Chapter 19
Cell Cycle Regulation by microRNAs in Stem Cells

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Abstract The ability to self-renew and to differentiate into at least one-cell lineage defines a stem cell. Self-renewal is a process by which stem cells proliferate without differentiation. Proliferation is achieved through a series of highly regulated events of the cell cycle. MicroRNAs (miRNAs) are a class of short noncoding RNAs whose importance in these events is becoming increasingly appreciated. In this chapter, we discuss the role of miRNAs in regulating the cell cycle in various stem cells with a focus on embryonic stem cells. We also present the evidence indicating that cell cycle-regulating miRNAs are incorporated into a large regulatory network to control the self-renewal of stem cells by inducing or inhibiting differentiation. In addition, we discuss the function of cell cycle-regulating miRNAs in cancer.

19.1 Introduction: Self-Renewal Process of Stem Cells

Self-renewal defines a process by which a stem cell generates one (asymmetric division) or two (symmetric division) daughter cells that have similar developmental potential as the mother cell. Different stem cells have different developmental potential, which are restricted by specific epigenetic programs, but generally a stem cell should have the ability to differentiate into at least one cell type. The rate of self-renewal strictly depends on the particular type of stem cell. Embryonic stem (ES) cells are cell lines derived from the inner cell mass of a developing blastocyst

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(Rossant 2008). Like their counterparts in the inner cell mass, ES cells are pluripotent stem cells. In other words, they can differentiate into all derivatives of three primary germ layers as well as germ cells. ES cells can self-renew rapidly and indefinitely; therefore, they can provide large amount of tissues for tissue replacement therapy. Late in embryonic development, transient fetal stem cells such as fetal hematopoietic and neural stem cells are generated, which have limited developmental potential but retain a high self-renewal rate to fulfill the needs of fetal growth (Mikkola and Orkin 2006; Kriegstein and Alvarez-Buylla 2009; He et al. 2009).

Eventually in a developed organism, adult stem cells replace fetal stem cells to maintain tissue homeostasis. Adult stem cells have limited developmental potential but many still have extensive self-renewal potential evidenced by their long-term maintenance both within their niche and even following serial transplantation experiments (Osawa et al. 1996; Li and Clevers 2010). However, the self-renewal rate of adult stem cells is generally slower than ES and fetal stem cells. In fact, many adult stem cells spend most of their life in quiescent state, which is a reversible cell cycle arrest stage. It is thought that quiescence is important for maintaining tissue homeostasis and preventing cancer growth. Cancer is a class of diseases in which a group of cells display unlimited growth, invasion, and eventually metastasis. How and in what cells cancer is initiated are not well understood. However, several features of somatic stem cells make them plausible sites for cancer initiation. Preserved replicative capacity may enable them to accumulate mutations over a prolonged life-span. In addition, the developmental potency of somatic stem cells may underlie the cellular heterogeneity of many human cancers (Reya et al. 2001; Harmes and DiRenzo 2009). Therefore, abnormalities in the self-renewal program of adult stem cells may lead to cancer initiation and progression. Understanding self-renewal mechanisms will provide fundamental insights into development and tumorigenesis, in turn leading to efficient methods to control stem cell self-renewal for advancing regenerative medicine and cancer therapy.

Self-renewal is accomplished by cell division. Cell division is achieved through a series of highly regulated events making up the cell cycle, including the alternating activities of various cyclin-dependent kinases (Cdks), which are activated only upon binding to specific cyclins. Higher organisms incorporate many protein modulators of Cdk activity such as Cdk inhibitors to differentially regulate cell cycle progression in different cells or in response to different environmental conditions. As in many other processes, these modulators are themselves regulated often at the posttranscriptional level. microRNAs (miRNAs) provide one important means of posttranscriptional regulation (Bartel 2009). Accumulating evidence shows that miRNAs are new players regulating many protein-coding genes and specific pathways in the cell cycle (Wang and Blelloch 2009). In this chapter, we summarize the data supporting the cell cycle regulating roles of miRNAs in ES cells, adult stem cells, and cancer cells. Furthermore, we describe how these cell cycle-regulating miRNAs affect self-renewal through their influence not only on proliferation but also on differentiation.

