SOX9 is a Target of miR-134-3p And miR-224-3p in Breast Cancer Cell Lines

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Abstract

SOX9 represents a transcription factor identified as an important mediator of cancer progression in breast cancer. miRNAs are small non-coding RNAs that can inhibit translation of target genes by binding to the 3'-UTR region of the respective mRNA molecule. Deregulated miRNA expression plays a pivotal role in hallmarks of cancer such as sustained proliferation and inhibition of apoptosis. In this study we investigated the miRNA-mediated regulation of SOX9 expression in two breast cancer cell lines providing further insights into cellular mechanisms driving breast cancer progression. The effect of miR-134-3p, miR-224-3p and miR-6859-3p on SOX9 expression was tested on mRNA as well as protein level in the human breast cancer cell line MDA-MB-231. Furthermore, direct binding of these miRNAs to the SOX9 3'-UTR was assessed by luciferase reporter assays and site-directed mutagenesis in MDA-MB-231 and MCF-7 cells. SOX9 expression was significantly reduced on mRNA and protein level by transfection of either miR-134-3p, miR-224-3p or miR-6859-3p. Luciferase reporter assays proved direct binding of miR-134-3p and miR-224-3p to the SOX9 3'-UTR in MDA-MB-231 and MCF-7 cells. Expression analysis performed *in silico* revealed reduced expression of both miRNAs in breast cancer tissues. We describe three novel miRNAs capable of targeting SOX9 in human breast cancer cell lines. For miR-134-3p and miR-224-3p direct interaction with the SOX9 3'-UTR was proven. Furthermore, miR-134-3p and miR-224-3p reduced breast cancer cell viability, which is in line with the tumorigenic effects reported for SOX9 in breast cancer.

Background

So far, 20 members of the SOX protein family have been described. Within this family, the SRY-Box Transcription Factor 9 (SOX9) belongs to the SoxE subgroup binding with its HMG domain to DNA regions containing the AACAAAT motif [1, 2]. SOX9 can form complexes with other SOX proteins or can associate with transcription factors such as members of the GLI zinc fingers family. Only complexed SOX9 can bind to respective target sites within the DNA. Depending on its intracellular binding partners and the target site within the DNA, SOX9 can function either as a transcriptional repressor or activator [2]. SOX9 was found highly expressed in different cancer types and its expression is associated with unfavorable clinical outcome [3]. Especially in breast cancer, SOX9 was identified as an important driver of cancer progression and has thus been referred to as “master regulator” of breast cancer cell fate [4]. In triple-negative breast cancer, representing the breast cancer subtype with worst prognosis, SOX9 is highly expressed together with SOX4, 6, 8, 10 and 11 [5]. Silencing of SOX9 reduced breast cancer cell viability as well as invasion *in vitro* and *in vivo* by enhancing the expression of apoptosis related genes such as FADD, while decreasing the expression of genes involved in epithelial–mesenchymal transition like ZEB1 or CTNNB1 [5]. Furthermore, high expression of SOX9 is associated with cancer stem-like cells [6-8].

miRNAs belong to the group of small non-coding RNAs with a size of approximately 20 nucleotides functioning as regulators of gene expression on post-transcriptional level. Argonaute proteins (AGO) are small RNA binding proteins involved miRNA processing and function. Among the four types of AGO proteins expressed in humans, AGO2 facilitates miRNA mediated repression of translation by targeting respective mRNA molecules [9, 10]. Upon association with AGO proteins, miRNAs form the RNA-induced

silencing complex (RISC) that binds via the 5 to 6 nucleotides comprising seed region to the 3'-UTR region of target mRNA molecules. Depending on the degree of complementary base pairing between the miRNA and its target mRNA, this will result in inhibited translation or degradation of the mRNA molecule [11-14]. Perfect binding of the miRNA to its target site activates the endonuclease activity of AGO2 resulting in target mRNA cleavage [14, 15]. Potentially, each miRNA can bind to several hundred different mRNA targets and each mRNA can be targeted by multiple miRNA species [14, 16, 17].

Cancer cells generally exhibit aberrant miRNA expression patterns [18], therefore miRNA expression profiling may gain clinical relevance, as miRNA expression patterns thus identified might resemble biomarkers in cancer therapy [18-21]. Understanding miRNA mediated regulation of gene expression in cancer cells might provide deeper insights into mechanisms up-regulating oncogenic genes, for example, by inhibited expression of tumor suppressive miRNAs or through enhanced expression of oncogenic miRNAs (OncomiRs) [22, 23]. In this study we investigated how expression of the “master regulator” of breast cancer cells, SOX9, can be inhibited by miRNAs.

