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Methods for assessing the effect of microRNA on stemness genes

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ABSTRACT

According to the latest concepts, for micrometastasis to develop into macrometastasis, differentiated cancer cells must revert to a dedifferentiated state. Activation of stemness genes plays a key role in this transition. Suppression of stemness gene expression using microRNAs can become the basis for the development of effective anti-metastatic drugs. This article provides an overview of the existing methods for assessing the effect of microRNAs on stemness genes and cancer cell dedifferentiation.

Keywords: microRNA, cancer, stemness genes

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Методы оценки влияния микроРНК на гены стволовой пластичности

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РЕЗЮМЕ

Согласно современным представлениям, для перехода микрометастаза в макрометастаз дифференцированная раковая клетка должна перейти в дедифференцированное состояние. Ключевую роль в данном переходе играет активация генов стволовой пластичности. Подавление экспрессии генов стволовой пластичности с использованием микроРНК может стать основой для разработки эффективных антиметастатических пре-

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паратов. Приведен обзор существующих методов оценки влияния микроРНК на гены стволовой пластичности и процесс дедифференцировки раковой клетки.

Ключевые слова: микроРНК, рак, гены стволовой пластичности

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INTRODUCTION

The study of fundamental mechanisms of carcinogenesis and the search for new drugs for therapy of malignant neoplasms are some of the leading tasks in oncology [1]. Metastatic cancer is a serious problem that complicates successful treatment of malignant neoplasms. Depending on the localization of the tumor and the stage of the disease, the proportion of patients who develop metastases ranges from 15 up to 90%. In the vast majority of tumor localizations, cancer patients die from metastases [2, 3]. At relatively early stages of tumor development, some cancer cells enter blood vessels and are further carried to various organs and tissues via the circulatory system, which leads to the formation of the so-called micrometastases. In micrometastases, cancer cells can remain dormant for a long time and under certain conditions trigger macrometastases. According to up-to-date concepts, for transition from micrometastasis to macrometastasis, differentiated cancer cells revert to a dedifferentiated state. Stemness genes play a key role in this transition.

It has been shown that stem cell plasticity is associated with the expression of a number of genes (*SOX2, MYC, OCT, NANOG*, etc.) [4–6]. Changes in the expression of these genes can be mediated by a wide range of factors, including non-coding RNAs (long non-coding RNA, short interfering RNA, microRNA) [7–9]. Currently, there are many studies on approaches to changing the expression of genes contributing to stem cell plasticity using microRNAs [10], since they can provide the basis for developing an effective anti-metastatic drug. This article provides an overview of existing methods for assessing the effect of microRNAs on stemness genes and dedifferentiation of cancer cells.

STEMNESS GENES

Transcription factors associated with stemness play a key role in the carcinogenesis. *SOX2, OCT4, NANOG*, and *MYC* genes are required for self-renewal and reprogramming of stem cells, as well as for maintenance of stem cell homeostasis. Overexpression of these transcription factor genes is associated with the development and progression of a wide range of cancers – breast cancer [11–14], melanoma [15–17], osteosarcoma [18–20], head and neck squamous cell carcinoma [21–23], colorectal cancer [24–26], cervical cancer [27, 28], pancreatic cancer [29–31], and stomach cancer [32–35]. At the same time, the highest level of *SOX2*, *OCT4, NANOG*, and *MYC* expression is typical of aggressive, metastatic, and chemoresistant tumors [17, 35–42].

Expression of stemness genes ensures maintenance of the phenotypic profile, self-renewal of cancer stem cells (CSCs), and proliferation of cancer progenitor cells and stimulates dedifferentiation of non-stem cancer cells [43, 44]. Stemness-related genes implement their functions by participating in almost all signaling pathways related to proliferation (WNT / B-catenin, Notch, Hedgehog (HH) and Sonic Hedgehog (SHH), NRF2, PI3K / AKT / mTOR, etc.) [45]. Activation of the self-renewal program is an integral part of CSC stemness, actively contributing to tumor progression and metastasis and causing high cell turnover and production of progenitor cells [46]. Moreover, the ability of CSCs to self-renew in the long term is one of the main reasons for the development of resistance to antitumor drugs [47, 48]. For example, the development of *SOX2*-mediated chemotherapy resistance was demonstrated for colorectal cancer, squamous cell carcinoma of the head and neck, pancreatic ductal adenocarcinoma, and breast cancer

