Original Paper

MiR-301a-3p Suppresses Estrogen Signaling by Directly Inhibiting *ESR1* in ER α Positive Breast Cancer

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Key Words

MiR-301a • ER α • Breast cancer • Estrogen dependence • Estrogen signaling

Abstract

Background/Aims: MiRNA-301a-3p is an oncogenic miRNA whose expression is associated with tumor development, metastases and overall poor prognosis. Estrogen receptor α (ER α) is one of the estrogen hormone-activated transcription factors, which regulates a large number of genes and is involved in the mammary gland development. Expression of ER α is considered to be a good indicator for endocrine therapy and breast cancer survival. Loss of ER α in breast cancer patients indicates invasiveness and poor prognosis. In this study, we focus on the regulation of ER α by miR-301a and its role in transition from estrogen-dependent to estrogen-independent breast cancer. **Methods:** Expression of miR-301a-3p was measured by qRT-PCR in tumor tissue samples from 111 patients with primary breast carcinoma and in mammospheres representing *in vitro* model of cancer stem-like cells. Dual reporter luciferase assay and complementary experiments were performed to validate *ESR1* as a direct target of miR-301a-3p. The effect of miR-301a-3p on estrogen signaling was evaluated on the level of gene and protein expression and growth response to estrogens. Finally, the effect of miR-301a-3p expression on tumor growth was studied in nude mice. **Results:** We identified *ESR1* as a direct target of miR-301a-3p. Ectopic miR-301a-3p causes a decrease in *ESR1* mRNA and protein level and modulates the expression of ER α target genes in ER α positive breast cancer cells. Consistently, miR-301a-3p causes a decrease in sensitivity of MCF7 cells to 17 β -estradiol and inhibits the growth of estrogen dependent tumor in nude mice. Yet, the mice tumors have significantly increased expression of genes related to cancer stem-like cells and epithelial to mesenchymal transition suggesting enrichment of the population of cells with more invasive properties, in line with our observation that miR-301a-3p expression is highly increased in mammospheres which show a decrease in estrogenic signaling. Importantly, miR-301a-3p level

is also increased in primary breast cancer samples exhibiting an ER/PR negative phenotype.

Conclusion: Our results confirm *ESR1* as a direct target of miR-301a-3p and suggest that miR-301a-3p likely contributes to development of estrogen independence, which leads to a more invasive phenotype of breast cancer.

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Introduction

Breast cancer is the most prevalent type of cancer in women worldwide [1, 2]. Up to 75 % of breast cancer subtypes are estrogen receptor α (ER α) and progesterone receptor (PR) positive. ER α and PR positive tumors are largely well-differentiated, less invasive and are associated with better prognosis in comparison with ER α and PR negative tumors [3–6].

ER α is one of the estradiol (E_2)-activated transcription factors, which regulates a large number of genes connected with proliferation, differentiation and migration [7] and plays a critical role in mammary gland biology and development [8]. E_2 /ER α signaling stimulates proliferation of ER α positive breast cancer cells and it is important for the growth of the primary tumor. Furthermore, the expression of ER α negatively correlates with the progressive grade of invasive ductal breast cancer [9]. Moreover, ER α signaling opposes pathways leading to epithelial to mesenchymal transition (EMT) and cancer stem-like cell phenotype [10, 11]. Thus, expression of ER α is connected with less invasive behavior of breast cancer and is considered to be a good indicator of endocrine therapy and breast cancer survival. This dependency on estrogenic signaling is exploited for treatment with selective estrogen receptor modulators (tamoxifen), selective estrogen receptor down regulators (fulvestrant) or aromatase inhibitors (letrozole) [12, 13]. However, resistance to hormonal therapies connected with transition from hormone-dependent to hormone-independent tumor and metastases formation is the main problem in clinical treatment occurring in almost 40% cases over 10 years of treatment [14, 15].

Until now, multiple mechanisms have been proposed to explain how breast cancer cells escape dependency on estrogen control and acquire hormone-independent, invasive and resistant phenotype. Among them, epigenetic modulation [16], transcription regulation [17], gene mutation [18] and alternative usage of splice variants [19], posttranslational modifications [20] or microRNA deregulation [21] have been described so far.

MicroRNAs (miRNAs) are small, 22 nucleotides long, non-coding RNAs that negatively regulate gene expression by complementary binding to their target mRNAs, which leads to repression of translation and mRNA degradation [22]. They target a wide spectrum of genes and deregulation of their function has important role in cancer progression [23]. MiR-301a-3p has recently emerged as an oncogenic miRNA whose expression is connected with progression and poor prognosis of pancreatic [24], gastric [25], hepatocellular [26], colorectal [27] and breast cancer [28].

In this study, we identified *ESR1* mRNA as a direct target of miR-301a-3p and we show that overexpression of miR-301a-3p leads to inhibition of estrogenic signaling in ER α positive breast cancer cell lines. Furthermore, our data suggest an important role of miR-301a in a shift from estrogen-dependent to estrogen-independent breast cancer and thus in progression of tumor growth.