Materials And Methods

Cell lines and cell culture

The human breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from ATCC. HEK293 cells were provided by the DKFZ. MDA-MB-231 and HEK293 cells were cultured in RPMI 1640 medium (Gibco, Carlsbad, CA), while MCF-7 cells were cultured in DMEM medium. The medium was supplemented with 10% FCS superior (Biochrom, Berlin, Germany) without antibiotics and cells were cultured at 37 °C and 5 % CO₂. Cells were regularly checked for mycoplasma contamination and authenticity of cell lines was verified by DNA fingerprinting.

Transfection, RNA extraction and qPCR

Cells were seeded in 12 well plates and cultured until 70-80% confluency was achieved. Subsequently, cells were transfected with 50 nM miRNA using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. Two days post transfection cells were harvested, and RNA was isolated using Qiagen RNeasy mini kit. RNA samples were measured using a Qubit™ 4 Fluorometer (Thermo Scientific, Boston, MA). A total of 500 ng RNA was reverse transcribed in a 20 µL reaction utilizing oligo(dT)18 primer using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturers protocol. All PCRs were run on a Veriti 96 well Thermal Cycler (Applied Biosystem, Foster City, CA). qPCRs were performed using primers listed in Supplementary Table S3 and PowerUp SYBR Green Mastermix (Thermo Scientific, Boston, USA). qPCRs were run on a QuantStudio 3 Real-Time-PCR System (Thermo Scientific). The ribosomal protein L19 (RPL19) encoding gene was used as house-keeping gene. For all samples, three technical replicates

were performed, and relative expression was calculated with the $2^{-\Delta\Delta CT}$ method relative to RPL19 according to the manufacturer's protocol.

Western Blot

Total protein was isolated from frozen cell pellets by dissociation with 200 μ L cell lysis buffer (Cell Signaling Technology, Cambridge, UK) supplemented with 1 mM phenylmethylsulfonylfluorid (PMSF). After 5 min incubation on ice, samples were centrifuged for 30 min at 13,000 rpm, 4 °C. Protein concentrations were measured using Qubit 4 Fluorometer (Thermo Scientific) and cell lysates were stored at -20 °C. Lysates were mixed with 5 x loading dye and heated for 5 min at 95 °C to denature cellular protein. A total of 20 μ g protein were loaded per slot on a 12 % polyacrylamide gel and separated by electrophoresis followed by electro-transfer onto a nitrocellulose membrane (Bio-Rad, Richmond, VA). After washing with TBS-T, the membrane was blocked for 1 hour using 5 % milk in TBS-T. The membrane was then incubated with the SOX9-specific primary antibody (AB5535, Sigma-Aldrich, St. Louis, MO) in 0.5 % blocking solution in TBS-T at 4 °C overnight. After extensive washing (four times 5 minutes with TBS-T) the membrane was incubated with the respective horseradish peroxidase-conjugated secondary antibody (SC-2054, Santa Cruz Biotechnology, Heidelberg, Germany) for one hour at room temperature. Followed by washing with TBS-T, protein bands were detected with an enhanced chemiluminescence (ECL) system (GE Healthcare, Buckinghamshire, UK) using a BioRad ChemiDoc XRS device. Densitometric quantification of protein bands was performed using ImageJ software. Actin was used as a positive control and the SOX9 protein level was normalized by division with actin protein expression levels. Full-size images of Western blots can be found in the supplements.

Luciferase-reporter assays

The pLS-SOX9 plasmid containing the SOX9 3'-UTR fused to renilla luciferase reporter gene was purchased from Active motif (Active motif, La Hulpe, Belgium). In reporter assays, 100 ng pLS-vector were co-transfected with 50 nM miRNA using the DharmaFect Duo transfection reagent (GE Dharmacon, Lafayette, CO) according to the manufacturer's protocol into cells seeded in 96-well cell culture plates on day prior to transfection. One day after transfection, luciferase signals were measured using Lightswitch Luciferase Assay kit (Active Motif, La Hulpe, Belgium) according to the manufacturer's protocol. Luminescence signal was assessed with CLARIOstar Plus reader (BMG LABTECH, Ortenberg, Germany). To verify direct binding, mutation of miRNA binding site within the SOX9 3'-UTR was performed. Therefore, the Quick-change mutagenesis II kit (Qiagen, Hilden, Germany) was used according to the manufacturer's protocol. Primers used for mutagenesis are listed in Supplementary Table 1.

