Acta Pharmacologica Sinica (2013) 34: 1374–1380
 1671-4083/13 $32.00
www.nature.com/aps

Review

MicroRNAs are involved in the self-renewal and differentiation of cancer stem cells

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MicroRNAs (miRNAs) are small non-coding RNA molecules, whose primary function is to regulate gene expression at the post-transcriptional/translational levels. MiRNAs play crucial roles in normal biological processes and are commonly dys-regulated in human diseases. Stem cells are regarded as the "mother" cells of all types of differentiated cells that comprise tissues and organs of the body. A novel hypothesis proposes that tumors are composed of heterogeneous cells derived from cancer stem cells, which have self-renewal and differentiation capabilities similar to those of normal stem cells. Cancer stem cells have been isolated and characterized from various tumors. Given recent studies supporting the critical regulatory roles of miRNAs in the self-renewal and differentiation of cancer stem cells, better understanding the functions of miRNAs will provide invaluable insights into the prevention of tumorigenesis and tumor progression. In this review, we will summarize the research progress in the study of miRNAs involved in the self-renewal and differentiation of cancer stem cells.

Keywords: cancer; stem cells; miRNAs; tumorigenesis; tumor progression; self-renewal; differentiation

Acta Pharmacologica Sinica (2013) 34: 1374–1380; doi: 10.1038/aps.2013.134; published online 14 Oct 2013

Introduction

Stem cell research can be dated back to at least the 1960s, when Becker *et al* illustrated the presence of self-renewing cells in mouse bone marrow[1]. In 1998, Thomson *et al* successfully isolated and cultured human embryonic stem (ES) cells for the first time[2]; this work is considered to be a milestone study in human stem cell research. The concept that cancer might arise from a rare population of cells with stem cell-like properties was proposed more than 150 years ago[3]. However, cancer stem cells (CSCs) were not confirmed to exist until they were discovered in acute myeloid leukemia (AML) in 1997[4]. CSCs have since been identified in most types of solid tumors[5].

MicroRNAs (miRNAs) are non-coding regulatory RNA molecules that are approximately 22 nucleotides long[6, 7]. After transcribed from the miRNA genes aided by RNA polymerase II or III[8, 9], pri-miRNAs with the hairpin structure are processed by Drosha and DiGeorge syndrome critical region gene 8 (DGCR8) to form pre-miRNAs, which are then exported out of the nucleus by Exportin-5[10–12]. In the cytoplasm, pre-miRNAs are subsequently cleaved into mature miRNA sequences by Dicer[13–17]. By incorporating with the RNA-induced silencing complex (RISC), miRNAs exert the repressive function through the translational repression of target genes and/or mediation of the target mRNA transcripts cleavage[18, 19]. Although the extent to which miRNAs regulate the human transcriptome has not yet been fully determined, increasing evidence now supports the crucial role of miRNAs in the regulation of gene expression.

In stem cell research, relatively fewer studies have examined non-coding miRNAs than protein-coding genes. Given the recent studies reporting that miRNAs play significant roles in the maintenance of stem cells in various cancers[20], we will summarize the research on miRNAs in CSCs, focusing on the processes of self-renewal and differentiation.

Cancer stem cells

CSCs, also called tumor-initiating cells, have been identified in various types of cancers[4, 5]. CSCs have the capacity to self-renew and produce the heterogeneous lineages of cancer cells that comprise the tumor[21]. Recent studies have found that CSCs account for resistance to chemotherapy in certain cancers, providing a novel insight into the mechanistic basis of chemoresistance[22].

In 1997, Bonnet *et al* first isolated and identified CSCs from AML[4], while subsequent studies found that solid tumors, including breast cancer, pancreatic cancer, colon cancer, brain

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Received 2013-07-21 Accepted 2013-08-22

cancer, liver cancer, head and neck cancer, ovarian cancer, and melanoma are also driven and sustained by CSCs[23–37]. Generally speaking, CSCs are only responsible for a very small portion of all tumor cells, although the percentage may vary depending on the tumor type. For instance, the CD133+ CSCs account for approximately 2.5% of the population of colorectal cancer cells[34]. However, recent studies support that CSCs play significant roles in tumor relapse and metastasis because they can differentiate into each of the diverse cell types that comprise the tumor through continuous self-renewal and differentiation[5]. As such, a better understanding of the CSC theory will shed light on the biology of tumorigenesis and aid in the development of novel therapeutic strategies to treat human cancer more efficaciously.

