

A microRNA expression signature characterizing the properties of tumor-initiating cells for breast cancer

LIXIN WANG¹, DAOQIANG ZHANG^{1,2}, CHANGWEN ZHANG¹,
SHUPING ZHANG¹, ZHE WANG¹, CHEN QU¹ and SIJIN LIU¹

¹State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085;

²Weifang Medical College, Wendeng Central Hospital, Weihai 264400, P.R. China

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Abstract. microRNAs (miRNAs) are involved in controlling tumor behaviors either as oncogenes or tumor suppressors. To elucidate the role of miRNAs in the regulation of tumor initiation, we delineated the microRNA expression signature characterizing the properties of tumor-initiating cells for breast cancer. A group of miRNAs were differentially expressed in MDA-MB-231 and SUM1315 cells (with a high proportion of breast cancer tumor-initiating cells, CD44⁺CD24^{-low} subpopulation) compared to MCF-7 cells (only a small proportion of CD44⁺CD24^{-low} cells). Among the differentially expressed miRNAs common to MDA-MB-231 and SUM1315, approximately 46% of them are suggested to regulate the 'stemness' of stem cells or progenitor cells. Taken together, these findings suggested that miRNAs contribute to the maintenance of tumor-initiating properties and indicate the potential value of the miRNA expression signature in characterizing or predicting the features (including metastasis) of breast cancer.

Introduction

The leading cause of death in breast cancer (BrCa) patients is not the primary tumor in the breast *per se*, but metastasis to distant organs. Metastasis accounts for over 90% of deaths in BrCa patients. Millions of cells are released from the primary

tumor into the blood circulation daily, but only a small minority of these cells survive and colonize on distant organs. The aim of the present study was to establish the identity of these cells. According to the current cancer stem cell hypothesis, tumors are organized in cell hierarchies composed of tumor-initiating cells (T-ICs), cancer cells and differentiated cells (1). T-ICs with stem cell-like properties, so-called cancer stem cells, are not only the source of the primary tumor; but are also responsible for tumor growth, metastasis and possible recurrence. The CD44⁺CD24^{-low} subpopulation in BrCa cells was reported to be enriched in BrCa T-ICs (2). This subpopulation of cells is capable of generating other cellular phenotypes observed in the original tumor and initiating metastases at distant sites from low numbers of cells (2). However, data on the characteristics of BrCa T-ICs and their molecular regulation is currently limited.

microRNAs (miRNAs) are small, non-coding RNAs that negatively regulate gene expression by binding to their target miRNAs. miRNAs regulate tumor development, prognosis and metastasis either as oncogenes or tumor suppressors (3,4). A burgeoning body of evidence showed that miRNAs modulate the properties of embryonic stem (ES) and tissue stem cells in a variety of eukaryotic organisms (5,6). A group of miRNAs may play specialized roles in stem cell regulation, as the Dicer (the enzyme that processes the pre-miRNAs into mature miRNAs) knockout in mice is embryonically lethal (7), and Dicer conditional knockout mouse ES cells grow more slowly than controls and fail to differentiate (8).

In the present study, we investigated the differences in miRNA expression profiling among BrCa cell lines with various proportions of T-ICs and the potential contribution of miRNAs to maintaining the 'stemness' of BrCa T-ICs. The results indicate that a miRNA expression signature may help identify the stemness of T-ICs, and that certain miRNAs may help predict clinical features, such as metastasis, for BrCa.

Materials and methods

Cell lines and cell culture. Human BrCa cell lines MCF-7, SUM149, SUM1315 and MDA-MB-231 were stored in the laboratory. The culture conditions for each cell line were identical to those used in our previous study (9).

Correspondence to: Professor Sijin Liu, State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, P.R. China
E-mail: sjliu@rcees.ac.cn

Abbreviations: miRNAs, microRNAs; BrCa, breast cancer; T-ICs, tumor-initiating cells; ES, embryonic stem; EC, embryocarcinoma; FACS, fluorescence-activated cell sorting; RT-PCR, reverse transcription polymerase chain reaction; RT, reverse transcription

Key words: microRNA, tumor-initiating cells, cancer stem cells, breast cancer, microarray

