure 2-3a**). The Oct4 shRNA induced substantial cell death; whereas, the Nanog shRNA resulted in a substantial reduction in AP positive colonies (**Representative Images**, **Figure 2-3b**). I followed up on this phenotype by performing AP staining and qRT-PCR for Oct4, Nanog, and Sox2 after transient transfection with a Nanog siRNA.

Table 2-1 Screen Positive miRNAs

miRNA	average screen score (high is more differentiated)	median survival score (high is more survival)
mmu-miR-122a	8	1
mmu-miR-345	8	6
mmu-miR-450	8	6
mmu-miR-149	7.3	5
mmu-miR-199a	7.3	6
mmu-miR-218	7.2	7
mmu-miR-127	7	4
mmu-miR-145	7	6
mmu-miR-199b	7	6
mmu-miR-216	6.7	3
mmu-miR-362	6.7	4
mmu-miR-452	6.7	6
mmu-miR-26a	6.7	5
mmu-miR-134	6.5	1
mmu-miR-193	6.5	2
mmu-let-7d	6.3	2
mmu-miR-128a	6.3	5
mmu-miR-34c	6.3	1
mmu-miR-409	6.3	3
mmu-miR-422b	6.3	2
mmu-miR-468	6.3	4
mmu-miR-129-5p	6.2	5
mmu-let-7b	6	3
mmu-let-7c	6	3
mmu-miR-100	6	5
mmu-miR-140	6	4
mmu-miR-18	6	2
mmu-miR-31	6	2
mmu-miR-384	6	5
mmu-miR-464	6	4
mmu-miR-465	6	2
mmu-miR-99b	6	4

Score represent relative number of AP positive cells as follows: 1, 100%; 2, > 75%; 3, 75%; 4, > 50%; 5, 50%; 6, < 50%; 7, 25%; 8, < 25%

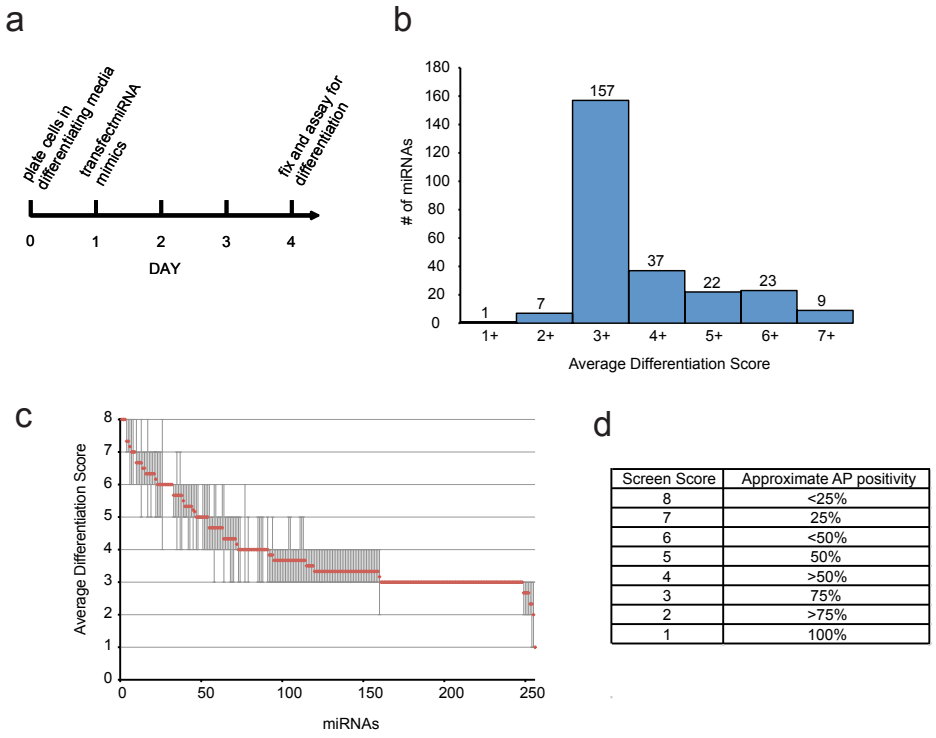
The Nanog siRNA in the absence of LIF led to a dramatic decrease in alkaline phosphatase positivity and mRNA levels of Oct4 and Nanog, 3 days post-transfection (**Figure 2-3c&d**). These data indicate that forced repression of the ESC transcriptional network overcomes the block in differentiation in *Dgrc8 -/-* ESCs. I hypothesized that miRNAs may perform a similar function to destabilize the ESC transcriptional network during differentiation.

Screening identifies miRNAs that silence ESC self-renewal in Dgcr8 -/- ESCs

The deficiency of *Dgcr8 -/-* ESCs to efficiently silence self-renewal indicated that miRNAs normally function to reinforce silencing of the ESC self-renewal program. Such miRNAs would be up-regulated in the developing embryo as pluripotent cells of the epiblast differentiate into more committed progenitor populations. To identify such miRNAs, I screened a library of miRNA mimics (Dharmacon Inc) for their ability to rescue differentiation of *Dgcr8 -/-* ESCs in the absence of LIF. A schematic of the strategy is depicted in **Figure 2-4a**. Briefly, in a 96 well format, cells were grown for 3 days in the absence of LIF after transfection of miRNA mimics. As a positive control in this screening assay I used Nanog siRNA. As a readout of differentiation I stained for alkaline phosphatase activity. Each mimic was scored for its ability to induce differentiation (% AP positive cells) and its toxicity/impact on proliferation (% confluency).

Scores for the top hits from the screen are contained in **Table 2-1**. This semi-quantitative strategy was the most feasible since many of the miRNA mimics we tested resulted in significant cell death. By this screening strategy I identified 54 miRNAs which were able to induce at least 50% differentiation in *Dgcr8 -/-* ESCs (screen score 5+) and 32 which were able to induce at least 62.5% differentiation (screen score 6+) (**Figure 2-4b&c, Table 2-1**). Notably, this set of miRNAs includes miR-134, which has been shown to silence the ESC self-renewing state in mouse ESCs(Y. Tay, et al., 2008; Y. M. S. Tay, et al., 2008), and miR-145, which similarly has been shown to silence the ESC self-renewing state in human ESCs(N. Xu, et al., 2009). miR-296 which was reported to silence ESC self-renewal in wild-type ESCs(Y. Tay, et al., 2008) had no effect in the

Figure 2-4



Screen Score	Approximate AP positivity
8	<25%
7	25%
6	<50%
5	50%
4	>50%
3	75%
2	>75%
1	100%

Screening identifies miRNAs that silence ESC self-renewal in *Dgcr8* -/- ESCs (a) a schematic of the screening strategy (b) Individual miRNAs were binned into categories based on their average score of n = 3 in the screening assays. A score of 1 means completely undifferentiated whereas a score of 8 means completely differentiated. (c) miRNA screen data plotted for individual miRNAs with the error representing the range of n = 3. (d)

screen. This could be a false negative for technical reasons. In some instances, miRNA mimics obtained independently of the screen plates gave differential results consistent with the these mimics in the screen being non-functional. MiR-470 which is also reported to silence ESC self-renewal in mouse ESCs(Y. Tay, et al., 2008) was not present on our screening plates.

Considering the large number of screen positive miRNAs and the large number of potential mRNA targets of any one miRNA, I feared that a large number of the screen

positive miRNAs reflected a non-developmentally relevant effect. To identify developmentally relevant miRNAs I sought to identify miRNAs that were expressed during multi-lineage ESC differentiation both in mouse and human. To identify those miRNAs up-regulated in mouse ESC differentiation, I performed miRNA microarray analysis of wild-type undifferentiated ESCs, wild-type ESCs treated with 1uM RA for 4 days, and wild-type ESCs removed from LIF for 4 days. RNA samples were collected and mRNA qPCR was performed to validate the extent of the differentiation achieved in each sample. For miRNA profiling, I used Exiqon microarrays of triplicate biological replicates. I found that a number of screen positive miRNAs were up-regulated upon ESC differentiation (**Figure 2-5a, Table 2-2**). For these miRNAs I compared miRNA expression data from high throughput small RNA sequencing of human ESC EB differentiation as well as mouse ESC derived NPCs and mouse embryonic fibroblasts (MEFs) to identify miRNAs(M. Bar, et al., 2008) that were commonly up-regulated upon both human and mouse ESC differentiation (**Table 2-2**). I reasoned that miRNAs with conserved expression patterns in mouse and human would more likely have conserved functions contributing to silencing of the self-renewing state. From this analysis I identified the let-7 family of miRNAs, the miR-99/100 family, miR-26a, miR-218, miR-193, and miR-199a-5p/miR-199b-5p. For a subset of these miRNAs, to validate the microarray data, I performed additional miRNA qPCR during mouse ESC differentiation (**Figure 2-5b**).

Of the candidate miRNAs/miRNA families, the let-7 family of miRNAs is among the most well studied in embryonic development. An increase in let-7 expression is

associated in diverse organisms with the transition from embryonic to adult tissue(Pasquinelli, et al., 2000). In mouse embryonic stem cells and in the developing embryo, it has been demonstrated that let-7 expression is regulated by degradation of the pre-miRNA transcript by a complex of the RNA binding protein Lin28 and the terminal uridyl transferase TUT4(Hagan, et al., 2009; Heo, et al., 2008; Heo, et al., 2009; Newman, Thomson, & Hammond, 2008; Rybak, et al., 2008; Thomson, et al., 2006; Viswanathan, et al., 2008). This regulation allows for a rapid increase in mature let-7 expression during ESC differentiation. Additionally, recent work has shown that an increase in let-7 expression in somatic breast stem cells and breast cancer tumor initiating cells plays a causal role in the differentiation of these cells(Ibarra, Erlich, Muthuswamy, Sachidanandam, & Hannon, 2007; F. Yu, et al., 2007). For these reasons, I chose to first study the role of the let-7 family in ESC differentiation.

Discussion

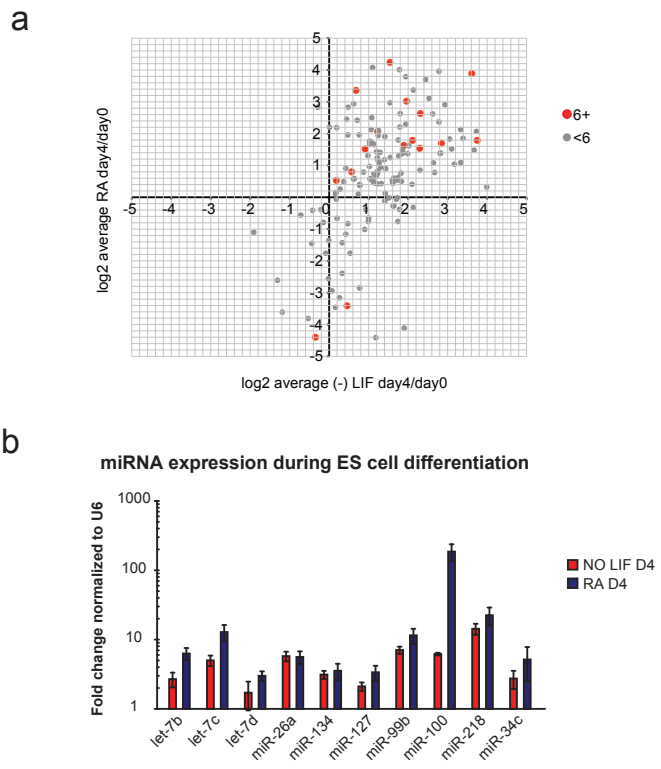
My findings in this chapter indicate that miRNAs are required for the silencing of the ESC self-renewal program during mouse ESC differentiation. These data suggest that miRNAs exist which actively silence ESC self-renewal during differentiation. Alternatively, miRNAs present in ESCs could prime ESCs for differentiation. Screening miRNAs that can silence ESC self-renewal in *Dgcr8 -/-* ESCs allowed me to identify a number of miRNAs, which can potently rescue silencing of ESC self-renewal in *Dgcr8 -/-* ESCs. Additionally, previous work identified miR-134, miR-296, and miR-470 as suppressors of Nanog, Pou5f1, and Sox2 in mouse ESCs(Y. Tay, et al., 2008; Y. M. S. Tay, et al., 2008). These miRNAs, however, are specifically up-regulated during RA

differentiation towards the neural lineage indicating that they may not broadly suppress ESC self-renewal down all lineages. My data indicate that a few miRNAs including the let-7 family are broadly up-regulated upon differentiation down all lineages and suppress the self-renewing ESC state.

Table 2-2

	Microarray log2 fold change		Deep Sequencing (counts per million reads)				
MiRNA	-LIF D4/D0	RA D4/D0	hES UD	hES Diff	mES UD	mEF	mNPC
mmu-miR-122a			267.9	607.1	4.0	0.0	2.9
mmu-miR-345			0.0	0.0	12.1	29.0	116.5
mmu-miR-450			0.0	0.0	0.0	0.0	0.0
mmu-miR-149			28.6	685.7	20.2	58.1	119.4
mmu-miR-199a			564.3	5878.6	2.0	8108.2	8.7
mmu-miR-218	1.2	2.1	0.0	1121.4	42.5	643.8	8.7
mmu-miR-127			0.0	0.0	890.3	4414.7	0.0
mmu-miR-145	-0.4	-4.4	3.6	364.3	24.3	914.9	11.7
mmu-miR-199b			17.9	457.1	2.0	271.1	2.9
mmu-miR-216			0.0	0.0	0.0	0.0	0.0
mmu-miR-362			0.0	42.9	18.2	101.7	102.0
mmu-miR-452	3.8	1.8	0.0	7.1	0.0	9.7	0.0
mmu-miR-26a	2.0	3.0	10.7	2664.3	2978.4	5232.8	11767.1
mmu-miR-134	2.1	1.8	0.0	0.0	91.1	643.8	0.0
mmu-miR-193	0.6	0.8	7.1	1471.4	28.3	227.5	23.3
mmu-let-7d	2.3	2.6	0.0	0.0	62.7	11206.2	12807.2
mmu-miR-128a			10.7	35.7	141.6	58.1	294.3
mmu-miR-34c	1.5	4.2	14.3	285.7	28.3	3741.9	116.5
mmu-miR-409			0.0	0.0	24.3	111.3	0.0
mmu-miR-422b			0.0	0.0	125.4	295.3	294.3
mmu-miR-468	0.9	1.5	0.0	0.0	0.0	0.0	0.0
mmu-miR-129-5p	2.8	1.7	0.0	0.0	4.0	4.8	64.1
mmu-let-7b	2.3	1.5	3.6	0.0	24.3	12058.2	16113.9
mmu-let-7c	1.9	1.6	3.6	85.7	261.0	18089.7	41544.7
mmu-miR-100			0.0	0.0	0.0	300.1	69.9
mmu-miR-140	0.2	0.5	0.0	0.0	135.6	503.4	215.6
mmu-miR-18					1161.4	784.2	652.6
mmu-miR-31	0.7	3.4	100.0	1742.9	8.1	585.7	2.9
mmu-miR-384			0.0	0.0	0.0	0.0	119.4
mmu-miR-464	0.5	-3.4	0.0	0.0	0.0	0.0	0.0
mmu-miR-465			0.0	0.0	0.0	0.0	0.0
mmu-miR-99b	3.6	3.9	2896.4	9871.4	56.7	609.9	492.4

Figure 2-5



miRNA profiling identifies screen positive miRNAs that are upregulated upon ESC differentiation. (a) miRNA microarray profiling in either RA or –LIF media. Data are plotted on a scatterplot. Red dots mark screen positive miRNAs. Gray mark all other miRNAs from the screen for which microarray data are present. (b) miRNA qRT-PCR for individual screen positive miRNAs to validate microarray data. Red marks fold change after 4 days in the absence of LIF and blue mark fold change after 4 days of exposure to RA. Fold change is relative to undifferentiated ESCs.

Chapter 3: Opposing microRNA families regulate mouse embryonic stem cell self-renewal

Summary

When embryonic stem cells (ESCs) differentiate, they must both silence the ESC self-renewal program as well as activate new tissue specific programs. In the absence of DGCR8 (*Dgcr8* -/-), a protein required for microRNA (miRNA) biogenesis, mouse ESCs are unable to silence self-renewal. Here, we find that the introduction of let-7 miRNAs, a family of miRNAs highly expressed in somatic cells, can suppress self-renewal in *Dgcr8* -/-, but not wild-type ESCs. Introduction of ESC cell cycle regulating (ESCC) miRNAs into the *Dgcr8* -/- ESCs, blocks the capacity of let-7 to suppress self-renewal. Profiling and bioinformatic analyses show that let-7 inhibits while ESCC miRNAs indirectly activate numerous self-renewal genes. Furthermore, inhibition of the let-7 family promotes de-differentiation of somatic cells to induced pluripotent stem (iPS) cells. Together, these findings show how the ESCC and let-7 miRNAs act through common pathways to alternatively stabilize the self-renewing versus differentiated cell fates.

Introduction

Mammalian development follows a carefully orchestrated unfolding of cell fate transitions leading to a complex set of highly specialized cell types. These cell fate transitions involve the silencing of previously active molecular programs along with the activation of new ones(Joshua E Babiarz & Blelloch, 2009; Hornstein & Shomron, 2006). MiRNAs are small non-coding RNAs that are well suited to suppress previously active

programs and, thereby, provide robustness to cell fate decisions. MiRNAs identify their targets via base pairing of nucleotides 2-8 of the miRNA (the seed sequence) with complementary sequences within the target mRNA's open reading frame (ORF) and 3′ untranslated region (UTR)(Joshua E Babiarz & Blelloch, 2009). This targeting is carried out in coordination with the RNA-induced silencing complex (RISC) and often results in both destabilization and translational inhibition of the targets. While inhibition of any one target is usually only partial, each miRNA binds and suppresses hundreds of mRNA targets, resulting in large overall changes in the molecular constitution of cells.

Removal of genes required for maturation of all miRNAs has shown that miRNAs play essential roles in the proliferation and differentiation of ESCs(Kanellopoulou, et al., 2005; Murchison, et al., 2005; Y. Wang, et al., 2007). For example, the loss of the RNA binding protein DGCR8, which is required for the production of all canonical miRNAs, results in a cell cycle defect and an inability to silence the self-renewal program of ESCs when they are placed in differentiation-inducing conditions(Y. Wang, et al., 2007). The introduction of individual members of a family of miRNAs, the ESCC miRNAs, into *Dgcr8 -/-* ESCs can rescue the cell cycle defect(Y. Wang & Blelloch, 2009). These same miRNAs are able to enhance the de-differentiation of somatic cells to iPS cells(Judson, et al., 2009). Here, we report the identification of another large family of miRNAs, the let-7 family, which performs the opposite role to the ESCC family. When introduced into *Dgcr8 -/-* ESCs, let-7 silences self-renewal by suppressing many of the same downstream targets that are indirectly activated by the ESCC family. Indeed, co-introduction of the

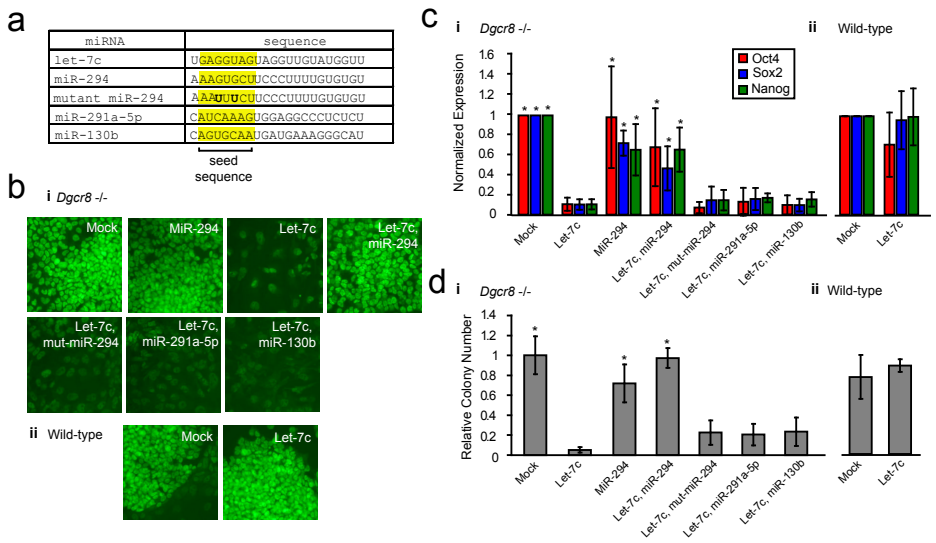
ESCC miRNAs inhibits the capacity of let-7 to silence self-renewal, and suppression of the let-7 family in somatic cells promotes de-differentiation.