Gene expression profiling

Gene expression profiling was performed on MDA-MB-231 cells transfected with mimic control-1, miR-134-3p, miR-224-3p and miR-6859-3p. Therefore, 2×10^5 cells/well cells were transfected with 50 nM miRNA in 12-well plate format, and 48 h post transfection RNA was isolated and sent to the DKFZ-Genomics and Proteomics Core Facility for microarray analysis using Affymetrix Clariom S human chip for all samples. Each condition was performed in triplicates. Processing of raw data was carried out by the Core Facility. Differential gene expression analysis was based on comparison to mimic control-1 samples. Venn diagrams were generated with webtool from Bioinformatics & Evolutionary Genomics Department of the Ghent University (<http://bioinformatics.psb.ugent.be/webtools/Venn/>). Gene-Set-Enrichment analysis was performed on genes significantly down- or up-regulated for all three investigated miRNAs using the GSEA-MSigDB webtool (<http://www.gsea-msigdb.org/gsea/index.jsp>) [24, 25].

Cell viability / proliferation assay

Cells were seeded in 96-well transparent culture plates to achieve 70-80 % confluence on the day of transfection. Twenty-four hours later, 50 nM miRNAs or siRNAs (mimic control-1, miR-134-3p, miR-224-3p, miR-6859-3p) were transfected. Cell viability assays were performed using XTT dye (Cell Signaling Technology, Cambridge, UK). 72 h post transfection cells were washed and incubated with XTT reagent at 37°C for 1 hour. The absorbance at 450 nm was measured using ClarioStar Plus reader (BMG LABTECH, Ortenberg, Germany).

Cell cycle analysis

MDA-MB-231 cells were seeded in 12 well plates and cultured until 70-80% confluency was reached at the day of transfection. Cells were transfected with 50 nM miRNA. After 72 hours of incubation, the medium was discarded and the cells were washed with phosphate-buffered saline (PBS), detached with trypsin, and washed once with cold PBS. Then, 10 mL cold 70% ethanol was added to the cell pellets dropwise while gently vortexing. The pellets were stored at -20 °C for 48 h before FACS analysis. At the day of cell cycle analysis using a FACS Canto II flow cytometry system (Becton Dickinson, Franklin Lakes, NJ), cells were washed three times with PBS, and stained with 100 µL of propidium iodide (PI) staining solution (50 µg/mL PI; 0.5 µg/mL RNase A) for 1 h at RT protected from light. Cell cycle stages were determined using FlowJo software.

miRNA expression level in breast cancer patients

Expression of miR-134 and miR-224 in breast cancer tissues of patients was assessed with OncomiR Cancer data base [26]. Analysis of miR-134 and miR-224 expression was based on 87 samples from normal tissue and 782 or 695 breast cancer derived samples, respectively. No data were available for miR-6859.

Results

miR-134-3p, miR-224-3p and miR-6859-3p are predicted regulators of SOX9 expression

We screened for miRNAs with known inhibitory effect on breast cancer cell proliferation, bearing a seed sequence specific for the 3'-UTR of the SOX9 encoding mRNA. Upon literature search we found miR-224-3p published to inhibit proliferation of MDA-MB-231 cells [27], and furthermore, based on the webtool miRmap [28], the SOX9 3'-UTR was predicted to contain one binding site for miR-224-3p seed region (see Supplementary Figure S1). Moreover, we found miR-134-3p described to be down-regulated in breast cancer [29] and of note, its overexpression was reported to reduce breast cancer cell proliferation by targeting apoptosis-inhibiting genes like BCL2 [30]. In addition, the 3'-UTR of the SOX9 mRNA exhibits one potential binding site for miR-134-3p (see Supplementary Figure S1). We therefore selected miR-134-3p and miR-224-3p for further analysis as well as miR-6859-3p, the latter predicted to bind through its seed region to the SOX9 3'-UTR.

miR-134-3p, miR-224-3p and miR-6859-3p mediated down regulation of SOX9 expression is detectable on mRNA and protein level in MDA-MB-231 cells

At first, we tested whether the selected miRNAs could reduce intracellular SOX9 mRNA levels. Therefore, we selected the triple negative breast cancer cell line MDA-MB-231 with sustained SOX9 expression. Two days after transfection with 50 nM miRNA we measured SOX9 mRNA levels by qPCR. As depicted in Figure 1a, all three miRNAs significantly reduced SOX9 mRNA levels with miR-134-3p showing the most significant effect (mean $\log_2(\text{FC}) = -0.61$, $p < 0.0001$). miR-224-3p had overall the strongest effect on SOX9 mRNA levels (mean $\log_2(\text{FC}) = -0.69$, $p = 0.0006$). miR-6859-3p induced variable effects on SOX9 mRNA levels with $\log_2(\text{FC})$ fold changes ranging between -1.88 and 0.02. Still, miR-6859-3p significantly reduced SOX9 mRNA levels in this cell line (mean $\log_2(\text{FC}) = -0.63$, $p = 0.02$).

