



OPEN ACCESS

Identification of *miR-200a* as a novel suppressor of connexin 43 in breast cancer cells

Jia Ming*¹, Yan Zhou†, Junze Du†, Shenghao Fan*, Beibei Pan*, Yinhuan Wang*, Lingjun Fan† and Jun Jiang†

*Department of Breast, Thyroid and Pancreas Surgery, the Second Affiliated Hospital of Chongqing Medical University, Chongqing 400010, China

†Breast Disease Center, the First Affiliated Hospital of The Third Military Medical University, Chongqing 400038, China

Synopsis

Both miRNAs (miRs) and connexin 43 (Cx43) were important regulators of the metastasis of breast cancer, whereas the miRs regulating Cx43 expression in breast cancer cells were still obscure. In the present study, we scanned and found *miR-1*, *miR-206*, *miR-200a*, *miR-381*, *miR-23a/b* and *miR-186* were functional suppressors of human Cx43 mRNA and protein expression. Specially, we demonstrated that only *miR-200a* could directly target the 3'-untranslated region (3'-UTR) of human Cx43 gene. Functionally, overexpression of Cx43 in MCF cells potentiated the migration activity, whereas additional *miR-200a* treatment notably prevented this effect. Finally, we demonstrated that decreased levels of *miR-200a* and elevated expression of Cx43 in the metastatic breast cancer tissues compared with the primary ones. Thus, we are the first to identify *miR-200a* as a novel and direct suppressor of human Cx43, indicating that *miR200a/Cx43* axis might be a useful diagnostic and therapeutic target of metastatic breast cancer.

Key words: breast cancer, connexin 43 (Cx43), metastasis, micro ribonucleic acid (*miR*)-200a, micro ribonucleic acid (*miR*)-1, 3'-untranslated region (3'-UTR).

Cite this article as: Bioscience Reports (2015) 35, e00251, doi:10.1042/BSR20150153

INTRODUCTION

Breast cancer is the most common malignant tumour in women and the second leading cause of cancer-related death among women worldwide [1–2]. Many breast tumours are not completely eradicated due to relapse, which results in metastasis at later stages leading to death [3]. Breast cancer metastasis is associated with an increase in connexin 43 (Cx43) expression and intercellular exchange disorders [4–6].

Connexin proteins form the gap junction, a membrane structure between adjacent cells, mediating mutual, direct electric and chemical signal communications. There are 21 members in the connexin family, which are named according to their relative molecular mass (M_r); for example, Cx43 refers to connexin with a M_r of 43000. Human breast tissues mainly express Cx26 and Cx43 [7]. Cx43 can mediate the adhesion of cancer cells with vascular endothelial cells [8], alter the cytoskeleton [9,10] and increase cell migration [11] through regulation of intercellular communications.

miRNAs (miRs) form one of the largest groups of post-transcriptional regulatory factors [12]. They had 2–8 bases at

the 5'-end that could bind with the 3'-UTR of the target gene so as to repress gene translation and reduce protein expression [13]. miRs are involved in a series of biological processes including cell cycle, growth, apoptosis, differentiation and stress response [14,15]. Especially, increasing evidences indicated that miRs were important regulators of breast cancer metastasis [16–20].

However, whether the miRs would regulate the metastasis of breast cancer cells via Cx43 was still not known. In the present study, we screened the 3'-UTR of human Cx43 gene and identified *miR-200a* as a direct suppressor of Cx43. We suggested that the *miR-200a/Cx43* axis might play an important role in the metastasis of breast cancer.

MATERIALS AND METHODS

Cell lines and culture conditions

The MDA-MB-231 is an aggressive breast cancer cell line that lacks the expression of estrogen receptor α (ER α), progesterone receptor (PR) and human epidermal growth factor receptor-2

Abbreviations: CDS, coding sequence; Cx43, connexin 43; DMEM, Dulbecco's Modified Eagle's Medium; ER α , estrogen receptor α ; HER-2, human epidermal growth factor receptor-2; miR, miRNA; PR, progesterone receptor; UTR, untranslated region; wt, wild type.

¹ To whom correspondence should be addressed (email mingjia1001@sina.com).



(HER-2) [21]. MCF-7 is a non-aggressive breast cancer cell line that has ER α and HER-2 expression [21]. Both the MDA-MB-231 and the MCF-7 cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. The cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Hyclone) containing 10% FBS (Gibco).

Tissue collection

From October 1, 2012 to July 1, 2013, 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. The age range of the patients was 32–67 years (median, 53 years) and the tumour diameter ranged from 1.5 to 4.7 cm (average, 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 to three pieces of the specimens from pulmonary metastases were obtained from metastatic patients during computed tomography-guided biopsy.

The present 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.

Real-time PCR

Total RNAs were extracted from tissues or cells with Trizol reagent (Invitrogen) according to the manufacture'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 glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Reactions were done in duplicate using Applied Biosystems Taqman Gene Expression Assays and Universal PCR Master Mix. The relative expression was calculated by the $2^{(-\Delta\Delta C_t)}$ method. All the primers used for PCR are available upon request.

Western blot analysis

Total proteins were extracted with cell lysis buffer. Then, 50 μ g of protein was separated using SDS/PAGE (12% gel), electro-transferred to PVDF membranes (Millipore) and incubated with rabbit anti-human Cx43 antibody (1:1000, Boster) and then anti-rabbit secondary antibody (1:2500, Beyotime). Protein bands were quantified from the membrane by densitometry using the Adobe Photoshop V7.01 imaging system.

Transwell migration assay

The MDA-MB-231 and MCF-7 cells were transfected with the Cx43 overexpression plasmids and/or miRs for 24 h and then

the cell suspension (200 μ l of cell suspension, 1×10^5 /ml) was sucked into the upper chamber of Transwell (PC membrane with 8.0- μ m pore size). After culture for 12 h, the upper chamber was air-dried, fixed with paraformaldehyde for 15 min and stained with 0.1% crystal violet and five fields of view were randomly selected to count cells under a microscope (200 \times). The cell migration activity was described as the relative cell numbers of the transmitted cells.

Molecular cloning experiments

Those experiments for plasmid construction, cell transfection, reporter gene assays and site-directed reporter gene mutation were performed as described in a previous study [22]. The wild-type (wt) or mutated human Cx43 3'-UTR sequences were subcloned into a pMIR-REPORT vector (Invitrogen) to form different reporter genes.