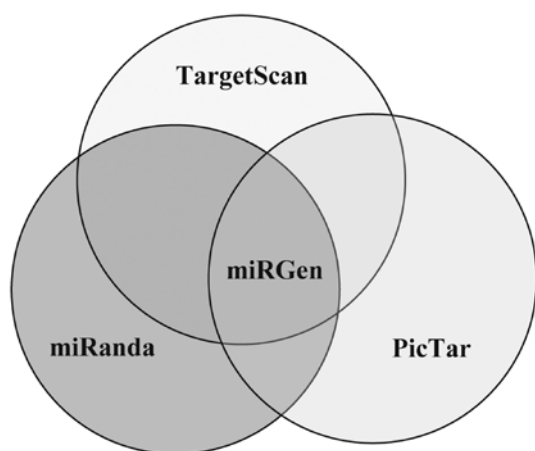


Figure 1. A summary of the online software miRGen for predicting target genes of miRNAs. miRGen provides the intersection of genes predicted by miRanda, TargetScan and PicTar.

Fluorescence-activated cell sorting (FACS) analysis. CD24 and CD44 expression of BrCa cells was examined by FACS analysis using anti-CD44 (FITC-conjugated) and anti-CD24 (PE-conjugated) antibodies as previously described (10). The two antibodies were obtained from BD Biosciences (San Jose, CA, USA).

miRNA microarray analysis. A MiRVana miRNA isolation kit (Ambion, Austin, TX, USA) was used to isolate total RNA with enriched miRNAs from cells according to the manufacturer's instructions. miRNA microarray was performed by LC Sciences (Houston, TX, USA; <http://www.lcsciences.com>) as previously described (9). The differentially expressed miRNAs in SUM1315 and MDA-MB-231 cells were those with a fold-change of >2 or <-2 and P-values of the t-test <0.05 compared to MCF-7 cells.

Reverse transcription polymerase chain reaction (RT-PCR) analyses. Total RNA with enriched miRNAs in BrCa cells was isolated as described above. Reverse transcription (RT) was performed using the RT primers for each individual miRNA from Ambion according to the manufacturer's instructions. Real-time PCR was performed using a miRVana qRT-PCR kit (Ambion) according to the manufacturer's instructions and the protocol used previously (11). PCR primers were purchased from Ambion.

miRNA selection and target gene prediction. To filter the miRNAs associated with stem cell-like properties, a search on various literature in the PubMed database was conducted using the keywords 'miR-xx' and 'stem cells' for each individual miRNA. Based on the literature, miRNAs clearly involved in stem/progenitor cell modulation were collected. Following that, the miRGen database (<http://www.diana.pcbi.upenn.edu/miRGen/v3/miRGen.html>) was used to predict target genes for selected miRNAs. The *Targets* interface of the miRGen database provides access to the union and intersection of the four widely used target prediction programs: miRBase, miRanda, TargetScan and PicTar. To obtain reliable target genes, the

intersection function (among miRanda, TargetScan and PicTar) was used to predict target genes (Fig. 1). The obtained gene list was further intersected with the target genes above the mean score from the PicTar prediction alone to obtain the final hits for each individual miRNA. Following this process, target genes documented to regulate stem cell-like properties were selected from PubMed.

Statistical analysis. The two-tailed Student's t-test was used to analyze the statistical significance of FACS and RT-PCR data. $P<0.05$ was considered to be statistically significant.

Results and Discussion

The prevalence of $CD44^+CD24^{-/low}$ cells in BrCa cell lines. To test the hypothesis that the prevalence of T-ICs in a BrCa cell population is associated with the metastatic phenotype of the BrCa cells, the proportion of the $CD44^+CD24^{-/low}$ subpopulation of cells was assessed in the tumorigenic/metastatic human BrCa cell lines, SUM1315 and MDA-MB-231, and the tumorigenic/non-metastatic human BrCa cell lines, MCF-7 and SUM149, by FACS analysis. Consistent with a previous report (12), the proportion of this subpopulation is relatively higher in SUM1315 (~60%) and MDA-MB-231 cells ($>90\%$) than in MCF-7 and SUM149 ones (0.05 and 3%, respectively; $P<0.001$) (Fig. 2A and B). The greater percentage of $CD44^+CD24^{-/low}$ cells in SUM1315 and MDA-MB-231 cells corresponds with the highly malignant and metastatic phenotypes of these cell lines (13,14). This finding is also consistent with our previous observation that these cell lines possess enhanced propensity to metastasize (9,13).

miRNA expression signature for tumor-initiating properties in BrCa cells. To investigate the role of miRNAs in the maintenance of BrCa T-ICs, total RNAs from MCF-7, SUM1315 and MDA-MB-231 cells were used as representatives to carry out a microarray analysis of miRNAs. The miRNA expression profiling was documented previously (9). There are 86 and 74 miRNAs that are differentially expressed (fold-change >2 or <-2 , $P<0.05$) in MDA-MB-231 and SUM1315 cells compared to MCF-7 cells, respectively, and there are 69 common miRNAs between the 86 and 74 differentially expressed miRNAs (9). Among the 69 miRNAs common to the two cell lines, approximately 46% ($n=32$) of them have been documented to regulate the stemness of stem/progenitor cells (Table I). The expression changes of miR-22, miR-93 and miR-203 were further validated by qRT-PCR (Fig. 3).