Let-7 and ESCCs regulate self-renewal

The let-7 miRNAs are broadly expressed across differentiated tissues(C. Chen, et al., 2007; Landgraf, et al., 2007) and are tightly regulated during ESC differentiation(Heo, et al., 2008; Newman, et al., 2008; Rybak, et al., 2008; Thomson, et al., 2006; Viswanathan, et al., 2008). Therefore, we hypothesized that the let-7 miRNAs could rescue the capacity of *Dgcr8 -/-* ESCs to silence ESC self-renewal when induced to differentiate. To test this hypothesis we introduced mimics of a representative let-7 family member, let-7c, into the *Dgcr8 -/-* ESCs **(Figure 3-1a)**. Let-7c silenced the ESC self-renewal program even when the ESCs were maintained in ESC culture conditions. Three days after treatment with let-7c, *Dgcr8 -/-* cells downregulated ESC associated markers including alkaline phosphatase activity (**Figure 3-2, panel i**), Pou5f1/Oct4 immunofluorescence staining (**Figure 3-1b, panel i**), and mRNA expression of Pou5f1/Oct4, Sox2, and Nanog (**Figure 3-1c, panel i**). Furthermore, the transfected cells showed a diminished capacity to reform ESC colonies in replating assays, a functional test of ESC self-renewal capacity (**Figure 3-1d, panel i**). Similar effects were observed with the introduction of let-7a, let-7b, let-7d, and let-7g (**Figure 3-3**) and these effects were observed over a range of concentrations, including levels normally found in more differentiated cell types (**Figure 3-4**).

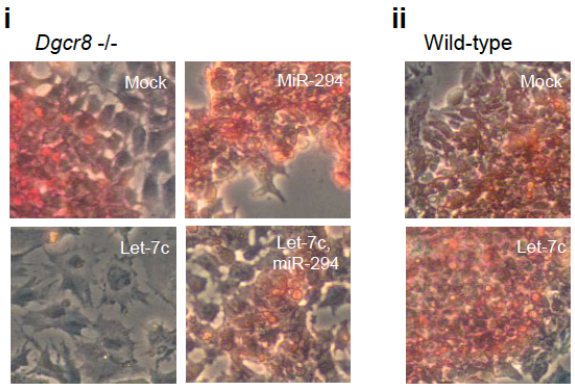
In contrast to the *Dgcr8* -/- ESCs, wild-type ESCs were resistant to let-7c (**Figure 3-1, panel ii & 1b-d, panel ii**). This finding suggested that other miRNAs normally expressed in wild-type ESCs inhibit let-7c-induced suppression of self-renewal. The ESCC miRNAs are likely candidates as they make up a majority of miRNA molecules in mouse ESCs(Calabrese, Seila, Yeo, & Sharp, 2007; Marson, et al., 2008), they are rapidly downregulated upon differentiation coincident with the upregulation of mature let-7 (**Figure 3-5**), and they promote the ESC fate(Judson, et al., 2009; Y. Wang, et al., 2008). Therefore, we introduced a representative member of this family, miR-294, to test if it could block let-7c-induced suppression of *Dgcr8* -/- ESC self-renewal. Three days after co-introduction of miR-294 and let-7c, *Dgcr8* -/- ESCs retained alkaline phosphatase activity (**Figure 3-2, panel i**), Pou5f1/Oct4 immunofluorescence staining (**Figure 3-1b, panel i),** and mRNA expression of Pou5f1/Oct4, Sox2, and Nanog (**Figure 3-1c, panel i**). Furthermore, miR-294 rescued the colony forming capacity of the *Dgcr8* -/- ESCs (**Figure 3-1d, panel i**). Control miRNAs (miR-294 with a seed mutation and other ESC expressed miRNAs, miR-291a-5p and miR-130b, that do not contain the ESCC miRNA seed sequence) did not antagonize the effects of let-7c (**Figure 3-1a-d**) showing that miR-294's effect is not simply secondary to competition for RISC complexes. Other members of the ESCC family miR-291a-3p, miR-291b-3p, and miR-295 were similarly able to block the effects of let-7c (**Figure 3-6**). These data indicate that the let-7 and ESCC families of miRNAs have opposing roles in the maintenance of ESC self-renewal.

Figure 3-1



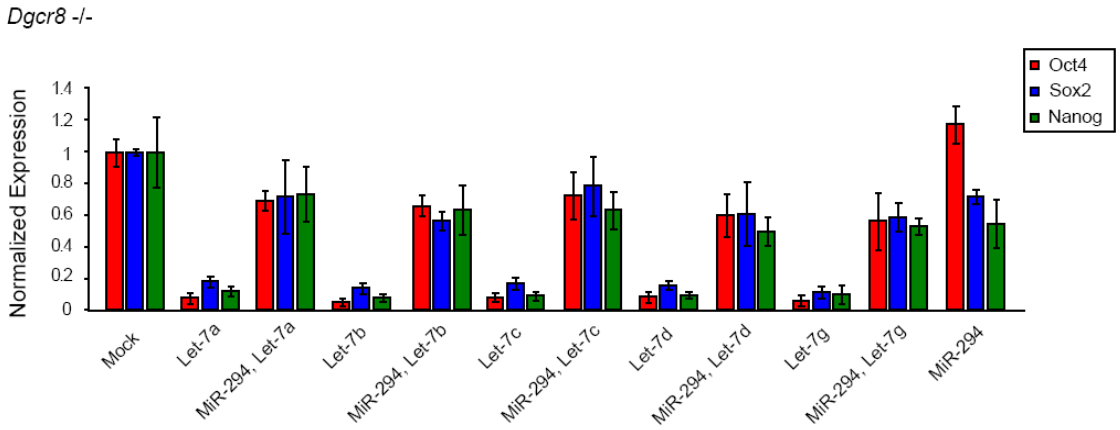
The let-7 and ESCC miRNA families have opposing roles in regulating ESC self-renewal. (a) Transfected miRNAs with the seed sequence highlighted. (b) Pou5f1/Oct4 immunofluorescence staining after transfection of let-7c, miR-294 and combinations of let-7c with miR-294, mutant-miR-294, miR-291a-5p, or miR-130b in *Dgcr8* -/- (i) and wild-type (ii) ESCs. Representative images, n = 3. (c) qRT-PCR for Pou5f1/Oct4, Sox2, and Nanog normalized to beta-actin after miRNA introduction as in b. n = 3-8. * indicates $p < 0.02$. (d) Colony reforming assays after miRNA introduction as in b and c. n = 3. * indicates $p < 0.05$. All p-values generated by Bonferroni corrected t-test of comparisons to let-7c treated. Error bars represent standard deviation.

Figure 3-2



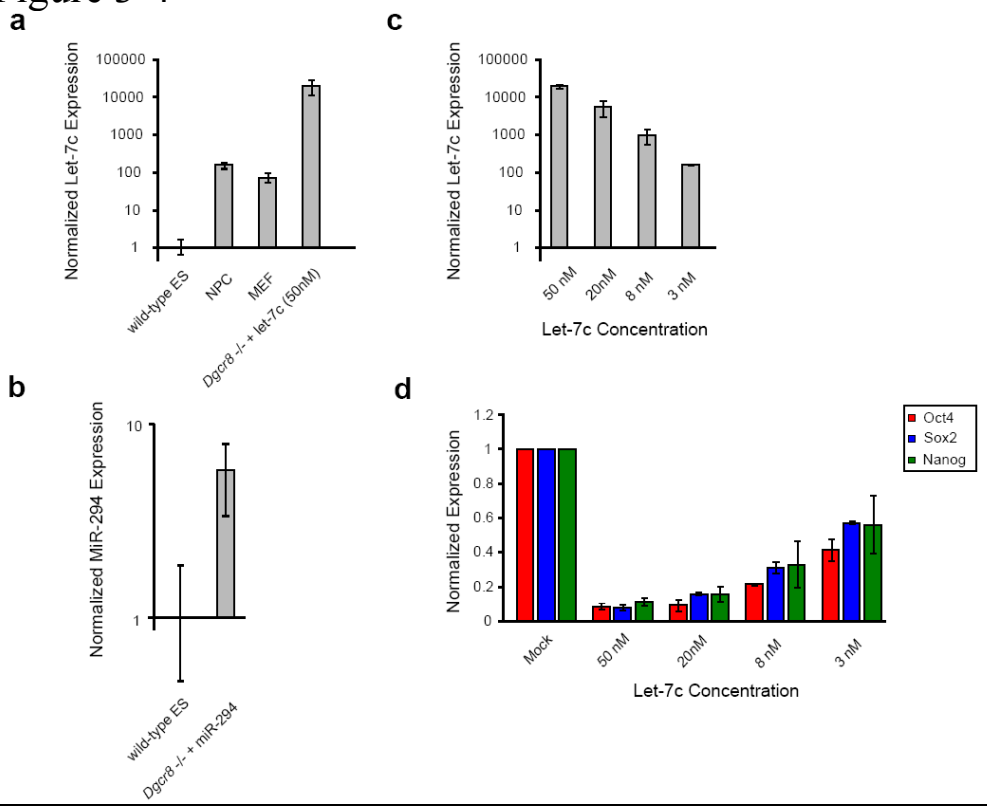
The let-7 and ESCC families of miRNAs have opposing roles in regulating ESC self-renewal. Alkaline phosphatase staining 3 days after transfection of miRNAs into *Dgcr8* -/- (i) and wild-type (ii) ESCs. Representative images, n = 3.

Figure 3-3



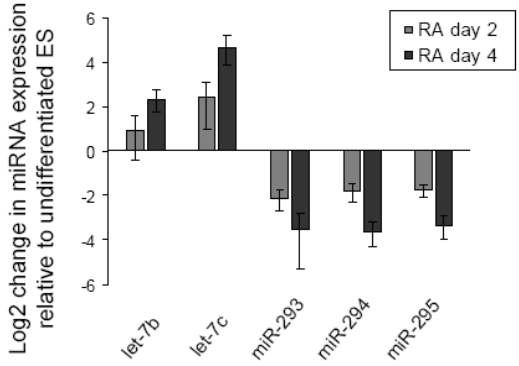
The let-7 family of miRNAs function to suppress self-renewal in *Dgcr8 -/-* ESCs. qRT-PCR for *Oct4*, *Sox2*, and *Nanog* normalized to *beta-actin* after transfection with different let-7 family members either alone or in combination with miR-294. n = 3, error bars represent standard deviation.

Figure 3-4



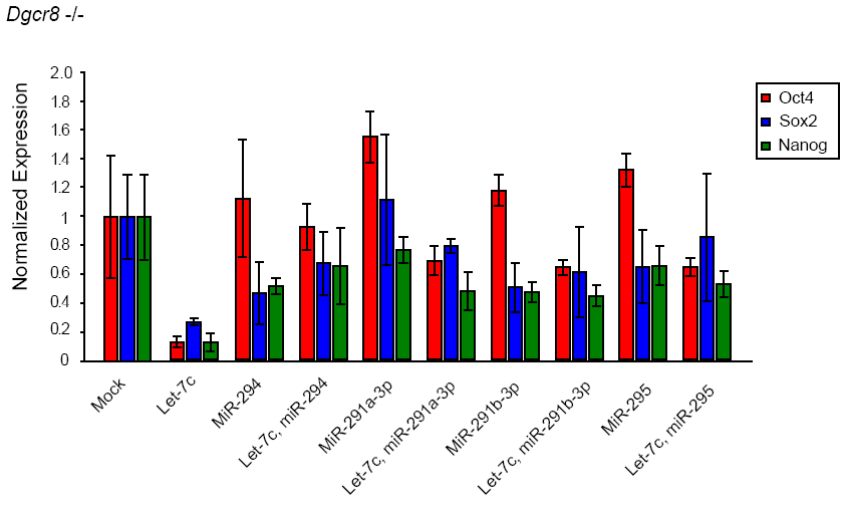
Let-7 and ESCC miRNAs exert their effects at physiologically relevant concentrations. (a) TaqMan miRNA qPCR day 3 after transfection for let-7c normalized to sno202 in wild-type ESCs, neural progenitor cells (NPCs), mouse embryonic fibroblasts (MEFs), and *Dgcr8*-/- ESCs transfected with let-7c at 50nM. Error represents range of n = 3. (b) TaqMan miRNA qPCR for miR-294 in wild-type ESCs and *Dgcr8*-/- ESCs transfected with miR-294 at 50nM day 3. Error represents range of n = 3. (c) TaqMan qRT-PCR for let-7c normalized to sno202 on day 3 of a dilutional series reducing the concentration of let-7c from 50nM to 3nM reduces the final concentration of let-7c to near NPC and MEF levels. Error represents range of n = 2. (d) qRT-PCR for *Oct4*, *Sox2*, and *Nanog* demonstrates that let-7c at reduced concentrations still silences self-renewal in *Dgcr8* -/- ESCs. Note, both NPCs and MEFs express many members of the let-7 family thus the physiologic levels of let-7 are achieved somewhere between 8 and 50nM.

Figure 3-5



Let-7 and ESCC miRNAs show inverse expression during ESC differentiation. polyA miRNA qPCR time course during retinoic acid induced differentiation of wild-type ESCs. Data are normalized to U6. Error represents standard deviation of n = 3.

Figure 3-6

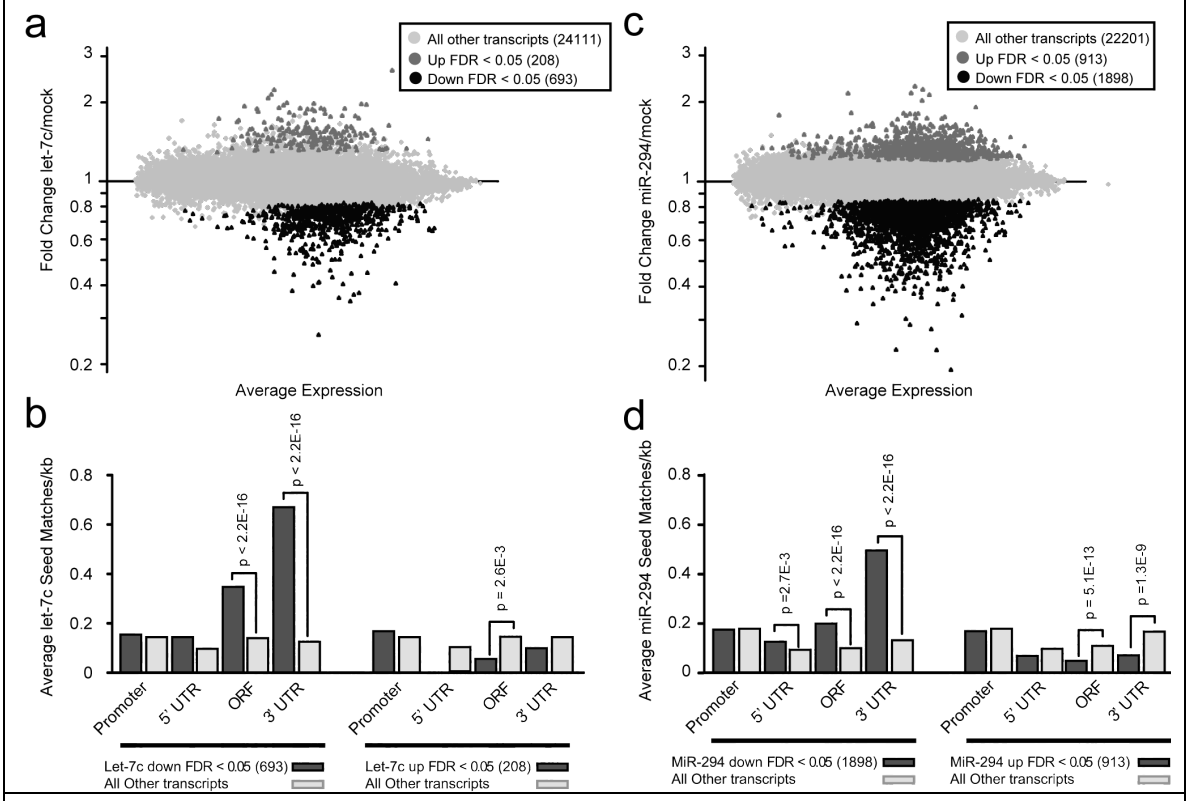


miR-290 cluster ESCC family members function to suppress let-7c induced silencing of ESC self-renewal in *Dgcr8 -/-* ESCs. qRT-PCR for *Oct4*, *Sox2*, and *Nanog* normalized to beta-actin after transfection with different ESCC family members either alone or in combination with let-7c. n = 3, error bars represent standard deviation.

Targeting through ORFs and 3'UTRs

The functional antagonism between let-7c and miR-294 on ESC self-renewal suggested opposing roles for these miRNAs on downstream molecular targets. To test this prediction, we sought to globally identify these targets using mRNA microarrays

Figure 3-7



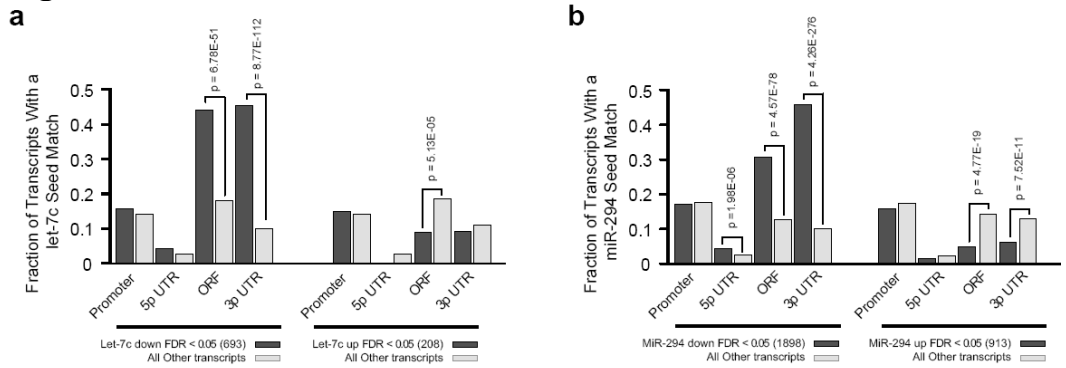
The let-7 and ESCC miRNAs suppress hundreds of transcripts by binding their ORF and/or 3'UTR. (a) Microarray analysis following introduction of let-7c alone. Upregulated transcripts are shown in dark grey, downregulated transcripts in black (FDR < 0.05). (b) Analysis of seed matches in the promoter, 5′UTR, ORF, and 3′UTR of let-7c-downregulated and upregulated transcripts. Presented are the mean number of seeds matches per kb of sequence for the listed groups of altered genes described in a. P-values calculated by the Wilcoxon Rank Sum Test and Bonferroni corrected are shown for $p < 0.01$. (c) Microarray analysis following introduction of miR-294 alone. Color labeling, as in a. (d) Seed analysis as in b for miR-294 up and downregulated transcripts.

following the introduction of let-7c or miR-294 into *Dgcr8* -/- ESCs. The introduction of the let-7c mimic led to downregulation of 693 and upregulation of 208 transcripts relative to mock treated cells with a false discovery rate (FDR) less than 5% (**Figure 3-7a**). Of the 693 downregulated transcripts, 294 contained a let-7c 7mer seed match in the 3′UTR, 287 contained a 7mer seed match in the ORF, and 113 contained both 3′UTR and ORF seed matches. The presence of these seed matches in the downregulated transcripts was highly enriched compared to the entire gene set (**Figure 3-7b, Figure 3-8a**). Similarly, the introduction of miR-294 led to a large number of upregulated and downregulated transcripts (**Figure 3-7c**). Again, downregulated transcripts were enriched for seed matches in the 3′UTR and ORF. In contrast, upregulated transcripts were depleted for seed matches in the 3′UTR and ORF (**Figure 3-7d, Figure 3-8b**). These findings suggest that miR-294 and let-7c functionally act through the downregulation of many targets by binding their ORF and/or 3′UTR.

Impact on ESC transcriptional network

To further investigate the mechanism for the opposing roles of let-7c and miR-294 on ESC self-renewal, we performed pathway analysis on the miRNA regulated transcript sets. Specifically, we searched for overlaps between the miRNA-regulated transcripts and genes identified by chromatin immunoprecipitation (ChIP) of pluripotency associated transcription factors(X. Chen, et al., 2008; Marson, et al., 2008). This analysis measures whether there is any influence of the let-7 or ESCC miRNAs on the transcription factors themselves (**Figure 3-9a, i&ii, & Supplementary Methods**) or the transcripts

Figure 3-8



Let-7c and miR-294 suppress direct targets through binding of ORF and 3′UTRs. This analysis is similar to the analysis presented in Fig. 2 except that there is no correction for sequence length. (a) Analysis of seed matches in the promoter, 5′UTR, ORF, and 3′UTR of let-7c downregulated and upregulated transcripts. Presented are the fraction of transcripts with a seed match in different regions (promoter, 5′UTR, ORF, and 3′UTR) for the listed groups of altered genes described in Fig. 2a. (b) An identical analysis to (a) but for miR-294 seed matches in miR-294 altered gene sets. Indicated p-values are calculated by Fischer's Exact Test and are presented only for $p < 0.001$

originating from the genes bound by the transcription factors (**Figure 3-9a, iii, & Supplementary Methods**).

