Curcumin rescues breast cells from epithelial-mesenchymal transition and invasion induced by anti-miR-34a

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Abstract. Breast cancer is the most prevalent type of cancer among women worldwide and it is characterized by a high morbidity. Curcumin is a naturally occurring compound derived from the rhizome of *Curcuma longa* and is known to have antioxidant and anticarcinogenic properties. Emerging evidence has indicated that microRNAs (miRNAs or miRs) function as oncogenes or tumor suppressor genes to control invasion and migration. The aim of this study was to evaluate the effects of curcumin on genes implicated in epithelial-mesenchymal transition (EMT) and to examine the involvement of *Rho-A* in the migration and invasion of MCF-10F and MDA-MB-231 breast cell lines. Furthermore, to the best of our knowledge, this is the first study to examine the effects of curcumin on *Rho-A* and on genes involved in EMT, such as *Axl, Slug* and *CD24* in order to determine whether the compound is able to prevent migration and invasion by targeting miRNAs as a regulator of such genes. Specifically, we focused on miR-34a which acts as a tumor suppressor gene in human breast cell lines. The present study demonstrated that the *Axl, Slug* and *CD24* genes were implicated in EMT, and *Rho-A* was also involved in the migration and invasion of MCF-10F and MDA-MB-231 cell lines. Curcumin also acted upon the miRNA as a regulator of genes implicated in EMT and upon Rho-A as well, affecting the migration and invasion of the cells. This occurred independently of their estrogen receptor (ER), progesterone receptor (PgR) and human epidermal growth factor receptor 2 (HER2) receptors in the non-malignant MCF-10F and malignant MDA-MB-231 breast cell lines, which are both negative for such receptors.

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Introduction

Breast cancer is the most prevalent type of cancer among women worldwide and it is characterized by a high morbidity (1). Metastasis is considered one of the most crucial stages of tumor progression as it accounts for >90% of all cancer-related deaths (2,3). There have been notable improvements in survival over the past 3 decades for the majority of cancer types and the 5-year relative survival rate for breast cancer patients has increased from 63% in the early 1960s to 91% between 2007 and 2013. However, the survival rate is low among women with more advanced stages of the disease at the time of diagnosis. Thus, the relative survival is 99% for localized disease, 85% for regional disease and 27% for distant-stage disease (4). According to the World Health Organization (WHO), the improvement of the survival of breast cancer patients by early detection remains the foundation of breast cancer regulations.

Epithelial-mesenchymal transition (EMT) is a biological process through which a polarized epithelial cell, which normally interacts with the basement membrane via its basal surface, which endows the cell with the ability to undergo multiple biochemical alterations. These alterations enable the cell to acquire a mesenchymal phenotype; consequently, the cell also acquires an enhanced migratory and invasive capacity, and increased resistance to apoptosis (5).

AXL is a receptor of the TAM family of receptor tyrosine kinases (Tyro3, Axl and Mer). It transduces signals from the extracellular matrix into the cytoplasm by binding to its main ligand, thus inducing dimerization and autophosphorylation and the activation of signaling pathways involved in a wide variety of cellular processes, including survival, proliferation, migration, invasion and EMT (6). AXL is considered to function as an oncogene and is known to be overexpressed in breast, lung, ovarian, gastric, pancreatic and prostate cancers. In addition, AXL is regulated by tumor suppressor microRNAs (miRNAs or miRs), such as miR-34a and it is associated with an enhanced metastatic potential (7), a poor prognosis (8) and resistance to therapy (9) in several types of cancer.

Slug is a C2H2-type zinc-finger transcription factor and is a member of the Snail family. It is known to play a key role in

the development of cancer and in cancer-related EMT. The Slug protein binds to E-box motifs in the promoter of downstream genes and has been shown to suppress E-cadherin transcription in breast cancer, gastric cancer and esophageal squamous cell carcinoma (10,11). It has also been demonstrated that Slug is negatively associated with estrogen receptor (ER)α in both breast and lung cancer (12,13).

Cluster of differentiation 24 (CD24) is a small GPI-linked membrane glycoprotein with glycosylation sites that bind P-selectin (14). CD24 is an adhesion molecule, and it has been shown to be widely expressed in a number of types of cancer, including renal, ovarian, lung and pancreatic cancer (15-17). A previous study also suggested that CD24 expression is a candidate marker for the prognosis of patients with breast cancer (18).

Ras homolog gene family member A (Rho-A) is a member of the Ras-related C3 botulinum toxin substrate (Rac) subfamily of the Rho family and is a small (~22 kDa) G protein/guanosine triphosphatase (19). Rho-A can induce the reorganization of the cell cytoskeleton and can regulate cell migration by activating effector proteins, such as Rho-associated coiled-coil kinase (ROCK) (20); these types of alterations are associated with tumor invasion and migration in several types of cancer cells (21,22). In breast cancer, an increased expression of Rho-A has been shown to be associated with cancer progression (23,24). In addition, an increased expression of Rho-A seems to promote an enhanced cell invasion and metastasis (24).

