miR-381 suppresses C/EBPα-dependent Cx43 expression in breast cancer cells

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Synopsis

Cx43 (connexin43) is an enhancer of the metastasis of breast cancer cells. Our previous study identified miR-381 as an indirect suppressor of Cx43 gene expression, with the precise mechanism being not understood. In the present study, using a reporter gene assay, we found that miR-381 suppressed Cx43 gene expression via the promoter region −500/−250. With site-directed gene mutation, we demonstrated that miR-381 could directly bind with the sequences CACUUGUAU in the 3′-UTR so as to inhibit C/EBPα (CCAAT/enhancer-binding protein α) expression. C/EBPα was further identified as a novel transcription factor by binding to a canonic element (AATTGTC) locating at −459/−453 in the promoter region of the Cx43 gene. Functionally, we demonstrated that miR-381 suppressed C/EBPα- and Cx43-dependent migration and invasion of breast cancer cells. Finally, we revealed that decreased levels of miR-381 as well as increased expression of C/EBPα and Cx43 in the metastatic breast cancer cells and tissues. Therefore we are the first to identify that miR-381 suppresses C/EBPα-dependent Cx43 expression in breast cancer cells. The miR-381–C/EBPα–Cx43 axis might be a useful diagnostic and therapeutic target of metastatic breast cancer.

Key words: C/EBPα, Cx43, MCF-7, MDA-MB-231, miR-381, 3′-UTR.

Cite this article as: Bioscience Reports (2015) 35, e00266, doi:10.1042/BSR20150167

INTRODUCTION

More than 1 million women are diagnosed with breast cancer every year worldwide, accounting for one-tenth of all new cancers and 23% of all female cancer cases [1,2]. Approximately 232340 new cases of invasive breast cancer and 39620 breast cancer deaths are expected to occur among U.S. women in 2013 [2]. One in eight women in the U.S.A. develop breast cancer in their lifetime [2]. The relapse and metastasis are the major causes of this disease-related death [3,4]. Emerging studies focus on the metastatic mechanisms of breast cancer, and the gap junction is suggested to be a major regulator of tumour metastasis [5].

The gap junction, located at the cell membrane, is basically comprised with different Cx (connexin) proteins, which are intimately correlated with diverse cell functions [6,7]. The Cx family include 21 members, among which Cx43 is abundantly expressed in the mammary gland [6]. Cx43 is reported to be a contributor to normal cell migration [8] and tumour cell invasion [9]. We [10] and others [11] also claim that Cx43 induces migration and invasion of breast cancer cells. Regulation of Cx43 expression provides promising strategies in regulating cell functions [11,12].

Cx43 gene expression is tightly regulated by different transcription factors in transcription level. According to previous studies, Sp1 (specificity protein 1), Sp3, AP-1 (activating protein 1) and c-Jun can bind directly to the promoter region of Cx43 so as to promote transcription activity [12,13]. In addition, Cx43 is also intimately regulated by miRNAs at the post-transcription level [10,11,14]. miRNAs are one of the largest groups of post-transcriptional regulators [15]. They are composed with two to eight bases at the 5′-end that could bind to the 3′-UTR of the target genes so as to inhibit mRNA levels and gene expression [15].

Abbreviations: CDS, coding sequence; C/EBPα, CCAAT/enhancer-binding protein α; Cx, connexin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GJIC, gap junctional intercellular communication; NC, negative control; Sp, specificity protein.

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By directly or indirectly regulating target gene expression, miRNAs regulate a series of biological processes including cell cycle [16], growth [17], apoptosis [17], differentiation [18] and stress reaction [19]. Emerging evidences indicate that miRNAs are important regulators of metastasis in breast cancer [20,21].

We and others have scanned and identified miR-1, miR-206, miR-200a, miR-381, miR-23a/b and miR-186 as potent suppressors of Cx43 [10,11,22]. However, the mechanism of miR-381-reduced Cx43 expression was still not revealed. In the present study, we provide a precise mechanism that miR-381 suppresses C/EBPα (CCAAT/enhancer-binding protein α)-dependent Cx43 expression. The miR-381–C/EBPα–Cx43 axis regulates the migration of breast cancer cells, which might shed light on the diagnosis and therapy of metastatic breast cancer.