A recent study of miRNA expression of mouse mammary progenitor cells revealed a characteristic group of miRNAs: miR-22 and miR-205 are highly expressed, and miR-93 and let-7 are decreased in progenitor cells compared to differentiated cells (25). In agreement with this study, the miRNA profiling data obtained in the present study showed that miR-22 was induced and miR-93 was reduced in MDA-MB-231 and SUM1315 cells compared to MCF-7 cells (Table I and Fig. 3). This result correlates with the high percentage of BrCa T-ICs in MDA-MB-231 and SUM1315 cells (Fig. 2). miR-21, regulated by REST (RE1-silencing transcription factor; also known as NRSF), specifically suppresses the self-renewal of mouse embryonic stem (ES) cells (23). miR-125a, along with let-7,

Table I. miRNAs associated with stem cell-like properties.

miRNA	MDA-MB-231 vs. MCF-7 cells	SUM1315 vs. MCF-7 cells	Function description	Representative target genes
miR-375	↓↓↓↓↓↓↓	↓↓↓↓↓↓↓	Regulates human ES cell differentiation (15)	RLF, OTP, INSM1
miR-203	↓↓↓↓↓↓↓	↓↓↓↓↓↓↓	Tumor suppressor, and regulates stem cell differentiation (16,17)	EDN1
miR-200c	↓↓↓↓↓↓↓	↓↓↓↓↓↓↓	Suppresses the ability of normal mammary stem cells to form mammary ducts and tumors (18)	FN1, ZFHX1B
miR-7	↓↓↓	↓↓↓↓	Regulates differentiation of germline stem cell lineage (19)	COL2A1, GLI3, KLF4
miR-98	↓↓	↓↓	Regulates differentiation of bronchoalveolar stem cells (20)	E2F5, HOXA9, SEMA4C
miR-183	↓	↓↓↓	Suppresses expression of stem cell factors in cancer and mouse ES cells (21)	ABCA1, LRP6, ITGB1
miR-26b	↓↓↓	↓	Significantly decreased in human ES cells (22)	JAG1, NLK, Ezh2
miR-21	↓↓	↓	Suppresses the self-renewal of ES cells (23)	SATB1, RHOB, PITX2
miR-148b	↓	↓↓	Regulates human mesenchymal stem cell differentiation (24)	CANX, CSF1, DNMT1
miR-93	↓	↓	Depleted in mammary progenitor cells (25)	LATS2, TXNIP, ARID4B
miR-125a	↓	↓	Regulates neural differentiation of stem cells (26)	TAZ, GPC4, PLAGL2
miR-106b	↓	↓	Altered expression in bronchoalveolar stem cells (27)	ARID4B, BCL2L2, EPHA4
miR-103	↓	↓	Modulates the self-renewal of mesenchymal stem cells (28)	BTG2, CDK6
miR-107	↓	↓	Modulates the self-renewal of mesenchymal stem cells (28)	OGT, RBM24, CDK6
miR-128	↓	↓	Promotes neural stem cell self-renewal (29)	BMI-1
miR-26a	↓	↓	Modulates the osteogenic differentiation of human adipose tissue-derived stem cells (30)	SMAD1, HOXA5, JAG1
miR-20a	↑	↑	Differentially expressed in developing mouse embryos, and controls differentiation of stem cells (31)	ABCA1, BCL11B, CD69
miR-23b	↑	↑	Regulates liver stem cell differentiation by targeting Smads (32)	PKNOX1, SRC1, ZIC1
miR-23a	↑	↑	Increased during replication and senescence of human cord blood-derived multipotent stem cells (33)	ELF5, SRC1
miR-92b	↑	↑	Controls the G1/S checkpoint gene p57 in human ES cells (34)	p57
miR-24	↑	↑↑	Inhibits endodermal differentiation of human ES cells (35)	CDX2
miR-22	↑↑	↑	Highly expressed in mammary progenitor cells (25)	LGALS1, PLAGL2, MECP2
miR-19a	↑↑	↑	Up-regulated by activin A in human ES cells (36)	CD164, ARHGAP1, WNT3
miR-27a	↑↑	↑↑	Reduces the differentiation of human mesenchymal stem cells (24)	HOXB8, APRIN, SEMA4C
miR-19b	↑↑	↑↑	Down-regulated by activin A in human ES cells (36)	NEUROD1, WNT3, CCNT2
miR-29c	↑↑↑	↑↑↑	Up-regulated in the course of replicative senescence of mesenchymal stem cells (37)	RLF, TRAF4, PHC1
miR-99a	↑↑↑	↑↑↑↑	Differentially expressed in human mesenchymal stem cells (38)	HOXA1
miR-29a	↑↑↑↑	↑↑↑↑	Highly expressed in hematopoietic stem cells and down-regulated in hematopoietic progenitors (39)	RLF, GDF8, TRAF4
miR-125b	↑↑↑↑	↑↑↑↑	Critical for the suppression of human U251 glioma stem cell proliferation (40)	GPC4, TAZ, MSI1