[21, 38, 49, 50]. Ectopic co-expression of Oct4 and Nanog results in non-small cell lung cancer (NSCLC) cells acquiring the properties of CSCs, including self-renewal, drug resistance, epithelial – mesenchymal transition (EMT), and high tumor-initiating activity [51]. Directly in the focus of possible metastasis, ectopic expression of stemness-related genes can provide acquisition of metastatic potential by differentiated cancer cells due to their dedifferentiation to CSCs [5, 52]. Thus, stemness-related genes can be considered key factors in tumor metastasis, resistance of cancer cells to treatment, and maintenance of the CSC population.

EFFECT OF microRNAS ON STEMNESS GENES

MicroRNAs (miRNAs) are short double-stranded RNAs containing 20-23 nucleotides which have a unique seed sequence of 6–7 nucleotides at 5' end. This sequence binds to mRNA, implementing post-transcriptional regulation of gene expression (Fig. 1, *a*). Currently, more than 2,500 miRNAs are known to be present in the human genome. These miRNAs regulate normal processes of development and homeostasis in cells. Information about miRNAs can be obtained from databases, such as https://www.mirbase.org/, where the description of the published miRNA sequences can be found, or https://miRTarBase.cuhk.edu.cn/ for experimentally confirmed target genes for miRNAs, etc.

Extracellular miRNAs in microvesicles, when getting into target cells, are capable of acting as autocrine, paracrine, and / or endocrine regulators of cellular processes [53]. Taken together, they can affect up to 60% of the cell transcriptome with a significant impact on both protein expression and on the function of the cell as a whole [54–56]. More information about the biogenesis and mechanism of action of miRNA can be found in the articles [57, 58].