MATERIALS AND METHODS

Reagents
The mimics and inhibitors for human miR-381 were synthesized by Shanghai GenePharma, as were the mimic NC (negative control) and anti-NC (inhibitor negative control). The detailed sequences of the mimics, inhibitors and controls were described in a previous study [23].

Cell lines and culture conditions
MDA-MB-231 is a highly aggressive breast cancer cell line, whereas MCF-7 is a non-aggressive one [24]. Both MDA-MB-231 and MCF-7 cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. The cells were cultured in high-glucose DMEM (Dulbecco’s modified Eagle’s medium) (HyClone) containing 10% (v/v) FBS (Gibco).

Tissue collection
The study was approved by the Ethics Committee of the First Affiliated Hospital of the Third Military Medical University and the Second Affiliated Hospital of Chongqing Medical University. All patient-derived tissues were obtained with their written informed consent.

We collected 20 cases of primary tissues and 20 cases of pulmonary metastases of breast cancer that were pathologically diagnosed at the First Affiliated Hospital of the Third Military Medical University and the Second Affiliated Hospital of Chongqing Medical University from 1 October 2012 to 1 July 2013. The age range of the patients was 32-67 years (median 53 years), and the tumour diameter ranged from 1.5 cm to 4.7 cm (mean 2.8 cm). The patients did not receive pre-operative neoadjuvant chemotherapy or endocrine therapy. The primary cancer tissues were obtained in a sterile manner by modified radical mastectomy. Two or three pieces of the specimens from pulmonary metastases were obtained from metastatic patients during CT (computed tomography)-guided biopsy.

Real-time PCR
Total RNAs were extracted from tissues or cells with TRIzol® reagent (Invitrogen) according to the manufacturer’s protocol. RNAs were transcribed into cDNAs using Omniscript (Qiagen). Quantitative real-time PCR was performed using the 7900HT Fast Real-time PCR system (Applied Biosystems). The mRNA expression levels were normalized to the expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Reactions were carried out in duplicate by using Applied Biosystems Taqman Gene Expression Assays and Universal PCR Master Mix. The relative expression was calculated by the 2(−ΔΔCT) method. All primer sequences used for PCR are available on request from J.M.

Immunoblot assay
Total proteins were extracted with cell lysis solution (P0013, Beyotime). Then, 40 μg of protein was separated using SDS/PAGE (10% gel), electrotransferred on to PVDF membranes (catalogue number VVL02500, Millipore), and incubated with rabbit anti-human Cx43 antibody (1:1000 dilution, catalogue number BA1727, Boster) or rabbit anti-human C/EBPα antibody (1:1000 dilution, catalogue number sc-61, Santa Cruz Biotechnology) at 4°C overnight. Then, anti-rabbit secondary antibody (1:2500 dilution, catalogue number P0110, Beyotime) was incubated for 1 h in room temperature. After three washes, protein bands were quantified from the membrane by densitometry using the Adobe Photoshop version 7.01 imaging system.

Transwell migration and invasion (Matrigel) assays
MDA-MB-231 and MCF-7 cells were transfected with the Cx43-overexpression plasmids, C/EBPα-overexpression plasmids and/or miRNAs for 36 h, and then the cells were digested and resuspended at 105 cells/ml.

In migration assay, 200 μl of cell suspension was sucked into each insert of the Transwell (polycarbonate membrane with 8.0-μm pore size; catalogue number 3422, BD Bioscience). After culture for 12 h, the upper inserts were air-dried, fixed with paraformaldehyde for 15 min, and stained with 0.1% Crystal Violet, and five fields of view were randomly selected for counting cells under a microscope (×200 magnification). The cell migration activity was described as the relative cell numbers of the transmitted cells. In invasion assay, the similar inserts covered with Matrigel (2 mg/ml; Becton Dickinson) were used to measure the cell invasion ability. Freshly trypsinized cells were suspended at 105 cells/ml. Aliquots of 200 μl cells suspension were plated in the inserts. The lower companion plate well contained RPMI 1640 medium plus 10% (v/v) FBS. After 48 h, the cells that migrated through the permeable membrane were fixed with paraformaldehyde, stained with haematoxylin and eosin, and finally counted.
miR-381 inhibits C/EBPα-dependent Cx43 expression