Materials and Methods

Cell lines and transfections

Human breast cancer cell lines ZR-751, MCF7, T47D and BT474 and non-malignant MCF10A cell line were obtained from American Type Culture Collection and were routinely cultivated in Dulbecco's Modified Eagle Medium (Sigma) supplemented with 10% fetal bovine serum (Thermo Scientific) and 100 U/ml penicillin, 100 μ g/ml streptomycin; in 5% CO₂ and 37°C. MCF10A cells were cultivated in DMEM/F12 (Lonza) with 5% horse serum and 100U/ml penicillin, 100 μ g/ml streptomycin, supplemented with 0.1 ng/

ml cholera toxin, 20 ng/ml epidermal growth factor (EGF, Thermo Scientific), 0.5 µg/ml hydrocortisone and 1 mg/ml insulin. For the generation of mammospheres, the advanced DMEM/F12 medium (Thermo Scientific) supplemented with 5% proliferation supplement (Stem Cell Technologies), 20 ng/mL EGF and 5 ng/mL fibroblast growth factor (FGF, Thermo Scientific), 4 µg/mL heparin (Sigma), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM glutamine and 100U/ml penicillin, 100 µg/ml streptomycin was used. The control medium contained 5% FBS, 10 mM HEPES, 1 mM glutamine and 100U/ml penicillin, 100 µg/ml streptomycin. MCF7 cells with inducible expression of miR-301a were prepared by stable transfection with trans-activator coded by pEF1-TET3G vector and with doxycycline-inducible pTREG-IRES vector containing either miR-301a gene or no insert (empty vector, EV), both from Clontech.

DNA constructs

miR-301a inducible vector

The pri-microRNA-301a sequence was amplified by PCR from cDNA obtained from MCF7 cells using Q5 hot start high-fidelity DNA polymerase (New England BioLabs) with the following primers: Forward 5' CCCTCGTAAAGTCGACTGCATGTTTCTGTTCTGAATG; Reverse 5' CAGTTACATTAGATCTGGGCAAGTAACTGCAGGAAA. The amplified sequence was then cloned into the Sall/BglII sites of pTREG-IRES vector (Clontech).

Luciferase vectors

The whole 3'UTR sequence of *ESR1* gene was amplified from MCF7 cDNA by using the following primers: Forward 5' TGCAAGTGAGCGGCCGCGAGCTCCCTGGCTCCACA; Reverse 3' TGCAAGTGAGCGGCCGCTTAGTTTAATTCTTTATTTGAACATC containing the NotI restriction sites. The amplified product was cloned into NotI site of pTK-Cypridina vector (Thermo Scientific). Vectors with deleted first or the second or both sites of predicted miR-301a-3p binding were created by site directed mutagenesis by using Q5 Hot start high-fidelity DNA polymerase (New England BioLabs) according to manufacturer's protocol with the following primers:

Site 1 Forward 5' TTGTTTTCTAAGTAATTGCTGCCTCTGTCTTTTGAGATTCAAGAAAAATTTTC;

Site 1 Reverse 5' GAAA-TTTTCTTGAATCTCAAAAGACAGAGGCAGCAATTACTTAGAAAAACAA;

Site 2 Forward 5' CATCCGC-TGGATTCTTTTCAATGTTTCATTAAACAAAGCAAAGC;

Site 2 Reverse 5' GCTTTGCTTTGTTTAATGAAACATTGAAAAAGAATCCAGCGGGATG; the thermal conditions for PCR were: 98°C for 5min; 5 cycles of 98°C for 15s, 80°C for 10 s, 70°C for 30 s and final extension at 72°C for 8 min then 15 cycles of 98°C for 10 s, 80°C for 10 s and 72°C for 8 min followed by final extension at 72°C for 10 min. Then the PCR reaction was incubated with DpnI Fast digest enzyme (Thermo Scientific) at 37°C for 30 min and transformed into TOP10 ultracompetent cells. All constructs were verified by Sanger sequencing (GATC Biotech).

Luciferase Assay

MCF7, T47D and BT474 cells were transfected with miR-301a-3p mimic (Sigma HMI0442), miR-301a-3p anti-miR (Ambion AM17000) and corresponding controls (Sigma HMC0003, Ambion AM17010) using INTERFERin transfection reagent according to manufacturer's protocol (Polyplus). After 24h, the second transfection with luciferase vectors and normalization pTK-Gussia-Dura Luc vector (Thermo Scientific) was performed using Lipofectamine LTX and Plus Reagent (Thermo Scientific) according to manufacturer's instructions. After 24h of incubation, medium was harvested to detect the activities of luciferase using the Pierce Gussia/Cypridina Glow Assay Kit (Thermo Scientific) using TECAN Infinity (TECAN).

MiR-301a mimic and anti-miR transfection

Cells were transfected with 40 nM miR-301a-3p mimic (Sigma HMI0442) or 80 nM miR-301a-3p anti-miR (Ambion AM17000) and corresponding controls (Sigma HMC0003, Ambion AM17010) using INTERFERin (Polyplus) according to manufacturer's instruction. After 72h of incubation cells were used for subsequent analysis.

qRT-PCR

The isolation of total RNA from cells was performed by RNazol reagent (Molecular Research Center) and RNA from mice tumors was isolated by using RNA Blue (Top-Bio) according to manufacturer's protocol.

cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Fermentas) using oligo-dT as primers. qRT-PCR was performed using 5x HOT FIREpol Eva Green qRT-PCR mix (Solis Biotec). 2.5 μ L of cDNA containing 10 ng of template RNA was mixed with 5 μ L of master mix (1.5 μ L Eva Green mix, 0.197 μ L of 10 μ M forward and reverse specific primers and 3.3 μ L of H_2O). The thermal profile for qRT-PCR was: 95°C for 12 min, 38 cycles of 95°C for 10 s, 60°C for 20 s and 72°C for 20 s. The sequences of primers used are in Table 1. The expression of hsa-miR-301a-3p was assessed using the TaqMan MicroRNA Assay (Applied Biosystems, TM000528); snU6 was used for normalization (Applied Biosystems, TM001973). Hsa-miR-301a-3p was transcribed by RevertAid First Strand cDNA Synthesis Kit (Fermentas) using a specific RT primer. One reaction contained: 3.5 μ L of RT master mix (1.5 μ L 5x reaction buffer, 0.095 μ L RNase inhibitor, 0.75 10 mM dNTPs, 0.5 μ L Reverse Transcriptase, 0.655 μ L H_2O), 2.5 μ L RNA of 2 ng/ μ L concentration and 1.5 μ L of oligo-dT primers. Thermal profile was: 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. The subsequent qRT-PCR was carried out using HOT FIREpol universal probe mastermix (Solis Biotec). 1.5 μ L of 5x mastermix, 0.375 μ L of TaqMan assay (20x), 3.125 μ L of H_2O and 2.5 μ L of 5x diluted cDNA was mixed and run for 95°C 10 min and 40 cycles of 95°C for 15 s and 60°C for 60 s. The data were analysed via GenEx software version 6, reference genes for normalization were identified by Normfinder.

Western blotting

Harvested cells were washed with phosphate-buffered saline (PBS) and lysed in RIPA buffer. Protein concentration was measured via the Pierce BCA protein Assay Kit (Thermo Scientific). 50 μ g of total protein was resolved on SDS polyacrylamide gels and blotted onto a nitrocellulose membrane (BioRad). After blocking with 5% non-fat milk (Serva)/ Tris-buffered saline with 0.05% Tween-20 (TBS-T) for 1h, the membrane was incubated overnight in 5% bovine serum albumin/TBS-T with primary antibody against ER α (sc-544, Santa Cruz), PR (8757S, Cell Signaling), GREB1 (HPA024616, Sigma), Cathepsin D (2284S, Cell Signaling), CXCL12 (3740S, Cell Signaling), BMP7 (ab129156, Abcam), β -Actin (5125S, Cell Signaling) diluted 1:1000. Next day, the membrane was washed with TBS-T and probed with secondary antibody conjugated with horseradish peroxidase in 1% milk/TBS-T. The membranes were then washed with TBS-T and incubated with Sirius ECL substrate (Advansta) and chemiluminescence was assessed with LAS4000.

Response of MCF7 cells to estrogen

The cells were washed several times with PBS and 2500 cells per well were seeded in 96-well plate in 100 μ L of DMEM media without phenol red (Sigma) supplemented with 10% charcoal stripped fetal bovine serum (Thermo Scientific) and 100U/ml penicillin, 100 μ g/ml streptomycin. Cells were incubated with the increasing concentration of 17- β -E₂ for 5 days. Cells were then fixed with 4 % paraformaldehyde in PBS and after paraformaldehyde removal, the cells were stained with 0.05 % crystal violet dye (Sigma). The unbound dye was washed away and the bound dye was dissolved in 1 % SDS. The absorbance was measured at 595 nm using TECAN infinity M200 (TECAN).

In vivo experiments

Female Balb/c nude athymic mice (CAN.N.Cg-Foxn1^{nu}/CrJ, Charles River) were implanted subcutaneously with 0.72 mg/90-day-release 17 β -E₂ pellet (Innovative Research of America, NE-121) and the following day injected subcutaneously with MCF7 cells inducibly expressing miR-301a or with an EV

Table 1. Sequences of used primers for qRT-PCR

Gene	Primer sequence
ESR1	Forward: 5' CCGGCTCCGCAAAATGCTACGA 3' Reverse: 5' AGCGGCTTGCCAAAGGTT 3'
GREB1	Forward: 5' GGACCAAGCTTCAGTCACCTT 3' Reverse: 5' CCAAGGGCTACCATTTGGGT 3'
PGR	Forward: 5' TGGTGTCTTACCTGTGGGA 3' Reverse: 5' CAGCCTGACAGCACTTCT 3'
BMP7	Forward: 5' ACAAGGCCGCTCTCAGTACC 3' Reverse: 5' GGTAGCGTGGGTGGAAGAT 3'
CTSD	Forward: 5' CTGGACATCGCTTGCTGGAT 3' Reverse: 5' TGCCTCTCCACTTTGACACC 3'
CXCL12	Forward: 5' GTGCCCTTCAGATTGTAGCCC 3' Reverse: 5' GCCCTTCCTAACACTGGTT 3'
HER2	Forward: 5' CACCCAAGTGTGACACCGGA 3' Reverse: 5' GCACGTAGCCCTGCACCTCC 3'
CD44	Forward: 5' GCTGACCTCTGCAAGGCTTTCAATAG 3' Reverse: 5' CTTCTTCGACTGTTGACTGCAATGCA 3'
ABCG2	Forward: 5' TCGTTATTAGATGTCTTAGCTGCAA 3' Reverse: 5' TTGTACCACGTAACTGAATTACA 3'
ALDH1	Forward: 5' ATGCTTCCGAGAGGGGGCA 3' Reverse: 5' CCAACCTGCACAGTAGCGCA 3'
VIMENTIN	Forward: 5' GCGACAACCTGGCCGAGGAC 3' Reverse: 5' GGTCAAGACGTGCCAGAGAGCGC 3'
VEGFA	Forward: 5' AGGGCAGAAATCATCAGCAAGTG 3' Reverse: 5' ATGTACTCGATCTCATCAGGGTACTC 3'
POLR2A	Forward: 5' TGCTCCGATTCGATCATGAACA 3' Reverse: 5' ATCTGTGTCAGCATGTTGGAATCGATG 3'
TBP	Forward: 5' TGTATCCACAGTGAATCTTGGTTGTAAG 3' Reverse: 5' CGTGGCTCTCTTATCCTCATGATTAC 3'
P0	Forward: 5' ATCAGAGAGGAACTCTGCATTCTCG 3' Reverse: 5' GATAGAATGGGGTACTGATGCAACAGTT 3'
ZEB1	Forward: 5' AACCCAACCTGAACGTGACA 3' Reverse: 5' ATTACACCCAGACTGGCTCA 3'
ZEB2	Forward: 5' TGCCCAACCATGAGTCTCTCCC 3' Reverse: 5' CGGTCTGGATCGTGGCTTCTGG 3'

