Non-coding RNA Research 7 (2022) 164–170

Contents lists available at ScienceDirect

Non-coding RNA Research

journal homepage: www.keaipublishing.com/en/journals/non-coding-rna-research

Original Research Article

Two lncRNAs, MACC1-AS1 and UCA1, co-mediate the expression of multiple mRNAs through interaction with individual miRNAs in breast cancer cells

Xiaona Zhang [a,1], Yanmei Zhu [a,1], Jun-Dong Wu [b], Yanchun Zhou [a], Weibing Chen [a], Wei Gu [a,*]

[a] Department of Pathophysiology, The Key Immunopathology Laboratory of Guangdong Province, Shantou University Medical College, Shantou, Guangdong Province, 515041, China
[b] Tumor Hospital, Shantou University Medical College, Shantou, Guangdong Province, 515041, China

ARTICLE INFO

Keywords:
lncRNA-miRNA-mRNA regulatory network
UCA1
MACC1-AS1

ABSTRACT

Background: Increasing studies have shown that lncRNAs often play roles through interaction with miRNAs to control gene expression by inhibiting translation or facilitating degradation of target mRNAs. Here, we report that two lncRNAs, MACC1-AS1 and UCA1 are coordinately expressed in breast cancer cells and share the ability to interact with multiple miRNAs to mediate the expression of different genes.
Methods: Targetscan, starBase and miRDB databases were used to predict the relationships of MACC1-AS1/UCA1-miRNA-mRNA network. qRT-PCR, and RNA sequencing were used to study the differential expression of lncRNAs and miRNA-targeted genes in breast cancer cells. RIP, RNA pull-down and luciferase assays were performed to confirm the molecular interactions of MACC1-AS1 or UCA1 with predicted miRNAs. The role of lncRNA-mediated miRNA-mRNA interactions in cell proliferation was examined by MTT assays following loss-of-function and gain-of-function effects.
Results: We identified a lncRNA-miRNA-mRNA regulatory network in breast cancer cells, in which a number of mRNAs can be co-regulated by MACC1-AS1 and UCA1 lncRNAs. Each lncRNA possesses the capacity as a ceRNA to compete with various mRNA-targeting miRNAs. Interaction of MACC1-AS1 or UCA1 with individual miRNAs is able to increase the expression of the same target mRNAs, such as TBL1X and MEF2D, thus affecting cancer-cell growth phenotype.
Conclusions: Our study suggests that in each cell type, there is a balance of interactions between certain lncRNAs and miRNAs. Disrupting the balance would eventually affect the expression of miRNA-targeted genes and cell proliferation.

1. Background

Breast cancer is one of the most common cancers worldwide and is the leading cause of cancer death among women [1]. The main cause of breast cancer-related death is metastasis, particularly to the lung, liver, and bone [2]. While significant progress has been made in understanding metastatic pathways, further research is necessary to evaluate breast cancer progression and prognosis, which might prevent metastasis and prompt cancer patients to take therapy at the early stage.

Advances in RNA-seq technology have revealed that the vast majority of the human genome is actively transcribed into non-coding RNAs (ncRNAs), in which microRNA (miRNA) is the most extensively studied class and plays important function during cell development and breast cancer progression [3,4]. MiRNAs post-transcriptionally silence gene expression by complementary base-pairing with target mRNAs. However, recent studies have identified new cellular mechanisms whereby miRNA-dependent gene regulation can be mediated by long non-coding RNAs (lncRNAs), another subtype of ncRNA transcribed from conserved genomic regions [5,6]. LncRNAs basically consist of at least 200 nucleotides and have limited protein-coding potential. Currently, the potential roles of the lncRNA-miRNA-mRNA network have become a focal point in understanding the pathogenesis and

* Corresponding author.
E-mail addresses: abc519088@qq.com (X. Zhang), 833091596@qq.com (Y. Zhu), wujun-dong@163.com (J.-D. Wu), zyc2013st@foxmail.com (Y. Zhou), 342284415@qq.com (W. Chen), weigu1@yahoo.com, weigu@stu.edu.cn (W. Gu).
[1] These authors contributed equally to this study.

https://doi.org/10.1016/j.ncrna.2022.06.003
Received 27 February 2022; Received in revised form 23 June 2022; Accepted 23 June 2022
Available online 4 July 2022

development of many human diseases including breast cancer, in which lncRNAs can serve as oncogenes or gene regulators to participate in certain gene expression pathways, depending on the circumstance [7,8].

LncRNAs can potentially function as competitive endogenous RNAs (ceRNAs) to interact with miRNAs through complementary base-pairing sequences. This interplay allows the shift of miRNA availability away from their target mRNAs to the lncRNAs. Thus, identifying lncRNA/miRNA/mRNA axes not only provides insights for studying oncogenic gene expression, but also offers novel targets for clinically relevant research. We have previously shown that UCA1 (human urothelial carcinoma associated 1), which is highly expressed in breast, colorectal and gastric tumors, can act as a ceRNA for endogenous miRNAs, such as miR-122-5p [9]. MACC1-AS1, a cognate antisense RNA for the sixth intron of MACC1 mRNA (metastasis-associated in colon cancer-1), is another example of a lncRNA that can serve as a molecular sponge for miRNAs to functionally affect the activity of RNA transcripts targeted by the miRNAs [10]. Interestingly, both UCA1 and MACC1-AS1 have the feature of competitively interacting with multiple miRNAs to simultaneously mediate the expression of different mRNAs originally silenced by the miRNAs to promote cell proliferation and invasion [9,10].