19.2 Cell Cycle Regulation

A cell division cycle usually consists of four major phases: the G1 phase in which cells prepare for DNA replication and respond to external signals by either remaining or exiting the cell cycle; the S phase in which cells replicate their DNA; the G2 phase in which cells prepare for division; and the M phase in which one cell divides into two cells. When cells exit the cell cycle at G1, they enter a nondividing state termed G0. Some cells such as neurons and muscle cells permanently exit cell cycle and become terminally differentiated. Others such as fibroblasts, hepatocytes, and adult stem cells usually stay in quiescence, a state in which cells stop proliferation but retain the ability to reenter cell cycle when needed. The duration of cell cycle time is highly variable among different cells (Dalton 2009). Mouse ES cells in culture have a cycle time as short as ~10 h. In contrast, stem cells in resting mouse skin may have a cycle time of more than 700 h (Bickenbach 1981). This difference is largely due to the varying length of G1, which is the most variable phase of the cell cycle.

During the G1 phase, a cell senses its environment for the presence of growth factors and nutrients as well as evaluates the integrity of its genome. These tasks are accomplished through a restriction or check point at the G1/S transition (Massagué 2004). Following the restriction point, a cell can pass through S phase and mitosis independent of mitogens and growth factors. The G1 restriction point requires the sequential activation of the Cdk4/6 and the Cdk2 kinases, which are expressed throughout the cell cycle but only activated upon binding to their specific cyclins. During the early G1 phase, the mitogenic factors stimulate the expression of the D-type cyclins. The Cdk4/6–Cyclin D complex then phosphorylates proteins of the retinoblastoma (pRb) family. This event leads to a partial inhibition of Rb and release of the E2F transcription factors, increasing the transcription of the E2F targets. Among the E2F targets, there are the E-type cyclins which activate Cdk2 further phosphorylating Rb. This feed-forward loop fully releases E2F, leading to the transcription of genes required for progression through S phase. In addition, the Cdk2–Cyclin E also phosphorylates several other targets important in the progression through S phase (Stein et al. 2006). Upstream inhibitors including members of the INK (p15, p16, and p18) and CIP families (p21, p27, and p57) modulate the activity of the Cdk–cyclin complexes. Some of these inhibitors are induced upon stresses such as nucleotide depletion and DNA damage. For example, the DNA damage checkpoint pathway activates the transcription of p21 through the posttranslational modification of p53, which arrests cells in the G1 phase until the attenuation of DNA damage signaling by the DNA repair machinery. Differential expression of the cell cycle regulatory factors shapes the G1/S transition kinetics in different cell types. Aberrations in the expression of these regulatory factors can lead to uncontrolled proliferation, the hallmark of cancer. Therefore, it is not surprising that most cancer cells have a faster G1/S transition compared to their normal counterparts.

19.3 miRNA Biogenesis

MiRNAs are ~22-nucleotide short RNAs that are produced by most eukaryotic cells to control gene expression. Despite their small size, they play important functions in a variety of developmental and physiological processes. The first miRNA was discovered in 1993 by Rosalind Lee, Rhonda Feinbaum, and Victor Ambros. In studying the function of the gene *lin-14* in *Caenorhabditis elegans* development, they discovered that the LIN-14 protein level was regulated by a short RNA product encoded by the *lin-4* gene (Lee et al. 1993). The lin-4 gene gives rise to a 22-nucleotide RNA containing sequences partially complementary to multiple sites at the 3′ untranslated region (UTR) of the *lin-14* mRNA. This complementarity was necessary and sufficient to inhibit the translation of *lin-14* mRNA into LIN-14 protein. At the time, this small RNA was thought to be an idiosyncrasy in the worm. However, in 2000 another miRNA, let-7, was identified (Reinhart et al. 2000). The let-7 represses *lin-41*, *lin-14*, *lin-28*, *lin-42*, and *daf-12* expression during developmental stage transitions in *C. elegans*. Interestingly, let-7 was found to be conserved in many species, indicating the existence of a wider phenomenon (Pasquinelli et al. 2000). Since then, thousands of miRNAs have been cloned and identified in various species. Around 700 miRNAs have been confirmed in humans, while more than 1,000 miRNAs are predicted to be encoded by human genomes (Creighton et al. 2010).