CSCs show greater tumorigenic potential than non-stem cancer cells and express specific markers. In 2003, Al-Hajj *et al* isolated and characterized CSCs from breast cancer cells based on the expression status of the specific cell surface markers CD44 and CD24, and this study was the first report showing the success of isolating CSCs from solid tumors[23]. Thereafter, CD133, CD166, epithelial cell adhesion molecule (EpCAM), and others were also used as the surface markers to identify and characterize CSCs in different tumors, such as brain cancer, prostate cancer, pancreatic cancer, colon cancer, and hepatocellular carcinoma[25, 34, 38–40]. Aldehyde dehydrogenase 1 (ALDH) was recently reported as a potential breast cancer stem/progenitor cell-specific marker[30]. In addition to identification of different CSCs in human tumors, the usage of these markers has been extended to evaluate the efficacy of chemotherapeutic drugs with the potential to target CSCs as well[41, 42].

The origin of CSCs remains elusive, but several hypotheses have been proposed. The cell fusion and horizontal gene transfer occurring in cell development and tissue repair process are considered to be the dominant origins of CSCs, although another opinion disputed that CSCs might arise from mutations in specific normal stem cells or early stem cell progenitors[43]. Interestingly, CSCs are also reported to be derived even from differentiated tumor cells in accordance to the report by Iliopoulos *et al*; they found that interleukin 6 (IL6) can convert non-stem cells to CSCs in breast and prostate cancer cell lines and in primary cells derived from human breast tumors[44].

Based on the ability of stem cells to grow in serum-free and non-adherent suspensions as spherical clusters, the tumorsphere culture technique has been developed to isolate and characterize CSCs[45, 46]. However, the ideal assay for CSC characterization would be serial transplantation in animal models in which cells are xenografted into an orthotopic site of an immunocompromised mouse for observing tumor formation. Given that there is very few drugs available that specifically target the unique machinery driving the renewal and differentiation of CSCs, the study of miRNAs in CSCs may provide a valuable insight into the development of novel strategies against human cancers.

MiRNAs are involved in the self-renewal and differentiation of cancer stem cells

Although the mechanism by which stem cells maintain self-renewal and differentiation remains unclear, it was shown that altered miRNA accumulation in murine ES cells with conditional knockout of *Dicer1* and *DGCR8* led to abnormalities in stem cell differentiation, suggesting that miRNAs may play important roles in stem cells[47, 48]. It was also reported that miR-134, miR-296, and miR-470 can directly inhibit the self-renewing state by suppressing several factors with the documented effects on pluripotency maintenance, such as *Nanog*, octamer-binding transcription factor 4 (*Oct4*), and sex determining region Y-box 2 (*Sox2*)[49]. In human ES cells, miR-145 can promote cell differentiation by directly targeting the mRNA transcripts of *Oct4*, *Sox2*, and kruppel-like factor 4 (*KLF4*)[50], and let-7 can translationally repress the expression of *Lin28*, which is a known factor to maintain cell pluripotency[51, 52]. In addition, miR-290 and miR-302a are reported to promote G_1-S transition that enables cellular rapid proliferation in human ES cells[53, 54]. In proliferating ventral midbrain/hindbrain (vMH) neural progenitors, miR-200 is required to promote cell cycle exit and neuronal differentiation by targeting the expression of *Sox2* and E2F transcription factor 3 (*E2F3*)[55]. These findings notably suggest that miRNAs can act as the upstream regulators of a panel of transcription factors that are involved in modulation of stem cell self-renewal and differentiation, such as *Oct4*, *Sox2*, *KLF4*, and *E2F3*.

On the other hand, miRNAs can also be regulated by some transcription factors and serve as downstream effectors in the signaling pathways associated with stem cell self-renewal and differentiation. For example, Lin *et al* reported that in ES cells, the expression of the miR-200 family was regulated by c-Myc. The transcriptional induction of these miRNAs by c-Myc significantly attenuated the down-regulation of pluripotency markers, which indicates that in ES cells, c-Myc acts, at least in part, through the miR-200 family to attenuate differentiation[56]. In addition, Wang *et al* found that during the reprogramming of somatic cells, *Oct4* and *Sox2* can induce the transcriptional activation of the miR-200 family, which can in turn promote mesenchymal-epithelial transition (MET) and generation of the induced pluripotent stem cells (iPSCs) by targeting zinc finger E-box binding homeobox 2 (*ZEB2*)[57]. It is also notable that *Oct4* and *Sox2* can transcriptionally regulate the expression of miR-302a that is involved in the cell cycle progression in human ES cells by targeting *cyclin D1*[54].