Table I. Continued.

miRNA	MDA-MB-231 vs. MCF-7 cells	SUM1315 vs. MCF-7 cells	Function description	Representative target genes
miR-100	↑↑↑↑↑↑↑↑	↑↑↑↑↑↑	Modulates differentiation of mouse ES cells (41)	SMARCA5, HOXA1
miR-222	↑↑↑↑↑↑↑↑	↑↑↑↑↑↑↑↑	Highly expressed at early developmental stages in the embryonic retina (42)	MAP3K10, PBX3
miR-221	↑↑↑↑↑↑↑↑↑↑	↑↑↑↑↑↑↑↑	Controls proliferation and differentiation of CD34-positive hematopoietic progenitor cells (43)	MAP3K10, CTCF

Fold changes: ↓↓↓↓↓↓↓↓, -512 to -256; ↓↓↓↓↓↓↓, -256 to -128; ↓↓↓↓↓↓, -128 to -64; ↓↓↓↓↓, -64 to -32; ↓↓↓↓, -32 to -16; ↓↓↓, -16 to -8; ↓↓, -8 to -4; ↓, -4 to -2; ↑, 2 to 4; ↑↑, 4-8; ↑↑↑, 8-16; ↑↑↑↑, 16-32; ↑↑↑↑↑, 32-64; ↑↑↑↑↑↑, 64-128; ↑↑↑↑↑↑↑, 128-256; ↑↑↑↑↑↑↑↑, 256-512; ↑↑↑↑↑↑↑↑↑, 512-1024.

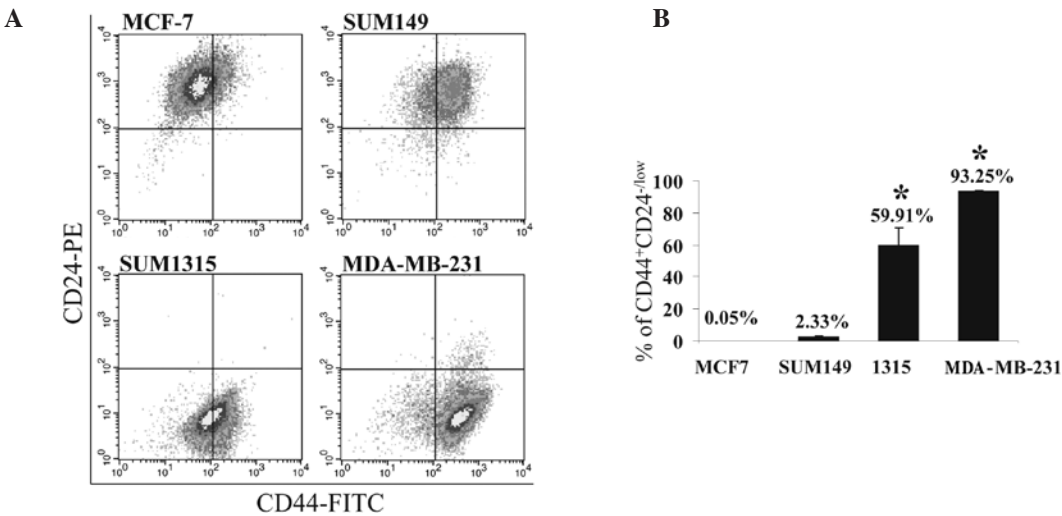


Figure 2. The proportion of T-ICs in BrCa cell lines. (A) FACS profiling of CD44 and CD24 expression in MCF-7, SUM149, SUM-1315 and MDA-MB-231 cells. (B) The average percentages of CD44⁺CD24^{-low} subpopulation in cells described above. Results are presented as the mean ± SEM (n=5-6). *P<0.001, compared with MCF-7 and SUM149 cells.