In ESCs, two Myc family members—nMyc and cMyc—are highly expressed and have largely overlapping ChIP target genes(X. Chen, et al., 2008). cMyc has previously been identified as a let-7 target in cancer cells(Madhu S. Kumar, Lu, Mercer, Golub, & Jacks, 2007), and we find that nMyc is significantly downregulated by let-7c in our array data. Consistent with let-7 directly targeting the Myc family, overlapping let-7c-regulated transcripts with Myc-bound genes showed an enrichment of Myc target genes in the let-7c-downregulated transcript set and a depletion in the let-7c-upregulated transcript set (**Figure 3-9b, Box I**). Furthermore, the enrichment was independent of the presence of seed sequence matches within the ORF or 3′UTR. This finding suggests that let-7 is

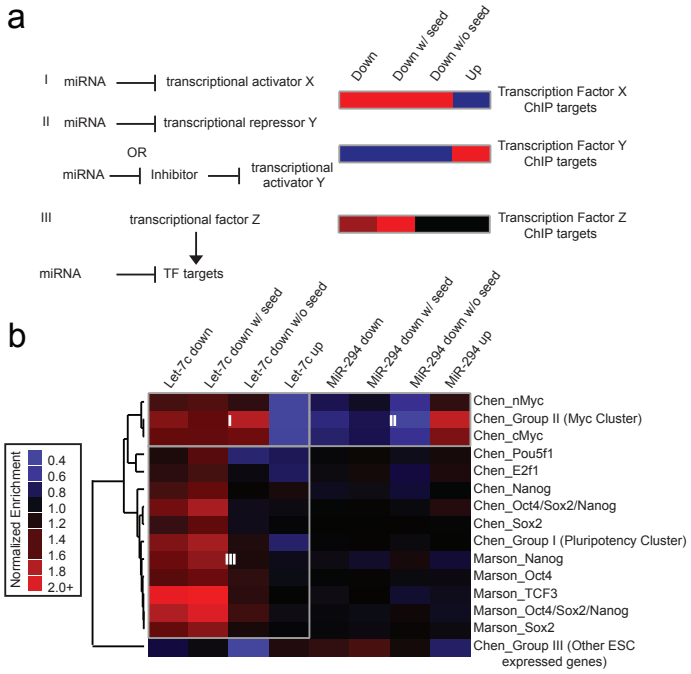
acting directly through Myc (cMyc and/or nMyc) rather than through Myc's downstream target genes (**Figure 3-9a, i)**.

Performing a similar analysis overlapping miR-294-regulated transcripts and Myc target genes showed the exact opposite pattern as the analysis with let-7c-regulated transcripts. There was a depletion for Myc targets in the miR-294-downregulated transcript set and an enrichment in the miR-294-upregulated transcript set (**Figure 3-9b, Box II**). This pattern suggests that miR-294 upregulates Myc activity (**Figure 3-9a, ii)**. Indeed, microarray data showed that miR-294 dramatically increased cMyc levels. As miR-294 itself suppresses its downstream targets (**Figure 3-8d**), the upregulation of cMyc must be indirect, through an unknown intermediate repressor (**Figure 3-9a, ii)**. These data show that the let-7 and ESCC families of miRNAs have opposing effects on Myc activity.

Overlap of the let-7c-regulated transcripts with ChIP target genes for the pluripotency transcription factors, Pou5f1/Oct4, Sox2, Nanog, and Tcf3 once again showed an enrichment among let-7c-downregulated transcript set (**Figure 3-9b, Box III**). However, this enrichment was limited to the downregulated transcripts with seed matches in their ORF or 3´UTR. These data suggest that rather than directly regulating the pluripotency transcription factors, let-7 targets transcripts originating from the genes bound by them (**Figure 3-9a, iii**). This pattern of enrichment is most clear for the ChIP target genes bound by Tcf3, cobound by Pou5f1/Oct4, Sox2, and Nanog, or bound by Chen et al.'s pluripotency cluster (a group of targets bound by Pou5f1/Oct4, Sox2, Nanog, Smad1, and STAT3). The latter results agree with recent reports showing that genes bound by

multiple pluripotency transcription factors are more likely to be transcriptionally activated(X. Chen, et al., 2008; J. Kim, Chu, Shen, Wang, & Orkin, 2008). There was no enrichment in the overlap between the miR-294-regulated transcripts and Pou5f1/Oct4, Sox2, Nanog, and Tcf3 bound genes. These data suggest that let-7c inhibits downstream targets of these pluripotency factors while miR-294 has no obvious effects on either the transcription factors themselves or on their downstream targets.

Figure 3-9



Enrichment/depletion of transcription factor bound genes among miRNA-regulated transcripts. (a) A schematic of hypothetical miRNA regulation of a transcription factor or its targets. Corresponding expected enrichment/ depletion of the transcription factor ChIP targets in miRNA-induced upregulated or downregulated transcript sets are displayed in a heat map. A key of color coding representing relative enrichment is given in b. (b) A heat map showing enrichment of the ChIP targets among the different sets of miRNA-regulated transcripts on the horizontal axis. Vertical axis represents the different ChIP data sets with first author and factor that was immunoprecipitated.

Opposing regulation of Myc, Lin28, and Sall4

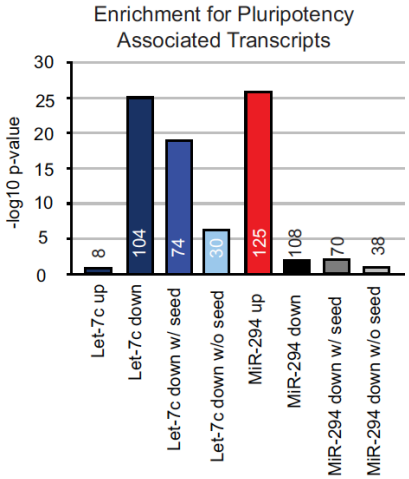
Having discovered that Myc activity was alternatively downregulated and upregulated by let-7c and miR-294, we sought to identify other factors that might be similarly regulated by these miRNAs. Indeed, gene ontology analysis showed an enrichment for ESC enriched genes among the let-7c-downregulated and miR-294-upregulated transcript sets (**Figure 3-10**). 88 transcripts were regulated in opposing directions by let-7c and miR-294, of which 44 contained a let-7c seed match (**Figure 3-11)**. Notably, this set of transcripts included the well-known pluripotency genes Lin28 and Sall4. Lin28 is an RNA binding protein that inhibits let-7 processing(Heo, et al., 2008; Newman, et al., 2008; Rybak, et al., 2008; Viswanathan, et al., 2008), but not transfected let-7 mimic (**Figure 3-12**). Sall4 is a transcription factor that promotes ESC self-renewal(Lim, et al., 2008; Q. Wu, et al., 2006; Zhang, et al., 2006). These findings show that the let-7 and ESCC families antagonistically regulate multiple genes with described roles in ESC self-renewal.

To verify our genomic analysis, we performed qRT-PCR, Western analysis, and reporter assays for a subset of the genes. qRT-PCR confirmed the opposing effects of let-7c and miR-294 on Lin28, Sall4, nMyc, and cMyc mRNA levels with a combination of the two miRNAs showing intermediate levels (**Figure 3-13a**). Western analysis showed similar results (**Figure 3-13b, Figure 3-14**). Of note, cMyc protein was dramatically reduced in *Dgcr8* -/- versus wild-type ESCs and was brought back to wild-type levels with the introduction of miR-294. MiR-294 had little effect on nMyc levels. In contrast, let-7c had little effect on cMyc, yet dramatically reduced nMyc levels. Therefore, the cumulative effect of the miRNAs on total Myc (cMyc + nMyc) protein levels followed a

strong pattern of opposing regulation. Similarly, the miRNAs showed significant opposing effects on Lin28 and Sall4 protein levels. Lin28 and cMyc are known targets of let-7(Madhu S. Kumar, et al., 2007; Rybak, et al., 2008) and luciferase assays confirmed that nMyc and Sall4 are also direct targets (**Figure 3-13c**).

Considering that cMyc was dramatically reduced in *Dgcr8* -/- cells and then increased with miR-294, we considered the possibility that the loss of cMyc alone could largely explain the sensitivity of *Dgcr8* -/- cells to let-7-induced silencing of ESC self-renewal. To test this possibility, we generated and evaluated *cMyc* -/- ESCs (**Figure 3-15**). The loss of cMyc led to decreased expression of Pou5f1/Oct4 relative to the parental cell line (**Figure 3-16a**). Introduction of let-7c into the *cMyc* -/- cells decreased the expression levels of Sox2 and Nanog (**Figure 3-16b&c**). However, levels were not reduced to the same degree as seen with the introduction of let-7c into *Dgcr8* -/- cells. These results

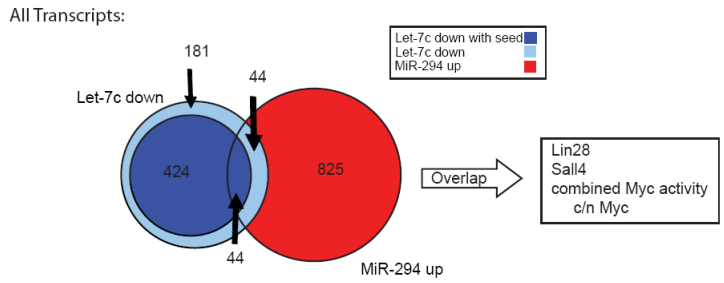
Figure 3-10



Let-7c down-regulates and miR-294 up-regulates transcripts highly enriched for pluripotency genes. Enrichment analysis for pluripotency associated transcripts among the different sets of miRNA up and downregulated transcripts. –log10 p-value is plotted and calculated by Fischer's exact test. In each column is the number of transcripts observed in each category.Figure S8

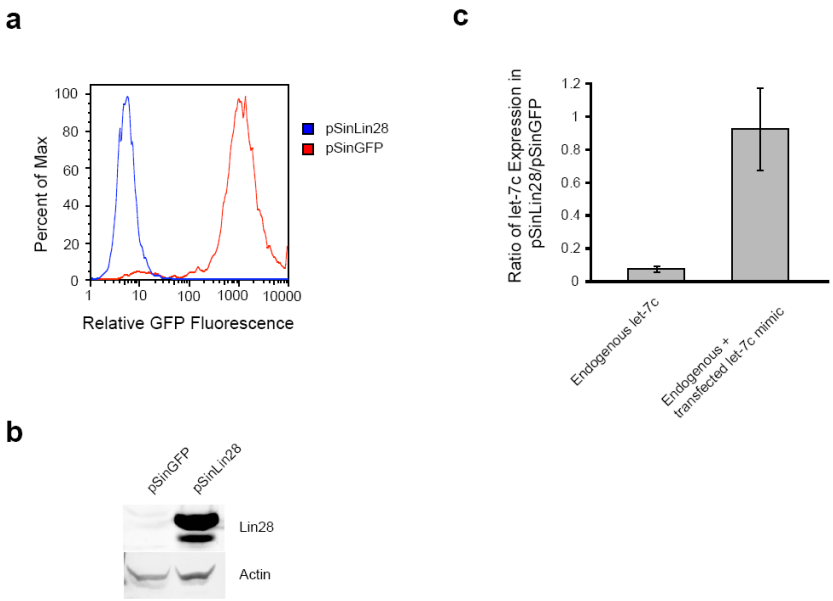
indicate that the decrease of cMyc in *Dgcr8* -/- cells alone cannot explain the sensitivity of these cells to let-7-induced silencing of ESC self-renewal.

Figure 3-11



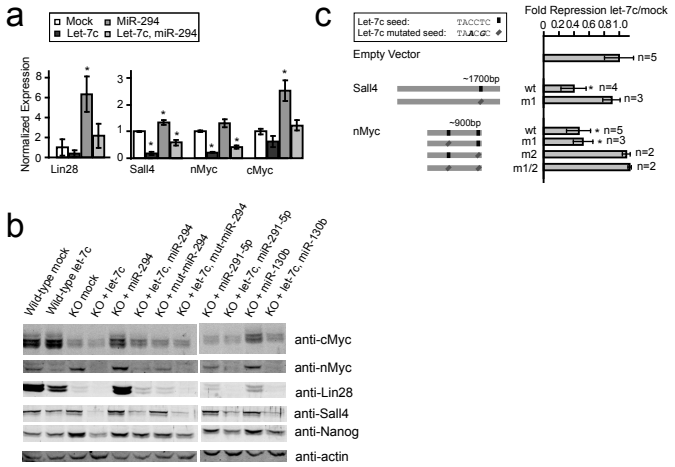
Overlap of transcripts downregulated by let-7c and upregulated by miR-294. A Venn diagram displaying the overlap between miR-294 upregulated and let-7c downregulated transcripts. *Lin28*, *Sall4*, and the combination of *c-Myc* and *n-Myc* are found in this overlap.

Figure 3-12



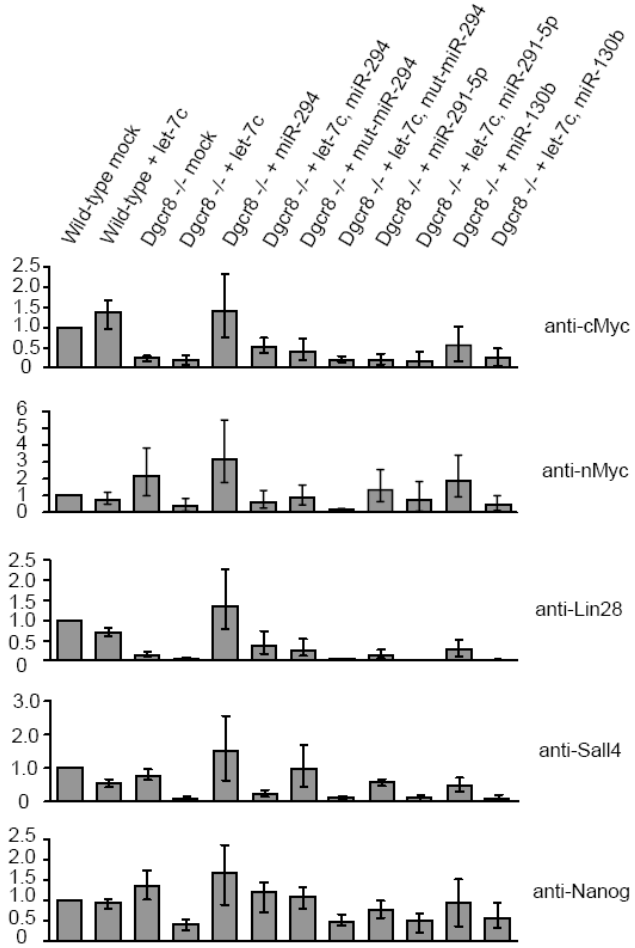
Lin28 does not promote degradation of let-7c mimic. (a) 293T cells were transfected with pSinLin28 and pSinGFP constructs and selected for puromycin resistance. Flow cytometry for GFP in GFP control cells demonstrates that following selection in puromycin most cell express the exogenous gene. (b) Western analysis shows that the 293T cells transfected with *Lin28* overexpresses Lin28 protein. (c) Taqman miRNA qPCR for mature let-7c normalized to sno202 shows that Lin28 represses maturation of endogenous let-7c but not transfected let-7c mimic. Error represents standard deviation of n = 3.

Figure 3-13



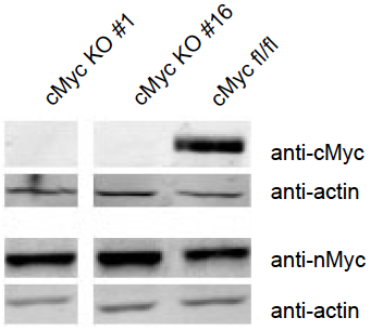
Let-7c and miR-294 regulate Lin28, Sall4, cMyc, and nMyc. (a) qRT-PCR for Lin28, Sall4, nMyc, and cMyc 12 hours after transfection with let-7c, miR-294, or a combination of the two. n = 3. (b) Representative Western blot analysis 48 hours after transfection with miRNAs. Quantitation shown in Figure S10 n = 3. (c) Luciferase analysis of Sall4 and nMyc 3′UTRs. Seed matches for let-7c in the 3′UTRs along with different mutant constructs are diagrammatically represented in the left panel. Luciferase results after co-transfection with let-7c mimic releative to mock transfected are shown in the right panel. All data are represented as mean +/- standard deviation. * indicates $p < 0.05$ by Bonferroni corrected t-test.

Figure 3-14



Opposing regulation of Myc, Lin28, and Sall4 protein levels by let-7c and miR-294. Quantification of Western analysis from Fig. 4b. To compare between replicates samples were normalized to set wild-type mock to one. Error represents standard deviation of n = 3 for these normalized samples.

Figure 3-15

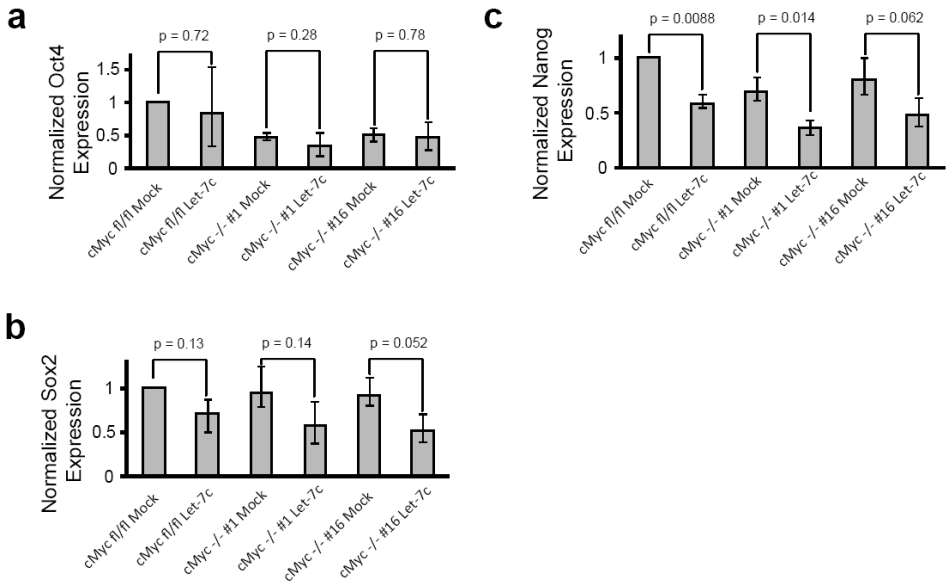


Loss of c-Myc in *c-Myc* -/- ESCs. Western analysis of c-Myc and n-Myc in two separate *c-Myc* -/- ESC lines and their parental cMyc fl/fl ESC line. For Western analysis, cells were taken off the irradiated MEF feeder layer for approximately 3-4 passages.

Inhibition of let-7 promotes de-differentiation

Having identified a pro-differentiation function of the let-7 family of miRNAs, we hypothesized that inhibition of this miRNA family would enhance reprogramming of somatic cells to iPS cells. Indeed, Lin28, among other activities(Balzer & Moss, 2007; Heo, et al., 2009; B. Xu, Zhang, & Huang, 2009), inhibits let-7 biogenesis(Hagan, et al., 2009; Heo, et al., 2008; Newman, et al., 2008; Rybak, et al., 2008; Viswanathan, et al., 2008) and promotes de-differentiation of human somatic cells to iPS cells(J. Yu, et al., 2007). Reprogramming to iPS cells is typically achieved by the introduction of virally expressed Pou5f1/Oct4, Sox2, and Klf4 with or without Myc into somatic cells such as

Figure 3-16



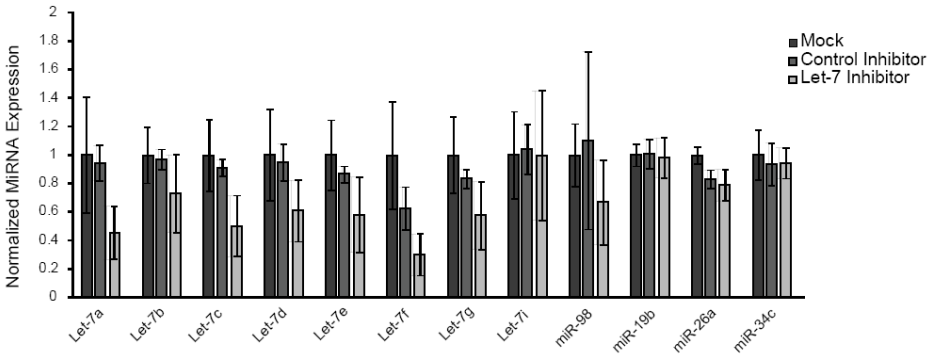
c-Myc -/- ESCs show partial susceptibility to let-7c induced silencing of ESC self-renewal. (a) qRT-PCR for *Oct4* in *c-Myc* fl/fl and two derived *c-Myc* -/- lines either mock or let-7c treated. *Oct-4* levels are reduced in *c-Myc* -/- cells and do not significantly change upon let-7c treatment. (b) qRT-PCR for *Sox2* and (c) *Nanog* as in a. Let-7c decreases expression of *Sox2* and *Nanog* in *c-Myc* -/- ESCs. Note, the decrease in *c-Myc* fl/fl control differs from V6.5 ESCs used in Fig. 1. This suggests slight differences between wild-type lines in their response to let-7. Uncorrected p-values were generated by t-test. Error bars represent range of n = 3.

mouse embryonic fibroblasts (MEFs). While Myc dramatically increases the efficiency of reprogramming, it is not essential(Nakagawa, et al., 2008; Wernig, Meissner, Cassady, & Jaenisch, 2008). To test the impact of let-7 family on reprogramming, we used a let-7 antisense inhibitor. This inhibitor was able to suppress multiple let-7 family members simultaneously (**Figure S3-13**).