For the cloning of human Cx43 overexpression constructs, the total cellular RNA was extracted from MDA-MB-231 cells. According to the instructions of the PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa), reverse transcription was performed to prepare the cDNA for the coding region or 3'-UTR of human Cx43.

The human Cx43 coding sequence (CDS) exons were subcloned into the pCDNA3.1 vector to construct a human Cx43 overexpression plasmid pCDNA-Cx43 Δ , which could not be inhibited by *miR-200a* due to lack of 3'-UTR. The primer sequence used for human Cx43 CDS exons cloning was: F: 5'-GGACTAGTATGGGTGACTGGAGCGCCTT-3'; R: 5'-CGACGCGTCTAGATCTCCAGGTCATCAG-3'. The SpeI and MuiI restriction enzyme cutting sites are underlined.

The human Cx43 CDS exons plus 3'-UTR were inserted into the pCDNA3.1 vector to construct a human Cx43 overexpression plasmid pCDNA-Cx43, which could be inhibited by *miR-200a* via the 3'-UTR. The primer sequence used for human Cx43 CDS exons plus UTR cloning was: F: 5'-GGACTAGTATGGGTGACTGGAGCGCCTT-3'; R: 5'-CGACGCGTTATGTTTATACTAAATTTAAA-3'. The SpeI and MuiI restriction enzyme cutting sites are underlined.

Statistics

Data are generated as mean \pm S.D. Comparisons were performed using ANOVA for multiple groups or Student's *t* test for two groups. $P < 0.05$ is considered statistically significant. Values not sharing a common superscript letter differ significantly ($P < 0.05$).

RESULTS

Prediction of potential miRs targeting in the 3'-UTR of Cx43 gene

To explore the miRs involvement in Cx43 gene expression, we screened the potential binding sites for different miRs

A	human	B	mouse	C	rat	D
	hsa-miR-1 <u>463</u> <u>671</u> <u>1594</u>		mmu-miR-1 <u>463</u> <u>1586</u>		rno-miR-1 <u>463</u> <u>1650</u>	miR-1
	hsa-miR-206 <u>464</u> <u>671</u> <u>1594</u>		mmu-miR-206 <u>463</u> <u>1586</u>		rno-miR-206 <u>463</u> <u>1650</u>	miR-206
	hsa-miR-613 <u>466</u> <u>1597</u>		mmu-miR-101b <u>1174</u> <u>1603</u>		rno-miR-101a <u>1221</u>	miR-101a
	hsa-miR-454 <u>480</u> <u>1362</u>		mmu-miR-101a <u>1174</u> <u>1603</u>		rno-miR-101b <u>1221</u>	miR-101b
	hsa-miR-130b <u>480</u> <u>1364</u>		mmu-miR-200a <u>469</u> <u>909</u>		rno-miR-221 <u>1134</u>	miR-200a
	hsa-miR-130a <u>480</u> <u>1363</u>		mmu-miR-141 <u>469</u> <u>909</u>		rno-miR-200a <u>469</u> <u>923</u>	miR-141
	hsa-miR-301a <u>483</u> <u>1362</u>		mmu-miR-590-3p <u>1685</u>		rno-miR-141 <u>469</u> <u>921</u>	miR-381
	hsa-miR-301b <u>483</u> <u>1368</u>		mmu-miR-224 <u>1479</u>		rno-miR-187 <u>550</u>	miR-130a
	hsa-miR-186 <u>625</u> <u>1065</u> <u>1680</u>		mmu-miR-222 <u>1090</u>		rno-miR-222 <u>1135</u>	miR-130b
	hsa-miR-19a <u>480</u> <u>1365</u>		mmu-miR-221 <u>1089</u>		rno-miR-539 <u>1077</u>	miR-301a
	hsa-miR-19b <u>480</u> <u>1365</u>		mmu-miR-7a <u>663</u>		rno-miR-381 <u>1368</u>	miR-301b
	hsa-miR-381 <u>1309</u>		mmu-miR-7b <u>664</u>		rno-miR-130a <u>1414</u>	miR-23a
	hsa-miR-300 <u>1309</u>		mmu-miR-23a <u>1202</u>		rno-miR-130b <u>1415</u>	miR-23b
	hsa-miR-93 <u>482</u>		mmu-miR-23b <u>1202</u>		rno-miR-301a <u>1418</u>	miR-30a
	hsa-miR-106b <u>479</u>		mmu-miR-301a <u>1370</u>		rno-miR-301b <u>1418</u>	miR-30b-5p
	hsa-miR-590-3p <u>477</u> <u>571</u> <u>1573</u>		mmu-miR-218 <u>1000</u>		rno-miR-455 <u>1462</u>	miR-30c
	hsa-miR-106a <u>476</u> <u>1523</u>		mmu-miR-381 <u>1317</u>		rno-miR-23a <u>1249</u>	miR-30d
	hsa-miR-17 <u>476</u> <u>1523</u>		mmu-miR-130a <u>1366</u>		rno-miR-23b <u>1249</u>	miR-30e
	hsa-miR-101 <u>1173</u>		mmu-miR-130b <u>1367</u>		rno-miR-218 <u>1009</u>	miR-186
	hsa-miR-23a <u>1201</u>		mmu-miR-30b <u>963</u>		rno-miR-216a <u>366</u>	miR-19a
	hsa-miR-23b <u>1201</u>		mmu-miR-30c <u>960</u>		rno-miR-30d <u>977</u>	miR-19b
	hsa-miR-185 <u>1076</u>		mmu-miR-384-5p <u>962</u>		rno-miR-30b-5p <u>975</u>	miR-144
	hsa-miR-214 <u>975</u>		mmu-miR-30e <u>961</u>		rno-miR-30c <u>974</u>	
	hsa-miR-653 <u>420</u>		mmu-miR-30d <u>965</u>		rno-miR-124 <u>664</u>	
	hsa-miR-20a <u>480</u>		mmu-miR-30a <u>965</u>		rno-miR-384-5p <u>974</u>	
	hsa-miR-20b <u>482</u>		mmu-miR-144 <u>1175</u> <u>1604</u>		rno-miR-30e <u>973</u>	
	hsa-miR-519d <u>484</u>		mmu-miR-216a <u>370</u>		rno-miR-30a <u>977</u>	
	hsa-miR-218 <u>993</u>		mmu-miR-290-5p <u>1382</u>		rno-miR-186 <u>1167</u>	
	hsa-miR-30a <u>959</u>		mmu-miR-292-5p <u>1382</u>		rno-miR-19a <u>1415</u>	
	hsa-miR-30b <u>957</u>		mmu-miR-186 <u>1077</u> <u>1673</u>		rno-miR-19b <u>1415</u>	
	hsa-miR-30c <u>956</u>		mmu-miR-19a <u>1367</u>		rno-miR-129 <u>1541</u>	
	hsa-miR-30e <u>959</u>		mmu-miR-135a <u>781</u>		rno-miR-374 <u>1070</u>	
	hsa-miR-30d <u>959</u>		mmu-miR-135b <u>781</u>		rno-miR-224 <u>1519</u>	
	hsa-miR-495 <u>821</u>		mmu-miR-19b <u>1367</u>		rno-miR-144 <u>1222</u>	
	hsa-miR-217 <u>1255</u>		mmu-miR-875-5p <u>193</u>			
	hsa-miR-135a <u>779</u>		mmu-miR-590-5p <u>1444</u>			
	hsa-miR-135b <u>781</u>		mmu-miR-21 <u>1447</u>			
	hsa-miR-144 <u>1174</u>		mmu-miR-376c <u>814</u>			
	hsa-miR-506 <u>1476</u>		mmu-miR-494 <u>1524</u>			
	hsa-miR-124 <u>1477</u>					
	hsa-miR-374a <u>234</u>					
	hsa-miR-374b <u>234</u>					
	hsa-miR-455-5p <u>895</u>					
	hsa-miR-758 <u>870</u>					
	hsa-miR-125a-5p <u>766</u>					
	hsa-miR-125b <u>768</u>					
	hsa-miR-141 <u>1675</u>					
	hsa-miR-200a <u>1675</u>					
	hsa-miR-421 <u>1443</u>					
	hsa-miR-219-5p <u>798</u>					