In this study, we identify a typical paradigm of a lncRNA-mediated mRNA regulatory network that could be involved in the underlying molecular mechanisms of breast cancer progression. We comprehensively analyze the crosstalk of MACC1-AS1/UCA1 with potential miRNAs and evaluate the consequence taking place from the crosstalk. By using RNA-seq databases and bioinformatic assays combined with biochemical analysis, we demonstrate that multiple mRNAs, such as TBL1X, NACC1 and MYO9A, could be simultaneously mediated by MACC1-AS1 or UCA1 via interaction with different miRNAs, revealing the complexity and multifunctional network of ncRNA in regulating gene expression and cell activities in breast cancer cells.

2. Methods

2.1. Bioinformatic studies

The potential regulatory relationships between MACC1-AS1/UCA1 and miRNAs, and the miRNA-targeted mRNAs were investigated. We predicted potential miRNA target sites on the transcripts of the two lncRNAs using three algorithms, TargetScan 7.2 (https://www.targetscan.org/vert_72/, released in March 2018), StarBase 3.0 (https://starbase.sysu.edu.cn/, released in Nov 2018) and miRDB (https://www.mirdb.org/, released in Dec 2018) from public databases. In addition, we also searched miRNA-targeted mRNAs from published literature [11,12]. Using this approach, seven mRNAs that could be co-regulated by MACC1-AS1 and UCA1 lncRNAs through interaction with ten miRNAs were characterized. The relationship of expression between MACC1-AS1 and UCA1 in human breast tumors was investigated using on line dataset, which comprised RNA-seq data from TCGA tumors (TCGA Data Portal at https://tcga-data.nci.nih.gov/tcga/). Spearman's correlation analysis was used to describe the correlation between quantitative variables without a normal distribution.

2.2. Reagents

PCR and qRT-PCR primers were purchased from IGE Biotech (Guangzhou, China) and are listed in Suppl Table S1. Primers for detection of the selected miRNAs were designed and synthesized in TianGen Biotech Co. Ltd (Beijing, China) and are listed in Suppl Table S2. Since the issue of related intellectual property, no information of the primer sequence was provided by the company. MiRNA mimics and siRNAs were purchased from Gene Pharma (Suzhou, China) and their sequences are listed in Suppl. Table S3. Lipofectamine 2000 reagent (Invitrogen, USA) was purchased from Thermo Fisher Scientific (Shanghai Branch, China). Promega's Dual-Luciferase Reporter Assay System (USA) was imported and sold by Accurate Biology (Hunan, China). SYBR Green Premix Pro Taq HS qPCR Kit (AG11701) was purchased from Accurate Biology. Amylose Resins (E8021L) and Q5® Site-Directed Mutagenesis Kit (E0552) were purchased from New England Biolab (MA, USA). MTT Cell Proliferation and Cytotoxicity Assay Kit (M1020) was purchased from Solarbio Life Sciences (Beijing, China). Recombinant MBP-MCP for MS2-tagged RNA pulldown was previously produced in our Lab [9]. Rabbit antibody against human Ago2 was purchased from Sangon Biotech (Shanghai, China).

2.3. Cell lines and culture

Primary MDA-MB-231, MCF7 and HEK293T cell lines were purchased from ATCC (USA), and cultured and passaged according to standard instructions of the ATCC. Cells were verified by determination of STR profiling through PCR following the instructions of the ATCC. The latest test was performed at October 2017. MDA-MB-231 stable cell lines expressing MS2-conjugated UCA1 or MACC1-AS1 and control cell lines were established previously [9,10]. In these stable cells, expressing levels for UCA1 is about 12 folds and for MACC1-AS1 is about 18 folds in contrast to control cells. Cells were grown in DMEM medium supplemented with 10% FBS, 100 units of penicillin/ml and 100 mg of streptomycin/ml. Cells were incubated at 37 °C and 5% CO_2 in a humidified chamber to a confluence of 90–95%. For large preparation, cells were expanded and passed two to three passages.

2.4. Cell transfection assays

Transfection of lentivirus vectors expressing MACC1-AS1, UCA1 or TBL1X mRNA, and transfection of siRNAs or miRNA mimics against MACC1-AS1, UCA1 or TBL1X mRNA were performed using Lipofectamine 2000 reagent based on the manufacture's instruction. At 48–72 h after transfection, cells were collected. Expression levels of the genes of interest in transfected cells were detected by RT-qPCR. Transfection assays were repeated for three times and data are statistically analyzed. A P value < 0.05 is considered significant.

2.5. Plasmid construction

Human TBL1X, PCDH1, MYO9A, MEF2D and NACC1 cDNAs were amplified by RT-PCR from total RNA isolated from MDA-MB-231 cells using the primers listed in Supp Table S1. TBL1X cDNA was cloned into the pCIP2 lentivirus plasmid [9](TBL1X vector) at the Not I and Bam HI sites. Mutant MACC1-AS1 or UCA1 was generated using Q5® Site-Directed Mutagenesis Kit. Mutations of the binding sites for potential miRNAs within MACC1-AS1 or UCA1 are indicated in Supp Fig. S5. To construct luciferase reporter plasmids, the DNA fragments of wild-type (WT), mutant MACC1-AS1 or UCA1 were cloned at the 3' of the Renilla luciferase gene of the psiCHECK-2 plasmid (Promega, USA). All the constructs were verified by sequencing.

2.6. MS2 pulldown assays to analyze MACC1-AS1 or UCA1 associated miRNAs

Pulldown assays of MS2-taged MACC1-AS1 or UCA1 were performed as previously described [9]. The pulldown assays used a recombinant fusion protein MBP-MCP that contains a maltose binding domain (MBP) and an MCP domain that recognizes the MS2 hairpins. Briefly, amylose beads (NEB, USA) were incubated with recombinant MBP-MCP for 1 h at 4 °C. Cell lysates prepared from cultured cells stably expressing $MS_2(6)$-tagged UCA1 or MACC1-AS1 were incubated with MBP-MCP coated amylose resins at 4 °C in the presence of RNase and protease inhibitors. After incubation for 5 h with gentle shaking, amylose resins were extensive washed and bound UCA1-MS2 or MACC1-AS1-MS2 RNP complexes were eluted with 100 μl lysis buffer containing 20 mM maltose. Total RNAs were extracted using TRIzol reagent (Invitrogen) and measured for enrichment of precipitated UCA1 or MACC1-AS1

RNAs by RT-qPCR. Associated miRNAs with precipitated RNAs were verified using the primers listed in Supp Table S2.