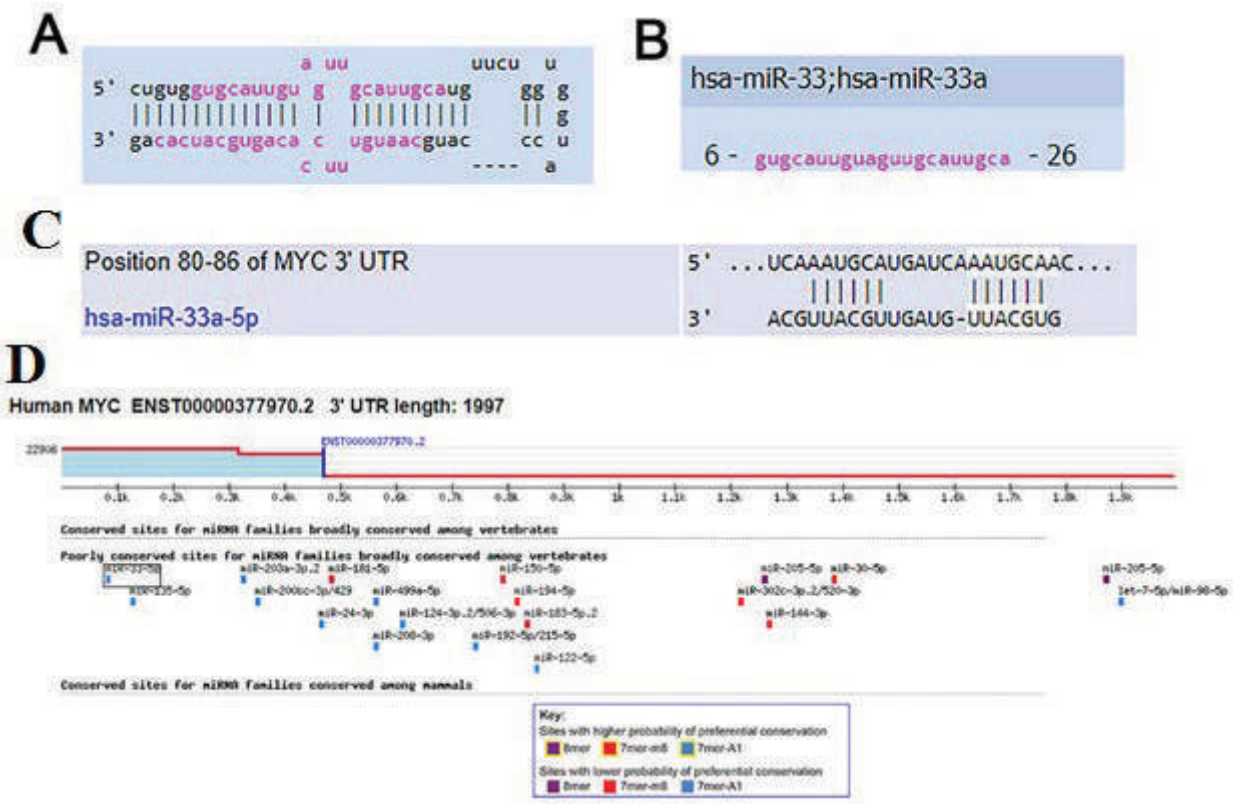

Fig. 1. Post-transcriptional regulation of gene expression: *a* – structure of immature miRNA; *b* – structure of mature miRNA, entering the active site of the Argonaute protein, hsa-miR-33 (miRBase database); *c* – hsa-miR-33 binding to a 3'-untranslated mRNA region encoding the c-Myc transcription factor; *d* – binding sites for various microRNAs in the 3'-untranslated region of the mRNA in the *MYC* gene predicted by TargetScan

Global microRNA dysregulation is observed in cancer cells [59]. There are published studies that confirm the involvement of miRNAs in the regulation of all processes leading to changes characteristic of cancer cells (Fig. 2) [60] – maintenance of proliferative signaling, avoidance of cellular growth suppression, resistance to cell death, unlimited cell division (cell immortality), induction of angiogenesis (growth of new vessels), activation of invasion (penetration into surrounding tissues) and metastasis, reprogramming of energy metabolism, avoidance of destruction by the immune system, epigenetic reprogramming, influence of polymorphic microbiomes, cellular aging, phenotypic plasticity, and closely related to it regulation of stemness genes.

It should be noted that identification of pro- or anti-oncogenic properties for each specific microRNA is a rather difficult task, since the patterns of their expression are tissue specific. Each type of miRNA can bind to multiple mRNA and, at the same time, one type of mRNA can be a target for a subset of miRNAs [61]. Moreover, one miRNA is able to regulate several targets that control different signaling pathways [62] (Fig. 1, *d*). The role of specific miRNA depends on the context – it can act as a tumor suppressor in one situation and as an oncogene in another. Miscellaneous tissues / cells express different sets of long non-coding RNAs (ncRNAs) and circular RNAs, as well as RNA-binding, RNA-modifying, and RNA-editing proteins [63]. Interaction with these proteins allows for microRNA binding sites on mRNA to become available or unavailable. Long ncRNAs and circular RNAs with a miRNA binding site can act as RNA sponges or traps that remove miRNA from the process. An additional factor is alternative polyadenylation, which affects the presence of a miRNA binding site [63].