Mature miRNAs are generated through two sequential cleavages by RNase III enzymes (Kim et al. 2009a). They are usually first transcribed as part of a long RNA transcript (pri-miRNA) by RNA polymerase II (Fig. 19.1). The first cleavage is conducted in the nucleus by the microprocessor complex (Gregory et al. 2004; Han et al. 2004, 2006) comprising the RNaseIII enzyme Drosha and its RNA binding partner Dgcr8. The cleavage generates a short hairpin (pre-miRNA) around 60–75 nucleotides. The pre-miRNA is then exported into the cytoplasm by Exportin 5 in a Ran-GTP dependent manner (Yi et al. 2003; Bohnsack et al. 2004; Lund et al. 2004). Another RNase III enzyme Dicer along with its partner TRBP conducts the second cleavage on the pre-miRNA to generate the mature miRNA duplex (Hutvágneret al. 2001). The duplex enters a third protein complex called the RNA induced silencing complex (RISC), which produces and directs the mature miRNA to its targets. miRNAs typically bind to the UTR of their mRNA targets and mediate the repression of translation and/or destabilization of these mRNA targets. The interaction between miRNAs and their mRNA targets is largely dependent on base-pairing between only a small fraction of the miRNA sequence (second to eighth position at the 5′ end, also known as seed sequence) and the 3′ UTR of their mRNA targets (Bartel 2009). Interestingly, recent studies suggest that miRNAs can also regulate mRNA targets by binding to the coding region and the 5′ UTR (Baek et al. 2008; Selbach et al. 2008; Forman and Coller 2010). The relatively loose requirement for the interaction between miRNAs and their mRNA targets enables a single miRNA to simultaneously target hundreds of mRNAs. In fact, around 60% of human genes are predicted to be regulated by miRNAs (Friedman et al. 2009).