Next, we examined whether the effect of the selected miRNAs on SOX9 protein expression and performed Western blot analysis on transfected MDA-MB-231 cells. Consistent with the qPCR data, miR-224-3p mediated the strongest effect also on SOX9 protein expression (mean $\log_2(\text{FC}) = -1.3$, $p = 0.0015$) (Figure 1b, c). Moreover, miR-134-3p significantly reduced SOX9 protein levels (mean $\log_2(\text{FC}) = -0.84$, $p = 0.0166$), whereas miR-6859-3p decreased SOX9 protein expression only to insignificant extent (mean $\log_2(\text{FC}) = -0.47$, ns).

miR-134-3p and miR-224-3p function through direct interaction with the SOX9 3'-UTR in breast cancer cell lines

According to the miRmap tool, each of the miRNAs investigated has one binding site for the SOX9 3'-UTR. Thus, the observed inhibitory effects on SOX9 mRNA and protein levels are likely caused by direct interaction of these miRNAs with the SOX9 3'-UTR, thereby leading to a block in translation and to destabilization of SOX9 mRNA. To prove direct interaction, we performed luciferase reporter assays using a pLS-SOX9 plasmid harbouring the SOX9 3'-UTR fused to the renilla luciferase gene (Supplementary Figure S2). Thus, binding of a miRNA to the SOX9 3'-UTR would inhibit luciferase expression resulting in reduced luminescence signals. We co-transfected MDA-MB-231 cells with 50 nM miRNA and pLS-SOX9 vector and measured luminescence intensity 24 h post transfection. To exclude possible viability effects caused by the transfected miRNAs *per se*, we monitored the cell viability of MDA-MB-231 cells by XTT assays performed under the same conditions as applied during the reporter assay (see Supplementary Figure S3). We observed no effect on cell viability by the transfected miRNAs in this setting. Regarding direct binding of the miRNAs to the SOX9 3'-UTR we found that miR-6859-3p reduced the luciferase signal with pLS-SOX9 in MDA-MB-231 cells by 77 % ($p < 0.0001$) (Figure 2a). Also, miR-224-3p ($p < 0.0001$) and miR-134-3p ($p = 0.0003$) significantly diminished the luminescence signal by 64 % and 47 %, respectively. To determine, whether direct binding of miR-134-3p, miR-224-3p and miR-6859-3p to the SOX9 3'-UTR was cell line dependent, we repeated the luciferase assay with an additional breast cancer cell line, MCF-7 and with human embryonic kidney cells (HEK 293). In accordance with the effects observed in MDA-MB-231 cells, we confirmed decreased luminescence signal intensity in MCF-7 and in HEK293 cells to be caused by miR-134-3p and miR-224-3p (Figure 2b, c), suggesting direct SOX9 targeting by these miRNAs across various breast cancer cell lines and HEK293 cells. For miR-6859-3p we noted a significant reduction of the luciferase signal in MCF-7 cells, however, this was not observed in HEK293 cells. An independent repetition of this assay with MDA-MB-231 and MCF-7 cells is shown in Supplementary Figure S4.

Next, direct binding of the miRNA to the SOX9 3'-UTR was investigated through site specific mutagenesis. Thus, for miR-134-3p, miR-224-3p and miR-6859-3p we generated individual nucleotide deletions at three different positions within the respective miRNA targeting sites of the SOX9 3'-UTR. The primer sequences used to insert the deletions are listed in Supplementary Table S3. Upon transfection of miR-134-3p a significant reduction of the luminescence activity was measured with the non-mutated plasmid, whereas deletions at position 1484 and 1486 restored the luciferase signal completely or close to the level of mimic control samples, respectively (Figure 3a). miR-224-3p caused a significant reduction in luciferase signal for the non-mutated pLS-SOX9 vector, which is in line with the results shown in Figure 2. Deletion of positions 474 and 475 respectively, not only restored the luciferase signal, but even led to an increased signal compared to mimic control (Figure 3b). Deletion at position 476 could not restore luciferase signal and miR-224-3p had a significant inhibitory effect comparable to the wild-type plasmid. To analyze direct binding of miR-6859-3p we used reporter constructs with deletions at the positions 683, 684 or 685 within the SOX9 3'-UTR. None of the deletions could restore the luciferase signal and the effects were still comparable to those observed after co-transfection of the construct containing the wild-type SOX9 3'-UTR (Figure 3c).