2.7. Total RNA extraction, reverse transcription and quantitative PCR (qPCR)

Total RNA from cultured cells and RNA pulldown precipitates was prepared using an RNA extraction kit (Tiangen, China). First-strand cDNA was synthesized using the PrimeScript RT reagent kit with gDNA Eraser (Takara, China). qPCR was performed using a SYBR Green Premix Pro qPCR kit and measured in an ABI Prism 7500 (Applied Biosystems, Foster City, CA). miRNA extraction was performed using a miRcute miRNA Isolation kit (Tiangen, China). Briefly, total RNAs were extracted and miRNAs were polyadenylated and reverse transcribed using the universal reverse PCR primer provided in the kit. The resulting cDNA was then subjected to qPCR. U6 snRNA was used as an internal control. The specific forward primers for candidate miRNAs and control U6 are listed in Suppl. Table S2. Each sample was analyzed in triplicate. The $2^{-\Delta\Delta ct}$ method was used to calculate the relative gene expression levels.

2.8. RNA immunoprecipitation assays (RIP)

Extracts of cultured MDA-MB-231 cells expressing UCA1 or MACC1-AS1 were prepared. Co-IP experiments were performed using antibodies against Ago2 and protein A resins (Sigma Aldrich, USA) following the manufacturer's instruction. Briefly, 30 μl of protein A resins were incubated with 200 μl cell extracts (5 mg/ml) on ice for 4 h with gentle agitation. After centrifugation, the resins were washed extensively. Aliquots of precipitated fractions were analyzed by western blots using anti-Ago2 antibodies. The rest was used for RNA extraction using TRIzol reagent. mRNAs co-precipitated with Ago2 were detected by RT–qPCR.

2.9. Luciferase assays

psiCHECK-2 reporter plasmids were transfected or co-transfected with individual mimic miRNAs into HEK-293T cells for 24–36 h using Lipofectamine 2000 reagent. After transfection, the cells were harvested for firefly/Renilla luciferase assays using Promega's Dual-Luciferase Reporter Assay System following the manufacturer's instruction. Luciferase activities were normalized to the empty psiCHECK-2 plasmid. The transfection and luciferase assays were repeated for three times and data are statistically analyzed.

2.10. Cell proliferation assays

Cell proliferation was determined by a 3-(4, 5-dimethylthiazolyl-2-yl)-2-5 diphenyltetrazolium bromide (MTT) assay using a MTT Cell Proliferation and Cytotoxicity Assay Kit [9]. Briefly, cells were seeded in 96-well plates at 5×10^3 cells per well in a final volume of 100 μl. Cells were incubated for 2, 24, 48 and 72 h, respectively, at 37 °C and 10 μl (5 mg/ml) of MTT solution was added to each well. After 4 h incubation, the supernatant was discarded and 150 μl of DMSO was added. Absorbance at 570 nm was measured by a microplate spectrophotometer (BioRad, USA). Each experiment was performed in triplicate and repeated for three times.

2.11. Statistical analysis

Student's t-test (two-tailed) was used for analyzing the significance of experimental results. One-way ANOVA followed by Tukey's multiple comparison test was used for analysis in more than two groups. For analyzing qPCR results, data from three independent experiments were calculated by the $2^{-\Delta\Delta Ct}$ method and represented as the means ± S.D. Only *P*-values lower than 0.05 were considered to be significant.

3. Results

3.1. A lncRNA-mediated regulatory network paradigm

Previously, we showed the oncogenic roles of lncRNAs MACC1-AS1 and UCA1 in promoting breast cancer cell proliferation, tumor growth and metastasis [9,10]. Both MACC1-AS1 and UCA1 contains multiple miRNA-response elements (MREs) and function as ceRNAs to post-transcriptionally mediate the expression of particular mRNAs [9,10]. Using three algorithms, TargetScan 7.2 (https://www.targetscan.org/vert_72/), starBase 3.0 (https://starbase.sysu.edu.cn/) and miRDB (https://www.mirdb.org/) from public databases to evaluate possible MACC1-AS1- or UCA1-bound miRNAs and searching published literature [11,12] for the miRNA-targeted mRNAs, we predicted a typical paradigm of a lncRNA-mediated regulatory network, in which MACC1-AS1 and UCA1 not only communicate with different miRNAs, but also co-regulate the same downstream target mRNAs via this communication (Fig. 1). To determine the potential of the MACC1-AS1/UCA1-mediated network, we examined expression levels of the five predicted mRNAs, TBL1X, NACC1, PCDH1, MYO9A and MEF2D, in MDA-MD-231 cell lines stabled overexpressing MACC1-AS1 or UCA1. All five mRNAs showed oncogenic characteristics. For examples, high expression of TBL1X mRNA correlates with poor prognosis and increased metastasis in breast tumor patients and MEF2D promotes cancer cell invasion and EMT [13–15]. RT-PCR experiments indicated that the cellular levels of the selected mRNAs were significantly increased in MACC1-AS1-overexpressing cells (Fig. 2A) or in UCA1-overexpressing cells with the exception of PCDH1 mRNA (Fig. 2B). In contrast, knockdown of endogenous MACC1-AS1 or UCA1 in MDA-MB-231 cells by siRNAs resulted in decreased levels of these mRNAs (Supp. Fig. S1). Enhanced expression of the mRNAs by MACC1-AS1 or UCA1 was also observed in less-invasive MCF7 breast cancer cells (Supp. Fig. S2). More interestingly, although MACC1-AS1 and UCA1 do not interact directly (Fig. 2C), expression of the two lncRNAs was mutually coordinated, since overexpression of either MACC1-AS1 or UCA1 increased levels of UCA1 or MACC1-AS1, respectively, in breast cancer cell lines (Fig. 2D and E). In consistent, a weak corelated expression of MACC1-AS1 and UCA1 was also shown in human breast tumors by analyzing TCGA RNA-seq database (https://tcga-data.nci.nih.gov/tcga/) (Fig. 2F, $R = 0.120$, $P < 0.001$). Based on the fact that both UCA1 and MACC1-AS1 can function as ceRNAs, we assume that the coordinated expression of UCA1 or MACC1-AS1 with these mRNAs would be through competitively sponging miRNAs originally bound to their target mRNAs.