There is evidence to suggest that the expression of miRNAs play a pivotal role in regulating the acquisition of the EMT phenotype (25). miRNAs are endogenous, non-coding RNAs approximately 21-23 nucleotides in length and have been identified as key negative regulators of gene expression through the endogenous RNA interference machinery. miRNAs are able to regulate gene expression by binding to miRNA recognition elements (MREs) located in the 3' untranslated region (3'-UTR) of target messenger RNAs (mRNAs), leading to their translational repression or degradation (25,26). It has been previously demonstrated either that miRNAs function as oncogenes or tumor suppressor genes, depending on their target genes of regulation, in order to control cell proliferation, invasion, migration, differentiation and apoptosis (27). Recently, the abnormal expression of miRNAs in several types of cancer has been confirmed, including colon cancer, hepatocellular carcinoma, lung cancer and breast cancer (28); in these types of cancer, miRNAs have been shown to be associated with tumor progression, invasion, metastasis and angiogenesis (28,29).

miRNAs as tumor suppressors have been found to be overexpressed in a variety of human cancers, contributing to tumor development. miRNA-34a (miR-34a) has been reported to function as tumor suppressor miRNA and has been shown to be downregulated in cancers (28,29); it also plays an important role in tumorigenesis and the progression of cancer (30).

It is known that certain herbs have the ability to regulate miRNAs associated with cancer (31). Among these, curcumin, a naturally occurring compound derived from the rhizome of *Curcuma longa*, and its analogs are known to exert potent anti-carcinogenic effects (32) through the regulation of multiple downstream cancer-related signaling molecules (33). Studies have demonstrated a protective role of curcumin in the oxidative stress of breast cells (34,35). It has been demonstrated that curcumin affects breast cancer cells transformed by low concentrations of radiation and estrogen (36). Among the genes involved in such processes, CD44 expression has been shown to be associated with oxidative stress in such cells (37). On the other hand, it has been demonstrated that curcumin suppresses the invasive capabilities of breast cancer cell lines through EMT (38).

MDA-MB-231 is a basal-like triple-negative breast cancer cell line. Triple-negative breast cancer [negative for ER, progesterone receptor (PgR) and human epidermal growth factor receptor 2 (HER2)] is the most aggressive breast cancer subtype and is associated with a poor prognosis. The aim of the present study was to determine whether curcumin modulates miRNA expression, whether it induces EMT-related changes, and thereof, whether it affects the motility of breast cells.

Materials and methods

Breast cell lines. MCF-10F human breast and MDA-MB-231 human breast cancer cell lines were obtained from the American Type Culture Collection (ATCC). The MCF-10F cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (all obtained from Life Technologies, Thermo Fisher Scientific) and 5% equine serum (Biofluids), 0.5 µg/ml hydrocortisone (Sigma) and 0.02 µg/ml epidermal growth factor (Collaborative Research). The MDA-MB-231 cells were maintained in RPMI-1640 medium (Life Technologies, Thermo Fisher Scientific) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), 0.1 mM non-essential amino acids, 0.2 mM glutamine, 1 mM pyruvate and 10% heat-inactivated fetal bovine serum, and incubated in a 5% CO_2 humidified atmosphere at 37˚C. The cells were grown to 80% confluence prior to the treatments.

Cell viability assay. The viability of the cells was determined in the presence of curcumin (Sigma-Aldrich; Merck KGaA). Firstly, the MCF-10F and MDA-MB-231 cell lines were seeded in 96-well microplates ($25x10^3$ cells/well) in a final volume of 100 µl and incubated in culture medium for 48 h at 37˚C and a 5% concentration of CO_2. Following overnight incubation at 37˚C, the cells were treated with 0, 5, 10, 15, 20, 25, 30 and 35 µM of curcumin. Curcumin was dissolved in 0.1% of DMSO solvent and used as a blank. Cells not treated curcumin functioned as the control group. Following 48 h of incubation at 37ºC, the metabolic activity of the living cells was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Sigma-Aldrich). The medium was removed, and the insoluble formazan crystals were dissolved in 200 µl of DMSO. Subsequently, 20 µl MTT (5 mg/ml) were added to each well. The cells were then incubated at 37˚C for a further 4 h. The reduction of MTT was determined following the manufacturer's instructions. The absorbance was determined at 570 nm (Autobio Labtec Instruments) in a microplate reader (Molecular Devices). The results were expressed as the percentage of cell survival relative to the control. The 50% inhibitory concentration (IC_{50}) was defined as the curcumin concentration that induced a 50% reduction in the viability of the cells compared to control, as previously described (34-39).