In summary, we prove direct binding of miR-134-3p and miR-224-3p to the SOX9 3'-UTR as underlying mechanism of their repressive effects on SOX9 expression. However, the observed effects of miR-6859-3p on SOX9 expression are most likely caused by indirect mechanisms.

miR-134-3p and miR-224-3p affect cell cycle processes in MDA-MB-231 cells

To determine the overall effects of miR-134-3p, miR-224-3p and miR-6859-3p in MDA-MB-231 cells, we performed gene expression profiling 48 h after individual miRNA transfection. Figure 4 shows the overlap of genes significantly down-regulated by these three miRNAs. The impact on SOX9 expression was significant as well (see Supplementary Figure S5), however, the fold change of SOX9 expression, especially in the case of miR-6859-3p, did not range among the genes maximally impacted. Genes collectively down-regulated ($FC < 0.5$) were selected for subsequent Gene-Set-Enrichment analysis. The enriched GO-terms and corresponding genes are listed in Table 1. In particular, pathways related to chromatin and its organization were enriched. Overall, miR-224-3p and miR-134-3p were more similar in their down-regulating expression pattern and shared 39 strongly decreased genes (Figure 4). We also performed pathway enrichment analysis for the genes exclusively inhibited by miR-134-3p and miR-224-3p, neglecting miR-6859-3p. These results are summarized in Table 2. Here, many GO-terms related to cell cycle, DNA repair and replication were significantly enriched. We also analyzed common up-regulated genes upon miRNA transfection (Supplementary Figure S6). Only the four genes RHOB, CHRNA1, MYO5B and TRIM21 showed significantly enhanced expression ($FC > 2$) for all three miRNAs and no enriched pathways were determined for these genes. RHOB is involved in apoptotic processes following DNA damage [31]. Its expression was strongly increased upon transfection of miR-134-3p ($FC = 3.2$) and miR-224-3p ($FC = 4.8$), respectively. Also, miR-6859-3p enhanced RHOB mRNA levels, albeit to lower extent ($FC = 2.1$).

Since the microarray data pointed towards an inhibitory function of miR-134-3p and miR-224-3p on cell cycle and proliferation, we performed cell viability assays and cell cycle analyses in transfected MDA-MB-231 cells. miR-134-3p and miR-224-3p significantly decreased the cell viability of MDA-MB-231 cells 72 h post transfection, whereas miR-6859-3p had no effect on viability compared to mimic control transfections (Figure 5a). Regarding cell cycle analysis, miR-134-3p, miR-6859-3p and miR-224-3p transfections resulted in a smaller percentage of cells within G2 phase ($\%G2_{miR-134} = 1.95$, $\%G2_{miR-6859} = 2.57$, $\%G2_{miR-224} = 2.82$) compared to mimic control transfection ($\%G2_{control} = 5.02$). However, transfection with miR-134-3p or miR-224-3p led to a higher proportion of cells in G1 phase ($\%G1_{miR-134} = 94.3$, $\%G1_{miR-224} = 91.6$) in comparison to the control ($\%G1_{control} = 87.9$). The proportion of cells in G1 phase after miR-6859-3p transfection was similar to the control ($\%G1_{miR-6859} = 86.6$). To note, upon miR-6859-3p transfection twice as many cells were in S phase ($\%S_{miR-6859} = 10.46$) when compared to control.

Expression of miR-134-3p and miR-224-3p is reduced in breast cancer samples

We then assessed the clinical relevance of the investigated miRNAs and compared their expression levels in breast cancer specimens to normal breast tissue. The data were obtained from OncomiR Cancer data base. miR-134 and miR-224 expression levels were found to be significantly decreased in breast cancer samples compared to normal tissue, as shown in Figure 6. Overall, miR-134 levels were higher than those of miR-224. The mean expression for miR-134 was 40 % lower compared to normal tissue ($\text{Normal}_{\text{miR134}} = 652$, $\text{BC}_{\text{miR134}} = 406$, $p = 0.0001$). miR-224 level was reduced about 45 % compared to normal tissue ($\text{Normal}_{\text{miR224}} = 104$, $\text{BC}_{\text{miR224}} = 57$, $p = 0.0005$). For miR-6859 no expression data was available.

Discussion

This paper shows for the first time the inhibitory effect of miR-134-3p, miR-224-3p and miR-6859-3p on SOX9 expression in human breast cancer cell lines. The suppressive effect on gene expression brought about by these miRNAs could be demonstrated on mRNA and protein level. Overall, miR-6859-3p had the lowest impact on SOX9 expression; still, in the reporter assays, miR-6859-3p showed inhibitory effects in both breast cancer cell lines tested, however not in HEK293 cells. Although miR-6859-3p was predicted to bind to the SOX9 3'-UTR, we were unable to prove direct binding in our assays using constructs containing site-specific point mutations. This strongly suggests that miR-6859-3p dependent repression of SOX9 expression is mediated indirectly and might offer an explanation for the different results observed with miR-6859-3p in the luciferase assays performed with the breast cancer cell lines and HEK293 cells, respectively, pointing towards a cell type specific function of this miRNA.

