

# The role of microRNAs in stemness of cancer stem cells

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## Abstract

Cancer is one of the most important diseases of humans, for which no cure has been found so far. Understanding the causes of cancer can pave the way for its treatment. Alteration in genetic elements such as oncogenes and tumor suppressor genes results in cancer. The most recent theory for the origin of cancer has been provided by cancer stem cells (CSCs). Tumor-initiating cells (T-ICs) or CSCs are a small population isolated from tumors and hematologic malignancies. Since CSCs are similar to embryonic stem cells (ESCs) in many aspects (such as pluripotency and self-renewal), recognizing the signaling pathways through which ESCs maintain their stemness can also help identify CSC signaling. One component of these signaling pathways is non-coding RNAs (ncRNAs). ncRNAs are classified in two groups: microRNAs (miRNAs) and long non-coding RNAs (lncRNAs). miRNAs undergo altered expression in cancer. In this regard, they are classified as Onco-miRNAs or tumor suppressor miRNAs. Some miRNAs play similar roles in ESCs and CSCs, such as let-7 and miR-302. This review

focuses on the miRNAs involved in stemness of ESCs and CSCs by presenting a summary of the role of miRNAs in other tumor cells.

## Introduction

Cancer stem cells form a small population of cells that are very similar to stem cells within the tumor tissue. One theory is that tumors arise from a small population of cells called tumor-initiating cells (T-ICs) or cancer stem cells (CSCs). CSCs were first isolated from hematologic malignancies,<sup>1</sup> and then from solid tumors and breast cancer.<sup>2</sup> In 1961, it was found that there are cells in tumor tissue capable of producing a variety of tumors.<sup>3</sup> There are three methods to isolate CSCs: i) isolation using flow cytometry based on Hoechst stain; ii) isolation based on surface marker expression; iii) sphere culture. Prominin-1 (CD133)<sup>4,5</sup> and CD44<sup>6,7</sup> are two major cell surface markers used to isolate CSCs. These cells must have three properties: tumorigenicity, self-renewal and pluripotency.<sup>8,9</sup> Classic treatments for cancer include chemotherapy, radiotherapy and antiangiogenic therapy against proliferating transit-amplifying cells. After a while, due to the presence of therapy-resistant cancer stem cells in tumor tissue, the cancer will appear again. Therefore, treatment should be performed simultaneously against these cells. For this purpose, the differentiation therapy strategy causes differentiation of these cells to proliferating transit-amplifying cells sensitive to classic treatments. This strategy, in which targeting of intracellular and extracellular signaling pathways causes disruption of stemness in CSCs, has proved to some extent to be successful.<sup>10,11</sup>

These cells undergo the same signaling pathways as embryonic stem cells (ESCs) such as Oct-4, Notch, Wnt, Sonic hedgehog (SHH) and Bmi-1.<sup>12,13</sup> Most of these signaling pathways are involved in self-renewal of ESCs or tissue adult stem cells and cancer. Mutation and deregulated expression of some components of these signaling pathways has been shown in many human tumors, and hyperactivation of these pathways mostly contribute to tumor regeneration.<sup>14-18</sup> Oct-4 is a core transcriptional regulatory circuitry in ESCs,<sup>19</sup> and is necessary for reprogramming somatic cells into pluripotent state;<sup>20</sup> it is also known as a pluripotency factor. Pluripotency helps CSCs to maintain an undifferentiated state, supporting the structure of tumor mass. Oct-4 and its partner Nanog have been shown to be expressed in many human tumors, and their downregulation causes a reduction in CSC stemness and resistance to chemotherapy.<sup>21-24</sup>

Contrary to mRNAs, as their name implies, non-coding RNAs (ncRNAs) do not encode for proteins. They are classified in two groups: those with less than 200 nucleotides (e.g. miRNAs), and those

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with more than 200 nucleotides, such as long non-coding RNAs (lncRNAs) and long intergenic non-coding RNAs (lincRNAs). Meanwhile, it has been shown that p53 activates a lincRNA known as lincRNA-p21, inhibiting the transcription of many genes involved in p53 response.<sup>25</sup> There is now an increasing trend to identify these lncRNAs and lincRNAs in cancers.<sup>26–28</sup>