at 2×10^6 cells per animal (4 mice per group and experiment was repeated twice). Mice were given doxycycline diet (200mg/kg, Bio-Serv) and the tumor growth was monitored twice a week, quantified by ultrasound imaging instrument Vevo770 (Visual Sonics). Mice were then sacrificed and tumors taken for further analysis (measurement of gene and protein expression by qRT-PCR and western blot). All animal studies were approved by Czech Academy of Sciences and conducted in accordance with Czech Council guidelines for the Care and Use of Animals in Research and Teaching.

Patient samples

Fresh frozen tumor tissue samples were obtained from total 111 patients with primary breast carcinoma diagnosed at the Motol University Hospital (Prague, Czech Republic), the Hospital Atlas (Zlin, Czech Republic), and the Faculty Hospital Kralovske Vinohrady (Prague, Czech Republic) between 2003 and 2014. Processing of the tissue samples was described in detail previously [29]. Histological classification of carcinomas was performed according to standard diagnostic procedures [30]. Expression of ER and PR was assessed immunohistochemically with the 1% cut-off value for classification of tumors as hormone receptor positive. ERBB2 (OMIM:164870) status was defined as positive in samples with immunohistochemical score 2+ or 3+ confirmed by fluorescence *in situ* hybridization (FISH) or silver *in situ* hybridization (SISH) analysis. Clinical characteristics of studied breast carcinoma patients are described in Table 2.

The expression level of hsa-miR-301a-3p and reference gene was assessed in patient samples by qRT-PCR as described above.

All patients were informed about the study and those who agreed and signed an informed consent participated in the study. The study was approved by the Ethical Commission of the National Institute of Public Health in Prague. The methods were carried out in accordance with guidelines approved by the above Ethical Commission.

Statistics

Results are represented as mean values $\pm$ S.E.M from at least three independent experiments. The Student's t-test was used to calculate the difference between groups and $P < 0.05$ was considered statistically significant.

Results

MiR-301a-3p is highly expressed in the in vitro model of cancer stem-like cells that show inhibition of estrogen signaling

There is increasing evidence that invasive properties of breast cancer rely on reprogramming of cancer cells into cancer stem-like cells [31], and recent studies suggest that miR-301a-3p contributes to EMT [32]. In order to better characterize the role of miR-301a-3p in cancer stem-like cells, we assessed the expression level of miR-301a-3p by TaqMan assay in our model of cancer stem-like cells based on growing cells as mammospheres in

Table 2. Clinical characteristics of studied breast carcinoma patients

Characteristics	Number of patients (%)
Menopausal status	
premenopausal	12 (10.8)
postmenopausal	93 (83.8)
not available	6 (5.4)
Histological type	
invasive ductal carcinoma	111 (100)
Tumor size, median $\pm$ S.D., mm	18.5 $\pm$ 9.8
Lymph node metastasis	
positive (pN1-3)	40 (36.0)
negative (pN0)	60 (54.1)
not determined (pNx)	11 (9.9)
Pathological stage	
I	39 (35.2)
II	50 (45.0)
III	11 (9.9)
not available	11 (9.9)
Histological grade	
G1	17 (15.3)
G2	54 (48.6)
G3	40 (36.0)
Estrogen receptor expression	
positive	55 (49.5)
negative	56 (50.5)
Progesterone receptor expression	
positive	55 (49.5)
negative	56 (50.5)
HER2 expression	
positive	46 (41.4)
negative	65 (58.6)
Pathological subgroup	
ER+, PR+, ERBB2+	22 (19.8)
ER+, PR+, ERBB2-	33 (29.7)
ER-, PR-, ERBB2+	24 (21.6)
ER-, PR-, ERBB2-	32 (28.8)

serum-free medium supplemented with EGF and FGF [33]. We generated mammospheres from several ER α positive breast cancer cell lines (ZR-751, MCF7, T-47D and BT474) and compared miR-301a-3p level between spheres and control adherent cells. We also included non-malignant MCF10A cell line of breast origin as a control representing normal breast epithelial cells. We detected significantly higher miR-301a-3p expression in sphere cells compared to control adherent cells in 3 of the tested cell lines (Fig. 1A). There was no difference between BT-474 control adherent cells and spheres which can be explained by already very high level of miR-301a-3p in adherent BT-474 cells in comparison with other tested adherent cell lines.

We further analyzed the estrogen signaling in the adherent cells and mammospheres derived from MCF7 cells. We detected a profound inhibition of ER signaling in spheres, as documented by western blot analysis showing a significant decrease in the ER α protein level and also a decrease in the amount of the ER α -regulated proteins such as Progesterone receptor (PR) and Growth regulation by estrogen in breast cancer 1 (GREB1) expression (Fig. 1B).

Collectively, our data suggest upregulation of miR-301a-3p in cells growing as mammospheres that represent an *in vitro* model of cancer stem-like cells. Importantly, these cells show a clear inhibition of estrogen signaling in our model, in accordance with published results [34].