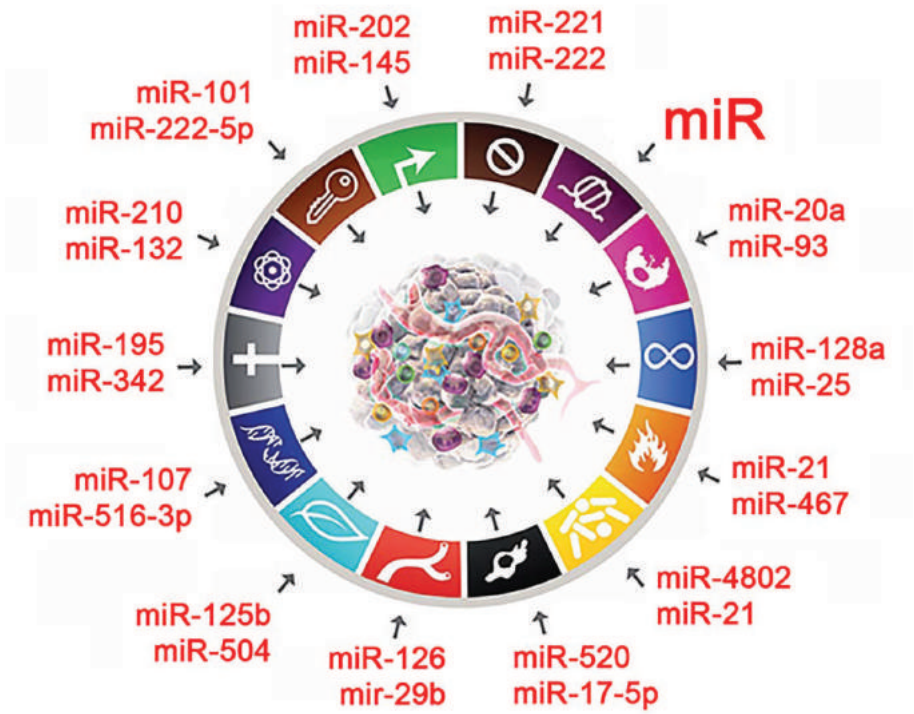

Fig. 2. Examples of microRNAs involved in the regulation of processes in the cell which are responsible for the difference between a normal cell and a cancer cell (adapted from [60])

METHODS FOR ASSESSING THE EFFECT OF MIRNAS ON TARGET GENES

The ability of miRNAs to maintain stem plasticity and regulate EMT, autophagy, resistance, and dedifferentiation of cancer cells is studied using a relatively small range of *in vitro* and *in vivo* methods (Table). At the first stage, to confirm the possible effect of a specific microRNA on a specific gene, researchers use a bioinformatic analysis of the presence of binding sites and a luciferase reporter assay allowing to validate microRNA target genes. The second phase includes an assessment of the effect of microRNA on the expression of the target gene (for example, by a real-time PCR) and, as a result, on protein production (for example, by the Western blotting). The third stage

includes an assessment of the effect of miRNAs on the functional cell activity – proliferation, mammosphere formation, migration, etc.

To predict the target genes of specific miRNAs, online analysis tools in databases, such as TargetScan (https://www.targetscan.org/) and miRDB (https://www.mirdb.org) [83, 84] are used. After conducting the bioinformatic analysis of the data to check for functional relevance of the predicted miRNA – mRNA pairs in the target gene, different modifications of the luciferase reporter assay are used, where luciferase acts as a reporter protein [85]. By gene cloning, the sequence of the 3'-untranslated region of the mRNA in the gene of interest is added at the 3' end of the luciferase gene. If the cell has successfully synthesized a reporter protein (luciferase), the addition of a substrate leads to a chemical reaction. The course of the reaction can be registered by the emergence of bioluminescence, and the intensity of bioluminescence is proportional to the number of successfully translated mRNAs [86]. Thus, the decrease in bioluminescence after miRNA treatment of cells indicates its interaction with mRNA.

To determine the level of gene expression under the effect of miRNAs, real-time PCR is widely used. It allows to evaluate gene expression based on data on the amount of mRNA in the sample [87]. The protein product of the target gene is determined mainly by the Western blotting. This method includes SDS-PAGE electrophoresis of the biosample, transfer of target proteins on a hydrophobic membrane, and their detection using specific antibodies [88]. To analyze the proliferative activity of cells under the effect of microRNA, different cell counting methods are used, ranging from basic techniques with trypan blue stain to more complex multi-stage protocols. One of the most common methods for assessing cell proliferative activity is an MTT assay based on enzymatic reduction of 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (yellow soluble tetrazolium dye) with mitochondrial dehydrogenases to purple – violet formazan crystals. The crystals are dissolved in DMSO, after which the optical density of the obtained samples is analyzed [89]. Analogs of the MTT assay are the MTS assay, which uses a different type of tetrazolium dye – (3- (4,5-dimethyl thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)), and the CCK-8 test using the tetrazolium salt WST-8. These types of dyes are water soluble and do not require a solubilization step, which reduces the probability of error in the analysis [90].