MEFs express high levels of mature let-7(Marson, et al., 2008) and, therefore, these cells should be responsive to any pro-reprogramming effects of let-7 downregulation. We used Oct4::GFP transgenic MEFs in order to quantify changes in reprogramming efficiencies as Oct4::GFP is activated late in the reprogramming process(Brambrink, et al., 2008; Stadtfeld, Maherali, Breault, & Hochedlinger, 2008). MEFs were transduced with retroviral vectors expressing Pou5f1/Oct4, Sox2, Klf4, with or without cMyc on day 0 as well as transfected with let-7 or a control inhibitor on days 0 and 6. When 3 transcription factors were used (minus cMyc), let-7 inhibition increased the number of GFP positive colonies on day 10 by 4.3 fold compared to mock whereas a control inhibitor had no significant effect (**Figure 3-5a, left panel**). In the presence of all four transcription factors, let-7 inhibition resulted in a 1.75 fold increase (**Figure 3-5a, right panel**). Immunofluorescence confirmed expression of Nanog in reprogrammed cells (**Figure S3-14**). Furthermore, the resulting iPS cells expressed endogenous pluripotency markers at levels similar to wild-type ESCs and did not express the exogenously introduced factors (**Figure S3-15&S4-16**), as expected for fully reprogrammed cells.(Stadtfeld, et al., 2008) The impact of the let-7 inhibitor is not due to enhanced proliferation of the MEFs as there was actually a subtle decrease in proliferation

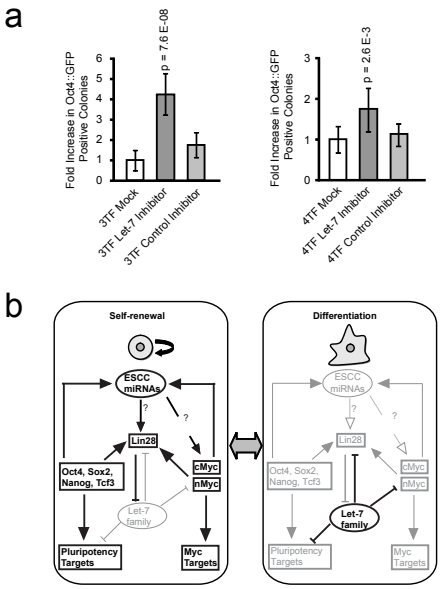
following transfection of either the let-7 or control inhibitor (**Figure S3-17**). These findings show that inhibition of let-7 family of miRNAs enhances the reprogramming of somatic cells. The finding that the enhancement was greater in absence of Myc is consistent with Myc activity being one, but not the only important downstream target of let-7 in stabilizing the somatic cell fate.

Figure 3-17



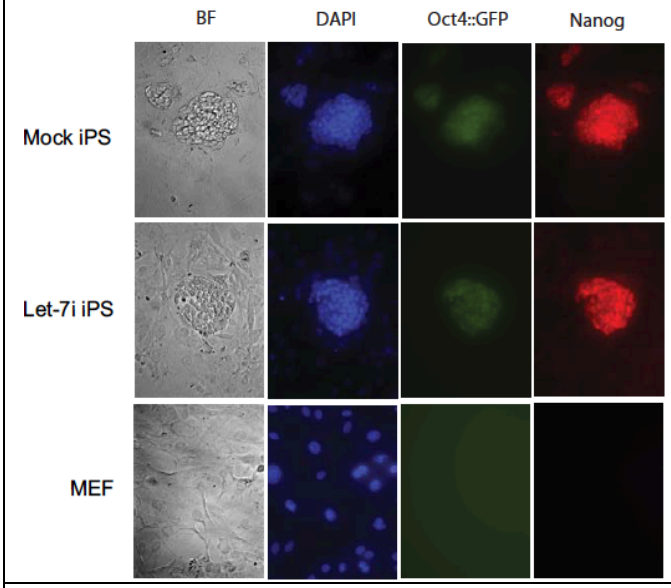
The let-7 inhibitor reduces levels of the majority of mature let-7 family miRNAs. polyA miRNA qPCR was performed in mock, control inhibitor, and let-7 inhibitor treated MEFs. Let-7 family miRNAs but not other highy expressed miRNAs (miR-19b, miR-26a, and miR-34c) have reduced levels in the presence of the let-7 inhibitor.

Figure 3-18



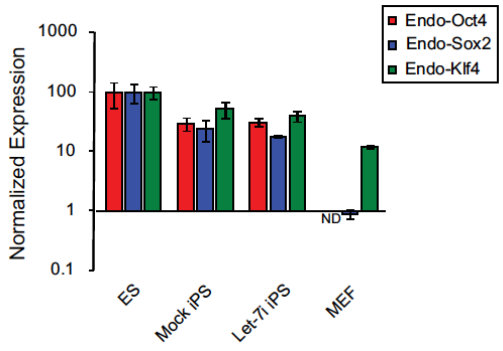
Inhibition of let-7 miRNAs promotes reprogramming to induced pluripotency (a) Fold increase of Oct4::GFP positive colonies in reprogramming with transduction of 3TFs (Pou5f1/Oct4, Sox2, and Klf4) or 4TFs (+ cMyc) after mock, let-7 inhibitor, or control inhibitor transfection. P-values are indicated for $p < 0.01$ calculated by Bonferroni corrected t-test. $n = 10$ for mock and let-7 inhibitor samples and $n = 6$ for control inhibitor samples (b) A model of the antagonism between the miR-294 and let-7c in the stabilization of the self-renewing and differentiated states. Bold and enlarged genes and arrows are active in the indicated state. Mechanisms of ESCC upregulation of Lin28 and cMyc are unknown and represented by a question mark.

Figure 3-19



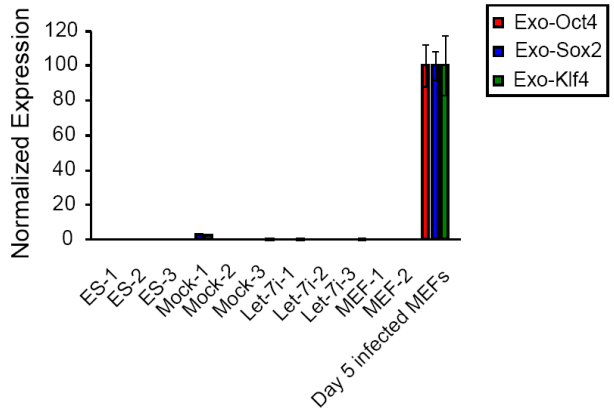
Oct4-GFP positive colonies co-express endogenous Nanog protein. Microscopy of 3TF mock and let-7 inhibitor reprogrammed cell lines for brightfield (BF), DAPI, Oct4-GFP, and Nanog immunostaining.

Figure 3-20



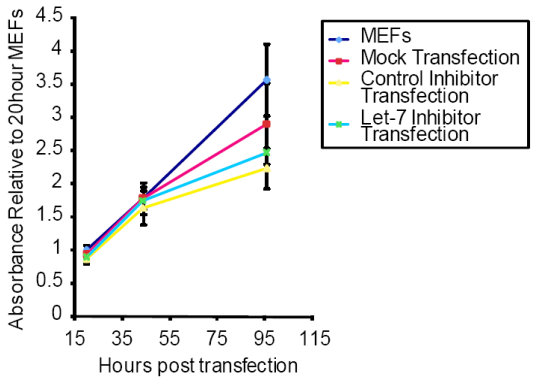
Oct4-GFP positive colonies express ESC-like levels of endogenous *Oct4*, *Sox2*, and *Klf4* mRNA. qRT-PCR for endogenous *Oct4*, *Sox2*, and *Klf4* in the indicated reprogrammed cell lines. Data are represented as mean +/- standard deviation for n=3. ND = not determined.

Figure 3-21



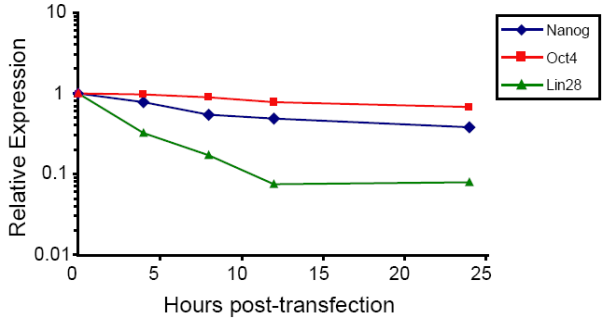
Oct4-GFP positive colonies have silenced exogenously introduced factors. qRT-PCR for exogenous *Oct4*, *Sox2*, and *Klf4* in the indicated reprogrammed cell lines. Data are represented as mean +/- standard deviation for n=3.

Figure 3-22



Treatment with the let-7 inhibitor does not increase proliferation in MEFs. MEFs untreated or after mock, control inhibitor, or let-7 inhibitor transfection were subjected to MTT assay as a surrogate marker of proliferation.

Figure 3-23



Let-7c induces maximal degradation of its target *Lin28* 12 hours after transfection without significant evidence of differentiation (as measured by *Nanog* and *Oct4* expression). qRT-PCR time course for *Oct4*, *Nanog*, and *Lin28* normalized to *beta-actin* is plotted after transfection of *Dgcr8* -/- ESCs. n = 1.

Discussion

Our findings show that the let-7 and ESCC miRNA families have opposing effects on ESC self-renewal. We propose that they act in self-reinforcing loops to maintain the ESC self-renewing versus differentiated cell states (**Figure 3-18b**). In the self-renewing state, ESCC miRNAs indirectly increase expression of Lin28 and cMyc. Lin28 functions to block the maturation of let-7(Heo, et al., 2008; Rybak, et al., 2008; Viswanathan, et al., 2008). Therefore, the ESCC miRNAs prevent co-expression of let-7 miRNAs. Additionally, ESCC-induced upregulation of cMyc forms a positive feedback loop in which cMyc and nMyc, along with Pou5f1/Oct4, Sox2, and Nanog, bind and activate expression of the ESCC miRNAs in the miR-290 miRNA cluster(Judson, et al., 2009; Marson, et al., 2008). As ESCs differentiate, Pou5f1/Oct4, Sox2, and Nanog are downregulated, resulting in the loss of ESCC and Lin28 expression. With the loss of Lin28, mature let-7 rapidly increases. This increase in let-7 is enhanced by a positive feedback loop in which let-7 suppresses its own negative regulator Lin28. In the differentiated state, downregulation of Myc activity by let-7 prevents co-expression of the

ESCC miRNAs. Furthermore, let-7 inhibits downstream targets of Pou5f1/Oct4, Sox2, Nanog, and Tcf3 to stabilize the differentiated state. Sall4, like Myc and Lin28, is positively regulated by the ESCC family and negatively regulated by let-7 family. Decreases in Myc, Sall4, and Lin28 all promote ESC differentiation(Cartwright, et al., 2005; Heo, et al., 2009; Lim, et al., 2008; Zhang, et al., 2006)

In the model we propose, the function of let-7 in repressing the self-renewing state is restricted to cells that do not express high levels of ESCC miRNAs. In fact, our model suggests that let-7 and ESCC miRNAs are never co-expressed at high levels. For this reason, we propose that the let-7 family does not function to initiate differentiation, but rather the antagonism between the let-7 and ESCC families stabilizes the switch between self-renewal and differentiation. Consistent with this model, the introduction of either ESCC miRNAs(Judson, et al., 2009) or let-7 inhibitors into somatic cells promotes their de-differentiation into iPS cells. Additionally, the ESCC and let-7 miRNAs make up a preponderance of the miRNAs in self-renewing ESCs and somatic cells respectively(Marson, et al., 2008), supporting a major role in influencing these alternative cell fates.

Other miRNAs have been reported to target the ESC transcriptional network(Y. M. S. Tay, et al., 2008, Tay, 2008 #383, Xu, 2009 #352). Unlike the let-7 family, these other miRNAs have a more limited tissue distribution(C. Chen, et al., 2007; Landgraf, et al.), suggesting that they may suppress self-renewal during differentiation along specific developmental pathways. Alternatively, these miRNAs may be involved in the early and

transient stages of ESC differentiation while the let-7 miRNAs are involved in stabilizing the resulting differentiated cell fate. miRNAs related to the ESCC family (miR-17, miR-20, miR-93, and miR-106) and let-7 miRNAs play analogous roles in cancer with the ESCC related miRNAs promoting and the let-7 miRNAs inhibiting cancer growth(Büssing, Slack, & Großhans, 2008; Mendell, 2008). It will be interesting to determine whether these miRNAs act through similar opposing pathways in cancer as in ESCs.

Chapter 4: Mechanisms by which microRNAs silence ESC self-renewal

Summary

When embryonic stem cells (ESCs) differentiate, they must both silence the ESC self-renewal program as well as activate new tissue specific programs. In the absence of DGCR8, a protein required for microRNA biogenesis, mouse ESCs are unable to silence ESC self-renewal. Previously, we found that the introduction of let-7 miRNAs, a family of miRNAs highly expressed in somatic cells, can suppress self-renewal in *Dgcr8* -/-, but not wild-type ESCs. Introduction of ESC cell cycle regulating (ESCC) miRNAs into the *Dgcr8* -/- ESCs, blocks the capacity of let-7 to suppress self-renewal. Here we identify additional miRNAs that silence ESC self-renewal in *Dgcr8* -/- ESCs. As with let-7, the ESCC miRNAs block the capacity of these miRNAs to suppress self-renewal. Profiling and bioinformatic analyses show that the ESC self-renewal silencing miRNAs share few common mRNA targets yet via disparate mRNA targets commonly impact a small number of molecular pathways. Notably, this set includes the G1/S cell cycle transition. As it is known that ESCC miRNAs have the opposite effect of promoting the G1/S transition(Y. Wang, et al., 2008), we hypothesize that the ESCC miRNAs promote whereas other miRNAs inhibit the G1/S transition to regulate differentiation. Inhibition of the cell cycle in ESCs by forced expression of P21 suppresses ESC self-renewal, suggesting that a rapid ESC cell cycle is required for the maintenance of self-renewal. These data indicate that a small but diverse complement of miRNAs can repress the ESC self-renewal program potentially via regulation of the ESC cell cycle.

Introduction

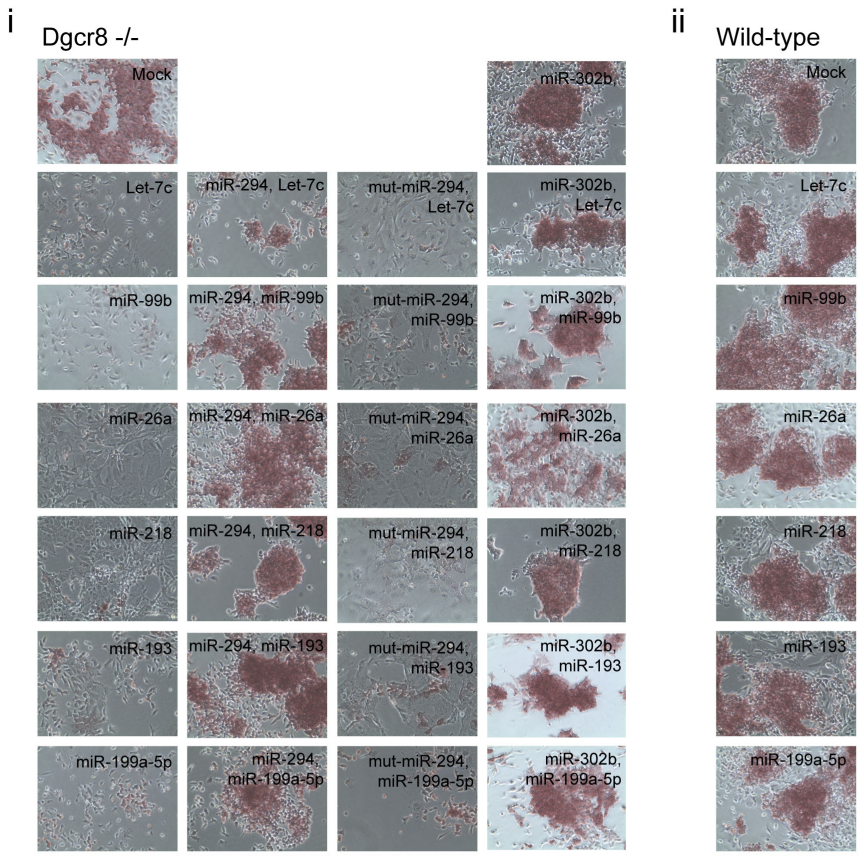
In **Chapter 2**, via a screening assay I identified a large number of miRNAs which silence ESC self-renewal in *Dgcr8 -/-* ESCs. To identify developmentally relevant miRNAs, I identified the subset of miRNAs from my screen that are expressed during differentiation of both mouse and human ESCs. MiRNAs which passed these criteria included the let-7 family of miRNAs, miR-99b, miR-26a, miR-218, miR-193, and miR-199a-5p. In **Chapter 3,** I explored the ability of let-7 to silence self-renewal in ESCs and found that its ability to silence ESC self-renewal was inhibited by ESCC miRNA expression. In this Chapter, I investigate the ability of miR-99b, miR-26a, miR-218, miR-193, and miR-199a-5p to silence ESC self-renewal in mouse ESCs both in the presence and absence of ESCC miRNAs.

miR-99b, miR-26a, miR-218, miR-193, and miR-199a-5p regulate self-renewal in *Dgcr8 -/- ESCs*

To assess their ability to silence ESC self-renewal, miR-99b, miR-26a, miR-218, miR-193, and miR-199a-5p were transfected into *Dgcr8 -/-* ESCs. Three days after transfection, cells down-regulated ESC associated markers including alkaline phosphatase activity and mRNA expression of Pou5f1 (Oct4), Sox2, and Nanog (**Figure 4-1a panel i, 4-1b panel i**). In contrast to the *Dgcr8 -/-* ESCs, wild-type ESCs were relatively resistant to silencing of ESC self-renewal by these miRNAs (**Figure 4-1a panel ii, 4-1b panel ii)**. Of the 5 miRNAs tested, miR-218 and miR-193 led to a small but reproducible decrease in expression of pluripotency markers in wild-type ESCs as

measured by qRT-PCR; however, this decrease was small compared to the impact of these same miRNAs in *Dgcr8 -/-* ESCs. Overall, these results matched those of let-7, which silences self-renewal in *Dgcr8 -/-* ESCs but not wild-type ESCs. As reported in **Chapter 3**, let-7's ability to silence ESC-self-renewal in *Dgcr8 -/-* ESCs is specifically blocked by co-transfection of ESCC miRNAs. Therefore, I tested the ability of ESCC miRNAs to block silencing of ESC self-renewal by miR-99b, miR-26a, miR-218, miR-193, and miR-199a-5p.