Table I. Sequences of primers used for RT-qPCR.

A. Sequence and annealing temperature of the primers used in the RT-qPCR reactions

Gene name	Primer sequence[a]	Annealing temperature
Axl	F: GTTTGGAGCTGTGATGGAAGGC	62.01
	R: CGCTTCACTCAGGAAATCCTCC	61
Slug	F: GACCCTGGTTGCTTCAAGGA	59.89
	R: TGTTGCAGTGAGGGCAAGAA	60.11
CD24	F: AACTAATGCCACCACCAGG	58.1
	R: GACGTTTCTTGGCCTGAGTC	58.9
Rho-A	F: CCATCATCCTGGTGGGGAAT	54.8
	R: CATCCCAAAAGCGCCA	56.1

B. Alignment of mature miR-200a, miR-200c, let-7a, let-7c, let-7b, miR-21 and miR-34a sequences

Assay ID	miR	Mature sequence[b]
000502	hsa-miR-200a	UAACACUGUCUGGUAACGAUGU
000505	hsa-miR-200c	UAAUACUGCCGGGUAAUGAUGG
000377	hsa-let-7a	UGAGGUAGUAGGUUGUAUAGUU
000379	hsa-let-7c	UGAGGUAGUAGGUUGUAUGGUU
000378	hsa-let-7b	UGAGGUAGUAGGUUGUGUGGUU
000397	hsa-miR-21	UAGCUUAUCAGACUGAUGUUGA
000425	hsa-miR-34a	UGGCAGUGUCUUAGCUGGUUGU

[a]Primer sequences are listed in the 5' to 3' orientation. [b]Mature sequences are listed in the 5' to 3' orientation. F, forward; R, reverse; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

RNA extraction and cDNA synthesis. For RNA isolation, two separate purification procedures were carried out. The measurement of small RNA (containing miRNA) and larger RNA (containing mRNA) from the cells was determined using the RNeasy Plus Mini kit (cat. no. 74204) and the Rneasy MinElute Cleanup kit (cat. no. 74134), according to the manufacturer's instructions (Qiagen). For mRNAs, 2 μg of the longer RNA fraction were reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). For miRNAs, reverse transcription was carried out using 2 to 10 ng of the small RNA fraction, the TaqMan® MicroRNA Reverse Transcription kit (cat. no. 4366596; Applied Biosystems) and gene-specific RT-primers according to the TaqMan MicroRNA assay protocol (cat. no. 4427975; Applied Biosystems).

RT-qPCR for the analysis of mRNA expression. RNA was obtained using RNeasy Plus Mini kit and RNeasy MinElute kit (Qiagen) following the manufacturer's protocol and for reverse transcription the High capacity cDNA Reverse Transcription (Applied Biosystems). An aliquot of a dilution 1/50 of cDNA (2 μl) was used in a 20 μl qPCR reaction containing SYBR-Green PCR Master Mix (Agilent Technologies) and 5 µM of each primer for the target genes, such as *Axl*, *Slug*, *CD24* and *Rho-A*. The primers for the selected genes are presented in Table IA. The reaction was carried out on a CFX 96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories) with the following conditions: 95˚C for 10 min and 40 cycles of a 2-step program of 95˚C for 10 sec and 61˚C for 45 sec when fluorescence-reading occurs. The reactions were carried out in triplicate and the threshold of the cycle was obtained using Bio-Rad CFX Manager 2.1 software and the average gene expression was normalized using the reference housekeeping gene β-actin and the relative expression level was calculated as previously described (36,38), using the $2^{-\Delta\Delta Cq}$ method (40).

RT-qPCR for the analysis of miRNA expression. It was done by using RNeasy Plus Mini kit and RNeasy MinElute Cleanup kit (Qiagen) according to the manufacturer's protocol. An aliquot of cDNA (2 μl) was used in 15 μl qPCR reaction containing TaqMan Small RNA Assays specific for each miRNA (cat. no. 4427975) and TaqMan Universal PCR Master Mix II (cat. no. 4440040; Applied Biosystems). The alignment of mature miR-200a, miR-200c, let-7a, let-7c, let-7b, miR-21 and miR-34a sequences is presented in Table IB. The reaction was carried out on a CFX 96 Touch Real-Time PCR Detection Systems (Bio-Rad Laboratories) with the following conditions; 50˚C for 2 min, 95˚C for 10 min and 40 cycles of a 2-step program of 95˚C for 15 sec and 60˚C for 60 sec when fluorescence-reading occurs. The reactions were carried out in triplicate and the threshold of the cycle was obtained using Bio-Rad CFX Manager 2.1 software and the average gene expression was normalized using the reference snRNA U6. The relative expression of each miRNA was normalized against