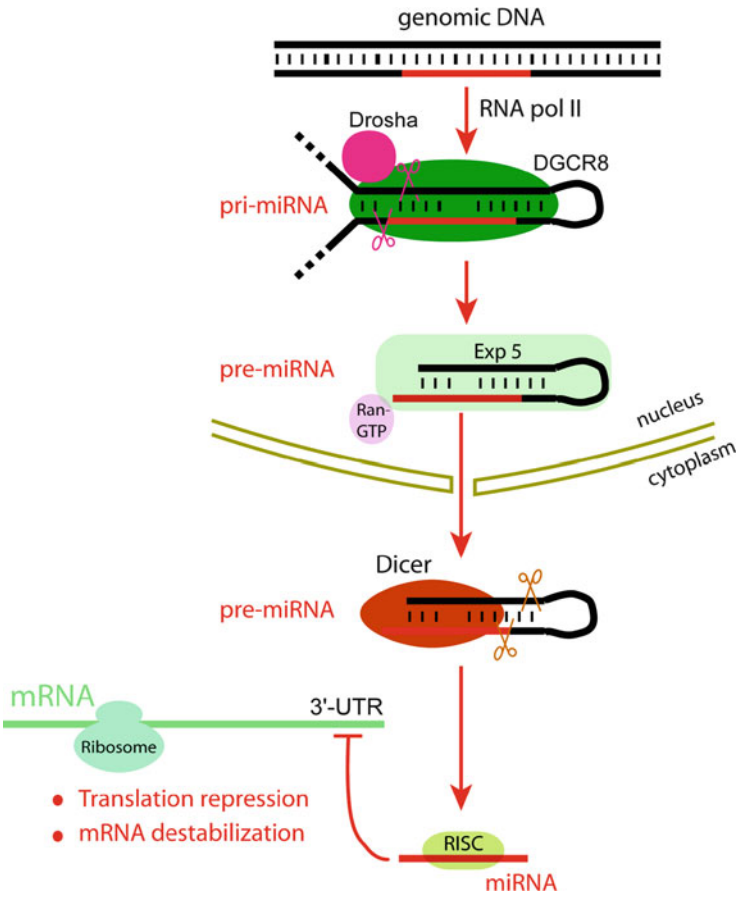

Fig. 19.1 miRNA biogenesis pathway. In the nucleus, the Drosha/Dgcr8 complex recognizes a stem loop structure of approximately 33 base pairs in length and cleaves the hairpin 11 base pairs from the stem-single strand RNA junction leaving a characteristic 2-nucleotide 3′ overhang. In the cytoplasm, the Dicer containing complex cleaves the pre-miRNA at the base of the hairpin loop to form a 2-nucleotide 3′ overhang and generates an approximately 22 nucleotide mature miRNA duplex. A single strand of the duplex is then loaded into the RISC

For these reasons, miRNAs are ideal candidates for regulating complicated processes such as cell proliferation that involves a large number of genes.

19.4 miRNAs Regulate the G1/S Transition in Mouse Embryonic Stem Cells

ES cells can self-renew rapidly and indefinitely (Dalton 2009). The proliferation rate of mouse ES cells is extremely fast with a cycling time of ~10 h. The length of cycling time is increased to more than 18 h in differentiated cells. The

rapid self-renewal of ES cells is largely due to their unique cell cycle structure. ES cells have an unusually short G1 phase (~2 h) with most (~70%) cells in S phase. In addition, unlike differentiated cells, ES cells lack a G1 restriction point or checkpoint, therefore can proliferate even in the absence of growth factors or mitogens (Jirmanova et al. 2002). This property is reminiscent of many cancer cells (Pardee 1974; Cherington et al. 1979; Berthet and Kaldis 2007), suggesting that cancer cells may hijack molecular factors used by ES cells for their proliferative advantages. In mouse ES cells, the Cdk4/Cdk6–Cyclin D complex is not present or active, while the Cdk2–Cyclin E complex is constitutively active throughout the cell cycle. During differentiation, the Cdk2–Cyclin E activity is decreased and becomes cell cycle dependent (Stead et al. 2002; Savatier et al. 1996). The decrease of Cdk2 activity is at least in part due to the upregulation of multiple G1/S transition inhibitors (e.g., p21, p27). The result is an elongated G1 phase and slower proliferation in the differentiated cells.

The molecular details resulting in high Cdk2 activity and, therefore, rapid G1/S transition in ES cells are not well understood. Studies in miRNA-deficient mouse ES cells suggest a central role for miRNAs (Murchison et al. 2005; Wang et al. 2007). Knockout of Dicer or Dgcr8, two essential proteins for miRNA biogenesis, results in slower proliferation in ES cells. Furthermore, both Dicer and Dgcr8 knockout ES cells accumulate in G1 phase, suggesting an important role of miRNAs for the G1/S transition in these cells (Fig. 19.2a). To identify specific miRNAs that promote the G1/S transition in ES cells, a screening strategy (Fig. 19.2b) was designed where chemically synthesized miRNA duplexes, called miRNA mimics, were individually transfected into the Dgcr8 knockout cells (Wang et al. 2008). The transfected cells were then evaluated for changes in their rate of cell proliferation. This unbiased screening approach identified multiple miRNAs that partially rescued the proliferation defect. These miRNAs include members of the miR-290 cluster (miR-291a-3p, miR-291b-3p, miR-294, and mir-295) and the miR-302a-d, and those with the slightly different seed sequence "AAAGUGC" including miR-20, miR-93, and miR-106 belonging to the miR-17/20/106 family. These results are consistent with the notion that members of miRNA families (defined by their common seed sequence) will often have overlapping roles in physiological processes (Miska et al. 2007; Ventura et al. 2008; Alvarez-Saavedra and Horvitz 2010). All these miRNAs are expressed in wild type ES cells with members of the miR-290 cluster being the highest. The mir-290 cluster alone makes up greater than 70% of the total quantity of miRNAs in ES cells (Marson et al. 2008). Importantly, expression of this cluster is rapidly downregulated upon differentiation, coincident with the elongation of cell cycle (Melton et al. 2010; Houbaviy et al. 2003).