3.2. Multiple miRNAs that target particular mRNAs can be sponged by MACC1-AS1 or UCA1

Next, we selected TBL1X mRNA as an example to address our hypothesis that particular mRNAs can be simultaneously mediated by different lncRNAs via competitively binding to the miRNAs. We first separately transfected MDA-MD-231 cells with miR-384, miR-181d-5p or miR-10b-5p. Based on the predicted model and our previous results, the three miRNAs were able to target TBL1X mRNA (Fig. 1), as well as bind to MACC1-AS1 (for miR-384 and miR-181d-5p) or UCA1 (miR-10b-5p) [9,10]. After transfection, all three miRNAs showed the ability to knockdown TBL1X mRNA by 30–50% (Fig. 3A). However, in MACC1-AS1- or UCA1-overexpressing cells, the knockdown efficacy caused by the miRNAs was significantly reduced, indicating lncRNA MACC1-AS1 or UCA1 interfered with miRNA-mediated degradation of TBL1X mRNA (Fig. 3B and C). To address the possibility that this interference resulted from sponging the miRNAs by MACC1-AS1 or UCA1, we performed RNA pulldown experiments to precipitate MS2-conjugated MACC1-AS1 or UCA1 chimeric RNA (Fig. 1) [9,10]. RT-qPCR assays of the RNA precipitates showed that all three miRNAs, miR-384, miR-181d-5p and miR-10b-5p, were preferentially

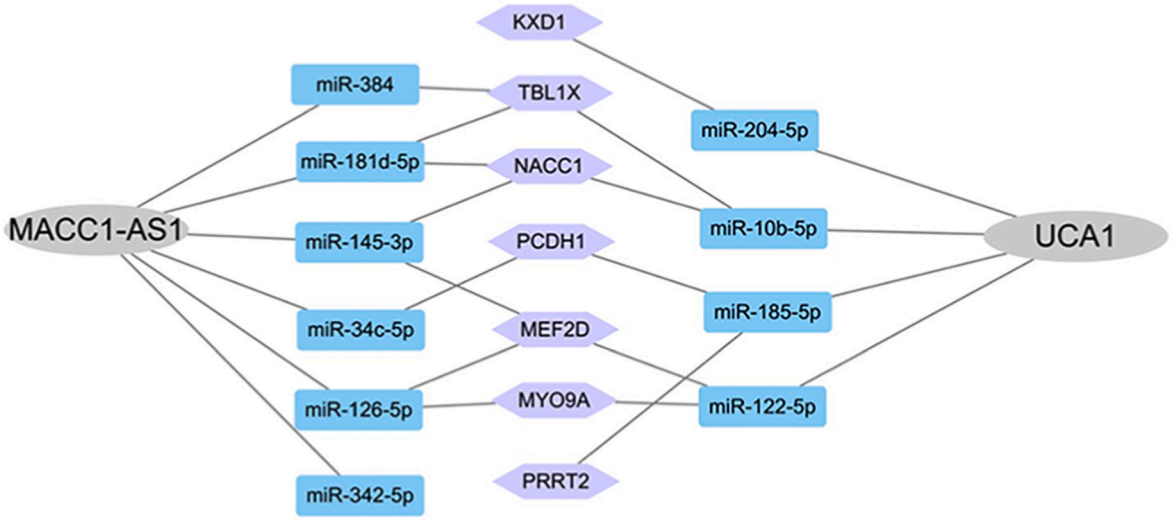

Fig. 1. Predicted model of a MACC1-AS1/UCA1-mediated regulatory network
A predicted lncRNA-mediated regulatory network showing that MACC1-AS1 and UCA1 can co-modulate the expression of a selected group of mRNAs through binding to multiple miRNAs.