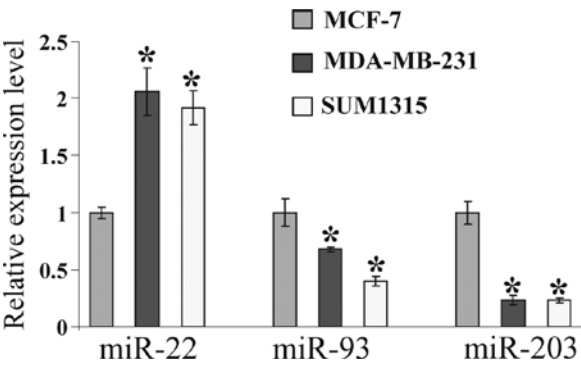


Figure 3. Real-time RT-PCR assays of miR-22, miR-93 and miR-203 expression. The relative expression level of miR-22, miR-93 and miR-203 in MCF-7, SUM1315 and MDA-MB-231 cells. Results are presented as the mean ± SEM (n=3). *P<0.05, compared with MCF-7 cells.

induces stem cell commitment by regulating the expression of the mammalian lin-28 (a marker for pluripotency in ES cells) (26,44) and its expression is significantly increased during the neural differentiation of ES and embryocarcinoma (EC) cells (26,44). The downregulation of miR-21 and

miR-125a (Table I) parallels the enriched progenitor cells in MDA-MB-231 and SUM1315 cells compared to MCF-7 cells (Fig. 2). miR-203 has been shown to control the keratinocyte differentiation of epithelial progenitor cells (16) and to be a tumor suppressor (17). This miRNA was depleted in a number of hematopoietic tumors due to hypermethylation, while its re-expression is capable of reducing tumor cell proliferation (17). Its expression was found to be markedly reduced in MDA-MB-231 and SUM1315 cells compared to MCF-7 cells (Table I and Fig. 3). miRNA depletion in metastatic MDA-MB-231 and SUM1315 cells correlates with the more aggressive phenotype of these cells compared to non-metastatic MCF-7 cells (14).

To shed light on the potential role of differentially expressed miRNAs in modulating tumor-initiating features, an analysis of target gene prediction was performed using the online miRGen database. Most miRNAs were predicted to have numerous target genes. miRNAs such as miR-99b, miR-200c and miR-7 have tens of predicted target genes, whereas other miRNAs (e.g., miR-148b, miR-375 and miR-20a) have hundreds of predicted target genes. Representative genes involved in the stemness are shown in Table I. LATS2, a potential target gene of miR-93, is

essential for embryonic development, proliferation control and genomic integrity (45). A possible target gene of miR-21, SATB1, contributes to embryonic stem cell differentiation and regulates Nanog expression (46). As a likely target gene of miR-22, PLAGL2 restrains differentiation in neural stem cells (47). Although the published data on miRNA profiles in T-ICs are limited, evidence suggests that certain miRNAs play a significant role in the generation and maintenance of T-ICs. In *C. elegans*, mutation of *lin-4* and *let-7* miRNAs results in the generation of stem cell-like cells (48). Mammalian *let-7* expression is depleted in human BrCa T-ICs and significantly increased in differentiated cells. *Let-7* suppresses self-renewal by targeting H-RAS and induces differentiation by targeting HMGA2 (10). The data from the present study further support the hypothesis that miRNAs are involved in the regulation of BrCa T-ICs. Future studies of the aberrantly expressed miRNAs in BrCa T-ICs may elucidate the mechanisms responsible for tumorigenesis, metastasis and cancer relapse, and may identify therapeutic molecular targets for anti-cancer drug discovery.

Taken together, these results indicate that BrCa cells with a higher prevalence of T-ICs possess distinct miRNA expression profiles that may contribute to the maintenance of stem cell properties. The mechanism by which miRNAs modulate BrCa T-ICs remains unknown. Moreover, our study suggests the potential diagnostic value of the miRNA expression signature in BrCa, including metastasis, although clinical evidence is required to confirm this hypothesis.

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