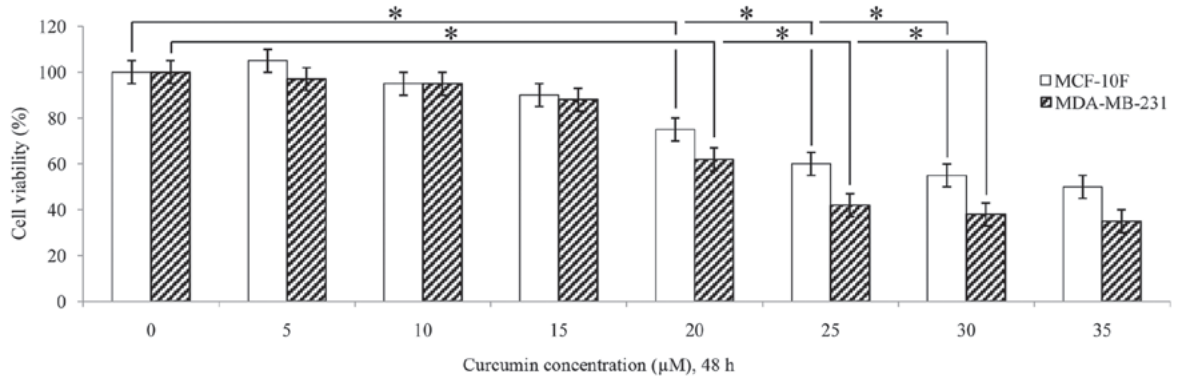

Figure 1. Cell viability assay by MTT. MCF-10F and MDA-MB-231 cell lines cultured in the presence of curcumin from 0 to 35 μM for IC_{50} determination (n=3) (*P<0.05).

snRNA U6 (ID no. 001973) expression using a Bio-Rad CFX Manager 2.1 software.

Oligonucleotide transfection. The MCF-10F and MDA-MB-231 cells were transfected with 75 pmol anti-miR-34a (cat. no. AM11030) or anti-miR negative control (cat. no. AM17010) (Life Technologies, Thermo Fisher Scientific) using Lipofectamine® 2000 (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions. The cells were assayed 24, 48 or 72 h post-transfection.

Cell migration and invasion assays. Migration and invasion assays were performed using modified Boyden's chambers (Corning, Inc.) constructed with multiwall cell culture plates and cell culture inserts as previously described (36,38). For the invasion assay, the upper chambers of Transwells with 8-μm membrane pores were pre-coated with Matrigel matrix gel (60 μl) (BD Biosciences) at least 1 h prior to the seeding of the tested cells. The cells were pre-treated with curcumin (30 µM) and cultured for 24 h. A total of $3x10^5$ cells in 100 μl of medium without fetal bovine serum was added to the upper chambers and 600 μl of medium with 10% FBS was placed in the lower chambers as a chemoattractant. The Matrigel invasion chamber was incubated for 16 h in a humidified tissue culture incubator. The upper chambers were then removed from the lower chambers and then wiped using cotton swabs. The invaded and migrated cells were fixed using 100% methanol at room temperature for 15 min, visualized and quantified using DAPI. Ten fields of each chamber were photographed using a fluorescence microscope (x40 magnification, Olympus Corp.). Migration assay was carried out the same way as the invasion assay; however, the Transwell insert was not coated with Matrigel. This experiment was independently repeated 3 times.

Protein expression by immunoperoxidase staining. Exponentially growing cells were plated on a glass chamber slide (Nunc Inc.) at a density of $1x10^4$ cells/ml of medium and allowed to grow for 2-3 days until 70% confluent as previously described (39). The cells were then fixed with buffered paraformaldehyde at room temperature, incubated with 1% H_2O_2 in methanol to block endogenous peroxidase and washed again twice with buffer solution. Subsequently, the cell cultures were then covered with normal horse serum for 30 min at room temperature and incubated with either anti-mouse or anti-goat monoclonal or polyclonal antibodies: Anti-Axl (mouse, sc-166269), anti-Slug (mouse, sc-166476), anti-CD24 (mouse, sc-65257) and anti-Rho-A (mouse, sc-418), (all from Santa Cruz Biotechnology, Inc.) at a 1:500 dilution overnight at 4˚C. The cells were subsequently incubated for 45 min with diluted biotinylated secondary antibody solution (Vector Laboratories Inc.) and Vectastin Elite ABC reagent (Vector Laboratories Inc.) was used. The experiments were repeated twice in cells with identical passages *in vitro*. A semi-quantitative estimation based on the relative staining intensity of protein expression both in the untreated control and treated cells was determined. The number of immunoreactive cells (30 cells/field) was counted in 5 randomly selected microscopy fields per sample and the percentage of relative fold protein expression was calculated.