Figure 1 miR-381 inhibits Cx43 expression by suppressing its promoter activity
(A) The mRNA levels of Cx43 in MDA-MB-231 cells treated with miR-381 (100 nM) or scrambled miRNAs (100 nM) as control (NC) for 36 h (n = 5, ** P < 0.01). (B) Immunoblot assay of Cx43 and GAPDH proteins in MDA-MB-231 cells treated with miR-381 (100 nM) or NC (100 nM) for 36 h. (C) Relative luciferase (Luc.) activity of MDA-MB-231 cells co-transfected with miR-381 (100 nM) or NC (100 nM) plus a miRNA reporter plasmid (0.4 μg/ml) harbouring the 3′-UTR (1731 bp) of the human Cx43 gene. (D) Relative luciferase (Luc.) activity of MDA-MB-231 cells co-transfected with miR-381 (100 nM) or NC (100 nM) plus a promoter reporter plasmid (0.4 μg/ml) harbouring the promoter sequences (−3000/+10) of the human Cx43 gene (n = 5, *** P < 0.005).

Reporter gene of human Cx43 promoter or 3′-UTR
To observe whether miR-381 would inhibit Cx43 expression via its promoter regions or 3′-UTR, the reporter gene of the human Cx43 promoter or 3′-UTR were constructed. Genomic DNA of MDA-MB-231 cells were extracted using a kit from Qiagen (catalogue number 19060). The sequences harbouring promoter regions (upstream of the transcription start site) from −3000 to +10 bp (−3000/+10) were cloned by PCR (catalogue number KOD-201, Toyobo) with the genomic DNA as the template. Likewise, the Cx43 promoter regions −1500/+10, −500/+10 and −250/+10 were also cloned. After PCR, all cloned DNA fragments were subjected to gel electrophoresis, gel recycling, enzyme digestion and linked into a reporter gene vector pGC4-basic (Invitrogen).

The wild-type or mutated human Cx43 3′-UTR sequences (1731 bp) were subcloned into the pMIR-REPORT vector (Invitrogen) to form different reporter genes. The sequences of all primers used in the PCR are available on request from J.M.

Molecular cloning experiments
For the cloning of human C/EBPα-overexpression constructs, the total cellular RNAs were extracted from MDA-MB-231 cells. According to the instructions of the PrimeScript First Strand cDNA Synthesis Kit (catalogue number 6110A, TaKaRa Bio), reverse transcription was performed to prepare the cDNA for the coding region or 3′-UTR of human C/EBPα.

The human C/EBPα CDS (coding sequence) exons were subcloned into the pCDNA3.1 vector to construct a human C/EBPα overexpression plasmid pCDNA-C/EBPαΔ, which could not be directly inhibited by miRNAs (such as miR-381). The human C/EBPα CDS exons plus 3′-UTR were inserted into the pCDNA3.1 vector to construct a human C/EBPα-overexpression plasmid pCDNA-C/EBPα, which could be inhibited by miRNAs (such as miR-381).

Cell transfection, site-directed reporter gene mutation and reporter gene assay
Site-directed reporter gene mutation, cell transfection and reporter gene assays were performed as described in [25]. The transfection was performed using the protocol of Lipofectamine™ 2000 (catalogue number 12566014, Invitrogen). The potential C/EBPα-binding site (AATTGTC) in the Cx43 promoter region was mutated to AGCTACC with a MutanBEST Kit.

siRNA-mediated silencing of C/EBPα expression
MDA-MB-231 cells were transfected with a scrambled siRNA (siNC, 20 nmol/ml) or human C/EBPα-specific siRNAs (si-C/EBPα-1 and si-C/EBPα-2, 20 nmol/ml) (catalogue number GS1050, Qiagen) for 36 h.