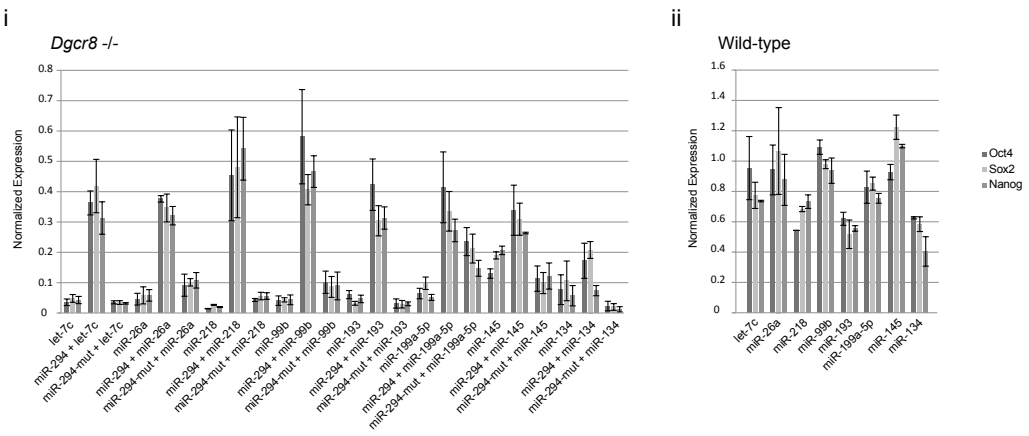
Figure 4-1 a



MiR-26a, miR-99b, miR-218, miR-193, and miR 199a-5p and ESCC miRNA families have opposing roles in regulating ESC self-renewal. (a) Alkaline phosphatase staining after transfection of let-7c, miR-26a, miR-99b, miR-218, miR-193, and miR-199a-5p alone or in combination with with miR-294 mutant-miR-294 or miR-302b. Transfections were done in *Dgcr8 -/-* ESCs (i) and wild-type ESCs (ii)

miR-99b, miR-26a, miR-218, miR-193, and miR-199a-5p were co-introduced into *Dgcr8 -/-* ESCs with either miR-294 or a miR-294 seed mutant. Three days after transfection, the cells were fixed for alkaline phosphatase staining or harvested for qRT-PCR. All miRNAs when co-introduced with miR-294 failed to silence ESC self-renewal as ascertained by alkaline phosphatase activity and mRNA expression of Oct4, Sox2, and Nanog (**Figure 4-1a panel i, 4-1b panel i)**. This effect was also observed for the closely related ESCC miRNA, miR-302b (**Figure 4-1a panel i)**. However, co-introduction of a miR-294 seed mutant with miR-99b, miR-26a, miR-218, or miR-193 did not block the ability of these miRNAs to silence ESC self-renewal (**Figure 4-1a panel i, 4-1b panel i)**. Notably, the ability of miR-199a-5p to silence ESC self-renewal was slightly reduced by the miR-294 seed mutant as indicated by qRT-PCR; however, the combination of miR-199a-5p and the miR-294 seed mutant caused significant death. Likely the increase in levels of pluripotency marker expression in this case is due at least in part to expression from the remaining live cells, which presumably are enriched for untransfected cells. In conclusion, these data indicate that miR-99b, miR-26a, miR-218, miR-193, and miR-199a-5p silence ESC self-renewal only in the absence of ESCC miRNAs. As with let-7, these ESC self-renewal silencing miRNAs and ESCC miRNAs have opposing roles in the maintenance of ESC self-renewal.

Figure 4-1 b



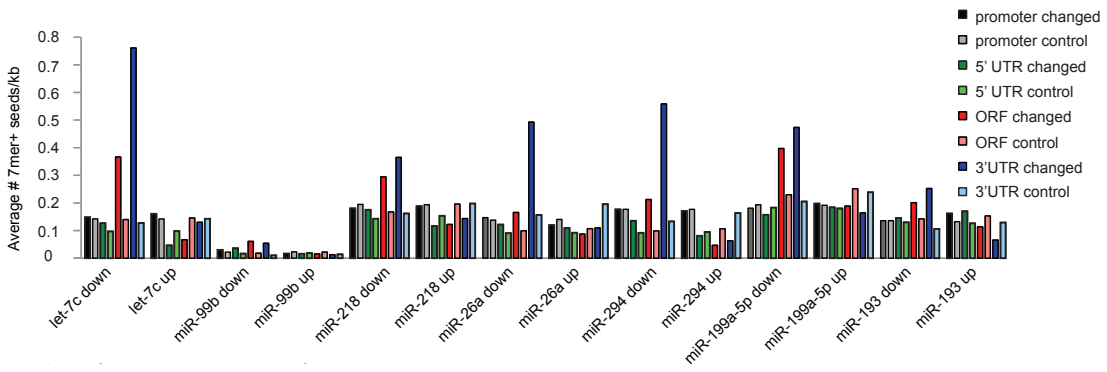
MiR-26a, miR-99b, miR-218, miR-193, and miR 199a-5p and ESCC miRNA families have opposing roles in regulating ESC self-renewal. (b) qRT-PCR for Pou5f1/Oct4, Sox2, and Nanog normalized first to beta-actin then to mock transfection after miRNA introduction as in a. n = 2.

Targeting through ORFs and 3'UTRs

The functional antagonism between ESC self-renewal silencing miRNAs and ESCC miRNAs suggested opposing roles for these miRNAs on downstream molecular targets, pathways, or cellular processes. Using a similar approach as taken with ESCC and let-7 miRNAs, I performed micorarray analysis 12 hours post-transfection of *Dgcr8* -/- ESCs with miR-99b, miR-26a, miR-218, miR-193, or miR-199a-5p in order to determine the immediate molecular changes induced by these miRNAs. The goal of this approach was to understand the molecular basis of miRNA suppression of ESC self-renewal. In particular, I sought to understand the similarities and differences among the various miRNA species that are able to suppress ESC self-renewal in the absence of ESCC miRNAs. I hypothesized that miRNAs that are able to silence ESC self-renewal in the absence of ESCCs would regulate a set of common genes or pathways and that these genes or pathways would be oppositely regulated by the ESCC miRNAs.

MiRNAs canonically interact with their target mRNAs through complementary base-pairing of nucleotides 2-8 on the 5' end of the miRNA with a target mRNA. The canonical miRNA/mRNA target interaction occurs in the 3' UTR; however, as others have reported and as I found previously for let-7c and miR-294 (**Chapter** 3), miRNAs often interact with the open reading frame. Our data set of miRNA regulated mRNA transcripts allowed us the opportunity to investigate the nature of miRNA/mRNA target interactions via the canonical seed sequence. This question is important for the identification of putative direct mRNA targets in my data set as well as for the general understanding of miRNA biology. As a first approach, I searched for exact 7mer seed matches within the promoters, 5' UTRs, ORFs, and 3' UTRs of the transcripts altered in response to various miRNA transfections (**Figure 4-2a**).

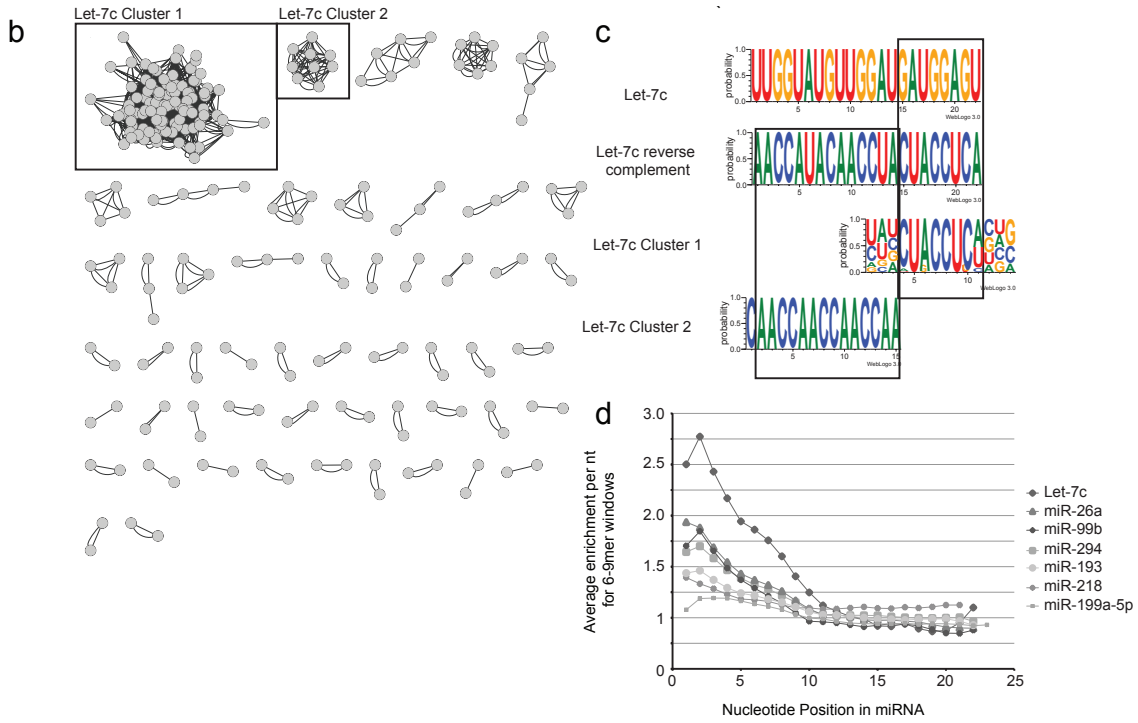
Figure 4-2 a



miRNAs suppress direct targets through binding of the seed sequence to ORFs and 3′UTRs. (a) Analysis of seed matches in the promoter, 5′UTR, ORF, and 3′UTR of miRNA down-regulated and up-regulated transcripts. Presented are average number of seeds per kb of transcript for different regions of the transcripts (promoter, 5′UTR, ORF, and 3′UTR). These averages are calculated for experimental lists of altered genes and control genes (everything not in the experimental set).

For the miRNAs tested I find considerable variability in the average enrichment of the 7mer seed sequence and in the location of the enrichment—ORF versus 3' UTR. All miRNAs have enrichment for seeds in the 3' UTR and ORF. Let-7c, miR-218, and miR-199a-5p seeds are most highly enriched in the 3' UTR and ORF. MiR-294 and miR-26a have a slight enrichment in the ORF and a much stronger enrichment in the 3' UTR. MiR-193 has a slight enrichment in both the ORF and 3' UTR. MiR-99b is unique in that

Figure 4-2 b-d



miRNAs suppress direct targets through binding of the seed sequence to ORFs and 3′UTRs. (b) Results of a motif enrichment analysis identifying all motifs 4 fold enriched in the 3' UTRs of let-7 down-regulated transcripts compared to all other transcripts. Boxed are clusters 1 and 2. Cluster 1 corresponds to sequences containing the sequence motif complementary to the let-7 seed. (c) WebLogo visualization of the aligned clusters 1 and 2 compared to the let-7 sequence. (d) An independent analysis to identify regions of the miRNA sequence enriched in the 3' UTRs of transcripts down-regulated by let-7c, miR-294, miR-26a, miR-99b, miR-218, miR-193, and miR-199a-5p. Motifs corresponding to 6-9 nucleotide windows across the miRNAs were analyzed for their enrichment in the 3' UTRs of down-regulated trancripts.

miR-99b seeds are enriched in both the ORF and 3' UTR but the overall number of seeds matches/kb is significantly lower than that of other miRNAs. It is unclear why this is the case. Seed sequences were not enriched in the 5' UTRs or promoters for any miRNA. Likewise, upregulated transcripts were not enriched for miRNA seed matches. These findings suggest (1) that miRNA target sites predominate in the ORF and 3' UTR, (2) that different miRNAs have different propensities to have target sites within either the ORF or 3' UTR, and (3) some miRNA seeds like that of miR-99b are found very rarely in the genome.

Motif analysis to identify the important functional nucleotide regions of the miRNA sequence

Because seed sequence enrichment varied greatly from one miRNA to the next, I sought to de novo identify motifs within the transcripts down-regulated upon miRNA transfection. In particular, I wanted to test whether an alternative miRNA sequence motif or a shortened seed sequence within the miRNA could be responsible for some of this variation between miRNAs. To perform this analysis I developed an algorithm which scans the sequences (3' UTRs and ORFs) of an experimental set of genes and a control set and constructs a library of sequence motifs which are enriched in the experimental set. This algorithm is paired with a clustering algorithm I developed in order to identify groups of related motifs. Briefly, sequences are compared pairwise and clustered if two sequences have 2 or fewer mismatches. The results of the clustering algorithm can be viewed either in tabular form or imported into Cytoscape(Shannon, et al., 2003) to be

viewed as a series of motif networks, where related motifs are visualized as nodes (circles) with edges (lines) delineating their relatedness.

I first used 3' UTRs of let-7 down-regulated transcripts as an experimental set and all other transcripts as control. I found two large clusters of enriched motifs with a 4 fold cutoff for enrichment. (**Figure 4-2b**). Alignment of the first cluster using ClustalW and visualization of this alignment using WebLogo indicated that this enriched cluster corresponds to the let-7c seed-sequence (**Figure 4-2c**). The second cluster when visualized in Cytoscape has significant complementarity to the 3' end of let-7c (**Figure 4-2c**). However this cluster is only represented in two down-regulated mRNAs suggesting that the 3' end of the miRNA has little contribution to the majority of let-7c targeting events.

Running the motif identification and clustering routine for the other miRNAs led to the identification of a large (10-100+ motifs) enriched cluster of motifs corresponding to the seed sequence from the 3' UTRs of transcripts down-regulated by miR-294, miR-26a, miR-99b, and miR-193. This routine failed to identify large clusters corresponding to the seed sequences for miR-199a-5p and miR-218. However, this was due to an excessively strict cutoff for fold enrichment. Using a 3 fold enrichment cutoff led to identification of large clusters corresponding to the seed sequences for these miRNAs. These analyses suggest that on a genome wide scale for all miRNAs tested, the miRNA seed sequence is the most important determinant of miRNA/mRNA target interaction.

Per nucleotide enrichment of miRNA sequence motifs

To further ascertain the contributions of miRNA nucleotides outside of the seed sequence to transcript targeting, I developed a novel algorithm to identify per nucleotide the enrichment of all miRNA sequence motifs from size 6-9 bases. For each miRNA, I found the set of all possible sequence motifs within the miRNA sequence from size 6-9 bases and then determined the enrichment of each of these motifs in the UTRs and ORFs of down-regulated transcripts. These motif data enrichments were mapped back to the miRNA sequence to generate an average enrichment per nucleotide. These data show enrichment within the seed sequence for all miRNAs tested (**Figure 4-2d**). The degree of enrichment varies from one miRNA to the next likely in part due to varying degrees of secondarily down-regulated transcripts in the different conditions. The observed 5' end enrichments occur beyond the 8^{th} nucleotide continuing on into the $10\text{-}11^{th}$ nucleotide for most miRNAs. These data confirm that the seed sequence at 5' end of the miRNA is the most important determinant of miRNA/mRNA target interactions.

Individual genes oppositely regulated by ESC self-renewal silencing miRNAs

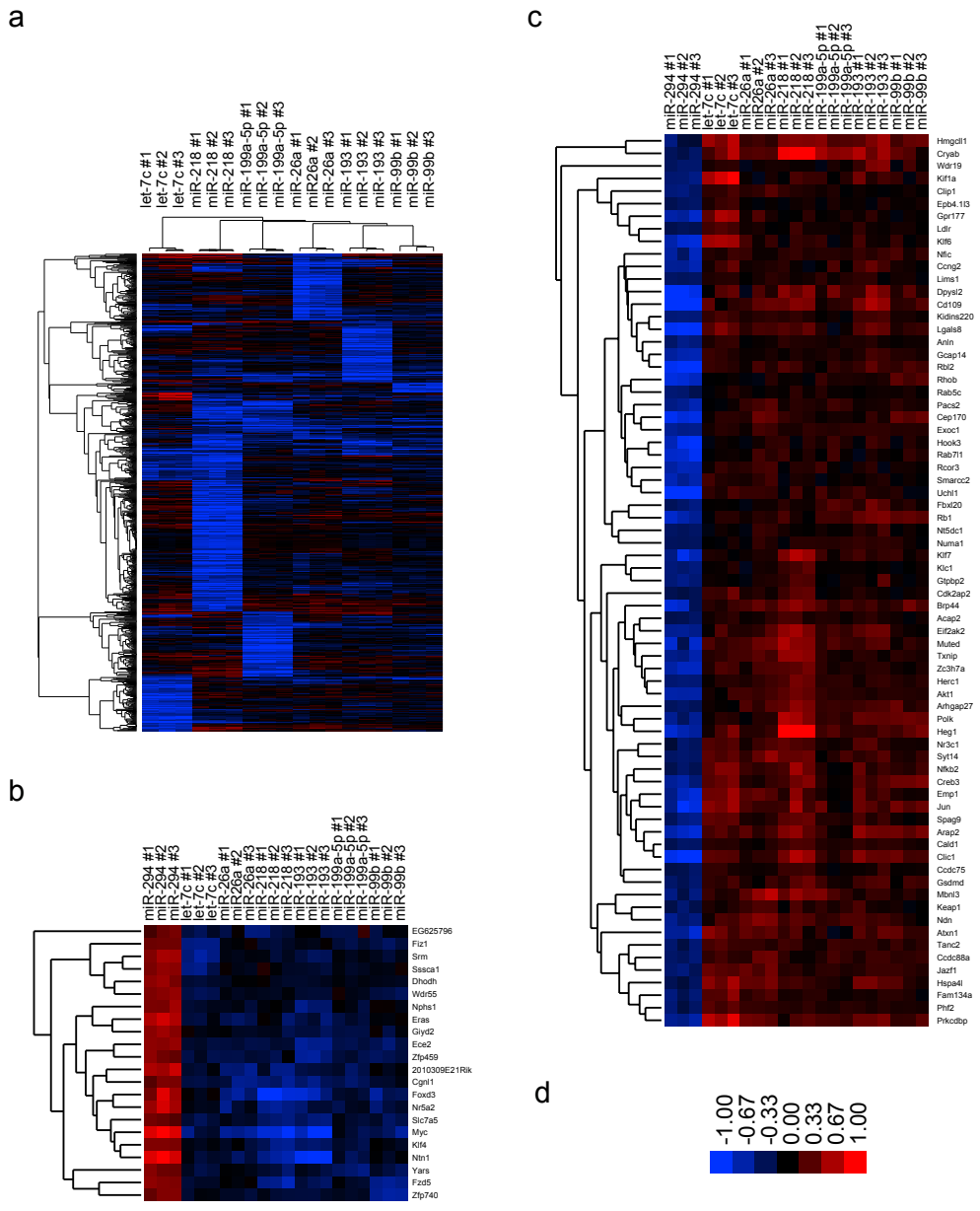
The impetus for identifying mRNAs directly affected by miRNA transfection including direct miRNA targets was to identify individual genes and/or pathways oppositely regulated by the ESCCs and ESC self-renewal silencing miRNAs. To address this hypothesis, I first searched for individual genes showing opposite expression patterns in response to miRNA transfection. Hierarchical average linkage clustering of genes significantly down-regulated with a seed match for at least one miRNA revealed diverse patterns of gene regulation. While each self-renewal silencing miRNA has many putative

mRNA targets (down with a seed match) very few of these mRNA targets are shared by multiple miRNAs (**Figure 4-3a**). These data suggest that the various miRNAs have largely distinct mRNA targets; however, it remained possible that a few common mRNA targets could explain the ability of these miRNAs to have similar effects on silencing ESC self-renewal.

To identify the mRNAs commonly regulated by the ESC self-renewal silencing miRNAs, I identified a cluster of genes that were up-regulated by miR-294 and down-regulated by ESC self-renewal silencing miRNAs (**Figure 4-3b**). This cluster includes cMyc, Klf4, Eras, Nr5a2, and Foxd3. Myc transcription factors are required for ESC self-renewal(Cartwright, et al., 2005), Eras promotes the rapid ESC cell-cycle(Takahashi, Mitsui, & Yamanaka, 2003), Foxd3 is required for ESC self-renewal(Hanna, Foreman, Tarasenko, Kessler, & Labosky, 2002), and Klf4 and Nr5a2 are important components of transcription factor cocktails used in iPS cell formation(Heng, et al., 2010; Takahashi & Yamanaka, 2006). An opposite cluster was also identified that contained genes down-regulated by miR-294 and up-regulated by ESC self-renewal silencing miRNAs (**Figure 4-3c**). This cluster includes the Rb family members Rb1 and Rbl2. While these data are intriguing, none of the genes in these clusters are strongly down or up-regulated (respectively) by all the miRNAs which silence ESC self-renewal. In each case a few or no miRNAs have a strong effect and the rest have a very subtle effect on expression levels of these genes. These data suggested that opposite regulation of cellular pathways and not individual genes by ESC self-renewal silencing miRNAs and ESCC miRNAs

may lay at the heart of the ability of ESC self-renewal silencing miRNAs to silence ESC self-renewal only in the absence of ESCCs.

Figure 4-3



ESCC and ESC self-renewal miRNA regulation of direct mRNA targets. (a) Heirarchical clustering heat map of transcripts down-regulated (< 0.67 fold) with a seed for at least one miRNA and (<1 fold) for all other miRNAs. (b) Heirarchical clustering heat map of transcripts up-regulated by miR-294 (FDR < 0.05) and down-regulated (<1 fold) for all other miRNAs. (c) Heirarchical clustering heat map of transcripts down-regulated by miR-294 (FDR < 0.05) and up-regulated (>1 fold) for all other miRNAs.