Figure 1 Prediction of potential miRNAs targeting at the 3'-UTR of Cx43 gene

(A–C). Prediction of the potential miRNAs targeting at the 3'-UTR of Cx43 gene in human (A), mouse (B) and rat (C). (D) The conserved miRNAs targeting at the 3'-UTR of human Cx43 gene. (A–C) The underlined numbers indicated the location of the miR-targeting sites in the 3'-UTR of Cx43 gene.

in the 3'-UTR of human (Figure 1A), mouse (Figure 1B) and rat (Figure 1C) Cx43 genes by using an online software (<http://www.microrna.org/microrna>). We selected out the common miRNAs from those three species (Figure 1D), since those conserved miRNAs might be evolutionarily selected as functional suppressors. Among the conserved ones, *miR-1* and *miR-206* had been identified as direct suppressors of Cx43 in a previous study [23]. However, the regulatory roles of other conserved miRNAs like *miR-101a/b*, *miR-200a*, *miR-141*, *miR-381*, *miR-130a/b*, *miR-*

301a/b, *miR-23a/b*, *miR-30a*, *miR-30b-5p*, *miR-30c*, *miR-30d*, *miR-30e*, *miR-186*, *miR-19a/b* and *miR-144* in Cx43 gene expression were still not identified.

Identification of multiple miRNAs in regulating Cx43 expression

To observe the regulatory role of the aforementioned miRNAs on Cx43 expression, we synthesized and transfected those miRNAs into

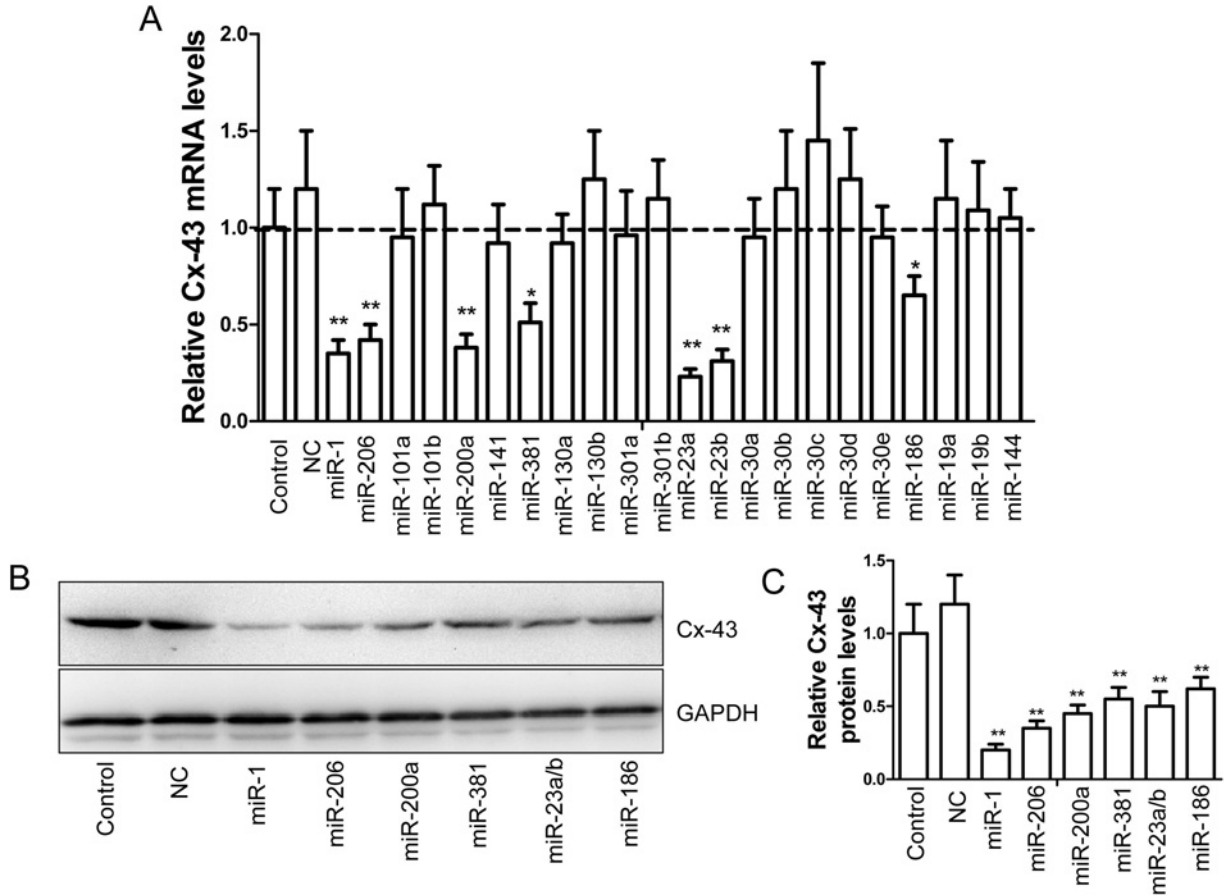


Figure 2 Identification of several miRNAs in regulating Cx43 expression

(A). The mRNA levels of Cx43 in the MDA-MB-231 cells transfected with different miRNAs (100 nM), control miR (NC, 100 nM) or mock as control for 24 h ($n=5$, $*P < 0.05$ and $**P < 0.01$). (B) Immunoblotting assay of Cx43 protein in the MDA-MB-231 cells treated as described above in (A). (C). Statistic of the relative protein levels displayed in (B) ($n=3$, $**P < 0.01$).