Transfection of miR-291a-3p, miR-291b-3p, miR-294, and miR-295 individually fully rescues the G1 accumulation phenotype along with enhanced proliferation suggesting that they are acting to promote the G1/S transition. Because of their function in regulating the cell cycle, these miRNAs were then termed the ESCC miRNAs for *E*mbryonic *S*tem cell enriched *C*ell *C*ycle-Regulating miRNAs (Wang et al. 2008). It was hypothesized that ESCC miRNAs promote the G1/S transition by suppressing inhibitors along the Cdk2–Cyclin E pathway as this is the key G1/S

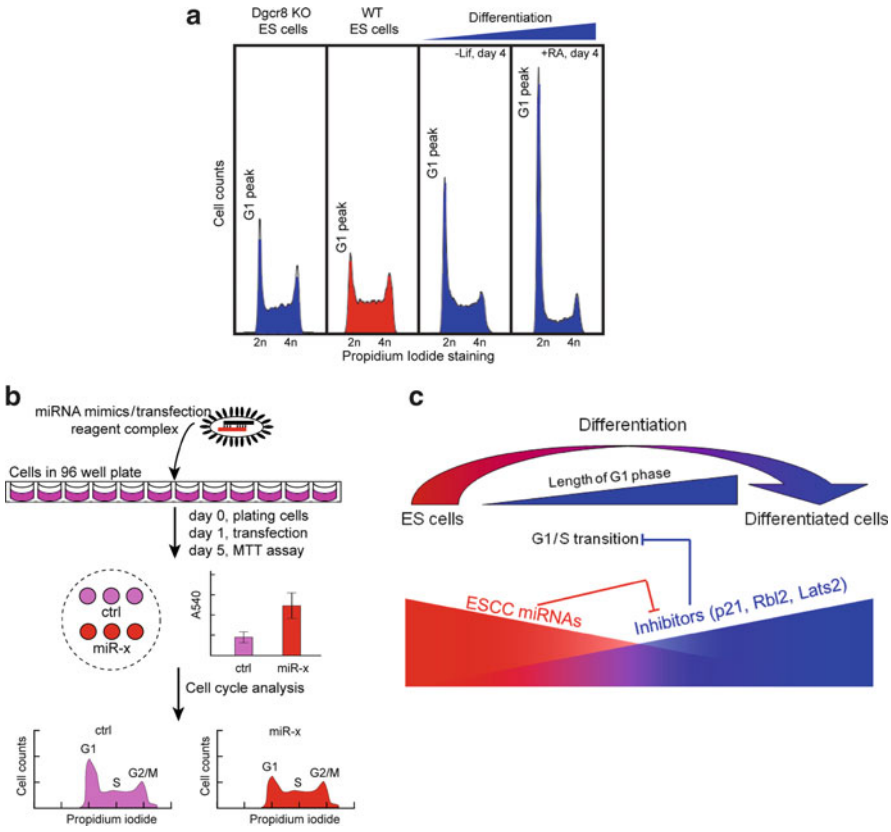

Fig. 19.2 miRNAs regulate the G1/S transition in mouse ES cells. (**a**) Cell cycle profiles of ES cells, differentiated ES cells, and miRNA-deficient ES cells. More cells accumulate in G1 phase in differentiated cells and miRNA-deficient ES cells than undifferentiated ES cells. (**b**) Screening strategy to identify cell cycle-regulating miRNAs in miRNA-deficient ES cell model (adapted from Wang et al. 2008). (**c**) ESCC miRNAs promote the G1/S transition in ES cells by repressing multiple inhibitors along the Cdk2-Cyclin E pathway (adapted from Wang and Blelloch 2009)