Another approach to assessment of the proliferative activity of cells is the analysis using the nucleoside analog BrdU, which is able to incorporate into a replicating DNA and can be determined using specific antibodies [91]. The impedance-based RTCA iCELLIgence instrument is a new technology for assessing cell proliferation and cell death in real time. This technique requires no dyes, and cells can be observed for a long time. However, this method is applicable only to adherent cell cultures [92]. To analyze survival of cells and their ability to proliferate after miRNA treatment, the clonogenic assay is used, which measures the capacity of single cells to form colonies. Cells are seeded at very low density and incubated for 1–3 weeks, after which formed colonies are fixed and stained with crystal violet, and their number is counted [93].

Cell viability in a number of studies is assessed by the presence or absence of flow cytometry signs of necrosis, apoptosis, and secondary necrosis using appropriate fluorescently labeled antibodies, such as Annexin V, caspase-Glo 3/7, and DNA-binding dyes, such as propidium iodide or 7-aminoactinomycin D [94]. It is also possible to use flow cytometry to determine the effect of miRNAs on phases of the cell cycle in pre-fixed cells using DNA-binding dyes [95].

To assess the phenotypic profile of cells, the expression of CSC markers CD24, CD44, CD133, and ALDH is studied using fluorescently labeled antibodies [96]. The expression of stem cell plasticity markers CD44, CD24, CD133, LGR5 and EMT markers Snail, Slug, Zeb1, Cloudin1, B-catenin, E-cadherin, and vimentin is estimated by immunocytochemistry and Western blotting.

Several tests are used to study cell migration. In one of the migration assay variations, after cells reach the monolayer, a streak is made across the bottom of the plate with the use of a cell scraper or pipette tip, and then the dynamics of cell migration and recovery confluency are assessed [97]. A Transwell cell migration assay is also quite popular. The bottom of Transwell inserts has pores that allow cells to move from the insert to the well of the plate. Thus, the assessment of cell migratory ability is based on the number of cells that have passed through the pores of the insert and reached the well of the plate [97]. Transwell inserts are also used to evaluate the ability of cells to invade. Before testing, the bottom of the insert is coated with solubilized extracellular matrix protein mixture Matrigel. During polymerization of

the matrix, a structure similar in composition and properties to the basement membrane is formed. As a result, the number of cells which are able to pass through Matrigel and end up in the main well correlates with their ability to invade [97].

The ability of cancer cells to initiate tumor growth is determined by the spheroid formation assay (for breast cancer cells – mammospheres). For this purpose, a suspension of single cells is placed in wells with hydrophobic coating of the bottom in a serum-free medium supplemented with growth factors or MammoCult medium. After 5–14 days, the number of formed spheroids with a diameter of 35–100 μm is assessed, depending on the cell line used and the design of the experiment [98, 99]. The formation of spheroids in this case is an *in vitro* prototype of metastasis. It is also possible to evaluate the ability of cells in the spheroids to self-renew by disaggregating primary spheroids and placing them again on low surface energy plastic in a serum-free medium for the formation of secondary and, similarly, tertiary spheroids [100]. To prevent migration of spheroids and their aggregation, spheroid formation in soft agar, a modification of the method, is used [101].

Several studies on the role of miRNAs in carcinogenesis are based on *in vivo* experiments on immunodeficient mice (nude, NOD / SCID). For a tumorigenicity assessment, cells transfected with miRNA mimics or genetically modified cell lines that express the target miRNA are used. After subcutaneous injection of cells, the rate of tumor growth, the histological structure of the tumor, and expression of markers of interest via immunohistochemical staining are evaluated [69, 71, 73, 75].