MDA-MB-231 cells, a highly invasive human breast cancer cell line [21]. We demonstrated that transfection of *miR-1*, *miR-206*, *miR-200a*, *miR-381*, *miR-23a/b* and *miR-186* notably suppressed the mRNA levels of human Cx43 respectively (Figure 2A). Likewise, the protein levels of Cx43 in MDA-MB-231 cells were also significantly inhibited by the treatment of aforementioned miRNAs (Figures 2B–2C).

miR-200a directly inhibits Cx43 expression via a canonic-binding element

We predicted the potential binding sites for *miR-23a/b* (1202), *miR-200* (469, 909), *miR-186* (1077, 1673) and *miR-381* (1317) in the 3'-UTR (from 1 to 1723 bp) of human Cx43 gene (Figure 3A). Then, we subcloned the 3'-UTR (from 1 to 1723 bp) or the ones with mutated miR-binding sites into a miR reporter plasmid to study the role of *miR-23a/b*, *miR-200*, *miR-186* and *miR-381* in Cx43 gene expression (Figure 3B). As expected, *miR-23a*, *miR-23b* and *miR-200a* dramatically suppressed Cx43

expression activity (Figures 3C–3F). However, mutation of the potential element for *miR-23a* or *miR-23b* could not rescue the inhibitory role of *miR-23a* or *miR-23b* in Cx43 expression (Figures 3C–3D), indicating that *miR-23a/b* did not suppress Cx43 expression in a direct manner via the predicted binding sites. Interestingly, the site-specific mutation at 909, but not 469 in the 3'-UTR of Cx43 gene could fully rescue the inhibitory effect of *miR-200a* on Cx43 gene expression (Figures 3E–3F). Unexpectedly, *miR-186* and *miR-381* could not suppress the wt or mutated reporter gene activity (Figures 3G–3H), indicating that those two miRNAs-inhibited Cx43 gene expression was not in a 3'-UTR dependent manner. Therefore, among the investigated miRNAs, we identified *miR-200a* as a novel and direct suppressor of Cx43 gene.

Overexpressing Cx43 in human breast cancer cells

The human Cx43 gene consists of 1149-bp CDS Exons and a 1723-bp 3'-UTR, in which we identified a canonic-binding

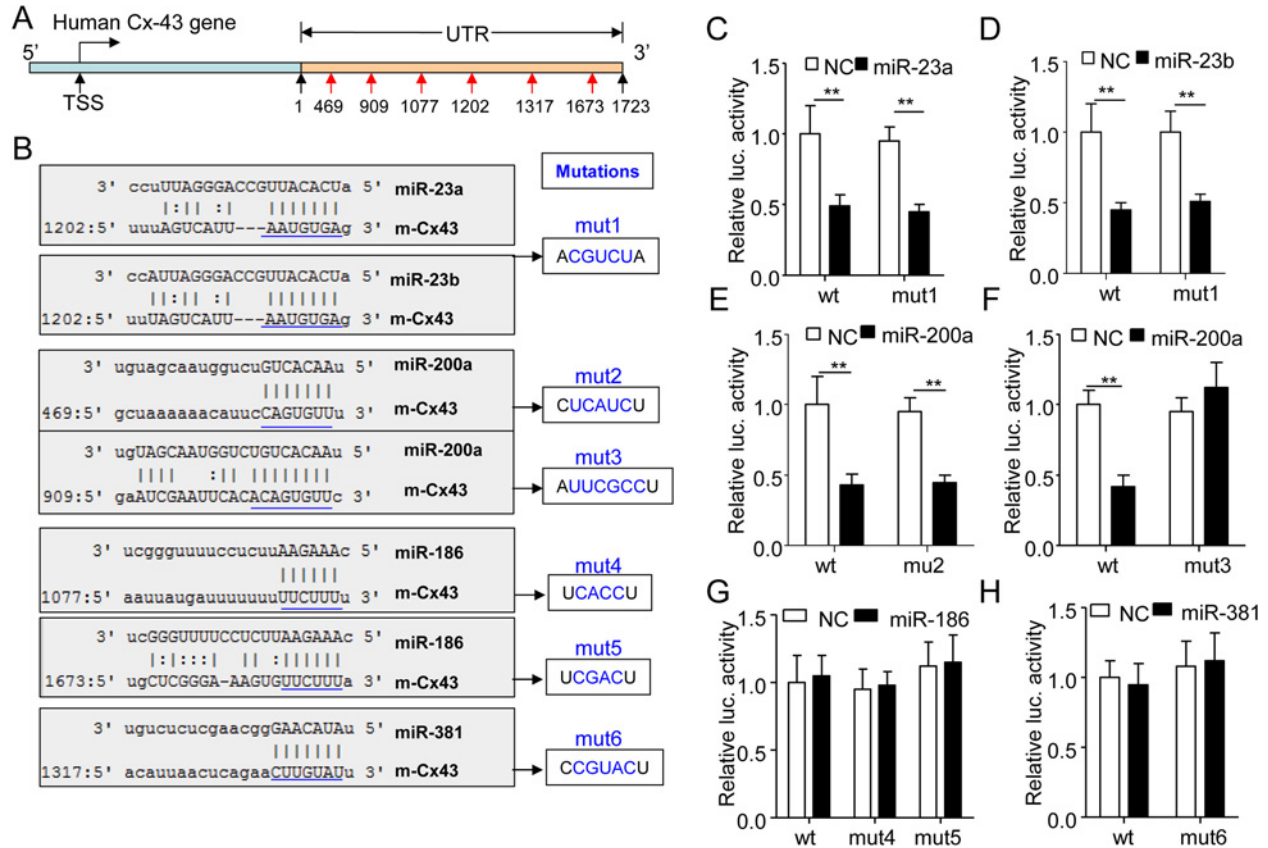


Figure 3 *miR-200a* suppressed human *Cx43* gene expression via directly targeting at a binding site locating at 909 in the 3'-UTR of human *Cx43* gene.