MiRNAs are a better-known group of ncRNAs. The first miRNA was discovered in *Caenorhabditis elegans*.<sup>29</sup> MiRNAs are non-coding RNAs, 20–25d-nt long, commonly binding the 3' untranslated region (3'UTR) of their targets, inhibiting their translation and regulating their stability.<sup>30</sup> It has also been shown that some miRNAs can activate gene expression in a specific manner named RNA activatory (RNAa) by targeting regulatory regions (e.g. promoters).<sup>31,32</sup> MiRNAs, like other genes, undergo altered expression in cancer (oncogenes/tumor suppressor genes). This altered expression is attributed to such mechanisms as chromosomal rearrangements, amplification, mutation and genomic deletion<sup>33</sup> which are epigenetic mechanisms usually in fragile sites of the genome.<sup>34,35</sup> The miRNAs are in the form of onco-miR in regions where they are amplified (like miR-17-19 cluster), and are in the form of tumor suppressor miR in the regions where they are deleted, such as miR-15a, 16-1 cluster. The first report on the relationship between miRNAs and cancer was published in 2002. It was found that deletion of a locus on chromosome 13, which contains miR-15 and miR-16-1 in chronic lymphocytic leukemia (CLL), is involved in pathogenesis of the disease. These two miRs bind the anti-apoptotic protein BCL2. In fact, the absence of these miRs inhibits the induction of apoptosis in these cells.<sup>34</sup>

Sometimes, miRNAs have a contradictory role in cancer cells. For example, miR-17-92 locus plays the role of tumor suppressor in human B-cell line by inhibiting proliferation,<sup>35</sup> and in another cell has an onco-miRNA role along with MYC and inhibition of apoptosis.<sup>36</sup> Studies have also shown that pri-miRs are involved in cancer independent of their active form (Table 1).<sup>37–61</sup>

## MicroRNAs and embryonic stem cells

Nearly 25% of the 110,000 known miRNAs are encoded by four clusters: miR-17-92 cluster, miR-21 loci, miR-290-295 cluster and miR-15b-16 cluster. The transcription factors Nanog/Oct4/Tcf3 bind the promoter of the miR-302, miR-290/371 cluster, miR-363 cluster, miR-148/152, miR-135b, miR-124, miR-615 and miR-708 clusters in the form of occupancy, and regulate their expression.<sup>62,63</sup> Core transcriptional regulatory circuitry in ESCs includes Nanog/Oct4/Sox2,<sup>19</sup> which along with Tcf3 causes the expression of Lin28, the latter subsequently inhibiting the processing of let-7. In this regulatory loop, let-7 has a negative effect on the expression of Lin-28. Incoherent feed forward is one of the regulatory mechanisms of miRNAs in ESCs. In fact, we can say that miRNAs are the regulatory arm of transcription factors in expression regulation of downstream genes.<sup>64</sup> Many studies have been performed on the effect of miRNAs in ESCs. MiR-145 inhibits pluripotency through inhibition of Oct-4, Sox2 and Klf4.<sup>65</sup> In addition to being able to target 3' UTR in mRNA, miRNAs can target the coding sequence (CDS). MiR-296, miR-134 and miR-470 can target CDS regions in Oct4, Nanog and Sox2.<sup>66</sup>

A subset of miR-290 clusters known as regulators of the cell cycle is called embryonic stem cell cycle (ESCC). This subset includes miR-291-3p, miR-294 and miR-295.<sup>67</sup> One theory about the origin of CSCs is reprogramming of somatic cells into dedifferentiation state and CSC formation. When the cells are divided, replication nucleosomes are temporarily removed, and transcription factors can access open chromatin. Therefore, active cell cycle can promote production of induced

pluripotency stem cells (iPS).<sup>68</sup> Some ESCC miRNAs can enhance iPS production by targeting cell cycle inhibitors. hsa-miR-302b and hsa-miR-372 promote iPS production by targeting G1-S inhibitors.<sup>69</sup> In another study, miR-92b promoted G1-S transition by targeting P57KIP2 as CDK inhibitor.<sup>70</sup>