ESC transcriptional pathways regulated by ESC self-renewal silencing miRNAs

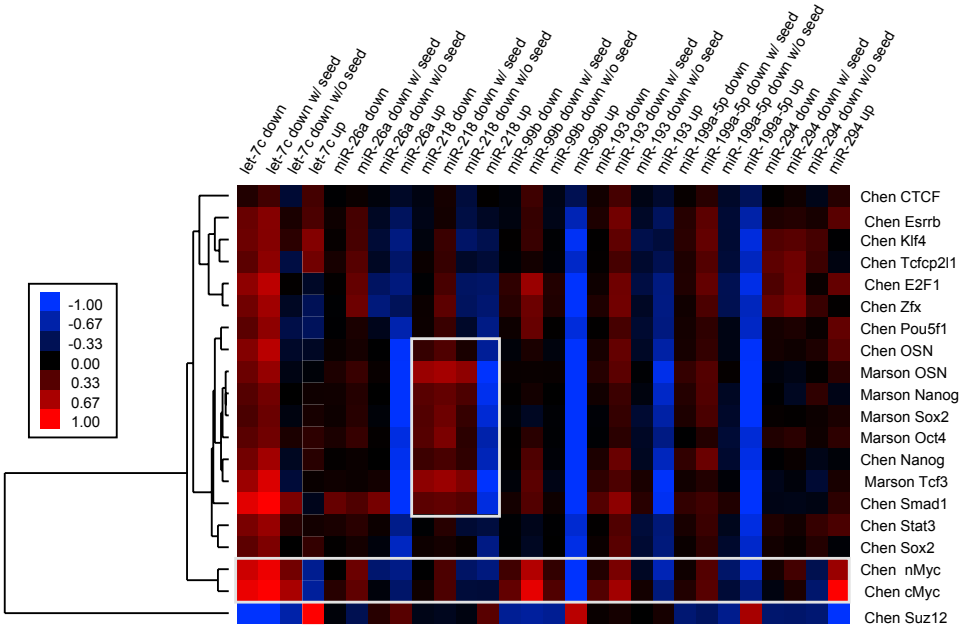
To further investigate the mechanism for the opposing roles of the ESCC and ESC self-renewal silencing miRNAs, I performed various pathway analyses on the miRNA regulated transcript sets. As in **Chapter 3**, I searched for overlaps between the miRNA-regulated transcripts and genes identified by chromatin immunoprecipitation (ChIP) of pluripotency associated transcription factors(X. Chen, et al., 2008; Marson, et al., 2008). This analysis measures whether there is any influence of the individual miRNAs on the transcription factors themselves (**Figure 3-3a, panels i&ii, & Methods**) or the transcripts originating from the genes bound by the transcription factors (**Figure 3-3a, panel iii, & Methods**).

This analysis previously revealed that let-7 preferentially down-regulated transcripts that were also directly regulated by the transcription factors Oct4, Sox2, Nanog, Tcf3, and Smad1. This was also true for miR-218 but not for miR-99b, miR-26a, miR-193, and miR-199a-5p (**Figure 4-4**). This enrichment of Oct4, Sox2, Nanog, Tcf3, and Smad1 targets within the miR-218 down-regulated transcripts was independent of a seed match suggesting that this enrichment was due to a inhibition of these transcription factors themselves and not through direct inhibition of transcripts regulated by these transcription factors. Consistent with this conclusion, we find that Oct4 is among the most down-regulated transcripts with miR-218 treatment. Oct4 contains a miR-218 seed match in its 3' UTR, making it a likely direct target of miR-218. This data suggest that miR-218 is similar to miRNAs like miR-134, miR-296, and miR-470 in its ability to

directly destabilize the ESC transcriptional network(Y. Tay, et al., 2008; Y. M. S. Tay, et al., 2008).

Previously in Chapter 2, I found that let-7 strongly down-regulated nMyc. This resulted in the down-regulation of Myc bound genes and therefore an enrichment of Myc targets among let-7 down-regulated transcripts. The only large enrichment among other ESC self-renewal silencing miRNAs was that of miR-99b down-regulated transcripts with a seed match. However, this may not mean much as there are very few direct miR-99b targets. This enrichment could simply be due to random chance given the small sample size. Interestingly, while additional ESC self-renewal silencing miRNAs, like let-7,

Figure 4-4



Enrichment/depletion of transcription factor bound genes among miRNA-regulated transcripts. A heat map showing enrichment of the ChIP targets among the different sets of miRNA-regulated transcripts on the horizontal axis. Vertical axis represents the different chromatin immunoprecipitation and sequencing data sets. Listed are the factor that was immunoprecipitated and the first author of the paper from which the data were obtained.

down-regulate both nMyc and cMyc at the transcript level, a corresponding strong down-regulation of Myc targets was not observed in the array data (**Figure 4-4**). Based on seed match analysis, miR-193 appears to be the only miRNA tested in addition to let-7 that directly targets nMyc. Down-regulation of nMyc by the other miRNAs is likely indirect. A delay in down-regulation of nMyc relative to let-7 could explain how nMyc transcript but not Myc targets are down-regulated at the 12 hour time point used in the array analysis.

Molecular pathways oppositely regulated by ESC self-renewal silencing miRNAs

With the possible exception of Myc—which could be a false negative result—the transcription factor pathway analysis failed to identify pathways oppositely regulated by ESCCs and ESC self-renewal silencing miRNAs. Therefore, I next investigated the effect of the miRNAs on canonical cellular pathways. For this analysis, sets of up and down-regulated transcripts for each miRNA were tested for enrichment in components of pathways found in MSigDB(Subramanian, et al., 2005). These pathways include Biocarta pathways (www.biocarta.com), Kegg pathways(Kanehisa & Goto, 2000), and Wikipathways(Pico, et al., 2008). Enrichment was determined for transcripts up and down-regulated by individual miRNAs. This pathway enrichment data was subjected to hierarchical clustering analysis and clusters with enrichments across multiple miRNA regulated gene sets were manually identified and are displayed graphically in **Figure 4-5**.

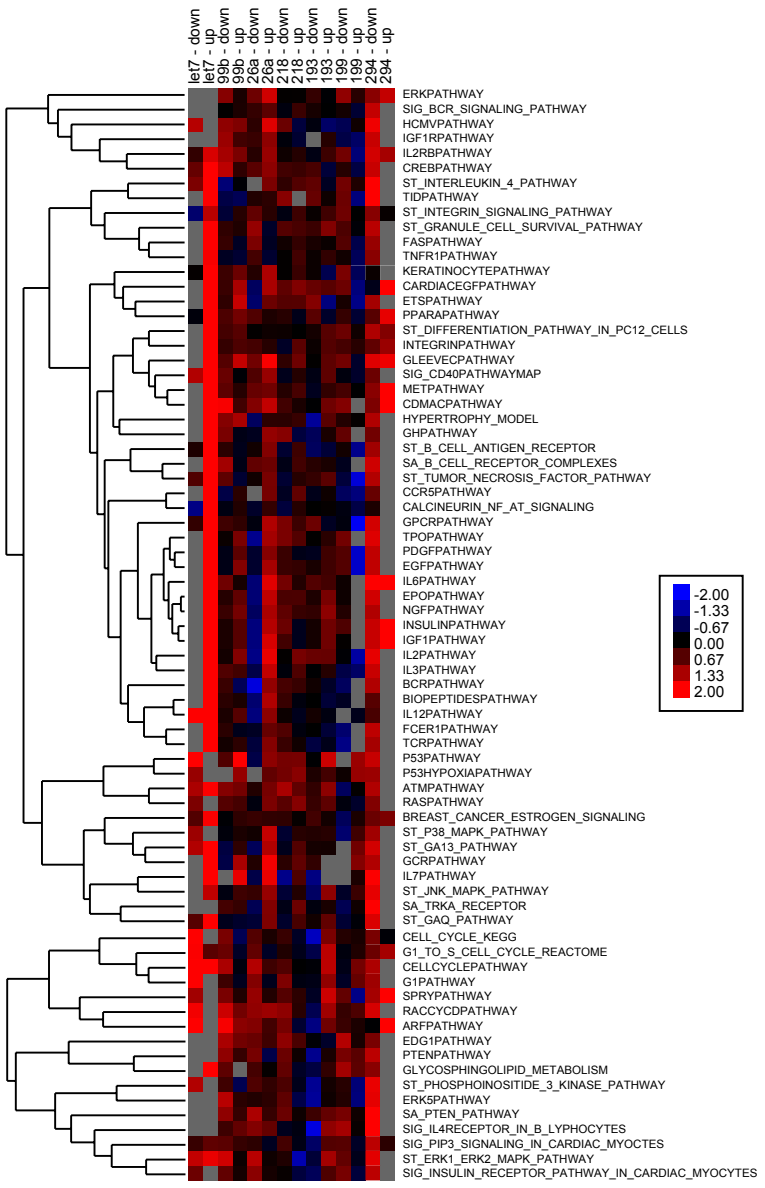
Many of the enriched pathways share molecular components and can be considered variants of a representative canonical pathway. To simplify this pathway enrichment

data, I identified pathway components represented in nine or more enriched pathways (**Table 4-1**). To identify which of these components was contributing to the enrichment, gene expression data for these components was extracted and clustered into a heat map (**Figure 4-6**). This representation allows for visual identification of key genes whose expression changes are strongly contributing to the enrichment of these pathways.

Many cell cycle components are altered by both ESCC and ESC self-renewal silencing miRNAs. ESCC miRNAs down-regulate the cell cycle inhibitors Rbl2, Rbl1, Rb1, and p21 and up-regulate the cell cycle activator c-Myc. In opposition to the ESCC miRNAs, miR-99b, miR-193, and miR-199a-5p up-regulate Rb. Additionally, miR-193 and miR-99b up-regulate Rbl2. Like let-7, miR-193 and miR-218 markedly down-regulate nMyc (~40% and ~50% decrease respectively) and have a seed sequence in the nMyc 3' UTR and ORF respectively. MiR-99b and miR-26a also significantly down-regulate nMyc although they do so to a lesser degree and lack seed sequence matches. MiR-193, miR-218, miR-99b, miR-26a, and miR-199a-5p down-regulate cMyc. miR-218 is the only miRNA with a seed sequence in cMyc. Max is the obligate binding partner of the Myc genes and is required for Myc transcriptional activity(Hurlin & Huang, 2006). MiR-193, miR-199a-5p, miR-26a, and miR-99b down-regulate Max. miR-193 and miR-199a-5p have seed sites in the 3' UTR and ORF respectively and down-regulate max (~50% and ~35% decrease respectively). These data indicate that ESC self-renewal silencing miRNAs can be divided into those that largely down-regulate Myc pathway components (let-7c, miR-193 and miR-218) and those that have significant but more attenuated effects (miR-26a, miR-99b, and miR-199a-5p) (**Fig 4-6**).

Another set of genes that appear to be a node of regulation is the G1 phase cyclins. In mouse ESCs the E type Cyclins, CyclinE1 and CyclinE2, are highly expressed and cooperate with Cdk2 to phosphorylate and inhibit Rb family proteins. This function leads

Figure 4-5



Clusters of enriched pathways among miRNA-regulated transcripts. A heat map showing log2 normalized enrichment of pathways among the different sets of miRNA-regulated transcripts on the horizontal axis. Vertical axis represents the different pathways.

to a rapid progression through the G1 phase. CyclinE1 is significantly down-regulated by miR-26a, miR-218, miR-193, let-7c, and miR-99b. MiR-26a causes the largest change (~40% decrease) in transcript levels of CyclinE1 consistent with the presence of a seed match in the 3' UTR. CyclinE2 is down-regulated by miR-26a, miR-193, and miR-99b. As with CyclinE1, miR-26a has the largest effect (~40% decrease) consistent again with the presence of a seed match in the 3' UTR. These data suggest that miR-26a can functionally regulate the CyclinEs to inhibit G1 phase progression in ESCs.

Previously, it was demonstrated that ESCC inihibition of the cdk inhibitor p21, facilitates the rapid cell cycle in ESCs(Y. Wang, et al., 2008). MiR-218, miR-193, and miR-99b significantly up-regulate p21 mRNA, with miR-218 having the largest effect.

In summary, the ESC self-renewal silencing miRNAs via disparate means all appear to negatively regulate the G1-S cell cycle progression. Let-7c, miR-193, and miR-218 inhibit the Myc proteins and/or Max. MiR-26a inhibits the CyclinEs. MiR218, miR-193, and miR-99b upregulate p21. The only miRNA without a clear means of regulating cell cycle progression is miR-199a-5p.

Summary of important miRNA targets

The analyses presented above and visual inspection of miRNA altered genes led to the identification of both direct and indirect transcription factor targets of ESC self-renewal silencing miRNAs (miR-218:Oct4; miR-199a-5p:Nanog; miR-99b&let-7c:Sall4; miR-218:Foxd3) that could help explain the ability of these miRNAs to silence ESC self-

Table 4-1: Most Represented Components Among Enriched Pathways

Gene	# of occurences	Representative pathway
MAPK3	41	MAPK
GRB2	37	Ras-Raf-Mek
PIK3CA	36	Akt
SHC1	36	Ras-Raf-Mek
SOS1	36	Ras-Raf-Mek
HRAS1	34	Ras-Raf-Mek
JUN	32	Cell cycle
RAF1	32	Ras-Raf-Mek
MAP2K1	30	MAPK
PIK3R1	30	Akt
MAPK1	30	MAPK
MAPK8	28	MAPK
AKT1	27	Akt
FOS	25	Ras-Raf-Mek
ELK1	19	Ras-Raf-Mek
NFKB1	17	Prkc
PRKCB1	16	Prkc
MAP2K4	16	MAPK
PLCG1	15	MAPK
PRKCA	15	Prkc
MAP3K1	13	MAPK
RELA	12	Ras-Raf-Mek
RB1	12	Cell cycle
AKT2	12	Akt
AKT3	12	Akt
NFKBIA	12	Prkc
STAT5A	12	jak stat
CDKN1A	11	Cell cycle
BAD	11	Apoptosis
CDKN2A	10	Cell cycle
JAK2	10	jak stat
TP53	10	Cell cycle
BCL2	10	Apoptosis
CSNK2A1	10	Ras-Raf-Mek
MAPK14	10	MAPK
PIK3CD	9	Akt
RPS6KA1	9	Ras-Raf-Mek
E2F1	9	Cell cycle
CREB1	9	Ras-Raf-Mek
STAT5B	9	jak stat
MYC	9	Cell cycle
MAP2K7	9	MAPK
SOS2	9	Ras-Raf-Mek

renewal(**Table 4-2**). However, these regulatory relationships fail to explain the ability of ESCC miRNAs to block differentiation by the ESC self-renewal silencing miRNAs. Through analysis of pathways altered by ESC self-renewal silencing miRNAs, I identified a number of cell cycle components regulated by the ESC self-renewal silencing miRNAs and ESCC miRNAs. The finding that miRNAs that silence ESC self-renewal also regulate the cell cycle, led me to hypothesize that the ESC self-renewal silencing miRNAs silence ESC self-renewal by inhibiting the G1/S cell-cycle progression.

Inhibition of the G1/S transition in wild-type ESCs promotes loss of ESC self-renewal but doesn't sensitize ESCs to miRNA induced silencing of ESC self-renewal

Previous studies in ESCs indicate that a fast G1/S cell-cycle progression is required to maintain ESC self-renewal and pluripotency. Mouse ESCs lacking Cdkap1, a negative regulator of Cdk2 activity, are resistant to ESC differentiation(Y. Kim, et al., 2009). Likewise, in human ESCs, inhibition of Cdk2 by RNAi promotes differentiation to extraembryonic lineages (Neganova, Zhang, Atkinson, & Lako, 2009). Furthermore, p53 activates p21 during human ESC differentiation and p53 is required for ESC differentiation in mouse (T. Lin, et al., 2005; Maimets, Neganova, Armstrong, & Lako, 2008). These findings suggest that p53 regulation of p21 and the cell cycle could be required for ESC differentiation.

To first test the hypothesis that a rapid G1/S cell cycle transition is required to maintain ESC self-renewal, I over-expressed the cell-cycle inhibitor p21 in wild-type mouse ESCs

Table 4-2: Notable mRNA targets of ESCC and ESC self-renewal silencing miRNAs

miR-294	up-regulated	Myc, Lin28, Trim71
	Down-regulated	Cdkn1a, Rbl2, Rb
let-7c	up-regulated	
	Down-regulated	Mycn, Lin28, Sall4, Trim71
miR-99b	up-regulated	Cdkn1a
	Down-regulated	Sall4 (no seed), Lin28 (no seed), Trim71 (no seed)
miR-26a	up-regulated	
	Down-regulated	Ccne1, Ccne2, Trim71 (no seed), Lin28 (no seed)
miR-218	up-regulated	Cdkn1a
	Down-regulated	Pou5f1, Mycn, Myc, Trim71, Lin28 (no seed)
miR-193	up-regulated	Cdkn1a
	Down-regulated	Myc, Mycn, Max
miR-199a-5p	up-regulated	
	Down-regulated	Max, Nanog (no seed)

using a doxycycline (dox) inducible system [unpublished data, Yangming Wang (YMW)]. Proliferation was clearly reduced in these cells by visual inspection after addition of dox to culture media. Previous characterization demonstrated that upon dox exposure these cells undergo a large increase in G1 phase cells indicating a delayed G1/S transition (YMW unpublished data). I find that after 4 days of exposure to dox in ESC media with LIF, mouse ESCs had an approximately 3-4 fold reduction in mRNA expression of Oct4, Sox2, and Nanog (**Figure 4-7**). These data indicate that a rapid G1/S transition is required to maintain ESC self-renewal.

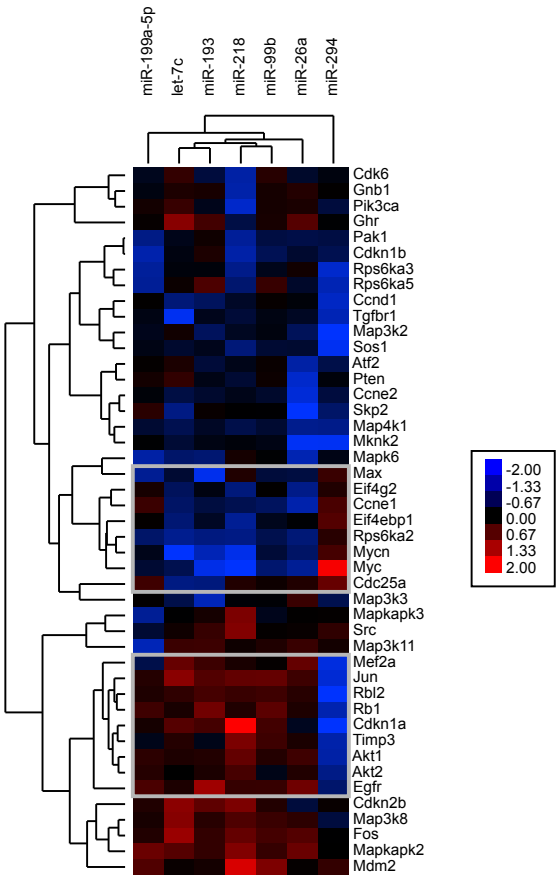
P21 over-expressing cells roughly mimic the increase in G1 phase cells in *Dgcr8 -/-* ESCs; therefore, we tested the effect of expressing ESC self-renewal silencing miRNAs in these cells. Three days after transfection of let-7c, miR-99b, miR-26a, miR-218, miR-193, and miR-199a-5p into Teto:p21 ESCs (with or without exposure to dox one day prior to transfection and for the duration of the experiment) had no significant effect on mRNA expression levels of Oct4, Sox2, and Nanog (**Figure 4-7**). ESC self-renewal silencing miRNA transfection into *Dgcr8 -/-* ESCs have a much more dramatic effect on silencing self-renewal than does the equivalent transfection into p21 overexpressing wild-type ESCs cells. Because inhibition of the G1/S transition does not sensitize wild-type ESCs to silencing of ESC self-renewal by ESC self-renewal silencing miRNAs, these data suggest that ESCC miRNAs or other miRNAs in ESCs may antagonize ESC self-renewal silencing miRNAs by mechanisms in addition to promoting a rapid G1/S transition. Nevertheless, the fact that inhibition of the G1/S transition leads to loss of

ESC self-renewal, suggests that miRNA inhibition of the cell cycle could result in silencing of ESC self-renewal.

Deregulating the G1/S transition in Dgcr8 -/- ESCs

To test whether inhibition of the G1/S transition is necessary for ESC self-renewal silencing miRNAs to silence ESC self-renewal, I sought to deregulate the G1/S transition in *Dgcr8 -/-* ESCs. To this end I engineered cells that were *p107 -/-*, *p130 -/-*, *Dgcr8 -/-*. P107, p130, and pRb are the three members of the Rb family and are important inhibitors

Figure 4-6

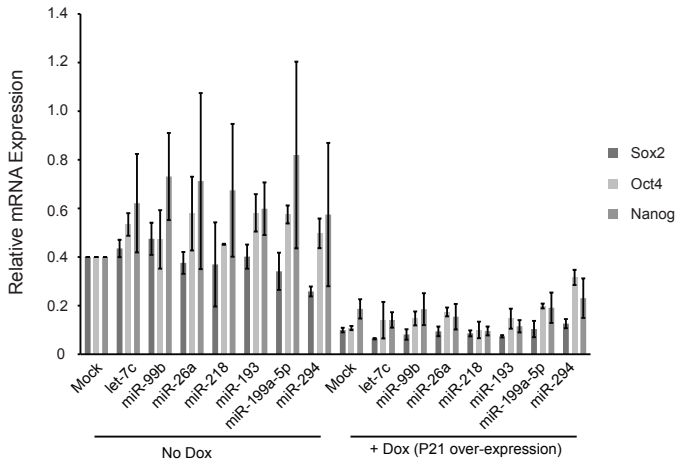


Clusters of altered components of enriched pathways. Heatmap of altered components (abs(log2(change))>0.5) among miRNA-regulated transcripts for components of MAPK signaling, Akt signaling, G1 cell cycle phase, mTor, p53, and ERK signaling. miRNA regulated groups on the horizontal axis. Vertical axis represents the different pathway components.

of the G1/S transition. When active, they bind to E2F family transcription factors to inhibit the transition from G1 to S. During the transition from G1 to S, Rb family members are hyper-phosphorylated and degraded(Giacinti & Giordano, 2006). Mouse embryonic fibroblasts (MEFs) deleted for the Rb family have a shortened G1 phase and avoid G1 arrest in response to contact inhibition or serum starvation(Sage, et al., 2000).