(A) The schematic diagram of potential binding sites for functional miRNAs in the 3'-UTR of human *Cx43* gene. (B) The 3'-UTR of human *Cx43* gene containing the wt or mutated (mut1–mut6) reporter binding sequences of potential miRNAs were subcloned into a miR reporter vector to form the wt or mutated (mut1–mut6) reporter genes as indicated. (C) The wt or mut1 reporter plasmid (0.4 $\mu\text{g}/\text{ml}$) was co-transfected with the control miR (NC, 100 nM) or *miR-23a* (100 nM) into the MDA-MB-231 cells for 24 h and then the cells were harvested for luciferase assay ($n=5$, $**P < 0.01$). (D) The luciferase activity of the MDA-MB-231 cells co-transfected with the reporter plasmids wt or mut1 plus NC or *miR-23b* (NC, 100 nM) for 24 h ($n=5$, $**P < 0.01$). (E) The luciferase activity of the MDA-MB-231 cells co-transfected with the wt or mut2 (0.4 $\mu\text{g}/\text{ml}$) plus NC or *miR-200a* (NC, 100 nM) for 24 h ($n=5$, $**P < 0.01$). (F) The reporter plasmids wt or mut3 (0.4 $\mu\text{g}/\text{ml}$) were co-transfected with the NC or *miR-200a* (100 nM) into MDA-MB-231 cells for 24 h and then the cells were subjected to luciferase assay ($n=5$, $**P < 0.01$). (G) The reporter plasmids wt, mut4 or mut5 (0.4 $\mu\text{g}/\text{ml}$) were co-transfected with the NC or *miR-186* (100 nM) into MDA-MB-231 cells for 24 h before cell collection and luciferase assay ($n=5$). (H) The luciferase activity of the MDA-MB-231 cells co-transfected with the reporter gene wt or mut6 (0.4 $\mu\text{g}/\text{ml}$) plus the NC (100 nM) or *miR-381* (100 nM) for 24 h ($n=5$).

site for *miR-200a* at 909-bp (Figure 4A). We subcloned the human *Cx43* CDS Exons only or *Cx43* CDS Exons plus UTR into pCDNA3.1 vector to construct human *Cx43* overexpression plasmids pCDNA-*Cx43* Δ or pCDNA-*Cx43*, respectively (Figure 4B). The latter construct-mediated *Cx43* overexpression was supposed to be inhibited by *miR-200a* via the 3'-UTR. As expected, the western blotting assay indicated that both the pCDNA-*Cx43* Δ and the pCDNA-*Cx43* plasmids could increase the expression of *Cx43* in a dose-dependent manner (Figure 4C). In addition, the pCDNA-*Cx43*, but not pCDNA-*Cx43* Δ -mediated *Cx43* overexpression was attenuated by *miR-200a* transfection (Figure 4D).

miR-200a/Cx43 axis-mediated migration of breast cancer cells

We further observed whether *miR-200a* could regulate cancer cell migration, since *Cx43* was an important contributor to the metastasis of breast cancer [6,24,25]. MCF-7 is a non-aggressive breast cancer cell line. The transwell assay revealed that overexpression of *Cx43* in MCF-7 cells with pCDNA-*Cx43* transfection potentiated the migration activity and additional *miR-200a* treatment notably prevented this effect (Figures 5A–5B). However, the pCDNA-*Cx43* Δ transfection-induced migration activity in MCF-7 cells could not be attenuated by *miR-200a* treatment (Figures 5C–5D). MDA-MB-231 is an aggressive breast cancer

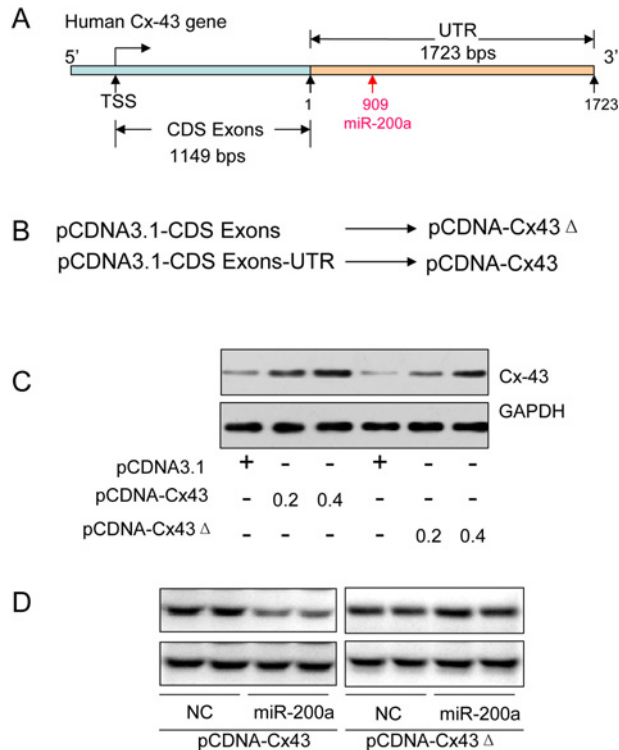


Figure 4 Overexpressing human Cx43 proteins in MCF-7 cells
(A) The schematic diagram of the human Cx43 gene structure containing the 1149-bp CDS Exons and 1723-bp 3'-UTR. **(B)** The pCDNA-Cx43, a human Cx43 protein overexpression plasmid, was obtained by subcloning the 1149-bp CDS Exons plus 1723-bp 3'-UTR into the pCDNA3.1 vector. The pCDNA-Cx43Δ construct was obtained by subcloning the 1149-bp CDS Exons only into the pCDNA3.1 vector. **(C)** Immunoblotting assay of human Cx43 in the MCF-7 cells transfected with different doses of pCDNA-Cx43 (0.2 or 0.4 μg/ml), pCDNA-Cx43Δ (0.2 or 0.4 μg/ml) or pCDNA3.1 vector as control for 24 h. **(D)** Western blotting assay of human Cx43 in the MCF-7 cells co-transfected with pCDNA-Cx43 (0.4 μg/ml) or pCDNA-Cx43Δ (0.4 μg/ml) plus NC (100 nM) or *miR-200a* (100 nM) for 24 h.

cell line. The migration activity of these cells could be attenuated by the treatment of *miR-200a* or *miR-1* (Figures 5E–5F). In consistence, both *miR-200a* and *miR-1* were direct suppressors of Cx43. Therefore, we concluded that *miR-200a* could inhibit the migration activity of breast cancer cells through suppression of Cx43 expression.