Functional miR-301a-3p seed sequences are present in the 3'UTR of ESR1 mRNA coding for ER α

In order to assess the importance of the miR-301a-3p in the ER α signaling, we searched for a "seed" sequence within the *ESR1* mRNA encoding ER α protein. Using three different publicly available miRNA databases (MiRanda, TargetScan, miRBase) we found that two miR-301a-3p seed sequences are present in the 3'UTR of *ESR1* mRNA (Fig. 2A).

To verify the prediction that miR-301a-3p negatively regulates ER α expression through binding to the 3'UTR of *ESR1* mRNA, we cloned a 4Kb long 3'UTR of the *ESR1* mRNA (containing 2 putative miR-301a-3p binding sites) downstream of the Cypridina luciferase gene in a reporter plasmid system. The reporter plasmid was transfected into MCF7 cells together with miR-301a-3p mimic and miR-301a-3p anti-miR and corresponding control mimic and anti-miR. The luciferase activity assay showed a significant reduction in luciferase activity in cells transfected with miR-301a-3p mimic and conversely significant increase of luciferase activity in cells transfected with miR-301a-3p anti-miR (Fig. 2B). Moreover, we did not detect any changes in luciferase activity when reporter plasmid with deleted miR-301a-3p binding sites was used (Fig. 2B). To determine which miR-301a-3p binding site is more important for regulation of ER α protein expression,

Fig. 1. MiR-301a-3p is highly upregulated in cancer stem-like cells with downregulated ER signaling. A, qRT-PCR was used to measure expression of miR-301a in different cell lines cultivated as mammospheres (Sph) and normal adherent cells (Ctrl). B, Western blot showing decreased expression of ER α , GREB1 and PR proteins in MCF7 control adherent cells and mammospheres. Results are represented as mean values $\pm$ S.E.M. (n=3). Statistical significance was calculated using the t-test, where the values obtained from the mammospheres were compared to the control values; * p<0.05, *** p<0.001.

Fig. 2. *ESR1* gene is a direct target of miR-301a-3p. A, Schematic diagram of miR-301a binding sites in the *ESR1* 3'UTR. *Cypridina* luciferase reporter vectors containing the wild type (WT), deleted first, second or both (DEL1, DEL2, DEL1+2) putative target sites in the 3'UTR of *ESR1* mRNA were generated. B, Relative luciferase activity of 3'UTR *ESR1* WT and DEL1+2 in MCF7 cells transfected with miR-301a and control mimic and miR-301a and control anti-miR. C, Relative luciferase activity of 3'UTR *ESR1* WT, DEL1, DEL2 and DEL1+2 in MCF7, BT474 and T47D cells. D, qRT-PCR showing miR-301a expression after transfection with miR-301a mimic and miR-301a anti-miR and expression of *ESR1* gene in MCF7, BT474 and T47D cells after transfection with miR-301a mimic and miR-301a anti-miR. E, Representative western blot showing expression of ER α protein in MCF7, BT474 and T47D cells after transfection with miR-301a mimic and miR-301a anti-miR. E, Results are represented as mean values $\pm$ S.E.M. (n=3). Statistical significance was calculated using the t-test; * p<0.05, ** p<0.01, *** p<0.001.

we constructed vectors with deleted first, second or both miR-301a-3p binding sites and transfected them into the MCF7, BT474 and T47D cells. We detected an increase in luciferase activity in both deletions meaning both sites can bind miR-301a-3p. However, the first site is more crucial given the fact that the increase in luciferase activity is almost identical when one or both sites are deleted while the deletion of the second motif only leads to a partial increase in luciferase activity (Fig. 2C).

MiR-301a-3p decreases abundance of ESR1 mRNA and reduces ER α protein level

In order to confirm the biological relevance of miR-301a-3p in the regulation of *ESR1*, we analyzed the effect of ectopic miR-301a-3p expression on the abundance of *ESR1* mRNA and also ER α protein. High expression of miR-301a-3p resulted in a significant decrease in *ESR1* mRNA level in MCF7, T47D and BT-474 cells as assessed by qRT-PCR (Fig. 2D). Also, ER α protein level tested by western blotting was significantly reduced in MCF7 and T47D cells, in BT474 cells the trend was similar but not statistically significant (Fig. 2E). Our data also show that the effect of downregulation of miR301a-3p by anti-miR was limited and was significant only in BT474 cells which is in line with the fact that these cells express significantly higher endogenous level of miR-301a-3p (Fig. 1A) and the downregulation was the most effective in this particular cell line.

In conclusion, our findings demonstrate that miR-301a-3p recognizes and binds to the 3'UTR of *ESR1* mRNA to suppress its translation and mRNA level.

MiR-301a-3p mimic downregulate the canonical ER pathway as evidenced by inhibition of genes positively regulated by ER α and activation of genes negatively regulated by ER α

ER α is a ligand-activated transcription factor that regulates expression of many target genes [35]. We assessed the expression level of several genes which are positively or negatively regulated by ER α transcription factor in MCF7, T47D and BT474 cells transfected with miR-301a-3p mimic and miR-301a-3p anti-miR and corresponding control RNAs. We found that increased expression of miR-301a-3p leads to a significant decrease in mRNA expression of ER α positively regulated genes as *PGR* (Progesterone receptor α), *GREB1* (Growth regulation by estrogen in breast cancer 1), *CXCL12* (C-x-c motif chemokine ligand 12) or *CSTD* (Cathepsin D) or to a significant increase in *BMP7* (Bone morphogenetic protein 7) which is negatively regulated by ER α (Fig. 3A). The changes in expression of these genes were also replicated on protein level (Fig. 3B, Fig. 4).