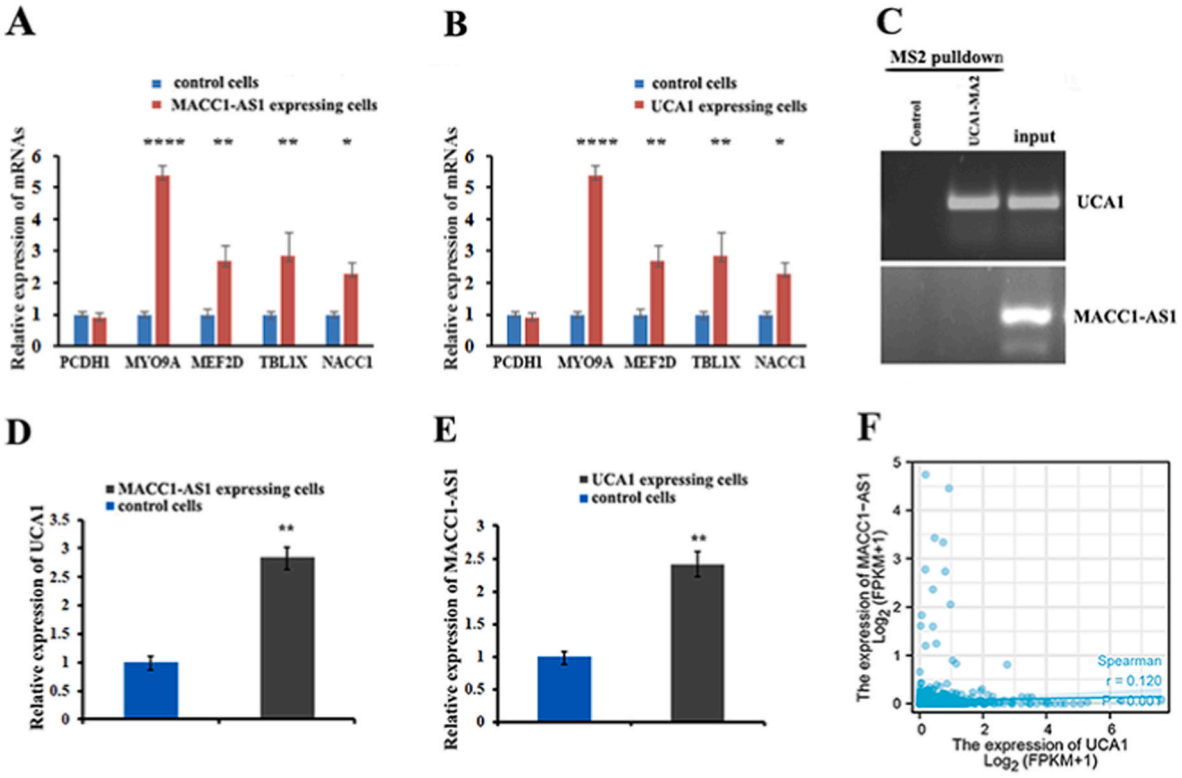

Fig. 2. Coordinated expression of UCA1 or MACC1-AS1 with their target mRNAs
(A) and (B) Total RNA was isolated from MDA-MB-231 cells stably overexpressing MACC1-AS1 or UCA1. Levels of five predicted mRNAs were examined by RT-qPCR. Relative levels of the mRNAs were normalized to GAPDH mRNA and the data are presented as means ± SD from three independent experiments: *P < 0.05, **P < 0.01 as determined by Student's *t*-test. (C) MS2 pulldown was performed in MDA-MB-231 cells expressing UCA1-MS2. Co-precipitated UCA1-MS2 (upper) and MACC1-AS1 (lower) were measured by RT-PCR and agarose gel electrophoresis. The 'control' indicates the cells without UCA1-MS2 expression. (D) and (E) Expression of MACC1-AS1 or UCA1 is mutually regulated. RNA was extracted from MACC1-AS1- or UCA1-overexpressing cells and the levels of lncRNAs were measured by RT-qPCR. Data are analyzed from three independent experiments. *$P < 0.05$, **$P < 0.01$ as determined by Student's t-test. (F) Spearman correlation analysis of UCA1 and MACC1-AS1 expression in human breast tumors using comprised mRNA-seq data from TCGA tumor database. A *p*-value of less than 0.05 was considered statistically significant.

coprecipitated with MACC1-AS1 or UCA1 (Fig. 3D and E), suggesting that such competitive binding decreases the abundance of the miRNAs and releases miRNA-mediated repression of their downstream targets.

To further test whether MACC1-AS1 or UCA1 would sequester miRNAs from binding to their downstream targets, we performed RIP experiments in the extracts of MDA231 cells using antibody against Ago2, a