Decreased *miR-200a* and elevated Cx43 expression in metastatic breast cancer tissues

Given the regulatory role of *miR-200a/Cx43* axis in the migration activity of breast cancer cells, we further observed the expression of *miR-200a* and Cx43 in 20 cases of primary and metastatic breast cancer tissues in human patients. We demonstrated that the levels of *miR-1* (Figure 6A) and *miR-200a* (Figure 6B) were significantly decreased, whereas the expression of Cx43 mRNA (Figure 6C) was notably elevated in the metastatic breast cancer tissues compared with the primary ones. Those results confirmed

the inhibitory role of *miR-200a* in Cx43 expression and indicated the possible pathophysiological effects of *miR-200a/Cx43* axis in metastatic breast cancer.

DISCUSSION

In the present study, we are the first to identify *miR-200a* as a novel suppressor of Cx43 gene by directly targeting in the 3'-UTR of Cx43 gene. Meanwhile, we also found that *miR-1*, *miR-206*, *miR-381*, *miR-23a*, *miR-23b* and *miR-186* could also inhibit human Cx43 expression in mRNA and protein levels. We did not further explore the targeting sequences of *miR-1* and *miR-206* in human Cx43 gene, since a previous study had reported the direct interaction between those two miRs and Cx43 gene [23].

With regard to *miR-23a* and *miR-23b*, we did not find their potential binding elements in the 3'-UTR of human Cx43 gene, although both miRs exerted a dramatically inhibitory role in Cx43 gene expression. We presumed that *miR-23a* and *miR-23b* might directly act on Cx43 gene via a non-canonic-binding site in the 3'-UTR, but not through the one we predicted or that those two miRs might indirectly suppress human Cx43 gene expression via regulating some other miRs which could interact with the 3'-UTR of human Cx43 gene.

As to *miR-186* and *miR-381*, both of them could inhibit the mRNA and protein levels of human Cx43, but they exerted no significant effects on the activity of Cx43 3'-UTR reporter gene. Those results indicated that *miR-186* and *miR-381* might not act directly on the 3'-UTR of human Cx43 gene. They might regulate Cx43 expression by acting on some transcription factors which could target Cx43 gene promoter. Therefore, the present study uncovered a complex network of miRs in the regulation of human Cx43 gene expression. The above presumptions need to be verified by further experiment in future.

Cx43 expression was associated with increased malignancy in multiple cancers [26–28]. Especially, a previous study reported that *miR-206* inhibited the migration and invasion of breast cancer by targeting at Cx43 [25]. Those observations indicated that the miRs we identified in the present study might be functional regulators of the migration and metastasis of breast cancer via regulating Cx43 expression. Our results verified these notions: (1) overexpressing Cx43 in a non-aggressive breast cancer cell line MCF-7 enhanced the cell migration activity; (2) Additional treatment of *miR-200a* could fully prevent Cx43-induced cell migration activity in MCF-7 cell; (3) Treatment of *miR-200a* or *miR-1*, both of which were direct suppressors of Cx43, could attenuate the migration activity of an aggressive breast cancer cell line MDA-MB-231.

To further correlate the expression of *miR-200a* and Cx43 to the physiopathology of breast cancer *in vivo*, we collected the primary and metastatic breast cancer tissues in clinics. We found the notably decreased levels of *miR-1* and *miR-200a*, as well as the increased mRNA expression of Cx43 in the metastatic breast cancer tissues compared with the primary cancer tissues.