MicroRNAs IN THE REGULATION OF STEMNESS GENES

Over the past 5–7 years, microRNAs have been widely studied as a component of tumor suppressor and oncogenic pathways. There are separate studies on stemness gene regulation using miRNAs in tumor tissues of various localizations. *SOX2* knockdown inhibits invasion and migration of breast cancer cells via antisense RNA [8]. MiR-302b, P21, and miR-145 miRNAs suppress expression of stemness genes *SOX2* and *MYC* in intestinal and diffuse gastric cancer [102]. Overexpression of miR-145 inhibits stem cell transcription factors SOX2, NANOG, and OCT4 in cervical CSCs and also reduces tumor invasion and colony formation *in vitro*. Knockdown of miR-145 leads to a significant decrease in cervical tumor growth in nude mice [9].

It has been shown that miR-148a inhibits stemness properties of glioblastoma cells and the metastatic potential of glioma through inhibition of *SOX2* and *OCT4.* In addition, decreased expression of pre-miR-29a, pre-miR-181, pre-miR-let7b, and pre-miR-124a is observed in CSCs expressing SOX2 and OCT4. MiR-148a has been recognized as one of the most significant microRNAs that negatively regulate in response to co-expression of Oct4 and Sox2. MiR-148a expression inhibits induced Oct4 / Sox2 CSC phenotypes in glioma, including *in vitro* sphere-formation and tumor formation *in vivo* [103]. It was shown that targets for miR-221-3p and miR-221-5p are 3'-untranslated regions of mRNA transcripts in the main transcription factors maintaining pluripotency (OCT4, Nanog, and Sox2) in mouse embryonic cells [104]. MiR-296, miR-470, and miR-134, as well as many others, regulate mouse embryonic stem cells by inhibiting Nanog, OCT4, and Sox2 [105]. MiR-371-5p is capable of suppressing EMT associated with stemness induction via the Wnt / beta-catenin signaling pathway. MiR-371-5p reduces spheroid formation by colorectal cancer cells. Demethylation of the *Sox17* gene induces miR-371-5 expression, which is suppressed by Sox2 expression. The Wnt inhibitory factor 1 (WIF1) is a member of the protein family that binds Wnt and inhibits transmission of the Wnt signaling pathway. WIF1 stimulates miR-200c expression, which reduces the expression of pluripotency and stemness markers *OCT4, c-MYC, c-KIT*, and *MYB*, as well as *BMI1, ZEB1* and *ZEB2,* which results in an increase in the expression of E-cadherin [106].

CONCLUSION

Currently, there are microRNAs known for their ability to regulate expression of stemness genes in embryonic stem cells, as well as in CSCs in different localizations. Extensive research in this area has been conducted since tumor origin from CSCs was proven. Significant achievements have been made in this area, which hopefully will result in the development of drugs that will regulate the activity of CSCs and thereby control tumor growth. In general, the current stage of research involves accumulation of knowledge about the mechanisms of regulation of the metastatic cascade and identification of targets for the development of RNA-based drugs for suppression of metastasis.

Table

Methods for the analysis of microRNA effects on stemness plasticity, EMT stimulation, and reprogramming of differentiated cancer cells.