C-Myc is an important factor for reprogramming, especially in its early stages,<sup>71</sup> controlling miRNAs expression by binding to their regulatory regions. It has been shown that c-Myc binds to miR-290-295,<sup>72</sup> miR-141, miR-200 and miR-429 promoters.<sup>73</sup> miRNAs profiles are changed during differentiation and reprogramming. Let-7 family, miR-210, miR-301, miR-136, miR-145, miR-29a/b, the miR-30 family and some other miRNAs are down-regulated during reprogramming,<sup>71,74</sup> and some are up-regulated, such as miR-17-92, miR-106b-25 clusters, miR-183<sup>71,74</sup> and miR-302 cluster.<sup>75</sup> Therefore, miRNAs promoting reprogramming and dedifferentiation can be proper candidates for knockdown and differentiation therapy studies. (For more details about the role of miRNAs in stemness and reprogramming, see<sup>52,62,76</sup>).

## MicroRNAs and cancer stem cells

As mentioned above, reprogramming of differentiated cells into a dedifferentiation state is a stage in CSCs development. Therefore, some mechanisms that take place during reprogramming are involved in tumor development and CSCs formation. Fully differentiated cells undergo epithelial-mesenchymal transition (EMT) by losing adherent molecules (e.g. E-cadherin) and becoming migratory cells. Some factors involved in EMT, such as TGF- $\beta$ , Notch1, Wnt, ZEB1/2 and Klf8 induce EMT by repressing E-cadherin expression.<sup>77</sup> These cells are metastatic, and if they undergo mesenchymal-epithelial transition (MET), they can form CSCs.<sup>78</sup>

A number of miRNAs are involved in EMT as inducers or repressors (Table 2). The most well-known miRNA inhibitor of EMT is the miR-200 family. The miR-200 family and miR-205 inhibit TGF- $\beta$  induced-EMT by targeting ZEB1 and SIP1, and downregulation of these miRNAs is required for induction of EMT in cancer cells.<sup>79</sup> It is interesting that ZEB1 also blocks the expression of the miR-200 family and miR-141 by binding to their promoters.<sup>48</sup> P53 is a repressor of EMT by binding to miR-200c promoter and activating its expression.<sup>49</sup> It has been shown that miR-200c is down-regulated in breast cancer stem cells.<sup>50</sup> By inhibiting klf4 transcription factor and polycomb repressor BMI1, miR-200c inhibits the stemness property of CSCs, the reduced expression of which is observed in breast cancer.<sup>50,51</sup> As it has previously been observed that p53 induces the differentiation of ESCs by binding to the regulatory region of Nanog gene,<sup>57</sup> it is worth noting that the inhibition of stemness by p53 is also indirectly carried out by activating miRNAs such as miR-200 family members that inhibit BMI1, Sox2, Klf4 and Notch signaling.<sup>50,51,58</sup> miR-22 indirectly inhibits miR-200 expression and EMT induction in breast cancer by targeting TET and demethylation inhibition of miR-200 promoter.<sup>59</sup> This study showed that miR-22 plays a role in epigenetic modification and enhances CSCs characteristics. In addition, it has been shown that miR-22 is up-regulated in myelodysplastic syndrome (MDS) and leukemia, and can be considered as a prognostic factor in these patients with low survival. miR-22 targets Methyl cytosine dioxygenase TET2, developing hematopoietic stem cell self-renewal and maintaining stemness.<sup>60</sup>

Another inducer of EMT is the miR-106b-25 cluster, which can promote EMT and CSCs characters in breast cancer model by targeting Smad7 (a negative regulator for TGF- $\beta$ /BMP signaling pathway).<sup>61</sup> miR-203 can revert the EMT back to MET by targeting NP63, a predominant p63 isoform and oncogene which promotes CSCs proliferation in breast

cancer cells. Forced expression of miR-203 caused reduction in CD44<sup>+</sup> CD24<sup>-</sup> CSC population, and increased differentiation to luminal epithelial cells.<sup>55,56</sup> (For more details about the role of miRNAs in EMT, see 53,54).