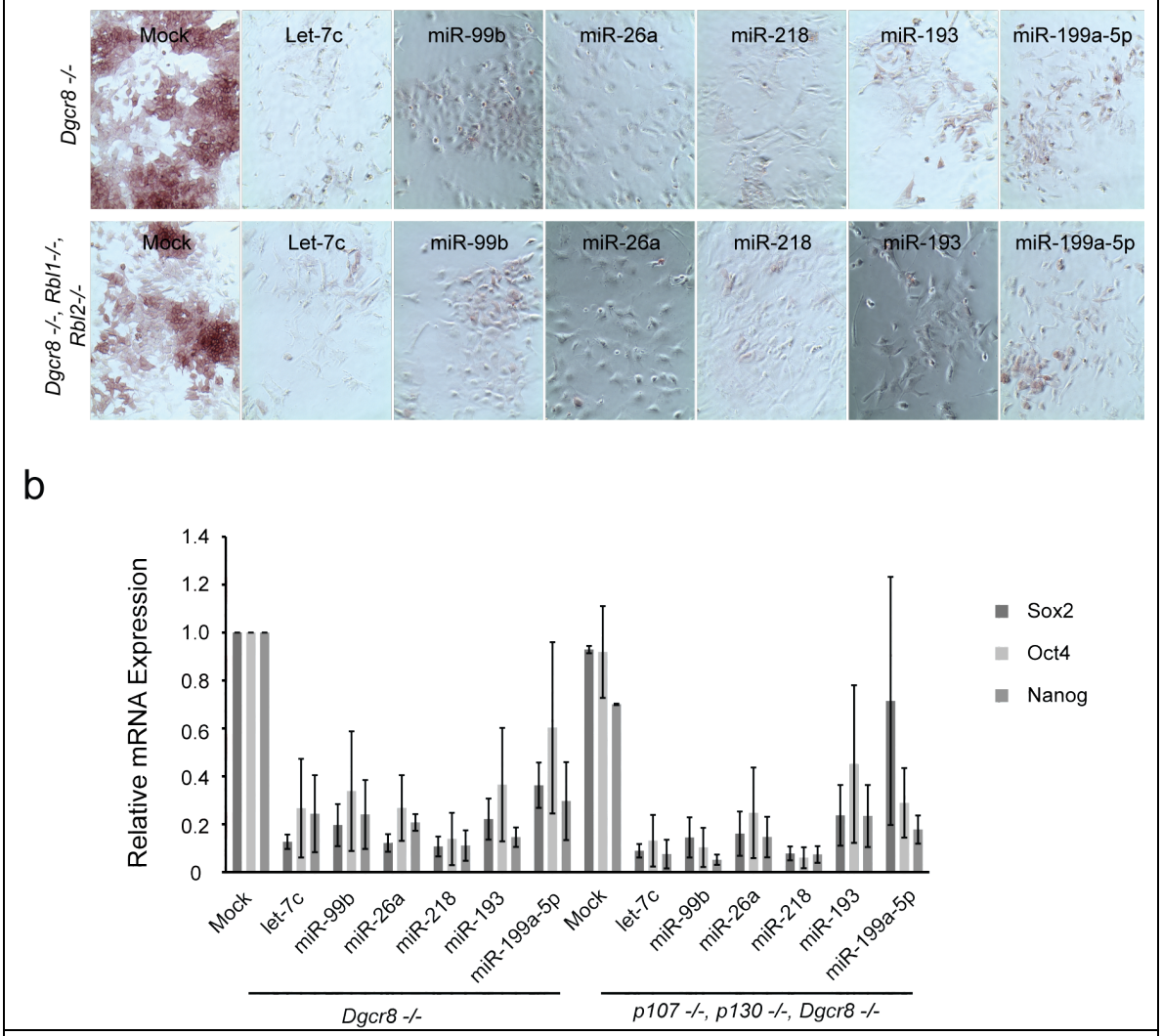
I first generated *p107 -/-*, *p130 -/-*, *Dgcr8 -/-* ESCs by gene targeting of *Dgcr8* in a *p107 -/-*, *p130 -/-* ESC line(Sage, et al., 2000). The genotype of these cells was verified by qRT-PCR for the deleted region of *Dgcr8* and the disrupted regions of p107 and p130. Surprisingly, these ESCs like *Dgcr8 -/-* ESCs proliferate slowly. Nevertheless, I tested whether the ESC self-renewal silencing miRNAs could silence self-renewal in these cells. Three days after transfection with miRNA mimics, these cells robustly silenced markers of ESC self-renewal including alkaline phosphatase activity and mRNA expression of

Figure 4-7



P21 over-expression differentiates ESCs. Rosa26 rtTA, Col1A Teto-p21 ESCs were cultured in ESC media with and without doxycycline to induce expression of p21 protein. miRNAs were transfected into these cells and mRNA expression of Sox2, Oct4, and Nanog was assessed by qRT-PCR.

Figure 4-8



Removal of *p107* and *p130* in *Dgcr8* -/- ESCs does not prevent let-7c, miR-99b, miR-26a, miR-218, miR-193, and miR-199a-5p from silencing ESC self-renwal. miRNAs were transfected into *Dgcr8* -/- or *p107* -/-, *p130* -/-, *Dgcr8* -/- ESCs and self-renewal was assessed by (a) AP staining and (b) qRT-PCR for Sox2, Oct4, and Nanog.

Oct4, Sox2, and Nanog (**Figure 4-8a&b**).

The susceptibility of *p107* -/-, *p130* -/-, *Dgcr8* -/- ESCs to silencing of self-renewal by ESC self-renewal silencing miRNAs could be due to the expression of the remaining Rb family member pRb. To test this hypothesis I propose to generate *pRb* -/-, *p107* -/-, *p130* -/-, *Dgcr8* -/- ESCs. This work is ongoing.

Discussion

The work presented in this chapter demonstrates that numerous miRNAs are able to silence the ESC self-renewal program in *Dgcr8 -/-* ESCs. These miRNAs, like let-7, are all unable to silence ESC self-renewal in the presence of ESCC miRNAs. This led us to hypothesize that common genes and/or molecular pathways are oppositely regulated by ESC self-renewal silencing miRNAs and ESCC miRNAs. I took a genomics level approach to address this hypothesis. I first identified the direct molecular changes induced by miRNA addition via mRNA microarray analysis. This allowed for identification of putative direct targets—down-regulated with a seed match—and indirect changes. Comparative analysis of genes directly and indirectly altered by miRNAs led to the finding that few genes are commonly impacted by these miRNAs. This led me to consider the possibility that either the miRNAs that silence ESC self-renewal act through different mechanisms or that these miRNAs impact common pathways via disparate targets. Comparative analsyis of pathways oppositely affected by the ESC self-renewal silencing miRNAs and ESCC miRNAs led to the identification of cell cycle components and in particular the G1/S transisiton as being oppositely regulated by ESC self-renewal silencing and ESCC miRNAs. Initial experiments suggest that regulation of the ESC cell cycle may be a critical event in the initiation of differentiation by ESC self-renewal silencing miRNAs.

The work in this chapter in addition to furthering our understanding of how miRNAs can either promote or inhibit ESC self-renewal, also is the first demonstration of using a

genomics level approach with multiple miRNAs to identify commonly affected molecular pathways. This kind of genomics level analysis allows for a less biased approach to identifying the key molecular changes induced by a particular set of miRNAs.

Chapter 5: Conclusions and Future Directions

Summary

The work presented in this thesis originates from the hypothesis that miRNAs are critical regulators of cell fate transitions due to their ability to quickly and globally alter a cell's proteome. MiRNAs are also well suited to stabilize a cell fate to prevent cell fate transitions. In Chapter 2, I found that ESCs fail to silence ESC self-renewal in differentiation inducing conditions. This led me to hypothesize that miRNAs specifically repress the ESC self-renewal program during differentiation. To identify such miRNAs, I performed a screening assay. I uncovered a small number of miRNAs that both rescue the differentiation defect in *Dgcr8 -/-* ESCs. These miRNAs are up-regulated during ESC differentiation consistent with their having biological functions in the ESC differentiation process. Of these miRNAs a number belonged to the let-7 family of miRNAs, which had known roles in promoting differentiation in a number of other model systems. In Chapter 3, I followed up on the mechanisms by which the let-7 family miRNAs silence ESC self-renewal. I found that let-7 miRNAs silence ESC self renewal only in the absence of ESCC miRNAs. ESCC miRNAs are highly expressed in ESCs and promote the ESC fate. I investigated the mechanisms underlying the antagonism between the let-7 and ESCC miRNAs and found that these miRNAs have opposing effects on the expression of Myc family proteins. These effects likely at least in part explain the antagonistic effects of these two miRNA families on ESC differentiation. In the 4th chapter of my thesis I investigate the mechanisms by which a number of other miRNAs induced silencing of ESC self-renewal in *Dgcr8 -/-* ESCs. These miRNAs like the let-7 miRNAs silence self-renewal only in the absence of the ESCC miRNAs.

Messenger RNA profiling followed by bioinformatics analysis uncovered a number of genes and pathways regulated in common by the pro-differentiation and opposed by the ESCC miRNAs. These analyses identified that the G1/S cell cycle transition is regulated by miRNAs that silence ESC self-renewal. Inhibition of the G1/S transition in wild-type ESCs inhibited ESC self-renewal. These analyses suggest that miRNA regulation of the ESC cell cycle is a critical event in the differentiation of ESCs.

Implications to miRNAs in somatic stem cells

miRNA function in somatic stem cells remains poorly studied. Indeed, aside from ESC derived neural progenitor cells (NPCs) no detailed analysis of the miRNA repertoires of pure somatic stem cell populations has been performed. In NPCs the let-7 miRNAs are the dominant miRNA species(Marson, et al., 2008). Interestingly, recent data suggest that the let-7 miRNAs are not required for the propagation but rather the differentiation of neural stem cells in the embryonic mouse brain(Schwamborn, et al., 2009). In this model, asymmetric divisions in neural stem cells segregates the RNA binding protein Trim32 into the daughter cell committed to differentiate further. Trim32, among other functions, increases the activity of let-7 in this cell to promote differentiation(Schwamborn, et al., 2009). It will be interesting to understand the roles of miRNAs in somatic cells. Do miRNAs promote self-renewal of somatic cells? Are miRNAs required for the differentiation of somatic stem cells? I would predict that as with ESCs, different somatic stem cell populations will be regulated by both pro and anti-self-renewal miRNAs. Antagonistic effects of two miRNA classes may be a common theme in cell fate transitions.

Implications to cancer

ESCC and related miRNAs act as oncogenes in cancer while miRNAs that silence ESC self-renewal act as tumor suppressors. ESCC miRNAs and the miR-17/20/106 family share a similar seed sequence. The miR-17/20/106 family has been shown to have important roles in cancer. For example, miR-93 and miR-106 miRNAs target p21 to deregulate the G1/S checkpoint and promote rapid cell proliferation in multiple tumor types(Ivanovska, et al., 2008; Petrocca, et al., 2008). Additionally, *in vivo* studies have shown important roles for these miRNAs in tumorigenesis. In particular, enforced expression of the miR-17-19b polycistron accelerates tumor formation and decreases apoptosis in an Eμ-Myc B cell lymphoma mouse(He, et al., 2005). The decreased apoptosis in this model is likely, at least in part, due to miR-17 family miRNAs targeting the pro-apoptotic protein Bim(Mendell, 2008). The miR-17/92 cluster also contributes to tumorigenesis by increasing angiogenesis in tumors(Dews, et al., 2006). The human miRNAs miR-372 and miR-373 are orthologs to the ESCC miRNAs. These miRNAs cooperate with oncogenic Ras to promote tumor formation in primary human fibroblasts and are highly expressed in germ cell tumors(Voorhoeve, et al., 2006). Collectively, these data demonstrate that miRNAs that share a similar seed sequence to the ESCC miRNAs, function as potent oncogenes often by acting through similar pathways normally seen in ESCs.

In contrast to the ESCC and related miRNAs, the let-7 miRNAs act as tumor suppressors. In a model of breast cancer, a subpopulation of the cancer cells, the tumor initiating cells

(TICs), can regenerate the tumor. When the TICs differentiate they are no longer capable of forming a full tumor. The let-7 miRNAs are sufficient for differentiation of these cells. In this setting, let-7 acts in part by suppressing Ras, to suppress proliferation, and HMGA2, to promote differentiation of the cancer cells(F. Yu, et al., 2007). Likewise, in a mouse model of K-Ras induced lung cancer and in xenograft models of established cancer cell lines, addition of exogenous let-7 miRNAs suppresses while inhibition of let-7 activity promotes tumorigenesis(Esquela-Kerscher, et al., 2008; M. S. Kumar, et al., 2008; Trang, et al., 2009). Furthermore, recent evidence suggests that Lin28 through inhibition of let-7 activity can promote tumor formation(Chang, et al., 2008; Dangi-Garimella, et al., 2009; Iliopoulos, Hirsch, & Struhl, 2009; Viswanathan, et al., 2009). Let-7 has been shown to target multiple oncogenes including K-Ras, N-Ras, Hmga2, cMyc, nMyc, and additional factors that collectively reduce cell proliferation(Büssing, et al., 2008). Together, these data strongly support a functional role for let-7 as a tumor suppressor.

Apart from the let-7 miRNAs, other miRNAs that silence ESC self-renewal have been less well studied in tumorigenesis. Of note, miR-99b family members miR-99a and miR-100 have been shown to target mTOR signaling in adrenocortical cancer(Doghman, et al., 2010) and miR-218 has been implicated as a tumor suppressor in metastatic gastric cancer(Tie, et al., 2010). In contrast, miR-26a has been reported as oncogenic in gliomas(Huse, et al., 2009). It remains to be seen how similar these other miRNAs behave in ESC biology and cancer biology.

In ESCs, we uncovered a biological network between ESCC and let-7 miRNAs whereby ESCCs inhibited the expression of let-7 miRNAs via Lin28. In cancer, Lin28 has been shown to possess oncogenic activity by suppressing let-7 miRNAs (see above). It remains to be seen whether ESCC or related miRNAs positively regulate expression of Lin28 in cancer. Antagonism between ESCC and let-7 miRNAs may be a common theme in diverse biological contexts.

Implications for iPS cell generation

ESCC miRNAs promote self-renewal in ESCs while the let-7 miRNAs promote silencing of ESC self-renewal. Reprogramming of somatic cells to induced pluripotent stem (iPS) cells can be achieved by nuclear transfer or by directed reprogramming with exogenously introduced transcription factors(Hochedlinger & Plath, 2009). Consistent with the role of ESCC miRNAs in promoting ESC self-renewal, addition of these miRNAs to directed reprogramming assays enhances reprogramming efficiency(Judson, et al., 2009). Likewise inhibition of the let-7 miRNAs enhances reprogramming(Melton, et al., 2010). The effects of inhibiting the direct miRNA suppressors of ESC self-renewal on reprogramming remain unknown. Together, these findings demonstrate that the same mechanisms that control ESC self-renewal and differentiation also govern the dedifferentiation process.

Additionally, the ability to reprogram with cocktails of transcriptions factors with and without Myc (either Sox2, Oct4, Klf4, cMyc or Sox2, Oct4, Klf4, no cMyc) has allowed for interrogation of the function of miRNAs and miRNA inhibitors in regard to whether

they function in the same or alternate pathways to each of these factors. For example, the ESCC miRNAs were shown to enhance reprogramming in the absence, but not in the presence of cMyc(Judson, et al., 2009). These findings suggest that ESCCs and Myc have redundant roles. Indeed it is now known that ESCCs induce the indirect upregulation of cMyc and that both cMyc and nMyc promote transcription of ESCC miRNAs(Judson, et al., 2009; Melton, et al., 2010). Additionally, it has been discovered that inhibition of let-7 promotes reprogramming more so in the absence than in the presence of Myc(Melton, et al., 2010). This finding suggests that let-7 in somatic cells in part acts to suppress ESC self-renewal through Myc. Indeed, both cMyc and nMyc are direct targets of let-7(Madhu S. Kumar, et al., 2007; Melton, et al., 2010). It will be important and interesting to understand if there exist miRNAs, which operate in the same pathways as the other pluripotency transcription factors and whether these miRNAs can replace these transcription factors in iPS cell reprogramming.

MiRNAs and synthetic miRNA inhibitors are attractive tools for iPS cell generation because they are only transiently expressed thereby leaving no trace of their existence in the resulting iPS cell line. This is advantageous, as many current tools for iPS generation require retroviral or lentiviral delivery. The integrated gene product can be reactivated during therapeutic differentiation and cause dysfunction of the cell type of interest or even promote cancer formation. The work presented in this thesis and elsewehere(Judson, et al., 2009) indicates addition of ESCC miRNAs and inhibition of let-7 miRNAs promotes iPS formation. Likely inhibition of other ESC self-renewal silencing miRNAs would also promote reprogramming.

Implications to the therapeutic generation of specialized cell types from ESCs

The work presented in this thesis suggests that specific miRNAs can stabilize specific cell types of interest. In this body of work, I demonstrate that ESCC miRNAs and let-7 miRNAs stabilized ESCs and somatic cells respectively. These results suggest that miRNA may also promote and stabilize the generation of specific therapeutically relevant cell types during ESC differentiation. Addition of miRNAs to differentiating cells could lead to higher yields and perhaps higher reproducibility of therapeutically relevant cells during ESC differentiation.

Implications to miRNA biology

The work in this thesis is among the first genomic analyses that implicate ORF seed matches as a major component of miRNA target site interactions. Recently a small number of papers have found functional miRNA target sites in ORFs(Bartel, 2009). Additionally, Baek *et al.* by proteomics analysis showed that mRNAs with seed matches in their ORFs are more likely to be downregulated at both the mRNA and protein level(Baek, et al., 2008). In this thesis I find that miRNA seed matches in the ORF are commonplace and are responsible for the miRNA target interactions of 100s of mRNAs. Recent work using IP-seq and IP-Chip have found similar results where miRNAs have target sites in both ORFs and 3' UTRs(Chi, Zang, Mele, & Darnell, 2009; W. X. Wang, et al., 2010).

The bioinformatics analysis to globally identify miRNA targets by miRNA addition in *Dgcr8 -/-* ESCs followed by microarray analysis is among the first studies to globally identify functional miRNA targets. The subsequent analysis to identify pathways—both transcription factor networks and canonical signaling pathways—that are regulated by specific miRNAs represents a novel approach to studying miRNA biology. Most miRNA studies focus on individual miRNA targets and make arguments that these individual targets can explain the bulk of the biological effects of the miRNA. My approach analyzing pathways affected by miRNAs takes an alternative view that the combined effects of many miRNA/mRNA target regulatory relationships can cumulatively have a much stronger effect than any single miRNA/mRNA target relationship.

Implications to developmental biology

The studies of this thesis were initiated to investigate the hypothesis that miRNAs are important regulators of cell fate transitions during cellular differentiation. I anticipated that miRNAs would be well suited to quickly alter the expression of many transcripts simultaneously and that this pleiotropic effect could destabilize the original cell fate during differentiation. Indeed, I have found that the let-7 and additional miRNAs have such an effect on promoting the transition from one cellular state to the next. Additionally, I have found that a second class of miRNAs, the ESCC miRNA family, has the opposite effect of stabilizing the original cell state and preventing transition to a more differentiated cell state. The ESCC miRNAs regulate negative regulators of the let-7 miRNAs to form a complex biological network that prevents expression of let-7 miRNAs

while ESCC miRNAs are expressed. These findings demonstrate that the changes to the miRNA repotoire are critical events during cell fate transitions.