Based on their roles, those functional miRNAs can be sorted to two subgroups: pluripotent miRNAs and pro-differentiation miRNAs. Pluripotent miRNAs are able to promote the self-renewal and proliferation of stem cells but inhibit cell differentiation. This class of miRNAs includes miR-137, miR-184, miR-200, miR-290, miR-302, and miR-9[54, 56, 58–62]. The pro-differentiation miRNAs that can initiate or stabilize differentiation include let-7, miR-122, miR-134, miR-145, miR-181, miR-296, and miR-470[49, 50, 52, 63–66]. These two types of miRNAs and their targets that have been validated to be involved in the self-renewal and differentiation of CSCs are summarized in

Table 1. Our recent study reported that miR-181 direct downregulation of *Lin28* can promote the megakaryocytic differentiation by disrupting the let-7/*Lin28* negative feedback loop in which let-7 translationally suppresses the expression of *Lin28,* whereas *Lin28* controls the maturation of let-7[52]. However, a recent report found that the overexpression of miR-122 could promote hepatic differentiation and maturation in murine ES cells through a miR-122/forkhead box protein A1(FoxA1)/hepatocyte nuclear factor 4 alpha (*HNF4a*) positive feedback loop[66]. Interestingly, *FoxA1* and *HNF4a* are not directly putative target genes of miR-122 but both of them play crucial roles in promoting the differentiation of hepatocytes. FoxA1 can induce HNF4α that further enables upregulation of miR-122 through the transcriptional modulation; miR-122 can indirectly elevate the expression of FoxA1 but the mechanism of such an action is still uncovered. The positive regulatory effects on the interactions among miR-122, FoxA1, and HNF4α lead to hepatic differentiation and maturation unremittingly[66]. Likely, these results support that the regulatory circuits consisting of miRNAs and pluripotency factors can provide more useful insights into understanding the molecular mechanisms by which the cells maintain the balance between stemness and differentiation.

The roles of miRNAs in various cancers have been examined in dozens of studies, but their functions in CSCs have not yet been observed intensively. The first study of miRNA expression in CSCs was carried out by Yu *et al*[67], who found that several miRNAs appeared at lower levels in breast CSCs, including let-7, miR-200a/b/c, miR-16, miR-107, miR-128, and miR-20b[67]. Among these small RNA molecules, let-7 emerged as the most consistently and significantly reduced miRNA, suggesting that let-7 acts to suppress CSC self-renewal. Shimono *et al* later identified 37 miRNAs that were differentially expressed in breast CSCs; the miR-200 family and the miR-183-96-182 cluster were among those significantly downregulated[68]. Notably, all five members of the miR-200 family (miR-200a, -200b, -200c, -141, and -429) were downregulated in human breast cancer stem cells and in normal human and murine mammary stem/progenitor cells[68].

Table 1. MiRNAs involved in stem cell pluripotency maintenance and differentiation promotion.

Subgroup	miRNAs	Validated target genes that are involved in the self-renewal and differentiation of CSCs
Pluripotent miRNAs	miR-137	*Mib1*
	miR-184	*Numbl*
	miR-200	*ZEB1; ZEB2*
	miR-290	*CDKN1a*
	miR-302	*Cyclin D1; AOF1; AOF2; MECP1-p66; MECP2*
	miR-9	*Stathmin*
Pro-differentiation miRNAs	let-7	*Lin28; Lin28B; IMP-1; HRAS; HMGA2*
	miR-122	*Not determined*
	miR-134	*Nanog; LRH1; Sox2*
	miR-145	*Oct4; Sox2; KLF4*
	miR-181	*Lin28*
	miR-296	*Nanog*
	miR-470	*Nanog; Oct4*

Abbreviations:
Mib1 (Mind bomb 1); Numbl (Numblike); ZEB (Zinc finger E-box binding homeobox); CDKN1a (Cyclin-dependent kinase inhibitor 1a); AOF1 (Lysine-specific demethylase 1B); AOF2 (Lysine-specific demethylase 1A); MECP1-p66 (Methyl CpG binding protein 1-p66 beta component); MECP2 (Methyl CpG binding protein 2); IMP-1 (Insulin-like growth factor 2 mRNA binding protein 1); HRAS (v-Ha-*ras* Harvey rat sarcoma viral oncogene homolog); HMGA2 (High mobility group AT-hook 2); LRH1 (Nuclear receptor subfamily 5, group A, member 2), Sox2 (Sex determining region Y-box 2); Oct 4 (Octamer-binding transcription factor 4); KLF4 (Kruppel-like factor 4).