Statistical analysis. Numerical data are expressed as the means ± standard error of the mean (SEM). Comparisons between 2 groups were made using the t-test and between several treatment groups and the controls by ANOVA with Dunnett's test. Data were analyzed using release IBM SPSS 22.0 (SPSS, Inc.). A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Cell viability of the cells following treatment with curcumin. Cell viability assay was performed using MTT assay to examine the effects of curcumin at various concentrations (fluctuating from 0 to 35 μM for 48 h) on the viability of the MCF-10F and MDA-MB-231 cells. As shown in Fig. 1, a significant (P<0.05) decrease in cell viability was observed in both cell lines following treatment with 20 and 30 μM curcumin for 48 h in comparison to the untreated cells (0 μM). The concentrations of 10 and 30 μM curcumin were used in several experiments.

Effects of curcumin on MCF-10F gene and protein expression levels. As shown in Fig. 2A, curcumin (30 μM for 48 h) significantly (P<0.05) decreased the *Axl* (39%), *Slug* (57%), *CD24* (62%) and *Rho-A* (53%) gene transcript levels. In addition,

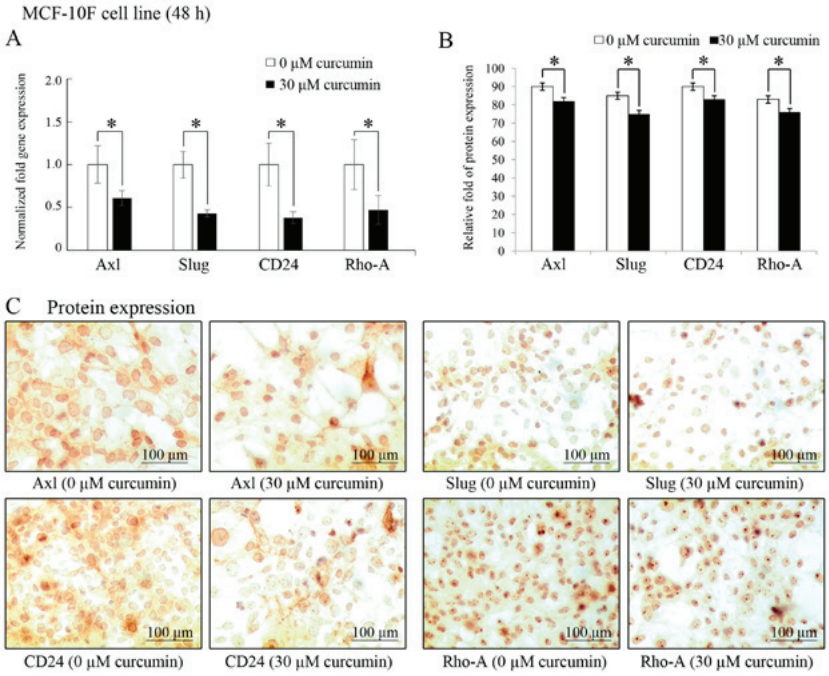

Figure 2. Graphs showing the effects of curcumin (30 μM for 48 h) on *Axl*, *Slug*, *CD24*, and *Rho-A* expression in the MCF-10 cell line. (A) Normalized fold gene transcript levels by RT-qPCR, and (B) relative protein expression (%) by immunocytochemistry in comparison to their own controls. Bars in the figure indicate the means ± standard error of the mean (n=3; *P<0.05). (C) Representative images of the effects of curcumin on protein expression levels in comparison to their own controls.