miR-34a, which is activated by p53,<sup>80</sup> directly inhibits CD44 in prostate cancer stem cells; it causes inhibition of metastasis and prevents regeneration of cancer.<sup>81</sup> In colon CSCs, tumor suppressor miR-34 targets Notch1 mRNA to disturb the balance between self-renewal and differentiation in Notch signaling. In this study, it was found that miR-34 determines cell fate in colon CSCs.<sup>82</sup>

miR-9 and miR-9\* (miR-9/9\*) are expressed in CD133<sup>+</sup> glioblastoma stem cells, and promote cell growth and maintain stemness by targeting Calmodulin-binding transcription activator 1 (CAMTA1), which is seen as a tumor suppressor.<sup>83</sup> Inhibition of miR-9/9\* causes glioblastoma stem cell differentiation.<sup>84</sup> It has been shown that miR-128 inhibits proliferation and self-renewal in glioma CSCs.<sup>85</sup> miR-130b promotes stemness and tumorigenicity in CD133<sup>+</sup> hepatocellular carcinoma (HCC) CSCs through inhibition of Tumor Protein P53 Inducible Nuclear Protein 1 (TP53INP1). Interestingly, forced expression of miR-130b in CD133<sup>-</sup> cells assists in self-renewal activity and chemotherapy resistance.<sup>86</sup>

As mentioned, miR-145 causes differentiation of ESCs by inhibiting the stemness factors.<sup>65</sup> Interestingly, the expression of miR-145 in prostate and renal cancer cells is inhibited.<sup>87,88</sup> In addition, miR-21 has been shown to induce stemness in colon cancer cells by modulating TGF $\beta$ R2.<sup>89</sup> Another miRNA down-regulated in CSCs is miR-140. This tumor suppressor miR reduces CSC self-renewal in breast CSCs by targeting Sox9 and ALDH1.<sup>90</sup> Reduced expression of miR-30 has been shown in CSCs in breast cancer. Increased expression of miR-30 will reduce self-renewal and will increase apoptosis in these cells.<sup>91</sup> miR-495, which up-regulates and targets E-cadherin, contributes to metastasis and DNA damage-inducible transcript 4 (DDIT4) to assist in proliferation and hypoxia resistance in breast CSCs.<sup>92</sup> (For more details about the role of miRNAs in breast CSCs, see<sup>93</sup>).

Altered expression of miR-142-3p, miR-451, miR-106a, miR-249 142-5p, miR-15b, miR-20a, miR-106b, miR-25, and miR-486 has been observed in lung cancer progenitor cells.<sup>94</sup>

It has been shown that the expression of miR-302, specific for ES cells, causes the expression of stemness factors such as Nanog, Oct-4 and Sox2 in cancer cells, conferring a stem cell-like property to them, likely reprogramming these cells to CSCs.<sup>95</sup> miR-328 helps in drug resistance and metastasis of colon CSCs by targeting ABCG2 and Matrix Metalloproteinase 16.<sup>96</sup>

**Table 1. miRNAs involved in cancer.**

MicroRNAs	Cancer correlation	Function	References
miR-15, miR-16	Induce apoptosis and decrease tumorigenicity, suppress Bcl-2	TS	38,39
miR-34a	Induce apoptosis, suppress E2F3, MYCN, CDK4	TS	40-42
miR-143, miR-145	Down-regulated in cancers	TS	43
miR-137, miR-193a	Suppress CDK6, E2F	TS	44
Let-7	Induce apoptosis and differentiation. Suppress Ras, c-Myc and HMGA2	TS	45-47
miR-21	Anti-apoptosis, targets Bcl-2, TPM1, PDCD4	OG	48-51
miR-155	Up-regulated in lymphoma and breast cancer	OG	52-54
miR-27	Targets ZBTB10, RYBP, MYT1	OG	55,56
miR-17-19 cluster	Up-regulated in lymphomas and many types of cancers. Targets E2F1, Bim, PTEN and Rb	TS/OG	57-61

TS, tumor suppressor; OG, oncogene.