Gene	miR	Tumor location	Cell line	Gene expression	Effects	Method	Ref.
c-MYC	miR-340	Breast cancer	MDA-MB231	↓ RT, WB	↓ proliferation ↓ migration ↓ invasion	MTT assay Transwell migration assay Transwell migration assay in Matrigel	64
	miR-320a	Hepatocellular carcinoma	HepG2, QGY-7703	↓ RT, WB	↓ proliferation ↓ invasion	MTT assay Transwell migration assay in Matrigel	65
	miR-320a	Breast cancer	MCF-7, T47D	↓ RT, WB	↓ proliferation ↓ resistance to tamoxifen ↓ number of mammospheres	MTT assay CCK-8-test Mammosphere formation assay in soft agar	66
	miR-145	Pancreatic cancer	PDAC	↓ RT, WB	↓ migration	Migration test	67
	miR-34a	Pancreatic cancer	MiaPaCa2	↓ WB	↓ colony formation	Colony formation assay	68
SOX2	miR-590-5p	Breast cancer	MCF-7, ZR75-1	↓ RT, WB	↓ ALDH expression ↓ number of mammospheres ↓ tumorigenicity *in vivo*	Flow cytometry Mammosphere formation assay Xenotransplantation in NOD / SCID mice	69
	miR-590-5p	Non-small cell lung cancer	A549, H1299	↓ RT, WB	↑ expression of E-cadherin ↓ expression of vimentin, Snail, Slug	WB WB	70
	miR-101	Breast cancer	MCF-7, MDA-MB231	↓ RT, WB	↓ colony formation ↓ migration ↓ invasion ↓ proliferation ↑ apoptosis ↓ tumorigenicity *in vivo*	Colony formation assay Migration test Transwell migration in Matrigel MTT assay Flow cytometry Xenotransplantation in nude mice	71
	miR-129-5p	Breast cancer	MCF-7, MDA-MB231	↓ RT	↓ migration ↓ invasion ↑ apoptosis ↓ colony formation ↓ resistance to adriamycin	Migration test Transwell migration in Matrigel Flow cytometry Colony formation assay CCK-8 test	72
NANOG	miR-760	Breast cancer	MCF-7, BT-549	↓ RT, WB	↓ proliferation ↓ migration ↓ tumorigenicity *in vivo*	MTT assay Transwell migration assay Xenotransplantation in nude mice	73
	miR-142-3p	Breast cancer	MCF-7, MDA-MB468	↓ RT	↓ colony formation ↓ number of mammospheres ↑ apoptosis ↑ cells in G2M phase ↓ SOX2, ALDH, c-MYC expression	Colony formation assay Mammosphere formation assay Flow cytometry Flow cytometry WB	74

	miR-134	Glioblastoma	U87	↓ RT, WB	↓ invasion ↓ migration ↓ tumorigenicity *in vivo*	Transwell-migration in Matrigel Transwell-migration Migration test Xenotransplantation in nude mice	75
	miR-128a	Prostate cancer	PC3, DU145, PPC-1, LAPC9, LNCaP, VCAP, NHP9	↓ RT, WB	↓ proliferation ↓ invasion ↓ number of cells in the S-phase ↓ number of mammospheres ↓ self-renewal ↓ tumorigenicity *in vivo*	BrdU assay Transwell migration in Matrigel Flow cytometry Spheroid formation assay Formation of secondary spheroids Xenotransplantation in NOD / SCID mice	76
	miR-203	Breast cancer	MDA-MB231	↓ RT	↓ population of $CD44^+CD24^-$ stem cells	Flow cytometry	77
	miR-150	Breast cancer	MDA-MB231	↑ RT	↑ population of $CD44^+CD24^-$ stem cells	Flow cytometry	77
	miR-126	Prostate cancer	PC3, DU145, LNCaP	↑ RT	↑ proliferation	MTS assay	78
	miR-149	Prostate cancer	PC3, DU145, LNCaP	↑ RT	↑ proliferation	MTS assay	78
Oct4	miR-598	Stomach cancer	MKN-45	↓ RT, WB	↓ proliferation ↓ colony formation ↓ migration ↓ invasion ↑apoptosis	MTT assay Colony formation assay Migration test Transwell migration in Matrigel Flow cytometry	79
	miR-299-3p	Breast cancer	MDA-MB231, HT1080	↓ RT, WB	↓ invasion ↓ proliferation ↑ apoptosis	LOC-analysis CellToxGreen Flow cytometry	80
	miR-145	Lung adenocarcinoma	A549	↓ RT, WB	↓ invasion ↓ proliferation ↓ number of cells in S-phase ↓ $CD133^+$ cells ↓ number of tumor spheres	Transwell migration in Matrigel CCK-8 test Flow cytometry Flow cytometry Mammosphere formation assay	81
	miR-3658	Colorectal cancer	SW480	↓ RT, WB	↓ CyclinD1, PCNA expression ↓ proliferation ↓ migration ↓ Snail, Zeb1 expression ↑ Cloudin1, B-catenin expression ↑ proportion of cells in the SubG1 phase	WB MTT assay Migration test WB WB Flow cytometry	82

Note: ↑ – increase, ↓ – decrease, RT – Real-time reverse transcription PCR, WB – Western blotting.

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