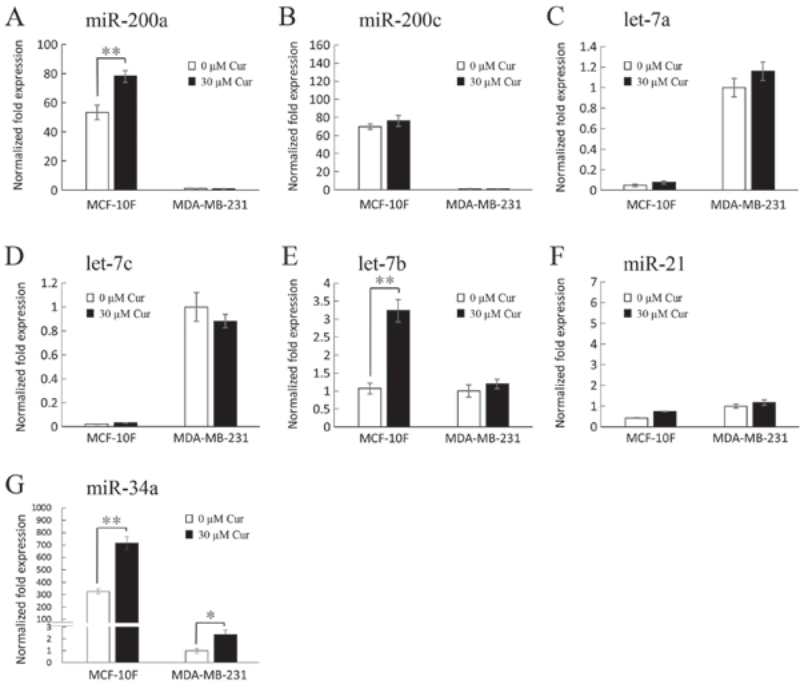

Figure 3. Effects of curcumin (Cur) (30 μM for 48 h) on (A) miR-200a, (B) miR-200c, (C) let-7a, (D) let-7c, (E) let-7b, (F) miR-21 and (G) miR-34a expression examined by RT-qPCR in the MCF-10F and MDA-MB-231 breast cell lines. Graphs show the fold change in miRNA expression in cells treated with Cur in comparison to their own controls. Bars in the figure indicate the means ± standard error of the mean (n=3; *P<0.05, **P<0.005).

protein expression was examined by immunocytochemistry in MCF-10F cell line in comparison to its own controls (Fig. 2B). Similar to the mRNA levels, curcumin also induced a decrease in the protein levels of Axl, Slug, CD24 and Rho-A in the cells. Representative images of the effects of curcumin on protein expression levels are presented in Fig. 2C.

Effects of curcumin on miRNA levels. As shown in Fig. 3, treatment with 30 μM curcumin significantly (46%, P<0.05) increased miR-200a expression in the MCF-10F cell line in comparison with its own control (Fig. 3A); however, it had no marked effect on the MDA-MB-231 cell line. In addition, curcumin did not exert any notable effects on the expression

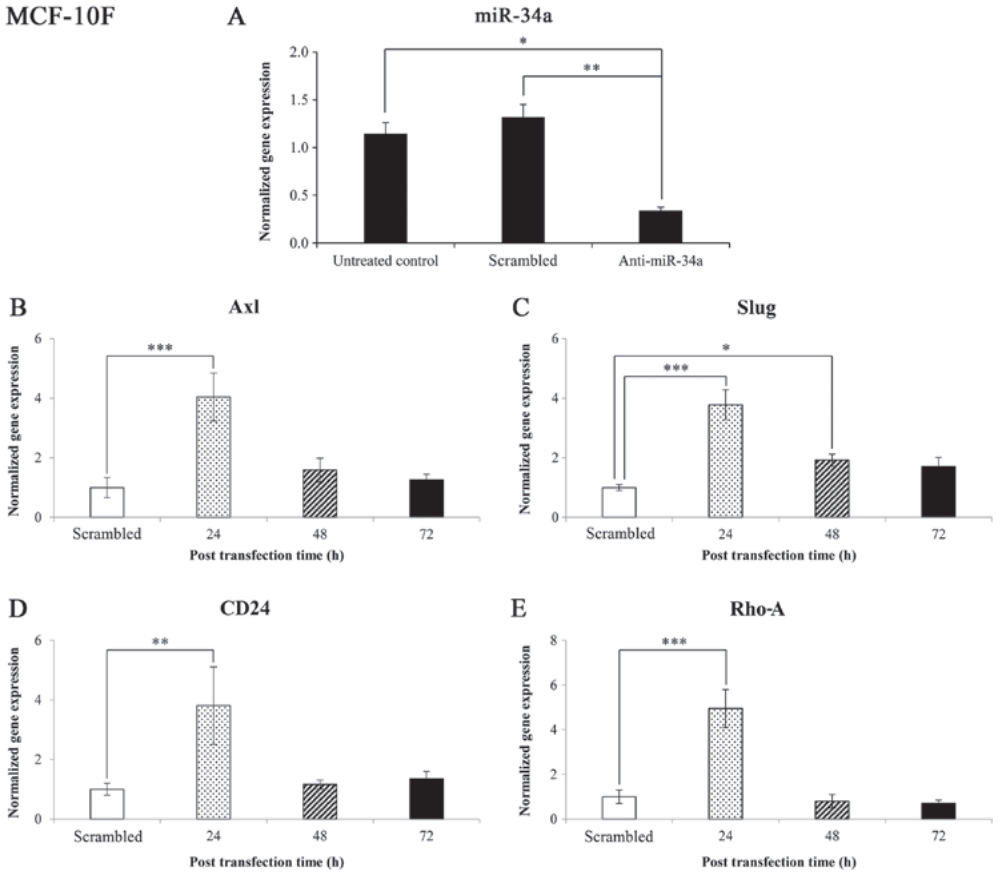

Figure 4. (A) Graph showing the fold change of miR-34a expression in the MCF-10F cell line in comparison to the untreated control and negative control (scrambled). (B-E) Graphs represent the effects of the time of post-transfection with anti-miR-34a in the MCF-10F cell line on (B) *Axl*, (C) *Slug*, (D) *CD24* and (E) *Rho-A* normalized gene expression after 24, 48 and 72 h. Bars represent the means ± standard error of the mean (n=3; *P<0.05, **P<0.005, ***P<0.001).