**Table 2. miRNAs involved in cancer stem cells.**

microRNA	Targets	Roles in cancer stem cells
miR-200 family	ZEB1 and SIP1, Bmi-1, Klf4	Inhibition induction of EMT, inhibit BMI1, Sox2, Klf4 and Notch signaling and reduce stemness in CSCs
Let-7	Lin28, H-RAS and HMGA2	Differentiation of CSCs
miR-302	Genes involved in differentiation	Facilitate dedifferentiation of human tumor cells
miR-30	ITGB3 and Ubc9	Reduce self-renewal and increases apoptosis
miR-140	Sox9 and ALDH1	Reduce CSCs self-renewal
miR-145	Oct-4, Sox2 and Klf4	Inhibit stemness properties
miR-128	Bmi-1	Disrupting self-renewal of CSCs
miR-34a	CD44 and Notch1	Inhibition of self-renewal
miR-203	$\Delta$ NP63 $\alpha$	Revert EMT to MET and reduction of CSCs population
miR-22	TET	Indirectly repress miR-200 expression and enhance EMT and self-renewal
miR-106b-25	Smad7	Cause EMT and promote CSCs properties
miR-9/9*	CAMTA1	Maintain stemness property
miR-130b	TP53INP1	Assist self-renewal activity and chemotherapy resistance
miR-21	TGF $\beta$ R2	Enhance stemness properties
miR-495	E-cadherin and DDIT4	Promote metastasis, proliferation and hypoxia resistance of CSCs
miR-328	ABCG2 and MMP16	Assist metastasis and drug resistance

CSCs, cancer stem cells; EMT, epithelial-mesenchymal transition; MET, mesenchymal-epithelial transition.

Finally, the reduced expression of let-7 has been demonstrated in many cancers.<sup>97–99</sup> Let-7 inhibits H-RAS and HMGA2.<sup>100</sup> This factor is controlled by Lin-28; however, methylation of its gene has been observed in lung cancer.<sup>101</sup> Differentiation of ES cells increases its level; therefore, its expression is expected to decrease in CSCs. As expected, let-7 is significantly decreased in breast CSCs.<sup>102</sup>

## Discussion

Recognizing CSCs as factors of resistance to chemotherapy and recurrence of tumor tissue requires identification of the mechanisms that regulate these cells for therapeutic targets. Since these cells show signaling similar to ESCs and have the two main features of stemness, including pluripotency and self-renewal, they appear to have similarities in expression of stemness factors. Thus, for differentiation therapy purposes, intra- and extracellular mechanisms that maintain this stemness should be further identified. Understanding transcriptome of pluripotency would help find more targets in differentiation therapy of cancer.

Since no specific research has been conducted to identify the miRNAs in various types of CSCs to identify miRNA profiling, we can resort to identifying the miRNAs of ESCs, which are very similar to CSCs. In addition, it has been shown that many of the miRNAs act in nucleus in upregulation of genes by binding to the promoters or antisense transcripts.<sup>103</sup> Therefore, it would be interesting if such miRNAs that cause upregulation of tumor suppressor genes or differentiation in CSCs can be found. An interesting study using RNAseq technique showed that most of the miRNA are transported to nucleus after maturation in cytoplasm.<sup>104</sup> The interesting point is that miRNAs that are important in maintaining stemness and differentiation induction in ESCs like mir-145, miR-302 and let-7 play similar roles in CSCs. Further studies are required to better understand the role of miRNAs in CSCs. Focusing on this topic can help find new drugs and more effective treatments for cancer. Finally, in line with basic research for finding new targets for treatment, attempts at specific delivery of miRNAs into tumor cells, especially CSCs, should also be approved. Use of active delivery instead of passive delivery by using targeted nanoparticles showed more specificity in drug delivery, and phase I/II clinical trials are ongoing.<sup>105</sup> (For more details about nanoparticle drug delivery, see <sup>106</sup>).

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