regulating pathway in ES cells (Fig. 19.2c). Indeed, careful analysis identified p21, Rbl2, and Lats2 as targets of ESCC miRNAs. mRNA profiling experiments suggest that dozens more cell cycle regulating genes are direct targets of ESCC miRNAs, since these genes are downregulated by the transfection of ESCC miRNAs and harbor miRNA binding sites at their 3′ UTRs (Yangming Wang and Robert Blelloch, unpublished data). p21 was confirmed as a functional target because ectopic expression of p21 without its 3′ UTR in wild-type ES cells lead to an increase in p21 protein levels and accumulation of cells in the G1 phase. However, it only partially phenocopies the G1 accumulation seen in the Dgcr8 knockout cells, indicating that ESCC miRNAs regulate G1/S transition through multiple targets. These findings show that miRNAs work through multiple targets reinforcing specific phenotypic

outcomes. That is, miRNAs act at a global scale to regulate a particular biological process.

ES cell differentiation is accompanied by an elongation of the G1 phase. In addition, the G1 regulatory molecules, such as p21 and p27, are exquisitely regulated during differentiation and in many models appear to play a pivotal role in differentiation (Savatier et al. 1996; Sabapathy et al. 1997; Parker et al. 1995; Ohnuma et al. 1999; Fujii-Yamamoto et al. 2005; Ullah et al. 2008). Therefore, it is expected that G1/S transition-promoting ESCC miRNAs may also play a role in promoting ES cell self-renewal by preventing differentiation. Indeed, a recent report (Melton et al. 2010) showed that ESCC miRNAs can prevent ES cell differentiation induced by another miRNA, let-7. let-7 preferentially regulates transcripts that are enriched in ES cells, including many transcripts that are regulated by the pluripotency transcription factors Oct4, Sox2, Nanog, and Tcf3. Interestingly, a number of the direct targets of let-7 are indirectly upregulated by the ESCC miRNAs, which may explain how the ESCC miRNAs antagonize let-7 induced differentiation. The targets oppositely regulated by let-7 and the ESCC miRNAs include known pluripotency regulators such as the Myc proteins, Sall4, and Lin28. At this point, how ESCC miRNAs upregulate these genes is still not clear. It will be interesting to figure out whether the upregulation is through direct regulation of cell cycle-regulating genes by ESCC miRNAs. Furthermore, it will be important to dissect the function of ES cell cycle structure in preventing differentiation.

19.5 Cell Cycle Regulation by miRNAs in Human Embryonic Stem Cells

Human ES cells were established by Thomson et al. in 1998 and hold great value for regenerative medicine and studying early human development (Thomson et al. 1998). There are significant differences between human and mouse ES cells in terms of morphology of cell colony, surface antigens, and growth factor requirements. Some of these differences may be species-specific. Alternatively, these differences could reflect different development stages of these ES cells. This hypothesis is supported by the derivation of mouse epiblast stem (EpiS) cells that share similar features with human ES cells (Tesar et al. 2007; Brons et al. 2007). Despite these differences, human and mouse ES cells share core similarities including unique cell cycle structure characterized by a short G1 phase, self-reinforcing transcriptional network, and a poised epigenetic state. Similarly, miRNAs have shared roles as regulators of the G1/S transition in human ES cells. A study by Deborah et al. found that miRNAs from the miR-302 cluster promote the G1/S transition in human ES cells (Card et al. 2008). This cluster is regulated by pluripotent transcriptional factors Oct4/Sox2 and is highly and specifically expressed in human ES cells. Moreover, they showed that miR-302 inhibits expression of Cyclin D1 in human ES cells. However, whether the inhibition of Cyclin D1 promotes the G1/S transition is not clear and needs more detailed investigation.