Mammalian development initiates with fertilization of the oocyte by a single sperm. During these first days of development the zygote rapidly replicates and undergoes massive epigenetic changes to form a compacted mass of undifferentiated cells termed the morula. It is thought that at the first cellular differentiation events occur at the morula stage with the specification of cells to become precursors of the trophectoderm lineage as well as precursors of the inner cell mass. Recently, it was demonstrated that this initial specification events can occur in the absence of both maternal and zygotic miRNAs(N. Suh, et al., 2010). miRNAs appear to be required subsequent to the blastocyst stage. In the blastocyst, a collection of cells known as the inner cell mass expand and give rise to the embryonic epiblast. The embryonic epiblast will give rise to the three embryonic germ layers: ectoderm, endoderm, and mesoderm. It is likely that cellular proliferation and differentiation of these cells is critically controlled by miRNA expression as both *Dicer* and *Dgcr8 -/-* embryos fail to gastrulate(Bernstein, et al., 2003; Y. Wang, et al., 2007). The ESCC miRNAs likely contribute to this defect. ESCC miRNAs of the miR-302 cluster are highly expressed in the mouse epiblast, and in the frog have been shown to be important for mesendodermal fate specification(Rosa, Spagnoli, & Brivanlou, 2009). It will be important to determine the functions of both the ESCC and let-7 miRNAs during *in vivo* mammalian development.

Let-7 miRNAs are evolutionarily quite old, being conserved across much of the animal kingdom. They first evolved in bilateria to function in neurosecretory cells of the foregut and later evolved to have important functions in developmental timing(Christodoulou, et al., 2010). This function of let-7 in developmental timing appears to be conserved across modern bilateria(Pasquinelli, et al., 2000). In Caenorhabditis elegans, for example, let-7 is required for terminal differentiation of a subset of cells termed seam cells(Reinhart, et al., 2000). In this thesis I show that let-7 function is conserved in mammals where it promotes differentiation of ESCs. In contrast to let-7, ESCC and related miRNAs are evolutionarily restricted to vertebrates including human, mouse, zebrafish, and frogs. ESCCs miRNAs have the opposite function of let-7 miRNAs; they promote rapid proliferation of undifferentiated cells. Additionally, in this thesis I demonstrate that ESCC miRNAs stabilize the undifferentiated state to prevent precocious differentiation by other miRNAs. Vertebrate development is slower and requires increased expansion of progenitor populations. It is interesting to speculate that ESCC miRNAs evolved specifically in vertebrates to promote the expansion of undifferentiated progenitor pools while simultaneously stabilizing these undifferentiated cells from differentiation by let-7 and other miRNAs. Likely, the antagonism between ESCC and let-7 miRNAs is not specific to mouse ESCs but rather is an evolutionarily phenomenon occurring across vertebrate species.

Methods

Tissue culture, transfection, and AP staining

ES cell lines and culture conditions were previously described[3]. ES cells were weaned off MEFs and maintained in MEF conditioned media. For ES cell differentiation assays 40,000 *Dgcr8* -/- or 12,000 wild-type ES cells were plated in gelatinized 12 well plates (or half the number of cells were plated on 24 well plates) on day 0 in LIF media. On day 1, miRIDIAN miRNA mimics (Dharmacon, ThermoFisher) were transfected at a concentration of 50nM using Dharmafect1 (Dharmacon, ThermoFisher) following the manufacturer's protocol. Media was changed daily. On the third day after transfection, cells were either lysed in Trizol (Invitrogen) for qRT-PCR analysis or fixed in 4% PFA for AP staining. AP staining was performed per the manufacturer's instructions (Vector Labs). iPS lines were maintained in ES media + 15% knock-out serum on irradiated MEF feeders. Colony reformation assays were performed as previously described[3]. Briefly, cells were exposed to miRNA mimics for 3 days then trypsinized and counted. A defined number of cells were replated on MEFs to form colonies for 5-7 days. The efficiency of colony reformation was determined by counting the number of AP positive colonies divided by the number of cells plated. Neural progenitor cells used in **Figure S3** were generated by in vitro differentiation of ES cells as described previously[42].

Animal use

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

ES Cell Derivation

Timed matings where set up for cMyc f/f mice[43]. ES cells were derived from embryos isolated at E3.5. These embryos were cultured on an irradiated MEF feeder layer in ES cell media supplemented with 50uM PD98059[44] and disassociated onto fresh feeders. ES cells were PCR genotyped as previously described[43]. A flox/flox line was grown out, infected with Ad5 Cre-IRES-GFP virus, sorted by FACs, and plated back onto MEF feeders. *cMyc -/-* colonies were grown out and verified by PCR genotyping and Western.

mRNA arrays

qRT-PCR showed that mRNA levels of a known let-7 target, Lin28[10], was maximally reduced 12 hours post-transfection prior to a large decline in Oct4 and Nanog (**Figure S18**). Therefore, we chose twelve hours for all microarray analysis to minimize secondary effects of let-7c-induced differentiation. 150,000 cells were plated in a 3.5cm dish on day 0. miRIDIAN miRNA mimics (Dharmacon, ThermoFisher) were transfected at a concentration of 50nM in media in the absence of LIF. At 12 hours post transfection cells were lysed in Trizol (Invitrogen) and RNA was prepared according to the manufacturer's protocol. Affymetrix Mouse Gene 1.0 ST arrays were probed by the Gladstone Genomics Core (www.gladstone.ucsf.edu/gladstone/site/genomicscore). Three biological samples were assayed for each treatment. Data were analyzed by Affymetrix Expression Console software. The Robust Multichip Analysis (RMA) algorithm was used to normalize the array signal across chips. SAM (http://www-stat.stanford.edu/~tibs/SAM/) was used to determine FDR cutoffs for significantly altered genes.

qRT-PCR analysis

RNA for all qRT-PCR analysis was prepared using Trizol (Invitrogen) and quantified on a Nanodrop Spectrophotometer (ThermoFisher). 500ng of RNA was DNAse treated using DNAseI amplification grade (Invitrogen). For qRT-PCR of mRNAs, DNAse treated samples were reverse transcribed using the Superscript™ III first-strand synthesis system for RT-PCR (Invitrogen). qPCR reactions on resulting cDNAs were performed on either an ABI Prism 7100 or ABI 7900HT (Applied Biosystems). For miRNAs, qRT-PCR was performed either by using TaqMan® miRNA assays (Applied Biosystems) or by polyadenylating the miRNAs and then using a modified oligodT reverse transcription primer as described previously[45].

Lin28 and GFP expression in 293T cells

Lin28 was cloned into an expression vector under the EF1alpha promoter and upstream of IRES Pac (puromycin resistance). A similarly constructed GFP expression construct was previously generated[6]. 293T cells were transfected with 5ug of each construct and selected with 0.6ug/mL puromycin for 12 days.

Luciferase reporter assays

Constructs were produced as follows. The nMyc and Sall4 3′ UTRs were amplified from ES cell cDNA and cloned into the NotI and XhoI sites in psiCheck™-2 vector (Promega). Mutant UTRs were generated by a two-step PCR strategy with overlapping mutated PCR primers. Products of two PCRs with mutations were used in a second PCR reaction to

generate full-length mutated inserts that were cut and ligated into cut empty vector. For transfections, 8000 *Dgcr8* -/- ES cells were plated in ES cell media in a 96 well plate pretreated with 0.2% gelatin. The next day, miRIDIAN miRNA mimics (Dharmacon, ThermoFisher) were transfected with Dharmafect1 (Dharmacon, ThermoFisher) following the manufacturer's protocol at a concentration of 100nM. Simultaneously, luciferase constructs were transfected into ES cells at a concentration of 200ng per well using FUGENE® 6 (Roche) transfection reagent following the manufacturers protocol. The following day, 14-18 hours later, cells were lysed and luciferase assays were performed using a Dual-Luciferase® Reporter Assay System (Promega) on a single automatic injection Mithras (Berthold technologies) luminometer following the manufacturer's protocol. Transfection of each construct was performed in triplicate in each assay. Ratios of Renilla luciferase readings to Firefly luciferase readings were averaged for each experiment. Replicates performed on separate days were mean centered with the common readings from the individual days.

Seed match analysis

Promoter (1000 base pairs from the transcriptional start), 5′ UTR, ORF, and 3′ UTRs for Ensembl Transcripts (mm9) and known genes (mm8) were downloaded separately from the UCSC Genome Browser Table Browser. Seed match analysis was performed on these transcripts using a custom Python script. 7mer seeds were defined as either 7mer-1A or 7mer-m8[46]. Seed match results were mapped to Affymetrix IDs. A Python script was then implemented to eliminate redundant transcripts as transcripts often mapped >1:1 with Affymetrix IDs. The transcript with the most 7mer seed matches was chosen to

produce a 1:1 transcript to Affymetrix ID mapping. This mapping was done separately for the promoters, 5′ UTRs, ORFs, and 3′ UTRs. In rare cases, duplicate Affymetrix IDs exist for the same gene. These were retained in our analyses. Microsoft Access (Microsoft) was used to generate list overlaps for analyses. P-values were calculated in **Figure 2b&d** with the # of seed matches per kb of transcript using the Wilcoxon Rank Sum test in R. P-values were calculated in **Figure S6a&b** using a binary 0 for no seed matches or 1 for a seed match using the hypergeometric distribution function in R.

ChIP target overlap analysis

ChIP targets were downloaded from the supplementary tables[15,19]. Scripts were written to convert provided transcript IDs to a non-redundant list of Affymetrix IDs. Microsoft Access (Microsoft) and custom Python scripts were used to perform comparisons between gene lists and ChIP gene target lists. ChIP data from Chen *et al.* was downloaded as an association score between any particular gene and the transcription factor of interest. These scores were used directly for enrichment. For the Oct4, Sox2, Nanog bound group from Chen *et al.* any score above 0 was counted as bound. For all data, enrichment for ChIP gene target sets in miRNA-regulated gene sets was performed relative to all genes analyzed to produce the miRNA-regulated gene sets (i.e. all genes with Affymetrix IDs mapping to coding transcripts). The enrichments for any given ChIP target set were median normalized with all the miRNA-regulated genes sets in **Figure 3b**. We performed this normalization because both the ChIP targets of the transcription factors and the miRNA-regulated gene sets in our analysis are enriched for more highly expressed genes[19]. We get a similar pattern of results without this

normalization although all comparisons appear more highly enriched due to the expression levels (data not shown)**.** Unnormalized enrichment is defined as (Genes in overlap of miRNA altered group and ChIP group/All genes in miRNA altered group)/(All genes in ChIP group/All genes used in analysis to generate miRNA altered groups).

Our enrichment analysis could yield a number of possible outcomes dependent on whether the miRNA targeted the transcription factor directly versus targeted transcripts downstream of the transcription factor. The following outcomes are presented in **Figure 3-3a**: (i) If a miRNA directly targets a specific transcriptional activator, this activator will be downregulated and thus its ChIP target genes will likewise tend to be downregulated. This will result in an enrichment of ChIP target genes within the miRNA's downregulated gene set independent of there being a seed match in these targets. Likewise, the ChIP target genes should be depleted in the miRNA's upregulated gene set (**Figure 3-3a, i**). (ii) If a miRNA directly targets a transcriptional repressor, there would be the inverse outcome; that is, the ChIP target genes should be enriched in the miRNA's upregulated gene set and depleted in the miRNA's downregulated gene set regardless of seed match (**Figure 3-3a, ii**). (iii) If a miRNA targets an activating transcription factor's downstream targets, but not the transcription factor itself, ChIP target genes would be enriched in the downregulated gene set with a seed match but not without a seed match. Furthermore, there should not be an enrichment in the upregulated transcripts (**Figure 3-3a, iii**).

Gene ontology

Stem cell associated genes (genes upregulated in ESCs relative to brain and bone marrow) were generated from data in Ramalho-Santos *et al.* 2002[47] and were downloaded as a list from MSigDB (http://www.broad.mit.edu/gsea/msigdb). Enrichment of these stem cell associated genes in miRNA altered gene sets was performed, and p-values were calculated by Fischer's exact test.

Immunohistochemistry

Cells were fixed with 4% paraformaldehyde and washed twice in 1xPBS with 0.1% Triton x-100 (PBT). PBT with 2% BSA and 1% goat-serum was used to block for one hour before addition of primary antibody against Oct4 (Santa Cruz, rabbit polyclonal, product # sc-9081) or Nanog (Calbiochem, rabbit polyclonal, product # sc-1000) which was incubated overnight at 4°C or at room temperature for approximately 2 hours. Cells were washed with PBT, blocked with PBT with 2% BSA and 10% goat-serum for 1 hour before addition of secondary antibodies (Invitrogen: Alexa Fluor 488 goat anti-rabbit IgG).

Western blots

On day 0, approximately 200,000 *Dgcr8* -/- or 50,000 wild-type ES cells were plated in a 6 well plate. The following day miRIDIAN miRNA mimics (Dharmacon, ThermoFisher) were transfected at a concentration of 50nM. Lysates were collected two days after transfection in EBC buffer (50mM Tris-HCl, pH 8.0, 120mM NaCl, 0.5% Nonidet P-40, 1mM EDTA) containing 1X protease inhibitor cocktail (Roche). Lysates were incubated at 4 degrees C for 45 minutes rocking then spun at 4 degrees and 15,000rpm in a table top

centrifuge. Protein was quantified using a Bio-Rad protein assay (Bio-Rad). 30ug of protein was resolved on an 8% SDS-PAGE gel. Proteins were transferred to Immobilon-FL (Millipore) and processed for immunodetection. Blots were scanned on a Licor Odyssey Scanner (Licor). The actin antibody was used at a 1:1000 dilution (Sigma, mouse monoclonal clone AC-40, Cat# A4700), the cMyc antibody was used at 1:500 (Epitomics, N-term rabbit monoclonal, Cat# 1472-1), the nMyc antibody was used at 1:500 (Calbiochem, mouse monoclonal, Cat# OP13), the Nanog antibody was used at 1:1000 (Abcam, rabbit polyclonal, Cat# ab21603), the Sall4 antibody was used at 1:500 (Abcam, rabbit polyclonal, Cat# ab29112), the Lin28 antibody was used at 1:1000 (Abcam, rabbit polyclonal, Cat# ab46020). Secondary IR antibodies from Licor were used at 1:10,000. Data were exported from the Licor Odyssey as jpg and quantified using ImageJ software (NIH).

MEF isolation

E13.5 embryos from Oct4::GFP/Rosa-26::β-galactosidase transgenic crosses were isolated by Caesarean section and washed in HBSS. Heads and visceral tissues were removed. Remaining tissue was washed in fresh HBSS, briefly rinsed with 70% ethanol, then submerged in 0.05mM trypsin / 1mM EDTA HBSS solution and incubated at 37°C for 10 minutes. Tissue was pipetted repeatedly to aid in tissue dissociation, then added to MEF media containing 10% FBS and plated (passage 0).

Retrovirus infection

The retroviral packaging vector pCL-ECO was transfected into 293T cells simultaneously with pMXs vectors containing Oct4, Sox2, Klf4, or cMyc cDNA (Addgene) using Fugene 6 (Roche)[48]. At 24 hours, the media was changed, and at 48 hours, the media was collected, filtered (0.45mM), and frozen in aliquots at -80°C. Retrovirus was never thawed more than once. To induce reprogramming, passage 3 Oct4-GFP, Rosa26-b-galactosidase/neo MEFs[49] were plated on gelatin-coated 12-well plates at 12 thousand cells per well. Retrovirus-containing media was added 24 hours later (Day 0). Cells were transfected with 16nM microRNA inhibitors (Dharmacon, ThermoFisher, Cat# I-310106-04 for let-7 inhibitor & Cat# IN-001000-01-05 for control inhibitor). Cells in reprogramming assays were transfected on days 0 and 6 post-retroviral-infection. Media was changed daily. Media was replaced with ES media + 15% FBS + LIF on day 2, and ES media + 15% knock-out serum replacement (Invitrogen) + LIF on day 6. GFP+ colonies were counted on day 10. Individual iPS colonies were picked and expanded for analysis between days 10 and 15.

Flow cytometry for SSEA1 and CD9

Cells were prepared by trypsinization and washed 2x in staining media [HBSS (Ca and Mg free wo phenol red) supplemented with 2% FBS]. Cells were resuspended in approximately 100 uL of primary antibody and incubated for 30 min on ice. SSEA1 (University of Iowa Developmental Studies Hybridoma Bank) was used at 1:50 and CD9 (BD pharmigen) at 1:100. Secondaries, PE anti-mouse IgM and streptavidin PE/Cy5 were used at 1:400. Cells were again spun down, rinsed in staining media then resuspended in secondary antibody. Cells were incubated covered on ice for 30 minutes.

Cells were again rinsed and spun down. Analysis was performed on either a BD FACs calibur or FACS Aria.

Bisulfite sequencing

3 ug of DNA were digested with EcoRV. DNA was purified by phenol chloroform extraction. 3ug digested DNA + 3ug yeast tRNA was denatured in 0.3M NaOH (final volume 30uL) for 20 minutes at 42 deg C. Bisulfite stock solution was prepared as follows: 10N NaOH solution was pepared. Hyrdoquinone solution (0.22g in 1mL H2O) was pepared. Sodium bisulfite solution was pepared by first mixing, 4.05 g sodium bislufite in 8 mL water. Subsequently 400uL 10N NaOH was added to achieve a pH of 5.0. Next 500uL hydroxyquinone solution was added then water to bring volume to 10mL. 330 uL of the final bisulfite solution was added to 30 uL denatured DNA and incubated at 65 deg in dark for 4 hours. DNA was purified using the Promega Wizard DNA cleaning kit final volume 100 uL in water. Purified DNA was desulfonated by adding 11uL f 3M NaOH and incubating at 37 deg C for 20 minutes. Desulfonating reaction was neutralized by addition of 47 uL of 10M Ammonium Acetate. DNA was ethanol precipitated and resuspended in Tris buffer. DNA was TOPO cloned and transformed into competent cells. Clones were isolated and sequenced.

Lentiviral preparation

Virus was generated by transfection of 293T cells with 2ug each of Gag, Pol, and Vsvg plasmids as well as 3 ug of viral vector of interest using Fugene6 (Roche) as per the

manufacturers protocol. After approximately 3 days supernatant was collected and concentrated by spinning at 25000g for approximately 1.5 hours.

miRNA screening

On day 0, 4000 *Dgcr8 -/-* ESCs were plated per well of a 96 well plate in media without LIF. On day 1, miRNA mimics were transfected 1 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).

miRNA arrays

0.4 10^6 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 manufacturers 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 was 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 1** for probes present both on the RA and (-) LIF arrays.

Motif finding algorithm and clustering

To identify enriched motifs the following algorithm was implemented. First two groups of transcripts experimental and control were defined—either stimulated and unaltered or repressed and unaltered. For each transcript in each of these groups the transcript was subdivided into all possible subsequences of length n. For example for n = 5 the sequences 1-5, 2-6, 3-7, etc were cataloged for each transcript. The outcome of this script is a dictionary with each subsequence corresponding to a count of the number of times that sequence was found in the experimental and control sets. This was repeated for n = 5-12. At the end of this procedure all subsequences, from now on referred to as motifs, were filtered to find motifs present > 0 times in control, > 5 times in the experimental, and where expt/control > 4. This filtered set of enriched motifs was then submitted to a clustering analysis.

Clustering analysis consists of a simple algorithm whereby all enriched motifs are compared pairwise to each other. If the two motifs have 2 or fewer mismatches they are placed together in a cluster. For motifs of unequal length 2 or fewer mismatches in the smaller of the two motifs is set as the criteria for clustering. This algorithm was used to generate clusters of related motifs.

These clusters of related motifs were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). These alignments were submitted to the WebLogo server (http://weblogo.berkeley.edu/) using the default DNA/RNA option.

Per nucleotide enrichments

6-9 base-pair windows were slid across each mature miRNA sequence. This generated a library of sequence motifs. For each motif the enrichment was calculated in the 3' UTRs of miRNA down-regulated versus all other transcripts. For each nucleotide of the mature miRNA sequence the enrichment of any motif containing that nucleotide was averaged to genenrate an enrichment score for that nucleotide. These data were then plotted in excel.

Gene clustering

Gene clustering analysis was performed via Cluster 3 (de Hoon, Imoto, Nolan, & Miyano, 2004).

Pathway enrichments

'Canonical pathways' were downloaded from MSigDB (https://www.broad.harvard.edu/gsea/msigdb). The overlap between components of these pathways and transcripts either up or down-regulated by specific miRNAs was determined and a median normalized enrichment of all miRNAs and all canonical pathways was determined. Pathways were filtered to remove pathways with few components, this was done by removing all pathways that had 0 components overlapping with any of the miRNA altered genes. Next, hierarchical clustering of the pathways

using Cluster identified pathways commonly altered by the miRNAs. The cluster with the most enriched categories is shown in figure 5-5.

P21 over-expression

Teto::P21, R26 rtTA ESCs were generated by Yangming Wang by flp/frt targeting into the collagen locus(Beard, Hochedlinger, Plath, Wutz, & Jaenisch, 2006). These cells were treated with 0.2 mg/mL doxycycline to induce expression of P21 protein.

Dgcr8 targeting in p107 -/-, p130 -/- ESCs

P107 -/-, p130 -/- were obtained from the laboratory of Julien Sage. These ESCs were targeted with R26CreER and Dgcr8 as has been previously described(Y. Wang, et al., 2007).

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