The effect of anti-miR-301a-3p treatment on ER α signaling pathway was significant only in the BT474 cells. As mentioned earlier, this cells line exhibits high basal expression of miR-301a-3p and the effect of anti-miR-301a-3p treatment was thus most evident there, resulting in significant upregulation of the *ESR1* mRNA and ER α level (Fig. 2D, E), accompanied by statistically significant increase in the mRNA transcripts regulated by the ER α such as *GREB1*, *PGR*, *CXCL12* reflected in the accumulation of the corresponding proteins (Fig 3A, Fig. 4).

Similar trend could also be seen in MCF7 and T47D cells but did not reach statistical significance and was weaker compared to BT-474 cells (Fig. 3B, Fig. 4). It might be explained by the fact that both MCF7 and T47D cells express relatively low levels of endogenous miR-301a and the effect of anti-miR-301a-3P treatment is thus limited compared to the BT474 cells.

In summary, our results show that miR-301a-3p regulates the activity of the ER pathway.

Upregulation of miR-301a-3p level in MCF7 cell line leads to a blunted response to 17- β estradiol

MCF7 cells transfected with miR-301a-3p mimic and anti-miR and corresponding controls were seeded in 96-well and cell number in estrogen-free medium as well as in the presence of increasing concentrations of 17- β E₂ was measured by crystal violet assay [36]. MCF7 cells transfected with miR-301a-3p mimic show significantly lower number of cells in

Fig. 3. MiR-301a-3p affects expression of genes and proteins regulated by ER α positively (*GREB1*, *PGR*, *CSTD*, *CXCL12*) and negatively (*BMP7*) in MCF7 cells. A, qRT-PCR showing expression of *GREB1*, *PGR*, *CSTD*, *CXCL12* and *BMP7* genes in MCF7, BT474 and T47D cells transfected with miR-301a mimic and miR-301a anti-miR. B, Representative western blots showing expression of GREB1, PR, Cathepsin D, CXCL12 and BMP7 proteins in MCF7 cells transfected with miR-301a mimic and miR-301a anti-miR and densitometry evaluation by image J. Results are represented as mean values $\pm$ S.E.M. (n=3). Statistical significance was calculated using the t-test; * p<0.05, **p<0.01, *** p<0.001.

response to $17\text{-}\beta\text{E}_2$ in comparison with MCF7 cells transfected with control mimic (Fig. 5A). We did not detect any statistically significant difference between cells transfected with miR-301a-3p anti-miR and control anti-miR (Fig. 5B), probably reflecting low basal level of miR-301a-3p in MCF7 cells.

The effect of miR-301a-3p mimic was also recapitulated in doxycycline inducible miR-301a-3p MCF7 cell line. After increasing miR-301a level by doxycycline addition, we observed similar decrease in number of cells in response to $17\text{-}\beta\text{E}_2$ (Fig. 5C). No such effect was seen when using MCF7 cells stably transfected with EV (Fig. 5D).

In conclusion, the decrease of ER α by miR-301a-3p overexpression results in a decreased response to $17\text{-}\beta\text{E}_2$.

MCF7 breast cancer cell line inducibly overexpressing miR-301a grow significantly slower in nude mice, show inhibition of ER signaling and exhibit enrichment of the cancer stem-like cells

To determine the role of miR-301a-3p in tumor growth, Balb/c nude athymic mice were implanted with slow-release estradiol pellet and injected subcutaneously with MCF7 cell line expressing miR-301a after induction by doxycycline (Fig. 6A) or with MCF7 cell line containing an empty vector (EV). Both miR-301a and EV clones exhibited similar growth potential without doxycycline induction *in vitro* as shown by confluency measurement by JULI FL cell analyzer and the changes in proliferation rates are thus not due to inherent properties of selected clones of MCF7 cells (Fig. 6B). The mice were given doxycycline diet and tumor growth was monitored twice a week with ultrasound imaging (Vevo770). Results show that the miR-301a overexpressing group exhibits significant inhibition of tumor growth in comparison with control group injected with cells carrying EV only (Fig. 7A). Both the tumor volume and weight from the miR-301a overexpressing group were significantly decreased in comparison with the control group (Fig. 7B, C).

Moreover, the overexpression of miR-301a in tumors led to a decrease in expression of ER α and PR proteins, as seen on western blot and also to inhibition of ER signaling measured

Fig. 4. MiR-301a-3p affects expression of proteins regulated by the ER α signaling in T47D and BT474 cells. Active ER α signaling induces certain proteins (GREB1, PR, CSTD, CXCL12) while others are downregulated (BMP7). Representative western blots showing expression of GREB1, PR, Cathepsin D, CXCL12 and BMP7 proteins in BT474 and T47D cells transfected with miR-301a mimic and miR-301a anti-miR.

Fig. 5. MiR-301a-3p modulates growth in response to 17β -estradiol. A, B, C, D Absorbance of crystal violet staining showing effect of 17β -estradiol on growth of MCF7 cells transfected with miR-301a mimic and miR-301a anti-miR and MCF7 cell line inducibly expressing miR-301a or EV after induction by doxycycline. Results are represented as mean values $\pm$ S.E.M. (n=3). Statistical significance was calculated using the t-test; * $p < 0.05$, ** $p < 0.01$.

by qRT-PCR. These results are in line with previous *in vitro* data and in accordance with the fact that MCF7 cells are dependent on estrogen signaling for their proliferation and once this signaling pathway is inhibited they do not grow (Fig. 7D, E). Importantly, we have seen a statistically significant upregulation of genes such as *CD44*, *ALDH1* (Aldehyde dehydrogenase 1), *ABCG2* (ATP binding cassette transporter subfamily G member 2), *VIM* (Vimentin), *ZEB1*, *ZEB2* (Zinc finger E-box-binding homeobox 1, 2), *HER2* (Erb-b2 receptor tyrosine kinase 2) and *VEGFA* (Vascular endothelial growth factor A) which are connected with cancer stem-like cell phenotype, EMT and metastasis (Fig. 7F).