levels of miR-200c (Fig. 3B), let-7a (Fig. 3C), let-7c (Fig. 3D) and miR-21 (Fig. 3F) in the MCF-10F and MDA-MB-231 cell lines; however, it significantly (P<0.05) increased let-7b expression (202%) in the MCF-10F cell line in comparison to its own control (Fig. 3E), although no notable effect was observed on the MDA-MB-231 cell line. On the other hand, as shown in Fig. 3G, curcumin significantly increased miR-34a expression in both the MCF-10F and MDA-MB-231 cell lines (P<0.005, 120% and P<0.05, 138%, respectively), when compared to their own controls.

Effects of anti-miR-34a and the time of post-transfection on gene expression levels in the MCF-10F cell line. The results of the miR-34a expression level in the MCF-10F cell line transfected with anti-miR-34a and negative control (scrambled) are shown in Fig. 4A. The results revealed that miR-34a expression significantly (P<0.005 and P<0.05) decreased in those cells transfected with anti-miR-34a compared to the scrambled and untreated control, respectively. The normalized gene expression levels following transfection with anti-miR-34a of genes that code for proteins involved in EMT in the MCF-10F cell line are shown in Fig. 4B-E. The effect of blocking miR-34a on *Axl*, *Slug*, *CD24*, and *Rho-A* gene expression was analyzed. The knockdown of miR-34a significantly (P<0.001) increased *Axl* (304%) expression after 24 h; no significant changes were observed after 48 and 72 h (Fig. 4B). The knockdown of miR-34a also significantly increased *Slug* expression after 24 and 48 h (278%, P<0.001; and 92%, P<0.05, respectively) (Fig. 4C). Following the knockdown of miR-34a, an increase was also observed in the *CD24* (281%, P<0.005) (Fig. 4D) *Rho-A* (395%, P<0.001) (Fig. 4E) expression levels following 24 h of transfection.

Effect of curcumin and anti-miR-34a on gene expression levels in the MCF-10F cell line. The effects of treatment with 30 μM curcumin for 48 h on the *Axl*, *Slug*, *CD24* and *Rho-A* gene expression levels in the MCF-10F cell line are shown in Fig. 5. The results revealed that curcumin significantly (P<0.05) decreased *Axl* (65%), *Slug* (50%), *CD24* (62%), and *Rho-A* (55%) gene expression following transfection of the cells with negative control (scrambled) in comparison with the curcumin-untreated cells. Transfection with anti-miR-34a significantly (P<0.05) increased the gene expression of *Axl* (133%), *Slug* (290%), *CD24* (282%) and *Rho-A* (380%); however, treatment with curcumin plus anti-miR-34a significantly (P<0.05) decreased the levels of the examined genes in comparison to the cells transfected with anti-miR-34a and not treated with curcumin (Fig. 5).

Effects of curcumin on the migratory and invasive capabilities of the MCF-10F cell line. The migratory and invasive capabilities were analyzed by migration and invasion assays carried out in a Boyden chamber (Fig. 6A and B). The results presented in Fig. 6A revealed that curcumin significantly (P<0.001)