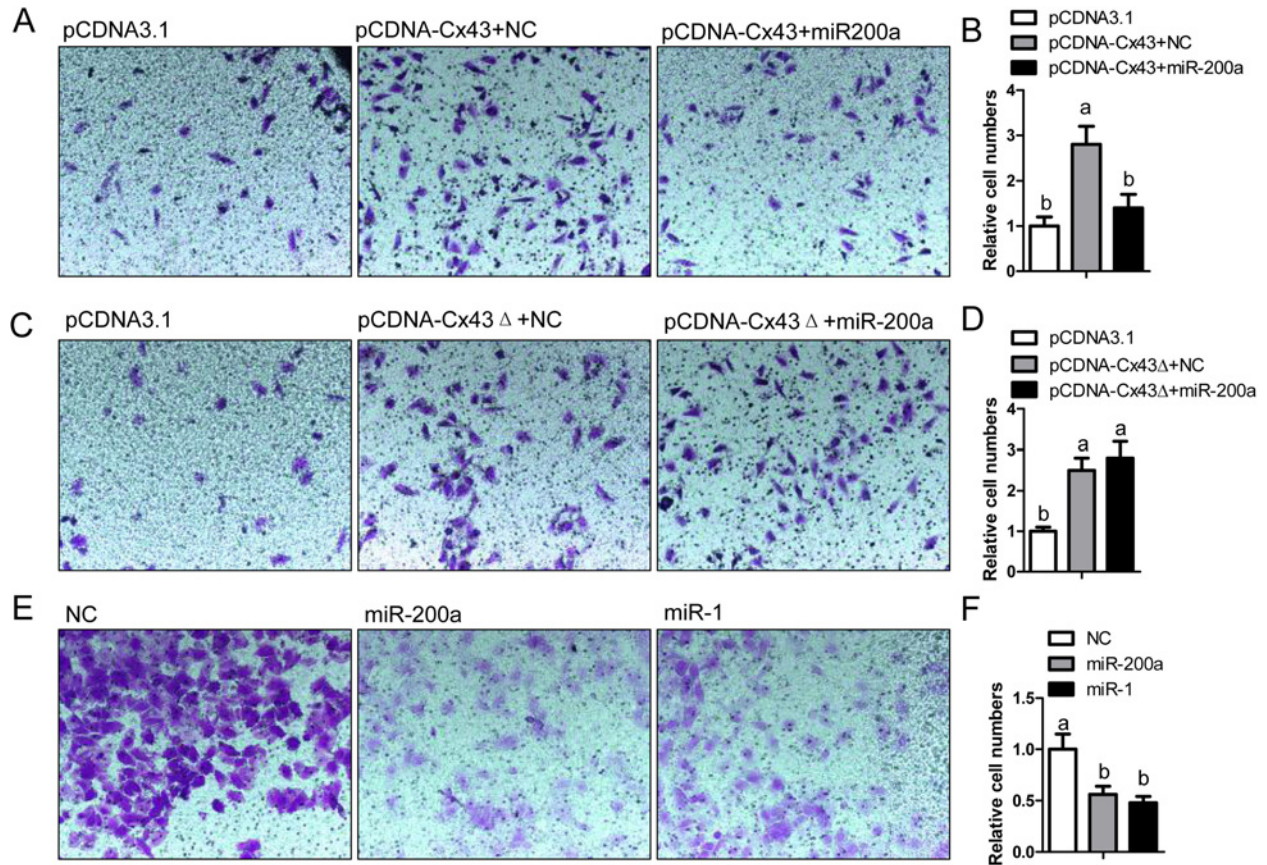


Figure 5 miR-200a/Cx43 axis regulated migration activity of breast cancer cells

(A) Migration activity of MCF-7 cells transfected with pCDNA3.1 (0.4 μ g/ml), pCDNA-Cx43 (0.4 μ g/ml) plus NC (100 nM) or pCDNA-Cx43 (0.4 μ g/ml) plus miR-200a (100 nM) was measured by transwell assay. (B) Relative transmitted cell numbers in the transwell assay in (A). (C) Transwell assay of MCF-7 cells transfected with pCDNA3.1 (0.4 μ g/ml), pCDNA-Cx43 Δ (0.4 μ g/ml) plus NC (100 nM) or pCDNA-Cx43 Δ (0.4 μ g/ml) plus miR-200a (100 nM). (D) Relative transmitted cell numbers in the transwell assay in (C). (E) Migration activity of MDA-MB-231 cells transfected with NC (100 nM), miR-200a (100 nM) or miR-1 (100 nM) was measured by transwell assay. (F) Relative numbers of migrated cells in the transwell assay in (E). (B, D and F) Values not sharing a common superscript letter differ significantly ($n=5$, $P < 0.05$).

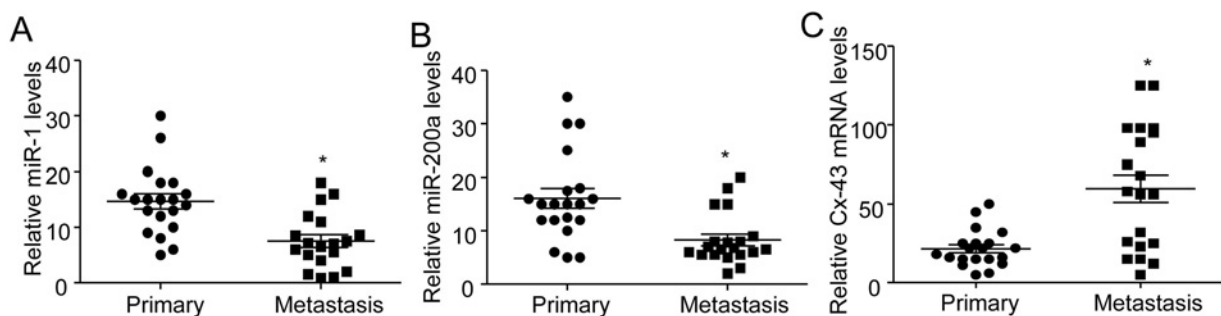


Figure 6 Decreased levels of miR-200a and miR-1 as well as elevated mRNA expression of Cx43 in the metastatic breast cancer tissues in patients

(A and B). Reduced levels of miR-1 (A) and miR-200a (B) in the metastatic breast cancer tissues compared with the primary ones ($n=20$, $*P < 0.05$). (C) Relative Cx43 mRNA levels in the primary and metastatic breast cancer tissues. ($n=20$, $*P < 0.05$)



These findings indicated that the *miR-1/Cx43* or *miR-200a/Cx43* axis might be promising targets for the diagnosis and therapy of metastatic breast cancer.

In conclusion, we identified *miR-200a* as a novel suppressor of Cx43 gene by directly targeting in the 3'-UTR. The *miR-200a/Cx43* axis regulated the migration activity of the breast cancer cells.

AUTHOR CONTRIBUTION

Jia Ming conducted the experiments, analysed the data and wrote the manuscript. 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 the present work, had full access to all the data and takes full responsibility for the integrity of data.

FUNDING

This work was supported by the National Natural Science Foundation of China [grant number 81172539]; and the Natural Science Foundation of Chongqing [grant number CSTC2011jjA10044].