Taken together, these data suggest that increased expression of miR-301a decreases the level of ER α which leads to inhibition of tumor growth of the estrogen-dependent MCF7 cell line *in vivo*, but on the other hand, it seems to induce an enrichment in the cancer stem-like cell population within the tumor.

MiR-301a-3p expression negatively correlates with ESR1 expression in breast cancer patient samples

MiR-301a-3p was found to be a crucial oncogene in human breast cancer acting through multiple pathways and mechanisms to promote tumor growth and metastasis [28, 37, 38]. To understand its role in breast cancer, we assessed the miR-301a-3p level in 111 human patient breast cancer samples clinically divided into four groups according to ER, PR and HER2 expression status, which was further confirmed by TaqMan qRT-PCR (data not shown). We found that expression of miR-301a-3p in ER+/PR+/HER2+ samples was 1.5-times lower when compared with the ER-/PR-/HER2+ patients and 2-times lower in comparison with ER-/PR-/HER2- samples (Fig. 8A). Similarly, miR-301a-3p was downregulated by 1.9 fold in ER+/PR+/HER2- samples relative to ER-/PR-/HER2+ samples and by 2.5-fold relative to ER-/PR-/HER2- samples (Fig. 8A). Unlike ER and PR, the expression of *HER2* has no statistically significant correlation with the expression of miR-301a-3p in studied samples (Fig. 8A). On the other hand, a statistically significant negative correlation between the miR-301a-3p and *ESR1* level was observed (Fig. 8B).

Collectively, observed results show a negative correlation between the expression of miR-301a-3p and ER/PR status in breast cancer patient samples, suggesting that higher expression of miR-301a-3p is connected with lower expression of *ESR1*.

Discussion

MiR-301a-3p has been shown as a negative prognostic marker in many human cancers since cells with high level of miR-301a-3p display higher metastatic potential [38, 39]. However, its role in ER-positive breast cancer has not been elucidated yet. Breast cancer is the most frequent malignancy in women worldwide. More than half of the patients are diagnosed with the estrogen positive cancer which has good prognosis and is often treated with the anti-estrogenic drug, tamoxifen. Although the drug is efficient and well tolerated,

Fig. 6. Characteristics of the miR-301a doxycycline inducible MCF7 cell line. (A) qRT-PCR showing expression of miR-301a-3p after doxycycline addition in miR-301a doxycycline-inducible MCF7 cell line and in MCF7 cell line carrying EV. (B) Graph showing confluency curve of MCF7 cell lines carrying doxycycline inducible miR-301a or EV measured without the addition of doxycycline by Juli cell analyzer. Results are represented as mean values $\pm$ S.E.M (n=3). Statistical significance was calculated using the t-test; * p<0.05, **p<0.01, *** p<0.001.

Fig. 7. MiR-301a-3p reduce tumor growth and inhibit ER signaling *in vivo*. A, Growth of MCF7 cell line inducibly expressing miR-301a after doxycycline addition in Balb/c nude mice (injected 2×10^6 cells per animal) which were implanted with slow-release estradiol pellet, given doxycycline diet; tumor volume was monitored twice a week and evaluated by ultrasound imaging instrument Vevo 770. B, Photographs showing representative tumors formed C, Tumor weight at the end of experiment D, Western blot showing expression of ER α and PR protein in tumor samples in control or miR-301a-3p overexpressing tumors and corresponding densitometry evaluation by image J. E, F, qRT-PCR showing expression of miR-301a-3p and *GREB1*, *PGR*, *CSTD*, *CXCL12*, *BMP7*, *CD44*, *ALDH1*, *ABCG2*, *VEGFA*, *VIM*, *ZEB1*, *ZEB2* and *HER2* genes in control or miR-301a-3p overexpressing tumors. Results are represented as mean values $\pm$ S.E.M. (n=7). Statistical significance was calculated using the t-test; * p<0.05, **p<0.01, *** p<0.001.

Fig. 8. MiR-301a-3p and *ESR1* expression inversely correlate in human breast cancer. A, qRT-PCR showing level of miR-301a-3p in breast cancer tissues divided according to their ER, PR and HER2 status into 4 groups. B, Pearson's correlation scatter plot of the correlation between *ESR1* gene and miR-301a-3p in breast cancer tissues. Results are represented as mean values $\pm$ S.E.M. Statistical significance was calculated using the t-test; * p<0.05, **p<0.01, *** p<0.001.

30-40 % of patients relapse with metastasis which do not response to endocrine therapy due to loss of ER [40, 41]. The transition from ER-positive to ER-negative breast cancer

thus represents a very important clinical problem and therefore finding markers predicting estrogen independence is of high importance.