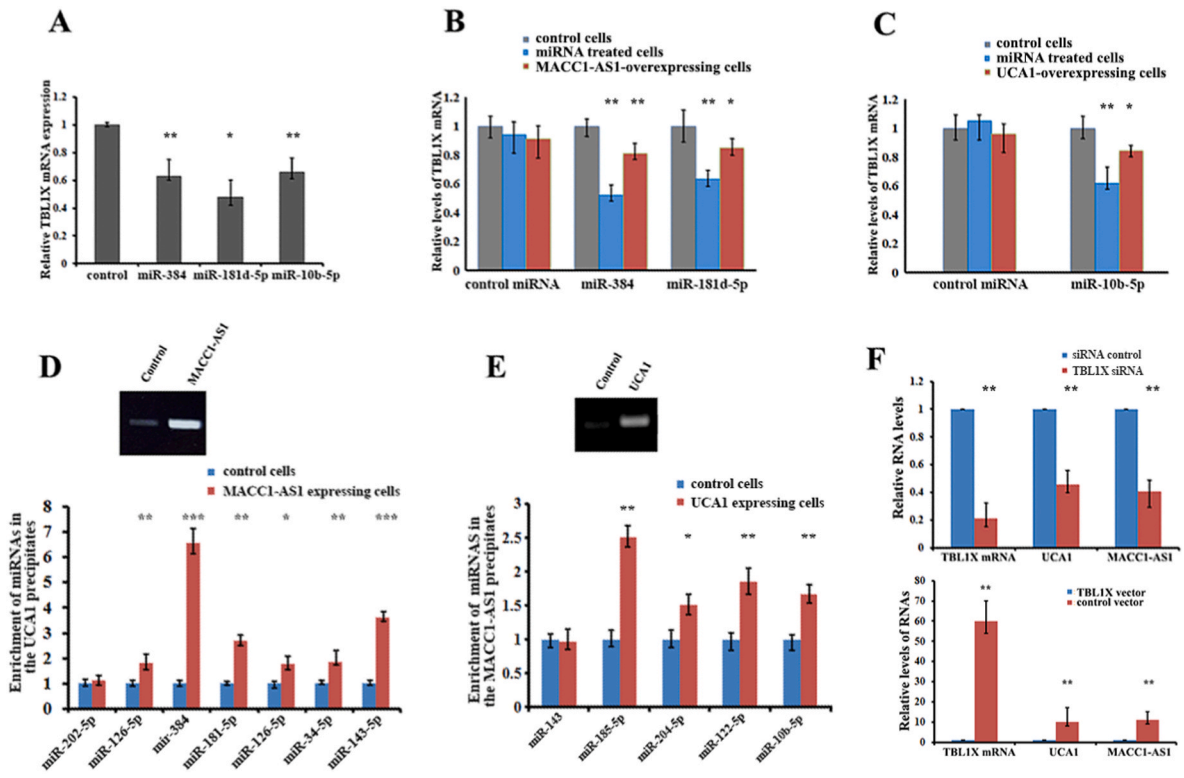

Fig. 3. Sponge effect of MACC1-AS1 or UCA1 on miRNAs affects the expression of their target mRNAs
(A) MDA-MB-231 cells were transfected with miR-384, miR-181d-5p or miR-10b-5p. RT-qPCR showing that expression of TBL1X mRNA was knocked down by each miRNA. (B) and (C) miR-384, miR-181d-5p or miR-10b-5p was transfected into MDA-MB-231 cells overexpressing MACC1-AS1 or UCA1. Relative levels of TBL1X mRNAs are the means ± SD from three independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ as determined by one-way ANOVA followed by Tukey's multiple comparison tests. (D) and (E) MS2 pulldown assays were performed in MDA-MB-231 cells overexpressing MACC1-AS1-MS2 or UCA1-MS2. Enrichment of MACC1-AS1 or UCA1 in the precipitates was measured by RT-PCR (upper panels). After MS2 pulldown, levels of individual miRNAs associated with MACC1-AS1 or UCA1 were measured by RT-qPCR. The data are presented as means ± SD from three independent experiments. $^{**}P < 0.01$ as determined by Student's t-test. (F) Upper: MDA-MB-231 cells were treated with TBL1X siRNA for 48 h, Lower: A vector expressing TBL1X mRNA was transfected into MDA-MB-231 cells for three days. Relative levels of TBL1X mRNA, MACC1-AS1 and UCA1 were measured by RT-qPCR. Data are presented as means ± SD from three independent experiments. $^{**}P < 0.01$ as determined by Student's t-test.

core component of RISC (RNA-induced silencing complex) and followed by RT-qPCR to analyze the levels of TBL1X, MYO9A and NACC1 mRNAs in the precipitate. Results indicated that the levels of three mRNAs were reduced by 30–70% when MACC1-AS1 or UCA1 was overexpressed. (Supp Fig. S3). In addition, the relative binding ability of a miRNA to a corresponding mRNA or lncRNA is reciprocal, since knockdown or increasing TBL1X mRNA also affected the levels of MACC1-AS1 or UCA1 lncRNA (Fig. 3F), indicating a miRNA can also shift from an mRNA to a lncRNA. These studies confirmed our predicted lncRNA-miRNA-mRNA network in which MACC1-AS1 or UCA1 does not only regulate the expression of particular genes through binding to multiple miRNAs, but also co-mediates the expression of a subset of genes, revealing the complexity of lncRNA-directed post-transcriptional regulation of mRNAs through restricting the function of miRNAs.

3.3. Crosstalk between lncRNA and miRNA changes the gene expression pattern and affect cell proliferation

The nature of interactions between lncRNA and miRNA is based on complementary base-pairing of the RNA sequences. Based on experimental results, predicted positions and the binding sites for potential miRNAs within MACC1-AS1 or UCA1 are indicated (upper panels of Fig. 4A and B, and Supp Fig. S4). To determine the biological consequence of the interactions between MACC1-AS1 or UCA1 and the miRNAs on gene expression, two groups of luciferase reporters were constructed. One group contained wild-type MACC1-AS1 or UCA1 that was fused to the luciferase gene as a 3′UTR. The other contained mutated MACC1-AS1 or UCA1 sequence in which each putative miRNA binding site in the lncRNA was individually mutated (Supp Fig. S5). After co-transfecting the mimic miRNAs with mutated luciferase reporters into HEK-293T cells, five of the six miRNAs were able to reduce the luciferase reporter activity by 30–40% (lower panels of Fig. 4A and B). These experiments indicate that the preferential binding of MACC1-AS1 or UCA1 to the miRNAs destabilizes the reporters and thus decrease the luciferase activity.

Since the oncogenic feature of MACC1-AS1 and UCA1, we assume that the crosstalk between the two lncRNAs and miRNA would eventually affect the growth phenotype of breast cancer cells through sequestering miRNAs from binding to their target mRNAs. This hypothesis was addressed by MTT experiments in which MACC1-AS1- or UCA1-induced cell proliferation could be repressed by overexpression of miR-181d-5p or miR-10b-5p, which silenced TBL1X mRNA, as well as by knockdown of TBL1X mRNA (Fig. 4C and D). These results suggest that interactions between MACC1-AS1 or UCA1 with miRNAs impact the expression of miRNA-targeted genes and influence cell proliferation.