Seeking explanations why the disruption of the miR-6859-3p binding site within the SOX9 3'-UTR had no effect, we focused on the predicted secondary RNA structure (Supplementary Figure S7). Interestingly, while the putative binding sites for miR-134-3p and miR-224-3p are located in unpaired regions, the predicted binding site for miR-6859-3p is found in a paired region within a loop structure, suggesting that binding of miR-6859-3p within SOX9 3'-UTR was prevented by structural hindrance. We therefore conclude, that miR-6859-3p suppresses SOX9 expression indirectly, e.g. by repressing a transcriptional activator of SOX9 or by altering endogenous miRNA expression. Interestingly, we found the gene MIR99AHG, which encodes the miR-99a/let-7c/miR-125b-2 miRNAs, to be significantly enhanced upon miR-6859-3p transfection in MDA-MB-231 cells. Both, miR-99a-3p and miR-125b-2-3p have a binding site for the SOX9 3'-UTR (see Supplementary Figure S8). Based on our microarray data and on correlation analysis using the NCI-60 data set [32], we identified PPARG as a transcription factor whose expression was negatively correlated with MIR99AHG mRNA levels and also repressed upon miR-6859-3p transfection (see Supplementary Figure S9). Indeed, PPARG has a binding site within MIR99AHG promoter and might be a transcriptional repressor of this miRNA gene. Since miR-6859-3p is not predicted to bind to PPARG 3'-UTR, we looked for transcription factors significantly reduced in miR-6859-3p transfected MDA-MB-231 cells. We found KLF5, a known transcriptional activator of PPARG [33, 34], to be

repressed by miR-6859-3p transfection. Since miR-6859-3p has a potential binding site for KLF5 3'-UTR, this miRNA might inhibit KLF5 expression causing lower levels of PPARG, which in turn enhances MIR99AHG expression and endogenous levels of miR-99a-3p as well as miR-125b-2-3p. The latter miRNAs potentially block SOX9 expression via binding to the SOX9 3'-UTR. This could explain the observed significant reduction in luciferase reporter assay in the two breast cancer cell lines tested upon miR-6859-3p transfection. To note, HEK293 cells exhibit very low levels of KLF5, which could explain the different effects detected among the breast cancer cell lines and HEK293 cells in the luciferase assays. Interestingly, KLF5 also has a potential binding site for the SOX9 promoter and its expression is positively correlated with SOX9 mRNA levels (see Supplementary Figure S9). But to date, this interaction has not been experimentally validated. Also, PPARG has a binding site within SOX9 promoter and in HCT-116 cells a significant increase in SOX9 levels upon treatment with rosiglitazone, a PPARG activator, was observed [35]. Thus, miR-6859-3p might inhibit transcriptional activation of SOX9 by reducing KLF5 and further downstream also of PPARG levels. Additionally, miR-6859-3p mediated inhibition of KLF5 expression might also enhance expression of the SOX9 targeting miRNAs miR-99a-3p and miR-125b-2-3p indirectly. This hypothesis together with the findings of our study are illustrated in Figure 7.

Regarding miR-224-3p and miR-134-3p we could prove that their SOX9 inhibitory effect was mediated via direct binding to the SOX9 3'-UTR. Furthermore, miR-134-3p and miR-224-3p transfection reduced the viability of MDA-MB-231 breast cancer cells and gene expression profiling confirmed the impact of these miRNAs on cell cycle associated pathways. Considering their suppressive effects on expression of the breast cancer master regulator SOX9 and their inhibitory impact on the cell viability of breast cancer cells, miR-224-3p and miR-134-3p could be considered as potential tumor suppressive miRNAs in breast cancer. Indeed, according to the OncomiR Cancer data base expression levels of these miRNAs are significantly reduced in breast cancer tissues compared to healthy tissue. Regarding miR-6859-3p, more experiments are needed to determine, whether this miRNA classifies as a tumor-suppressive miRNA.