ESR1 mRNA has a long 3'UTR region of about 4.3 kb bearing many evolutionarily conserved miRNA target sites, and several miRNAs such as miR-22 [42], -206 [43, 44], -145 [45] have already been reported to regulate ER α protein expression. In this report, we analyzed the *cis* regulatory sequences in the 3'UTR of *ESR1* gene mRNA and identified 2 seed sequences, which are able to bind miR-301a-3p. We have further generated a reporter vector where *Cypridina* luciferase gene is coupled to the 3'UTR of *ESR1* and demonstrated that deletion of these two sites results in higher expression of the luciferase, thus confirming the role of these two sites in the negative regulation of the ER α protein expression. Interestingly, site deletion showed that the first site closer to the end of translation is a preferred binding site for miR-301a-3p as its deletion resulted in identical phenotype to the deletion of both binding sites. Yet, the deletion of the second site also led to an increase in reporter luciferase expression suggesting that this site can be utilized by miR-301a-3p as well. We showed that miR-301a-3p downregulates *ESR1* mRNA and ER α protein level and, more importantly, inhibits the canonical estrogen signaling pathway in MCF7, T47D and BT474 breast cancer cell lines. This leads to lower expression of genes positively regulated by ER α (*PGRA*, *GREB1*, *CXCL12*, *CSTD*) and to induction of genes negatively regulated by ER α (*BMP7*). Moreover, we demonstrated that overexpression of miR-301a-3p caused inhibition of proliferation of estrogen-dependent MCF7 cells *in vitro* and suppressed the growth of miR-301a-3p overexpressing MCF7 tumors in nude mice. These results further confirmed that *ESR1* is a direct target of miR-301a-3p *in vitro* and *in vivo* and are in line with already published results referring to miRNAs targeting ER α [44, 45].

We did not detect any significant changes when using miR-301a-3p anti-miR with exception of BT474 cells that express high levels of miR-301a-3p. This suggests that the effect is predominantly caused by the translational inhibition of *ESR1* mRNA and could be detected only in cells with higher endogenous miR-301a-3p levels where the anti-miR treatment is more effective. An alternative explanation is that *ESR1* mRNA is relatively abundant and contains long 3'UTR with many other regulatory miRNA sites [46]. In that situation, the impact of miR-301a-3p anti-miR could be limited by low expression of miR-301a-3P in comparison with other abundant and non-inhibited regulatory miRNAs as suggested by Androsavich and Chau [47].

Interestingly, we detected an increase in the expression of *HER2* gene in miR-301a-3p overexpressing tumors. Our data are in line with observation that *HER2* is upregulated in tamoxifen-resistant MCF7 cells with decreased ER α signaling [48] and also in breast cancer stem-like cells [49]. Apart from *HER2*, we observed upregulation of other markers related to cancer stem-like cells, EMT and metastatic phenotype such as *CD44*, *ALDH1*, *ABCG2*, *VIM*, *ZEB1*, *ZEB2* and *VEGFA* in cells from miR-301a-3p overexpressing tumors. The cancer stem-like cell properties of miR-301a-3p overexpressing cells were further confirmed by analysis of mammospheres, the *in vitro* model of cancer stem-like cells [33], which expressed dramatically higher level of miR-301a-3p together with significantly lower ER α protein level, accompanied by inhibition of ER α targets GREB1 and PR. Furthermore, the other ER α -regulating miRNAs such as miR-22, -145 and -206 were not significantly and consistently elevated in sphere samples (data not shown). Given that Shi et al [28]. showed that miR-301a-3p renders breast cancer nonresponsive to the anti-estrogenic drug tamoxifen, we hypothesized that upregulation of miR-301a-3p may represent a feasible mechanism contributing to the phenotypical shift of primarily ER α dependent cells toward tumor cells relying on other proliferative signals. More importantly, since miR-301a-3p overexpressing cells acquire properties of cancer stem-like cells, which are highly invasive and resistant to treatment [50–52], they might represent the subpopulation that survives endocrine therapy and gives rise to relapsing metastasis.

In order to define whether the expression of miR-301a-3p in the samples of real human breast tumors is correlated with ER α expression, we analyzed primary human breast cancer biopsies and found significantly higher miR-301a-3p level in patient samples that were ER-

negative. Similarly, we have detected a significant negative correlation between miR-301a-3p expression and *ESR1* expression. Since miR-301a-3p is able to regulate ER α signaling it might serve as a biomarker of breast cancer prognosis and response to endocrine treatment. Our findings are thus in line with findings that higher expression of miR-301a-3p is significantly associated with larger tumor size and lymph node metastases in triple negative breast cancer [37]. Importantly, as miR-301a-3p also regulates other signaling pathways that are important in the progression of breast cancer such as PTEN/Akt or NF κ B signaling [28, 38, 53] it can be utilized as a general marker of poor prognosis.

In summary, our study provides functional evidence that miR-301a-3p is able to downregulate ER signaling in the ER positive breast cancer cells *in vitro* as well as *in vivo*, thus forcing them to become estrogen-independent with high selection pressure to activate alternative survival/pro-proliferative pathways in order to proliferate. The transition of estrogen-dependent cancer into the estrogen-independent cancer is one of the crucial steps in carcinogenesis and together with documented effects of miR-301a-3p on cancer motility and metastasis, our results suggest that miR-301a-3p might be used as a marker of poor patient prognosis and higher chance to become hormone-insensitive and resistant to tamoxifen.

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SL and JT developed the ideas and drafted the manuscripts. SL, JB, JT, DV, MV and VB conducted experiments and contributed to analysis of data. All authors contributed to revise the manuscript and approved the final version for publication.

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The ethics approval statements for animal work were approved by Czech Academy of Sciences and conducted in accordance with Czech Council guidelines for the Care and Use of Animals in Research and Teaching. Written informed consent was obtained from all participant and the study was approved by the Ethical Commission of the National Institute of Public Health in Prague.

Disclosure Statement

The authors declare that they have no conflicts of interest.

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