In another study by Qi et al., knocking down Dicer or Drosha leads to the accumulation of human ES cells in G1 phase (Qi et al. 2009), reminiscent of the phenotype observed in Dicer or Dgcr8 knockout mouse ES cells. In addition, this study shows that transfection of miR-372, an ortholog of ESCC miRNAs in human, rescued the G1 accumulation defect. p21 was also identified as a target of miR-372, suggesting that ESCC miRNAs-p21 axis promoting G1/S transition is conserved in both human and mouse ES cells (Fig. 19.3). Interestingly, the authors showed that another two miRNAs with similar seed sequence as miR-372, miR-106a, and miR-302c did not rescue the G1 accumulation defect. In contrast, miR-106a and miR-302c promotes the G1/S transition in the Dgcr8 knockout model (Wang et al. 2008). It should be interesting to investigate the molecular mechanisms that cause this difference in different species and how miRNAs with similar seed sequences may lead to different functional outcomes. This is particularly important for miR-302 as the previous study showed that miR-302 can promote G1/S transition in human ES cells. A simple explanation could be that synthetic RNA oligos in Qi et al.'s study do not guarantee the incorporation of only sense miRNA strand into the RISC. The incorporation of antisense strand may complicate the phenotypic outcome.

Another important finding in Qi et al.'s study is that miRNAs are also important for the G2/M transition of human ES cells. Knocking down Dicer or Drosha leads to significant accumulation of cells in the G2 phase. Further analysis showed that miR-195 can rescue this defect by regulating WEE1 kinase. WEE1 kinase is an inhibitor for the Cdk1–Cyclin B complex, which is important for the G2/M transition. This regulation may be specific for human ES cells because no significant accumulation of Dicer or Dgcr8 knockout mouse ES cells in G2 is observed, and miR-195 does not promote proliferation of Dgcr8 knockout mouse ES cells and is

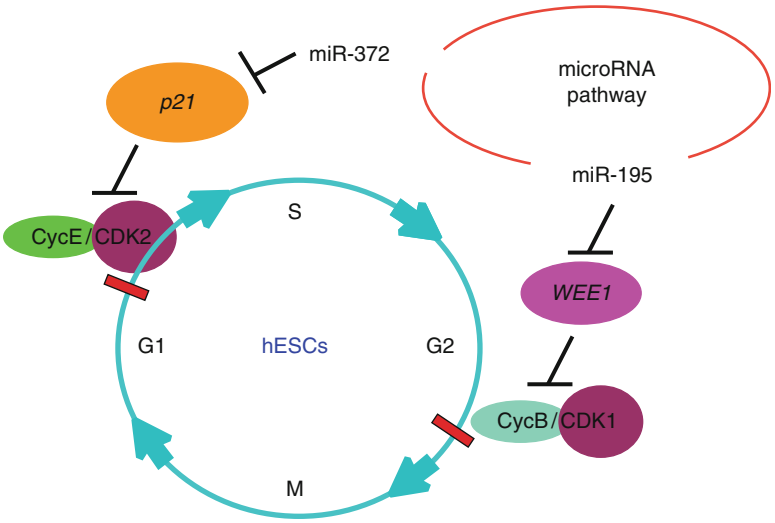

Fig. 19.3 miRNAs regulate the G1/S and G2/M transition in human ES cells. This figure was previously published in Qi et al. (2009)

not highly expressed in mouse ES cells (Yangming Wang and Robert Blelloch, unpublished data). Since mouse EpiS cells share more similarities and may be developmentally closer with human ES cells (Tesar et al. 2007; Brons et al. 2007), it would be interesting to investigate whether miR-195 can also regulate G2/M transition in mouse EpiS cells.