Interestingly, miR-134-3p has been described by others to inhibit cancer progression. For example, in ovarian cancer it was demonstrated that miR-134-3p overexpression lowered ovarian cancer cell proliferation and caused cell cycle arrest as well as migration and invasion [36]. With respect to ovarian cancer, the effects were partially caused by down-regulated expression of FEN1 mediated by miR-134-3p [36]. Also, for small-cell lung cancer, a tumor-suppressive function of miR-134 was shown. In the study conducted by Qin *et al.* the inhibited cell proliferation caused by miR-134 expression was partly mediated through direct targeting of ITGB1 [37]. These findings are in accordance with our results demonstrating that miR-134-3p might be considered as tumor-suppressive miRNA in breast cancer, ovarian and small-cell lung cancer. However, further functional *in vitro* and *in vivo* studies, are necessary to verify this notion. In contrast, oncogenic features of miR-134 have been described for other cancer types, thus whether miR-134 causes cancer progression or suppression might depend on the tumor entity [38].

In osteosarcoma it has been described, that the long non-coding RNA (lncRNA) SNHG4 enhances cancer progression by sponging miR-224-3p. The cancer-promoting effects of SNHG4 could be reversed by overexpression of miR-224-3p [39]. In contrast, for non-small lung cancer it was found that sponging of

miR-224-3p by the lncRNA HCG11 enhances cancer cell proliferation and inhibits apoptosis [40]. However, our findings together with previous data obtained from breast cancer studies [27] support a possible tumor-suppressive role of miR-224-3p for breast cancer.

Conclusion

We demonstrate that miR-134-3p, miR-224-3p and miR-6859-3p diminish SOX9 expression in human breast cancer cells. This finding might help to understand breast cancer specific up-regulation of SOX9 expression accompanied by enhanced tumor cell proliferation and increased tumor growth. The down-regulation of miR-134-3p and miR-224-3p expression might represent initial events in breast cancer development, leading to elevated SOX9 levels that will drive breast cancer progression.

Abbreviations

AGO Argonaute protein

FC Fold change

lncRNA long non-coding RNA

miRNA microRNA

OncomiR oncogenic miRNA

PCC Pearson's Correlation Coefficient

RISC RNA-induced silencing complex

RPL19 Ribosomal Protein L19

SOX9 SRY-Box Transcription Factor 9

UTR Untranslated region

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Original data are stored at the senior author's lab and can be provided upon request.

Competing interests

The authors declare no conflicts of interest.

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Authors' contributions

Conception and design: SBE, TK

Development of methodology: TK

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): TYC, TK

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): TYC, TK, WO, SBE

Writing, review, and/or revision of the manuscript: TK, WO, SBE

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Tables

Table 1. GSEA analysis of genes commonly down-regulated by miR-134-3p, miR-224-3p and miR-6859.3p (FC < 0.5).

Gene Set name	Genes in overlap	FDR q-value
DNA packaging complex	H2AC16, H2AC17, H2AC21, H2AC19, H2BC9, H2BC11, H2BC13	1.18 e-13
Protein DNA complex	H2AC16, H2AC17, H2AC21, H2AC19, H2BC9, H2BC11, H2BC13	3.57 e-12
Protein heterodimerization activity	H2AC16, H2AC17, H2AC21, H2AC19, H2BC9, H2BC11, H2BC13	5.48 e-11
Chromatin organization	H2AC16, H2AC17, H2AC21, H2AC19, H2BC9, H2BC11, H2BC13	2.51 e-8
Protein dimerization activity	H2AC16, H2AC17, H2AC21, H2AC19, H2BC9, H2BC11, H2BC13	1.13 e-7
Chromatin	H2AC16, H2AC17, H2AC21, H2AC19, H2BC9, H2BC11, H2BC13	2.97 e-7
Chromosome organization	H2AC16, H2AC17, H2AC21, H2AC19, H2BC9, H2BC11, H2BC13	2.97 e-7
Chromatin silencing	H2AC16, H2AC17, H2AC21, H2AC19	1.09 e-6
Chromosome	H2AC16, H2AC17, H2AC21, H2AC19, H2BC9, H2BC11, H2BC13	2.84 e-6
Negative regulation of gene expression epigenetic	H2AC16, H2AC17, H2AC21, H2AC19	6.22 e-6

Table 2. GSEA analysis of genes down-regulated by miR-134-3p and miR-224-3p but not miR-6859-3p. (FC < 0.5).