REFERENCES

- Coughlin, S.S. and Ekwueme, D.U. (2009) Breast cancer as a global health concern. *Cancer Epidemiol.* **33**, 315–318 [CrossRef PubMed](#)
- Jemal, A., Center, M.M., DeSantis, C. and Ward, E.M. (2010) Global patterns of cancer incidence and mortality rates and trends. *Cancer Epidemiol. Biomarkers Prev.* **19**, 1893–1907 [CrossRef PubMed](#)
- Lin, S.X., Chen, J., Mazumdar, M., Poirier, D., Wang, C., Azzi, A. and Zhou, M. (2010) Molecular therapy of breast cancer: progress and future directions. *Nat. Rev. Endocrinol.* **6**, 485–493 [CrossRef PubMed](#)
- Park, S.Y., Lee, H.E., Li, H., Shipitsin, M., Gelman, R. and Polyak, K. (2010) Heterogeneity for stem cell-related markers according to tumor subtype and histologic stage in breast cancer. *Clin. Cancer Res.* **16**, 876–887 [CrossRef PubMed](#)
- Elzarrad, M.K., Haroon, A., Willecke, K., Dobrowolski, R., Gillespie, M.N. and Al-Mehdi, A.B. (2008) Connexin-43 upregulation in micrometastases and tumor vasculature and its role in tumor cell attachment to pulmonary endothelium. *BMC Med.* **6**, 20 [CrossRef PubMed](#)
- Kanczuga-Koda, L., Sulkowski, S., Lenczewski, A., Koda, M., Wincewicz, A., Baltaziak, M. and Sulkowska, M. (2006) Increased expression of connexins 26 and 43 in lymph node metastases of breast cancer. *J. Clin. Pathol.* **59**, 429–433 [CrossRef PubMed](#)
- Conklin, C.M., Bechberger, J.F., MacFabe, D., Guthrie, N., Kurowska, E.M. and Naus, C.C. (2007) Genistein and quercetin increase connexin43 and suppress growth of breast cancer cells. *Carcinogenesis* **28**, 93–100 [CrossRef PubMed](#)
- Cotrina, M.L., Lin, J.H. and Nedergaard, M. (2008) Adhesive properties of connexin hemichannels. *Glia* **56**, 1791–1798 [CrossRef PubMed](#)
- Xu, X., Francis, R., Wei, C.J., Linask, K.L. and Lo, C.W. (2006) Connexin 43-mediated modulation of polarized cell movement and the directional migration of cardiac neural crest cells. *Development* **133**, 3629–3639 [CrossRef PubMed](#)
- Homkajorn, B., Sims, N.R. and Muyderman, H. (2010) Connexin 43 regulates astrocytic migration and proliferation in response to injury. *Neurosci. Lett.* **486**, 197–201 [CrossRef PubMed](#)
- Behrens, J., Kameritsch, P., Wallner, S., Pohl, U. and Pogoda, K. (2010) The carboxyl tail of Cx43 augments p38 mediated cell migration in a gap junction-independent manner. *Eur. J. Cell Biol.* **89**, 828–838 [CrossRef PubMed](#)
- Petri, A., Lindow, M. and Kauppinen, S. (2009) MicroRNA silencing in primates: towards development of novel therapeutics. *Cancer Res.* **69**, 393–395 [CrossRef PubMed](#)
- Zhang, H., Li, Y. and Lai, M. (2010) The microRNA network and tumor metastasis. *Oncogene* **29**, 937–948 [CrossRef PubMed](#)
- Nana-Sinkam, S.P. and Croce, C.M. (2013) Clinical applications for microRNAs in cancer. *Clin. Pharmacol. Ther.* **93**, 98–104 [CrossRef PubMed](#)
- Jovanovic, M. and Hengartner, M.O. (2006) miRNAs and apoptosis: RNAs to die for. *Oncogene* **25**, 6176–6187 [CrossRef PubMed](#)
- Knezevic, J., Pfefferle, A.D., Petrovic, I., Greene, S.B., Perou, C.M. and Rosen, J.M. (2015) Expression of miR-200c in claudin-low breast cancer alters stem cell functionality, enhances chemosensitivity and reduces metastatic potential. *Oncogene* [CrossRef](#)
- Lin, Y., Liu, A.Y., Fan, C., Zheng, H., Li, Y., Zhang, C., Wu, S., Yu, D., Huang, Z., Liu, F. et al. (2015) MicroRNA-33b inhibits breast cancer metastasis by targeting HMGA2, SALL4 and Twist1. *Sci. Rep.* **5**, 9995 [CrossRef PubMed](#)
- Benaich, N., Woodhouse, S., Goldie, S.J., Mishra, A., Quist, S.R. and Watt, F.M. (2014) Rewiring of an epithelial differentiation factor, miR-203, to inhibit human squamous cell carcinoma metastasis. *Cell Rep.* **9**, 104–117 [CrossRef PubMed](#)
- Cheng, J., Guo, S., Chen, S., Mastroianni, S.J., Liu, C., D'Alessio, A.C., Hysolli, E., Guo, Y., Yao, H., Megyola, C.M. et al. (2013) An extensive network of TET2-targeting MicroRNAs regulates malignant hematopoiesis. *Cell Rep.* **5**, 471–481 [CrossRef PubMed](#)
- Han, C., Liu, Y., Wan, G., Choi, H.J., Zhao, L., Ivan, C., He, X., Sood, A.K., Zhang, X. and Lu, X. (2014) The RNA-binding protein DDX1 promotes primary microRNA maturation and inhibits ovarian tumor progression. *Cell Rep.* **8**, 1447–1460 [CrossRef PubMed](#)
- Zhao, H., Chen, D., Wang, J., Yin, Y., Gao, Q. and Zhang, Y. (2014) Downregulation of the transcription factor, FoxD3, is associated with lymph node metastases in invasive ductal carcinomas of the breast. *Int. J. Clin. Exp. Pathol.* **7**, 670–676 [PubMed](#)
- Miao, H., Zhang, Y., Lu, Z., Yu, L. and Gan, L. (2012) FOXO1 increases CCL20 to promote NF-kappaB-dependent lymphocyte chemotaxis. *Mol. Endocrinol.* **26**, 423–437 [CrossRef PubMed](#)
- Anderson, C., Catoe, H. and Werner, R. (2006) MIR-206 regulates connexin43 expression during skeletal muscle development. *Nucleic Acids Res.* **34**, 5863–5871 [CrossRef PubMed](#)
- Li, Z., Zhou, Z. and Donahue, H.J. (2008) Alterations in Cx43 and OB-cadherin affect breast cancer cell metastatic potential. *Clin. Exp. Metastasis* **25**, 265–272 [CrossRef PubMed](#)
- Fu, Y., Jiang, B.Q., Wu, Y., Li, Z.D. and Zhuang, Z.G. (2013) Hsa-miR-206 inhibits the migration and invasion of breast cancer by targeting Cx43. *Zhonghua Yi Xue Za Zhi* **93**, 2890–2894 [PubMed](#)
- Grek, C.L., Rhett, J.M., Bruce, J.S., Abt, M.A., Ghatnekar, G.S. and Yeh, E.S. (2015) Targeting connexin 43 with alpha-connexin carboxyl-terminal (ACT1) peptide enhances the activity of the targeted inhibitors, tamoxifen and lapatinib, in breast cancer: clinical implication for ACT1. *BMC Cancer* **15**, 296 [CrossRef PubMed](#)

27 Zhang, A., Hitomi, M., Bar-Shain, N., Dalimov, Z., Ellis, L., Velpula, K.K., Fraizer, G.C., Gourdie, R.G. and Lathia, J.D. (2015) Connexin 43 expression is associated with increased malignancy in prostate cancer cell lines and functions to promote migration. *Oncotarget* **6**, 11640–11651 [PubMed](#)

28 Zhao, J.Q., Sun, F.J., Liu, S.S., Yang, J., Wu, Y.Q., Li, G.S., Chen, Q.Y. and Wang, J.X. (2013) Expression of connexin 43 and E-cadherin protein and mRNA in non-small cell lung cancers in Chinese patients. *Asian Pac. J. Cancer Prev.* **14**, 639–643 [CrossRef](#) [PubMed](#)

Received 10 June 2015/3 August 2015; accepted 4 August 2015

Accepted Manuscript online 14 August 2015, doi 10.1042/BSR20150153