4. Discussion

In recent years. the functions and regulatory network of lncRNA and miRNAs are continuously becoming more deeply understood [16,17]. Analogical to miRNAs and protein-coding genes, lncRNAs can play important role in cancer progression. Several well-known lncRNAs have been identified to be aberrantly expressed and involved in breast tumor metastasis, such as *HOTAIR* and lncRNA *H19* [18,19]. In this work, using

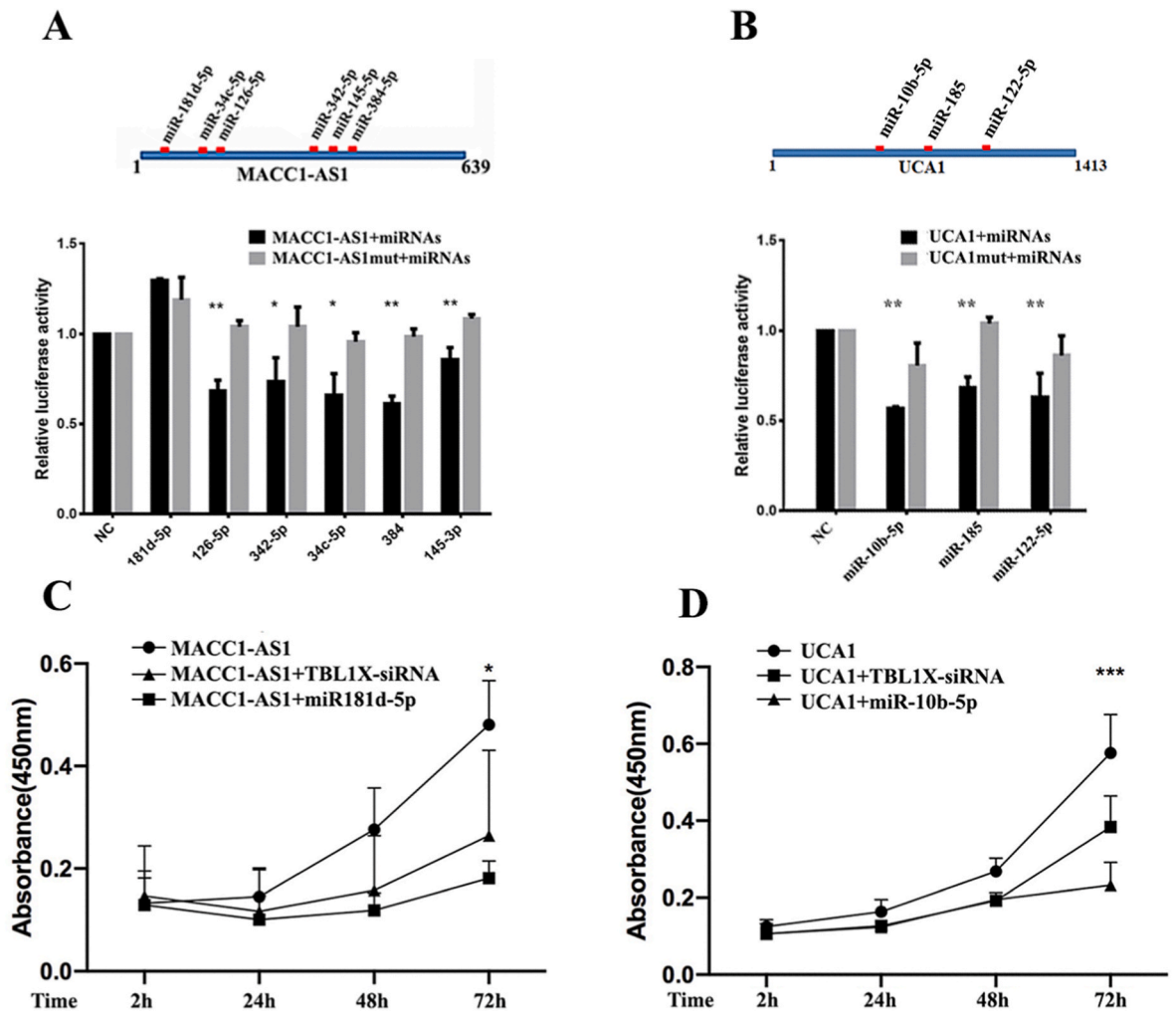

Fig. 4. Crosstalk between lncRNA and miRNA changes gene expression pattern and affects cell proliferation
(A) and (B) Upper panels: schematic representation of the putative binding sites for individual miRNAs within MACC1-AS1 or UCA1 lncRNA. Lower panels: the entire MACC1-AS1 or UCA1 sequence, as well as the mutant sequences, in which the putative miRNA biding sites were individually mutated, were cloned downstream of the Renilla luciferase gene of the PsiCHECK 2 dual luciferase reporter construct. HEK293T cells were co-transfected with the reporters, or the mutant reporters with individual miRNAs. Luciferase activity was determined in 24 h. Activity of Renilla luciferase was normalized to the activity of firefly luciferase. Data from three independent experiments were statistically analyzed. $^{**}P < 0.01$, $^{*}P < 0.05$ as determined by Student's t-test. (C) and (D) MTT assays were used to measure cell proliferation in MACC1-AS1- or UCA1-overexpressing cells in the presence of individual miRNAs or TBL1X siRNA. Data are repeated for three times and presented as means ± SD. $^{*}P < 0.05$, $^{***}P < 0.001$.

bioinformatic study based on three different RNA-seq databases, we predicted a potential regulatory network mediated by MACC1-AS1/UCA1 lncRNAs, both of which are largely oncogenic in breast cancer cells [9,10]. This prediction was further determined by biochemical experiments and cell proliferation assays. We demonstrate that the expression of a number of mRNAs can be coordinately regulated by MACC1-AS1 and UCA1 through sponging multiple miRNAs that target different mRNAs. Similar to how miRNAs can target multiple mRNAs [20,21], our multiplex results show that MACC1-AS1 and UCA1, which possess the capacity to function as ceRNAs, compete with various mRNA-targeting miRNAs. Thus, lncRNAs and mRNAs build complicated networks through their ability to interact with miRNAs.