Gene Set name	Genes in overlap	FDR q-value
Chromosome organization	19	1.76 e-17
DNA metabolic process	17	1.32 e-16
Chromosome	20	1.32 e-16
Cell cycle	18	2.27 e-13
DNA replication	11	2.27 e-13
DNA conformation change	11	2.37 e-12
DNA repair	11	3.84 e-10
Cell cycle process	14	7.81 e-10
Telomere organization	8	8.4 e-10
Cellular response to DNA damage	12	9.22 e-10

Figures

Figure 1

miR-134-3p, miR-224-3p and miR-6859-3p reduce SOX9 mRNA and protein levels in MDA-MB-231 cells. (a) MDA-BM-231 cells were transfected with 50 nM miRNA and 48 h later cells were harvested for RNA isolation and qPCR analysis. Fold changes in expression were calculated compared to mimic control-1 samples. RPL19 was used as housekeeping gene for qPCRs since none of the tested miRNAs exhibit a binding site for this gene according to miRmap. For each miRNA, at least seven independent experiments were performed. (b) Western Blot analysis performed with cellular protein isolated 72 h after transfection.

The relative SOX9 protein expression was calculated compared to beta-actin as loading control since none of the miRNAs have predicted binding sites for the ACTB gene according to miRmap. (c) Quantification of protein levels was performed with ImageJ. One representative Western blot image is shown. At least three independent experiments were performed per condition. Fold changes were compared to mimic control-1 samples. Significance was assessed by one-sample T-test. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$

Figure 2

miR-134-3p and miR-224-3p directly bind to the SOX9 3'-UTR in breast cancer cell lines and in HEK293 cells. MDA-MB-231 (a), MCF-7 (b) or human embryonic kidney cells HEK293 (c) cells were transfected with 50 nM miRNA and 24 h later luciferase activity was assessed by luminescence measurement. Per condition five individual transfections were performed. Significance was assessed by one-way ANOVA using Dunnett's multiple comparison test. All samples were compared to mimic control-1 samples. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$

Figure 3

miR-134-3p and miR-224-3p, but not miR-6859-3p are direct binders of the SOX9 3'-UTR. MDA-BM-231 cells were co-transfected with 50 nM miRNA and luciferase encoding pLS-SOX9 constructs. Twenty-four hours later luciferase activity was quantified by luminescence measurement. (a) miR-134-3p mediated decrease in luciferase activity is abolished upon deletion of individual nucleotides at positions 1484 or 1486, respectively. (b) Decreased luciferase activity mediated by miR-224-3p is reversed by deletion of

individual nucleotides at positions 474 or 475 within the SOX9 3'-UTR. (c) None of the deletions generated within the SOX9 3'-UTR restored the diminished luminescence signal caused by miR-6859-3p. Per condition five individual transfections were performed. Significance was assessed by one-way ANOVA using Dunnett's multiple comparison test. All samples were compared to the respective mimic control samples. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$

Figure 4

Venn diagram of genes showing decreased expression upon transfection of miR-134-3p, miR-224-3p and miR-6859-3p. MDA-MB-231 cells were transfected with 50 nM miRNA and subsequent gene expression profiling was performed. Genes with significantly decreased expression levels compared to mimic control-1 transfections with a fold change < 0.5 were considered

Figure 5

Overexpression of miR-134 or miR-224 in MDA-BM-231 cells decreases viability and enhances the proportion of cells in G1 phase. MDA-BM-231 cells were transfected with 50 nM miRNA and cell viability as well as cell cycle stages were assessed 72 h post transfection by XTT assay (a) or FACS (b), respectively. Four transfections were performed per condition for XTT assays. Significance was assessed by one-way ANOVA using Dunnett's multiple comparison test. All samples were compared to the respective mimic control-1 samples. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$

Figure 6

miR-134 and miR-224 expression levels in healthy tissue vs. breast cancer samples. Expression levels of miR-134 and miR-224 across tissue samples from healthy donors and breast cancer patients were compared. Both miRNAs are expressed at significantly lower level in breast cancer samples compared to normal breast tissue. Analysis of miR-134 and miR-224 expression was based on 87 samples from normal tissue and 782 or 695 breast cancer derived samples, respectively. No data were available for miR-6859. Significance was assessed by unpaired T-test with Welch correction. ***: $p < 0.001$

Figure 7

Graphical summary of study. miR-134-3p and miR-224-3p were shown to directly inhibit SOX9 expression by binding to SOX9 3'-UTR. miR-6859-3p could also inhibit SOX9 at protein and mRNA level, but the effect was rather mediated indirectly and not by binding of miR-6859-3p to SOX9 3'-UTR. The expression of MIR9AHG, encoding for miR-125b-2-3p and miR-99a-3p, both exhibiting a binding site for SOX9 3'-UTR, was up-regulated after miR-6859-3p transfection in MDA-MB-231 cells. KLF5 and PPARG were significantly down-regulated upon miR-6859-3p transfection and miR-6859-3p has one potential binding site for KLF5 3'-UTR. We propose, that miR-6859-3p might directly inhibit KLF5, which in turn lowers PPARG levels, which normally suppresses MIR99AHG transcription

Supplementary Files

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