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miR-381 suppresses human Cx43 expression through a potential C/EBPα-binding element in the promoter region of the Cx43 gene

(A) The promoter region from −3000 to +10 bp (−3000/+10) in the human Cx43 gene was subcloned into the pGL4-basic vector to construct a reporter gene PC-1. Likewise, the regions −1500/+10, −500/+10 and −250/+10 were subcloned to construct the reporter genes PC-2, PC-3 and PC-4 respectively. (B) Reporter gene assay of PC-1 (0.4 μg/ml), PC-2 (0.4 μg/ml), PC-3 (0.4 μg/ml) or PC-4 (0.4 μg/ml) co-transfected with NC (100 nM) or miR-381 (100 nM) for 36 h in MDA-MB-231 cells (n = 4, **P < 0.01). (C) Alignment of the human and mouse promoter regions (−3000/0) of Cx43 using the online software on the NCBI website. Two conserved regions (−1064/−935 and −497/−1) in the human Cx43 gene) were displayed. mCx43, mouse Cx43; hCx43, human Cx43. (D) Two potential binding elements of C/EBPα located at −459/−453 (AATTGTC) and −229/−254 (TATTGTA) in the reporter gene PC-1 (WT) were mutated to form the mutated reporter genes PC-1-MUT1 and PC-MUT2 respectively. (E) Relative luciferase (Luc.) activity of MDA-MB-231 cells co-transfected with miR-381 (100 nM) or NC (100 nM) plus PC-1-WT (0.4 μg/ml), PC-1-MUT1 (0.4 μg/ml) or PC-1-MUT2 (0.4 μg/ml) for 36 h. (n = 5, **P < 0.01). (F) miR-381 inhibited human Cx43 promoter activity via the 3′-UTR of human CEBPA. The pCDNA3.1 (0.4 μg/ml), pCDNA-C/EBPαΔ (0.4 μg/ml) or pCDNA-C/EBPα (0.4 μg/ml)-transfected MDA-MB-231 cells were co-transfected with miR-381 (100 nM) or NC (100 nM) plus the promoter reporter plasmid PC-1 (0.4 μg/ml) for 36 h. Then, the relative luciferase (Luc.) activity was measured. (n = 5, **P < 0.01). pCDNA3.1, empty expression plasmid; pCDNA-C/EBPαΔ, overexpression plasmid harbouring the human CEBPA mRNA lacking the 3′-UTR; pCDNA-C/EBPα, an overexpression plasmid containing the human CEBPA mRNA containing the 3′-UTR.

ChIP assay

A ChIP assay was used to measure miR-381-mediated binding between C/EBPα protein and Cx43 promoter DNA. In brief, the cultured MDA-MB-231 cells were fixed with 1% (w/v) formaldehyde, followed by sonication. The supernatants with equal amounts of protein were immunoprecipitated with 1 μg of anti-mouse C/EBPα antibody or anti-rabbit IgG as control by using the ChIP Kit (catalogue number 17-10460, Millipore) according to the manufacturer’s protocol. The immunoprecipitates were analysed by PCR to detect the co-immunoprecipitated DNA containing the functional C/EBPα-binding site. The ChIP primers were designed as: forward, 5′-ATTTTTGAAATCTCTCTCC-3′, reverse, 5′-GTGTGAGTGACCTGTTTGAT-3′. The length of the desired product was 100 bp.

Statistics

Results are shown as means±S.D. Comparisons were performed by using ANOVA for multiple groups or Student’s t test for two groups. P < 0.05 was considered statistically significant. Values

(catalogue number 401, TaKaRa Bio). The potential miR-381-binding site (CACUUGUAU) in the 3′-UTR of the human CEBPA gene was mutated to CAGGAUCAU. The luciferase activities of the cell lysates were evaluated according to the manufacturer’s instruction (catalogue number E1910, Promega) and the total protein concentration in each assay was measured as an internal control.
miR-381 inhibits C/EBPα-dependent Cx43 expression