5. Conclusions

We identified a lncRNA-miRNA-mRNA regulatory network in breast cancer cells, in which a number of mRNAs are co-regulated by MACC1-AS1 and UCA1 lncRNAs. This regulation is accomplished through cellular crosstalk of the lncRNAs with particular miRNAs that have the ability to destabilize their corresponding mRNAs via complementary base-pairing. Using TBL1X mRNA as an example, we show that an mRNA can not only be regulated by two different lncRNAs through interaction with individual miRNAs, but can also perform a role in reciprocally mediating the levels of lncRNAs (Fig. 2). The data suggest that in each cell type, there could be a balance of interactions between certain lncRNAs and miRNAs or mRNAs and miRNAs. Disrupting the balance will eventually affect downstream gene expression and cell proliferation.

Ethics approval and consent to participate

Not applicable.

Funding

Not Applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

CRediT authorship contribution statement

Xiaona Zhang: carried out most of the molecular and biochemistry experiments, and performed cell biology studies, carried out bioinformatic study, data collection and statistical analysis. **Yanmei Zhu:** carried out most of the molecular and biochemistry experiments, and performed cell biology studies. **Jun-Dong Wu:** carried out bioinformatic study, data collection and statistical analysis. **Yanchun Zhou:** carried out bioinformatic study, data collection and statistical analysis, performed cell proliferation and imaging experiments. **Weibing Chen:** performed cell proliferation and imaging experiments. **Wei Gu:** designed research experiments, coordinated studies, and wrote the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (grant number 31171209) to W.G.

Abbreviations

LncRNA	Long non-coding RNAs
MACC1-AS1	MACC1 antisense RNA 1
UCA1	Urothelial cancer associated 1
DMEM	Dulbecco's modified Eagle's medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
TBL1X	Transducin beta like 1 X-linked
MEF2D	Myocyte enhancer factor 2D
PCDH1	Protocadherin 1
MYO9A	Myosin IXA
NACC1	Nucleus accumbent associated 1
ceRNA	Competing endogenous RNAs
RIP	RNA immunoprecipitation

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ncrna.2022.06.003.

References

[1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, CA A Cancer J. Clin. 70 (1) (2020) 7–30, 2020.
[2] S. Kimbung, et al., Transcriptional profiling of breast cancer metastases identifies liver metastasis-selective genes associated with adverse outcome in luminal A primary breast cancer, Clin. Cancer Res. 22 (1) (2016) 146–157.
[3] H. Ling, M. Fabbri, G.A. Calin, MicroRNAs and other non-coding RNAs as targets for anticancer drug development, Nat. Rev. Drug Discov. 12 (11) (2013) 847–865.
[4] B.J. Petri, C.M. Klinge, Regulation of breast cancer metastasis signaling by miRNAs, Cancer Metastasis Rev. 39 (3) (2020) 837–886.
[5] J. Liz, et al., Regulation of pri-miRNA processing by a long noncoding RNA transcribed from an ultraconserved region, Mol. Cell 55 (1) (2014) 138–147.
[6] S. Panni, et al., Non-coding RNA regulatory networks, Biochim. Biophys. Acta. Gene Regul. Mech. 1863 (6) (2020), 194417.
[7] M. Jadaliha, et al., A natural antisense lncRNA controls breast cancer progression by promoting tumor suppressor gene mRNA stability, PLoS Genet. 14 (11) (2018) e1007802.
[8] M.D. Wasson, et al., Datasets exploring putative lncRNA-miRNA-mRNA axes in breast cancer cell lines, Data Brief 37 (2021), 107241.
[9] Y. Zhou, et al., IMP1 regulates UCA1-mediated cell invasion through facilitating UCA1 decay and decreasing the sponge effect of UCA1 for miR-122-5p, Breast Cancer Res. 20 (1) (2018) 32.
[10] X. Zhang, et al., LncRNA MACC1-AS1 sponges multiple miRNAs and RNA-binding protein PTBP1, Oncogenesis 8 (12) (2019) 73.
[11] A. Talukder, X. Li, H. Hu, Position-wise binding preference is important for miRNA target site prediction, Bioinformatics 36 (12) (2020) 3680–3686.
[12] G. Riolo, et al., miRNA targets: from prediction tools to experimental validation, Methods Protoc. 4 (1) (2020).
[13] S. Rivero, et al., TBL1 is required for the mesenchymal phenotype of transformed breast cancer cells, Cell Death Dis. 10 (2) (2019) 95.
[14] W. Shan, et al., miR-339-5p inhibits migration and invasion in ovarian cancer cell lines by targeting NACC1 and BCL6, Tumour Biol. 37 (4) (2016) 5203–5211.
[15] L. Su, et al., MEF2D transduces microenvironment stimuli to ZEB1 to promote epithelial-mesenchymal transition and metastasis in colorectal cancer, Cancer Res. 76 (17) (2016) 5054–5067.
[16] J.S. Mattick, I.V. Makunin, Non-coding RNA, Hum. Mol. Genet. (1) (2006) R17–R29.
[17] J. Venkatesh, et al., LncRNA-miRNA axes in breast cancer: novel points of interaction for strategic attack, Cancer Lett. 509 (2021) 81–88.
[18] Y. Ren, et al., Paracrine and epigenetic control of CAF-induced metastasis: the role of HOTAIR stimulated by TGF-ss1 secretion, Mol. Cancer 17 (1) (2018) 5.
[19] W. Zhou, et al., The lncRNA H19 mediates breast cancer cell plasticity during EMT and MET plasticity by differentially sponging miR-200b/c and let-7b, Sci. Signal. 10 (483) (2017).
[20] M.S. Ebert, P.A. Sharp, Emerging roles for natural microRNA sponges, Curr. Biol. 20 (19) (2010) R858–R861.
[21] M.S. Ebert, J.R. Neilson, P.A. Sharp, MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells, Nat. Methods 4 (9) (2007) 721–726.