The miRNA profiles in CSCs have been examined in various tumor types in addition to breast cancer. In glioblastoma multiforme, the levels of miRNAs including miR-451, miR-486, miR-425, miR-16, miR-103, miR-107, and miR-185 were decreased in the stem cell (CD133+) population compared to the non-stem (CD133-) cell populations[69]. The overexpression of miR-451 inhibited neurosphere formation and cell growth[69]. In hepatocellular carcinoma, CSCs show a unique miRNA signature characterized by the upregulation of miR-181a-1, miR-181a-2, miR-181b-1, miR-181b-2, miR-181c, miR-17, miR-20a, miR-25, miR-92, miR-93, and miR-106b[70]. The inhibition of miR-181 led to a reduction in the CSC (EpCAM+) cell number and tumor initiating ability, whereas the expression of exogenous miR-181 resulted in an enrichment of CSCs (EpCAM+)[70]. In prostate cancer stem or progenitor cell populations that are enriched for CD44, CD133, or α2β1, the expression of miR-34a was low[71]. However, the forced expression of miR-34a can obviously inhibit prostate cell proliferation, tumor regeneration and metastasis by directly repressing *CD44*[71]. In colon CSCs (CD133+), 11 miRNAs (miR-16-2*, miR-744, miR-185, miR-455-3p, miR-155, miR-455-5p, miR-105, miR-494, miR-1826, miR-423-5p, and miR-181b) were upregulated, whereas 8 miRNAs (miR-221, miR-548d-5p, miR-636, miR-31, miR-320d, miR-151-3p, miR-429, and miR-151-5p) were downregulated[72]. Nam *et al* evaluated the miRNA expression profiles of ovarian CSCs (CD133+) and found that 34 miRNAs were significantly upregulated and 3 miRNAs were downregulated[73]. These differentially expressed miRNAs in CSCs indicated the crucial regulatory roles of miRNA in CSCs biological processes (Table 2), while let-7 and miR-200 are noted to be the mostly studied miRNAs in CSCs.

As described above, the expression of let-7 was significantly reduced in breast CSCs compared to non-stem cancer cells[67]. The upregulation of let-7 in breast CSCs reduced proliferation, mammosphere formation, the proportion of undifferentiated cells *in vitro*, and tumor formation and metastasis *in vivo*, while the downregulation of let-7 enhanced the *in vitro* self-renewal of non-stem cancer cells[67]. Further research indicated that let-7 targets v-Ha-*ras* Harvey rat sarcoma viral oncogene homolog (*HRAS*) and high mobility group AT-hook

Table 2. Aberrant expression of miRNAs in various human CSCs.

CSC	Expression	miRNAs	References
Breast cancer	Down	let-7a, let-7b, let-7c, let-7d, let-7e, let-7g, let-7i, miR-103, miR-107, miR-10a, miR-128a, miR-128b, miR-130a, miR-138, miR-141, miR-15a, miR-15b, miR-16, miR-17, miR-181b, miR-182, miR-183, miR-193b, miR-196a, miR-200a, miR-200a*, miR-200b, miR-200c, miR-20b, miR-210, miR-215, miR-22, miR-96	Yu *et al*[67]; Shimono *et al*[68]
	Up	miR-125b, miR-127, miR-132, miR-142-3p, miR-146b, miR-150, miR-155, miR-199a, miR-199a*, miR-199b, miR-212, miR-214, miR-221, miR-222, miR-223, miR-299-5p, miR-31, miR-409-3p, miR-432, miR-495	Gal *et al*[69]
Glioblastoma	Down	miR-103, miR-107, miR-16, miR-185, miR-425-5p, miR-451, miR-486	Ji *et al*[70]
	Up	N/A	
Hepatic cancer	Down	N/A	
	Up	miR-106b, miR-17, miR-181a, miR-181b, miR-181c, miR-20a, miR-25, miR-92, miR-93	Liu *et al*[71]
Prostate cancer	Down	miR-34a	
	Up	N/A	
Ovarian cancer	Down	miR-1181, miR-1202, miR-1207-5p	Nam *et al*[73]
	Up	let-7f, miR-100, miR-107, miR-135b, miR-146a, miR-181a, miR-183, miR-193a-3p, miR-200a, miR-200b, miR-205, miR-21, miR-210, miR-26b, miR-29b, miR-33a, miR-34a, miR-340, miR-340*, miR-365, miR-424, miR-425, miR-449a, miR-455-3p, miR-494, miR-516a-5p, miR-517a, miR-517c, miR-522, miR-7, miR-886-3p, miR-96	
Colon cancer	Down	miR-151-3p, miR-151-5p, miR-221, miR-31, miR-320d, miR-429, miR-548d-5p, miR-636	Zhang *et al*[72]
	Up	miR-105, miR-155, miR-16-2*, miR-181b, miR-1826, miR-185, miR-423-5p, miR-455-3p, miR-455-5p, miR-494, miR-744	

Underlined miRNAs represent those miRNAs that show similar dysregulation (up or down) in more than one type of cancer stem cells.