RESULTS

miR-381 inhibits Cx43 expression by suppressing its promoter activity

To explore the regulatory role of miR-381 in Cx43 expression, we determined miR-381-regulated Cx43 expression at the promoter, mRNA and protein levels. We demonstrated that miR-381 transfection in MDA-MB-231 cells dramatically suppressed the mRNA (Figure 1A) and protein (Figure 1B) levels of Cx43. Unexpectedly, miR-381 treatment could not inhibit the luciferase activity of the miRNA reporter gene, which contained the 3′-UTR of the Cx43 gene (Figure 1C). These results indicate that miR-381 might regulate Cx43 expression at the transcriptional level. As expected, we demonstrated that transfection of miR-381 notably suppressed the promoter activity of the Cx43 gene in MDA-MB-231 cells (Figure 1D). Thus we concluded that miR-381 inhibited Cx43 expression via suppressing its promoter activity.

miR-381 suppresses human Cx43 expression through a potential C/EBPα-binding element in the promoter region of the Cx43 gene

To determine further the precise mechanism of miR-381-reduced promoter activity of Cx43, we constructed a series of mutated reporter genes harbouring different lengths (−3000/+10, −1500/+10, −500/+10 and −250/+10) of promoter sequences in the human Cx43 gene (Figure 2A). Using a reporter gene assay, we found that miR-381 reduced Cx43 promoter activity via the −500/+250 region (Figure 2B). Interestingly, by alignment of the human and mouse promoter regions (−3000/0) of Cx43 with software on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&LINK_LOC=align2seq), two conserved regions, −1064/−935 and −497/−1, in the human Cx43 gene were discovered (Figure 2C). Consistent with the conserved regions, the −500/−250 sequences might contain some important transcription factor-binding elements. Using online software (http://alggen.lsi.upc.es/), we predicted that two potential binding elements for C/EBPα, AATTGTC located at −459/−453 and TATTGTA located at −324/−318 (Figure 2D and Supplementary Figure 1), might be responsible for miR-381-reduced promoter activity of human Cx43. To verify this presumption,
miR-381 suppresses C/EBPα expression via binding directly to a consensus sites in the 3′-UTR of the CEBPA gene

(A) Relative mRNA levels of CEBPA in MDA-MB-231 cells transfected with NC (100 nM) or miR-381 (100 nM) for 36 h \((n = 5, \, **p < 0.01)\).

(B) Immunoblot assay of C/EBPα in MDA-MB-231 cells transfected with NC (100 nM) or miR-381 (100 nM) for 36 h.

(C) A potential binding element of miR-381 in the 3′-UTR of CEBPA was mutated to obtain a mutated miRNA reporter gene (MUT). (D) The wild-type (WT, 0.4 μg/ml) or the mutated (MUT, 0.4 μg/ml) miRNA reporter genes of human CEBPA plus the NC (100 nM) or miR-381 (100 nM) were co-transfected into MDA-MB-231 cells for 36 h, and then the cells were harvested for luciferase (Luc.) activity assay \((n = 5, \, **p < 0.01)\).

(E) ChIP assay of MDA-MB-231 cells transfected with NC (100 nM) or miR-381 (100 nM) for 36 h. The binding activity between C/EBPα protein and Cx43 promoter DNA was tested.

we performed site-specific gene mutation of these elements, and checked their function using a reporter gene assay. Results showed that miR-381 notably decreased the luciferase activity of PC-1-WT and PC-1-MUT2, whereas PC-1-MUT1 fully rescued this effect (Figure 2E). These results indicate that miR-381 inhibited Cx43 promoter activity via the C/EBPα-binding element AATTGTC located at −459/−453 in the promoter region. To verify further the regulatory role of C/EBPα in miR-381-reduced Cx43 expression, we overexpressed C/EBPα in MDA-MB-231 cells. We demonstrated that overexpression of C/EBPα without the 3′-UTR could fully rescue miR-381-inhibited Cx43 expression activity, but overexpression of that with 3′-UTR did not have this effect (Figure 2F).