19.6 Cell Cycle Regulation by miRNAs in Somatic Stem Cells

Compared to their role in ES cells, miRNA function in somatic stem cells is less clear. However, analysis of tissue specific knockouts of Dgcr8 or Dicer suggests that miRNAs play essential roles in the proliferation, survival, and differentiation of somatic stem cells. For example, Dicer knockout in the epidermis increases the number of BrdU-positive and phosphohistone-H3-positive mitotic suprabasal cells relative to wild type control (Yi et al. 2008). Further analysis by flow cytometry revealed an approximately threefold increase in the number of G2/M suprabasal cells. This response may be secondary to an increasing need to replace lost cells. Alternatively, it may indicate that miRNAs regulate the cell cycle exit in skin stem cells. Indeed, miR-203 was found to induce cell cycle exit in skin stem cells by inhibiting expression of p63, an essential regulator of stem-cell maintenance in stratified epithelial tissues.

In adult neural stem cells, let-7b and miR-9 were found to inhibit proliferation and induce differentiation (Zhao et al. 2009, 2010). Both miRNAs target Tlx, an important nuclear receptor maintaining self-renewal of neural stem cells (Qu et al. 2010). Let-7b was also found to inhibit expression of Cyclin D1 which is important for the G1/S transition. In a recent study, miR-184 was shown to positively regulate neural stem cell proliferation (Liu et al. 2010). Interestingly, miR-184 is directly repressed by methyl-CpG binding protein 1 (Mbd1) which inhibits gene expression via a DNA methylation-mediated epigenetic mechanism. Further analysis revealed that Mbd1, miR-184, and Numbl, a target of miR-184, formed a regulatory network controlling the balance between proliferation and differentiation of neural stem cells. This study supports the hypothesis that factors promoting proliferation of stem cells may also help prevent stem cell differentiation and vice-versa.

19.7 Cell Cycle Regulation by miRNAs in Cancer

miRNA profiling experiments in normal and tumor tissues revealed that a large number of miRNAs are dysregulated in tumors (Lu et al. 2005), suggesting potential roles of miRNAs in tumor progression including tumor initiation, proliferation, and metastasis. Some cancers share very similar molecular characteristics as ES cells (Ben-Porath et al. 2008); therefore, it is not surprising that molecular mechanisms promoting ES cell proliferation are also used by cancer cells to achieve

growth advantages over normal tissues. The miR-372 and miR-373, two orthologs of ESCC miRNAs in human, can promote tumor formation in human primary fibroblasts in cooperation with oncogenic Ras (Voorhoeve et al. 2006). Interestingly, the two miRNAs are also highly expressed in human germ cell tumors. These miRNAs target Lats2, an inhibitor of Cdk2–Cyclin E complex that is important for the G1/S transition. Tumors also use other miRNAs with similar seed sequences as ESCC miRNAs to promote proliferation. For example, members of the miR-106b family promote the G1/S transition in breast cancer cell lines by targeting p21 (Ivanovska et al. 2008); in gastric cancer, miR-93 and miR-106b were shown to promote cell cycle progression by targeting p21, in addition to their role in preventing apoptosis by targeting E2F1 and Bim (Petrocca et al. 2008; Kim et al. 2009b). In addition, miR-221/2 can promote cell proliferation in various cancers by targeting G1/S transition inhibitors such as p27 and p57 (le Sage et al. 2007; Galardi et al. 2007; Fornari et al. 2008). Together, these data suggest that miRNAs can serve as potent oncogenes to promote cancer cell proliferation by targeting various cell cycle inhibitors.

19.8 Conclusion

The data summarized in this chapter support essential roles of miRNAs in cell cycle regulation in ES, somatic stem, and cancer cells. In addition, cell cycle-regulating miRNAs in stem cells serve as mediators that couple the self-renewal maintenance and cell cycle regulation. As miRNA knockout mouse projects are carrying on in many research laboratories, we expect the discovery of more cell cycle-regulating miRNAs in different stem cells and cancers. The exciting challenge will be how to manipulate these tiny molecules to control cell proliferation for advancing regenerative medicine and cancer therapy.

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