2 (*HMGA2*); the silencing of *HRAS* in breast CSCs reduced self-renewal with little effect on differentiation, whereas the silencing of *HMGA2* enhanced differentiation but not self-renewal[67].

In one of our recent studies, we investigated the mechanism by which let-7 regulates cell differentiation using bipotent K562 human leukemia cells and human CD34+ hematopoietic progenitor cells as research models[52]. We found that let-7 and Lin28 appear to play contrary roles in megakaryocytic (MK) differentiation and maintain a dynamic balance through a reciprocal regulatory loop (Figure 1). As discussed earlier, *Lin28* is one of the direct targets of let-7 and can also influence the biogenesis of let-7 by recruiting terminal uridylyl transferase-4 (TUT4) to add a uracil residue to the 3′ end of pre-let-7; this modification results in the degradation of pre-let-7 and a blockade of let-7 maturation[74, 75]. Interestingly, when miR-181 is introduced to translationally downregulated *Lin28*, the let-7/*Lin28* loop is disrupted and let-7 expression is thereby induced that lead to the promotion of MK differentiation. Our results are consistent with the observation that miRNAs play important roles in the control of cell differentiation.

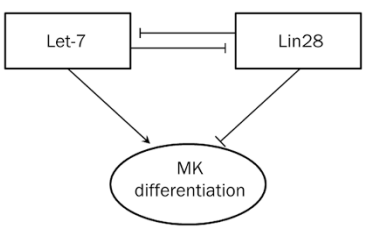

Figure 1. The reciprocal regulatory loop of let-7 and Lin28 in the control of megakaryocytic (MK) differentiation.

The first report of miR-200 in stem cells was published in *Cell* in 2009[68]. In this study, the authors found that all the miR-200 family members (miR-200a, -200b, -200c, -141, and -429) were downregulated in breast CSCs compared to non-stem cancer cells. By targeting BMI1 polycomb ring finger oncogene (*BMI1*), miR-200c can inhibit the clonal expansion of breast cancer cells and suppress the growth of embryonal carcinoma cells *in vitro*. Moreover, miR-200c can strongly suppress tumor formation driven by breast CSCs *in vivo*[68]. In support of these results, Iliopoulos *et al* reported that miR-200b can suppress the formation and maintenance of mammospheres *in vivo*, which may, at least in part, attribute to the repression of the target gene named suppressor of zeste 12 homolog (*Suz12*)[76]. Moreover, Lim *et al* reported that the conversion of immortalized human mammary epithelial cells from a non-stem phenotype to a stem-like phenotype was accompanied by the loss of miR-200 expression. The restoration of miR-200 expression in these cells decreased their stem-like properties while promoting their transition to an epithelial phenotype, suggesting a negative role of miR-200 in CSC tumorigenesis[77].

Conclusion and perspective

MiRNAs, as the master post-transcriptional and translational regulators on gene expression, have been reported to play important roles in stem cells and tumorigenesis. CSCs are

now believed to be responsible for tumor relapse and chemotherapy failure in many cancers. Recent studies show that miRNAs are significantly involved in the CSC self-renewal and differentiation. Given that the dysregulation of miRNAs has been intimately implicated in tumor development, the modulation of CSC properties may contribute to the underlying mechanisms by which miRNAs regulate tumorigenesis. For examples, let-7 controls the cell cycle progression and differentiation of CSCs, miR-200c modulates the self-renewal of CSCs by targeting *BMI1*, and miR-34a restricts the migratory and invasive properties of prostate CSCs by directly repressing *CD44*, which have been discussed earlier in details. These findings support the crucial roles of miRNAs in the regulation of CSCs. As such, further studies on this topic are expected to provide more insights into our understanding of tumorigenesis and aid in the development of new strategies against chemoresistance by targeting CSCs.

Acknowledgements

This study is supported by an American Cancer Society Research Scholar Grant (RSG-13-265-01-RMC, to Yaguang XI) and an NIH/NCI R21 Grant (5R21CA160280, to Yaguang XI). We sincerely apologize to those colleagues whose work have not be cited due to time and space constraints.

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