C/EBPα up-regulates Cx43 expression via directly binding to the promoter region of Cx43

To verify further that Cx43 is a transcriptional target of C/EBPα, we demonstrated that overexpression of C/EBPα dramatically induced the mRNA (Figure 3A) and protein (Figure 3C) levels of Cx43 in MCF-7 cells. Likewise, silence of C/EBPα by siRNAs notably decreased the mRNA (Figure 3B) and protein (Figure 3D) levels of Cx43 in MDA-MB-231 cells. To determine the direct binding effect between C/EBPα and Cx43 promoter DNA in vivo, a ChIP assay was performed. The results showed that overexpression of C/EBPα increased the binding activity between C/EBPα and Cx43 promoter DNA (Figure 3E). In addition, siRNA-mediated silence of C/EBPα obtained identical results (Figure 3E).

miR-381 suppresses C/EBPα expression via directly binding to a consensus site in the 3′-UTR of the CEBPA gene

Aforementioned results revealed that miR-381 suppressed C/EBPα-dependent Cx43 expression in breast cancer cells, whereas the mechanism of miR-381-regulated C/EBPα expression was still obscure. We found that miR-381 treatment significantly decreased the mRNA (Figure 4A) and protein (Figure 4B) levels of C/EBPα in MDA-MB-231 cells. Using online software (http://www.microrna.org/microrna/getMirnaForm.do), we predicted that C/EBPα might be a direct target of miR-381 (Figure 4C). A potential binding element of miR-381 in the 3′-UTR of human CEBPA mRNA was mutated from CACUGUAU (WT) to CAGGAUCAU (MUT) to form the miRNA reporter genes (Figure 4C). The reporter gene assay indicated that mutations of the potential binding element could fully rescue miR-381-reduced C/EBPα expression activity (Figure 4D). Likewise,
miR-381 transfection significantly attenuated the binding activity between C/EBPα and Cx43 promoter DNA, whereas this effect was fully rescued by overexpression of an exogenous C/EBPα without the 3′-UTR in MDA-MB-231 cells (Figure 4E).

miR-381 inhibits C/EBPα/Cx43-dependent migration and invasion activity of breast cancer cells

Cx43 was previously identified as a regulator of cell migration [8] and invasion [9]. Thus we presumed that miR-381-regulated Cx43 expression would also affect the migration and invasion activity of breast cancer cells. A previous study revealed that MDA-MB-231 cells were highly aggressive, whereas MCF-7 cells were non-aggressive [24]. We found that miR-381 treatment notably inhibited the migration and invasion activity of MDA-MB-231 cells, and overexpression of Cx43 could fully rescue this effect (Figures 5A and 5B, and Supplementary Figures S2A and S2B). As expected, C/EBPα overexpression produced identical results as Cx43 (Figures 5C and 5D, and Supplementary Figures 2C and 2D). We also confirmed that blockage of miR-381 in MCF-7 cells robustly induced cell migration and invasion, whereas siRNA-mediated silence of C/EBPα prevented this effect (Figures 5E and 5F, and Supplementary Figures 2E and 2F).

Elevated levels of C/EBPα and Cx43 as well as decreased expression of miR-381 in aggressive breast cancer cells and tissues

Given the regulatory role of the miR-381–C/EBPα–Cx43 axis in the migration activity of breast cancer cells, we further observed the expression of miR-381, C/EBPα and Cx43 in aggressive and non-aggressive breast cancer cells and tissues. The aggressive cells MDA-MB-231 expressed much higher levels of Cx43 (Figure 6A) and C/EBPα (Figure 6C), as well as lower levels of miR-381 (Figure 6B) compared with the non-aggressive MCF-7 cells. Similarly, the metastatic breast cancer tissues expressed much more Cx43 (Figure 6D) and C/EBPα (Figure 6F), as well...
as much less miR-381 (Figure 6E) compared with the primary breast cancer tissues.

**DISCUSSION**

In the present study, we were the first to identify miR-381 as a suppressor of C/EBPα by binding directly to the 3′-UTR of the CEBPA gene. Meanwhile, we also demonstrated that C/EBPα was a novel transcription factor of Cx43. The miR-381–C/EBPα–Cx43 axis could regulate the migration and invasion of breast cancer cells (Figure 7).

Cx43 is an important regulator of the metastasis of multiple cancers [9,26–31]. However, whether it exerted a protective or promoting role was still controversial in different cancers. Forster et al. [27] reported that the absence of Cx43 prevented GJIC (gap junctional intercellular communication) and enhanced aggressiveness in pancreatic cancer [27]. Yu et al. [31] suggested that Cx43 sensitized the chemotherapy of non-small-cell lung cancer by inhibiting epithelial–mesenchymal transition. These studies supported the promoting role of Cx43 in tumour metastasis. In contrast, Ghosh et al. [26] reported that Cx43 regulated p38-mediated cell migration and invasion in tumour cells by low doses of γ-radiation in an ERK1/2 (extracellular-signal-regulated kinase 1/2)-independent manner. Likewise, another study suggested that the abnormal expression of Cx43 played an essential role in peritoneal metastasis and that Cx43-mediated heterocellular GJIC between gastric cancer cells and mesothelial cells might be an important regulatory step during metastasis [30]. Therefore, these studies supported the promoting role of Cx43 in tumour metastasis. For breast cancer, we were the first to demonstrate that Cx43 might be an enhancer of metastasis by providing several pieces of preliminary evidence: (i) Cx43 was highly expressed in aggressive breast cancer cells and tissues compared with the non-aggressive cancer cells; (ii) overexpression of Cx43 potentiated migration and invasion of breast cancer cells; and (iii) suppressing Cx43 expression by miR-381 largely inhibited the migration activity of breast cancer cells. It should therefore be pointed out that the diagnostic and therapeutic strategies based on Cx43 need to be designed individually in each specific type of cancer.

The roles of miR-381 were also controversial in multiple tumours and the related reports were rather limited [32,33]. In line with our findings in the present study, a previous study demonstrated that down-regulation of miR-381 contributed to the metastatic potential of lung adenocarcinomas via targeting the inhibitor of differentiation 1 [32]. However, Tang et al. [33] suggested that miR-381 was an ‘oncomir’ in the glioma through suppressing the glioma suppressor leucine-rich repeat C4. These studies indicate that miR-381 had diverse and complex functions in tumour biology, since miR-381 might have different targets in different tumours. Whether the role of the miR-381–Cx43 axis identified in breast cancer cells in the present study is compatible with other tumours needs to be explored further.
miR-381 inhibits C/EBPα-dependent Cx43 expression

Figure 7 Proposed hypothesis for the miR-381–C/EBPα–Cx43 axis in regulating the migration of breast cancer cells

As a transcription factor, C/EBPα could translocate into the nucleus and regulate further a variety of genes directly or indirectly, and have involvement in the regulation of cell differentiation [34,35], proliferation [36] and apoptosis [37]. With regard to cancer research, C/EBPα was suggested to be a tumour suppressor, especially in acute myeloid leukaemia [35]. However, C/EBPα could also be a predictor of poor prognosis in hepatocellular carcinoma [38]. These studies uncovered the contradictory role of C/EBPα in cancer biology. In the present study, by directly targeting Cx43, C/EBPα functioned as a suppressor of metastasis of breast cancer cells. These studies indicate that the role of C/EBPα relies on the special environment of tumours.

The expression activity of Cx43 was regulated at multiple levels, including transcription [13,39], post-transcription [11] and protein phosphorylation [40]. Multiple transcription factors and miRNAs were identified as potent regulators of Cx43 in different cell types. The complex regulatory network of Cx43 expression resulted in its diverse and contradictory functions. More work needs to be performed to verify the unique role of Cx43 in a specific type of tumour.

In conclusion, we have demonstrated that miR-381 suppressed Cx43 expression by directly targeting the 3′-UTR of CEBPA, a novel transcription factor of Cx43 in human breast cancer cells. The miR-381–Cx43 axis might be a useful diagnostic and therapeutic target of metastatic breast cancer.

**AUTHOR CONTRIBUTION**

Jia Ming conducted the experiments, analysed the data, and wrote the paper. Yan Zhou, Junze Du, Shenghao Fan, Beibei Pan, Yinhuan Wang, Lingjun Fan and Jun Jiang designed experiments and discussed the data. Jia Ming is the guarantor of this work, had full access to all the data and takes full responsibility for the integrity of data.

**FUNDING**

This study was supported by the National Natural Science Foundation of China [grant number 81172539] and the Natural Science Foundation of Chongqing [grant number CSTC2011jaA10044].

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