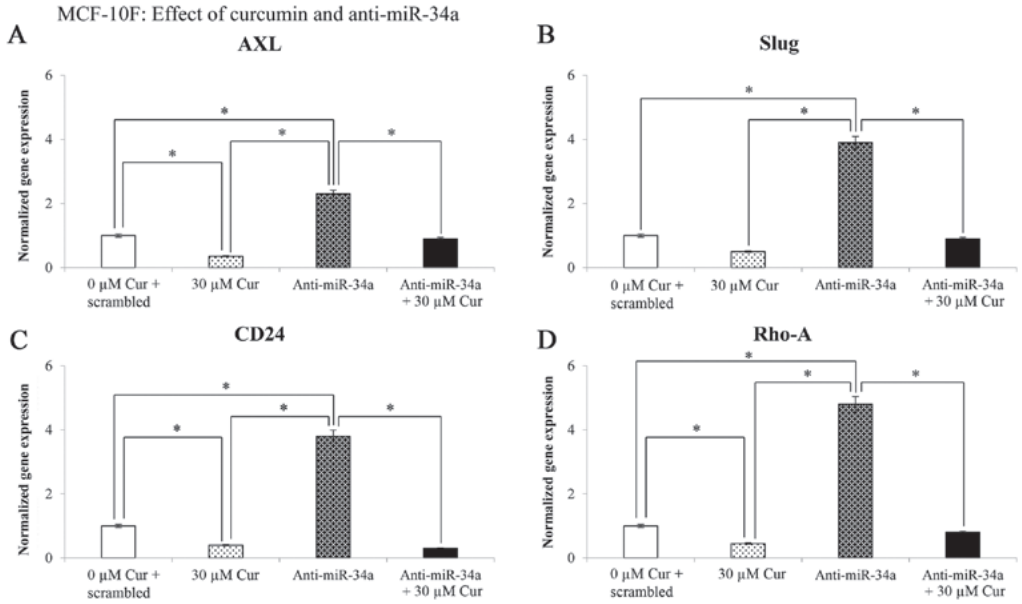

Figure 5. Graphs showing the effects of curcumin (Cur) (30 μM for 48 h) on (A) *Axl*, (B) *Slug*, (C) *CD24* and (D) *Rho-A* normalized gene expression, and the transfection with anti-miR 34a analyzed by RT-qPCR. Bars in the figure indicate the means ± standard error of the mean (n=3; *P<0.05).

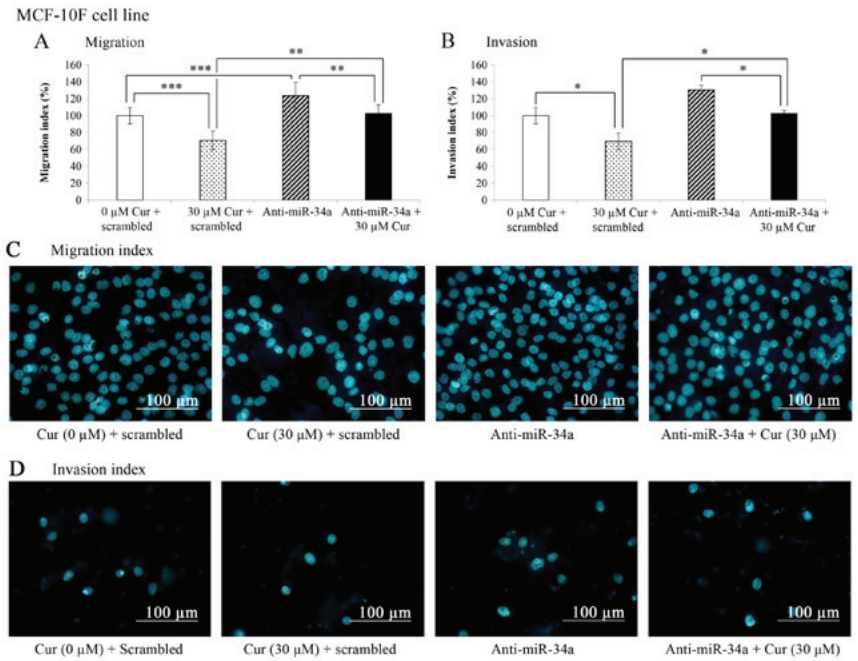

Figure 6. Graphs showing the effects of curcumin (Cur) (30 μM for 48 h) and Anti-miR-34a on (A) migration (B) invasion in the MCF-10F cell line (%). Bars represent the means ± standard error of the mean (n=3; *P<0.05, **P<0.005, ***P<0.001). Representative images are shown of the (C) migration index and (D) invasion index.

decreased the migration (29.3%) of the MCF-10F cells transfected with the negative control (scrambled) in comparison with the untreated control cells (scrambled + 0 μM Cur). In addition, a significant increase (24%, P<0.001) in the migration of the MCF-10F cells transfected with anti-miR-34a in comparison with the untreated control cells (scrambled + 0 μM Cur) was observed. Curcumin plus anti-miR-34a significantly (P<0.005) decreased the migration of the MCF-10F cell line in comparison to anti-miR-34a alone. The percentage of migrated cells treated with curcumin plus anti-miR-34a was similar to the untreated control (scrambled + 0 μM Cur) following transfection. When miR-34a was blocked, the migratory capability of the cells decreased.

The effects of the knockdown of miR-34a on the invasive ability of the MCF-10F cells were also evaluated. As shown in Fig. 6B, curcumin significantly (P>0.05) decreased the invasion (30.5%) of the MCF-10F cells transfected with the negative control (scrambled) in comparison with the untreated control cells (scrambled + 0 μM Cur). The invasive ability was significantly (P<0.05) decreased (39.1%) in the MCF-10F cells transfected with anti-miR-34a and treated with curcumin compared with anti-miR-34a alone. Cell invasion decreased

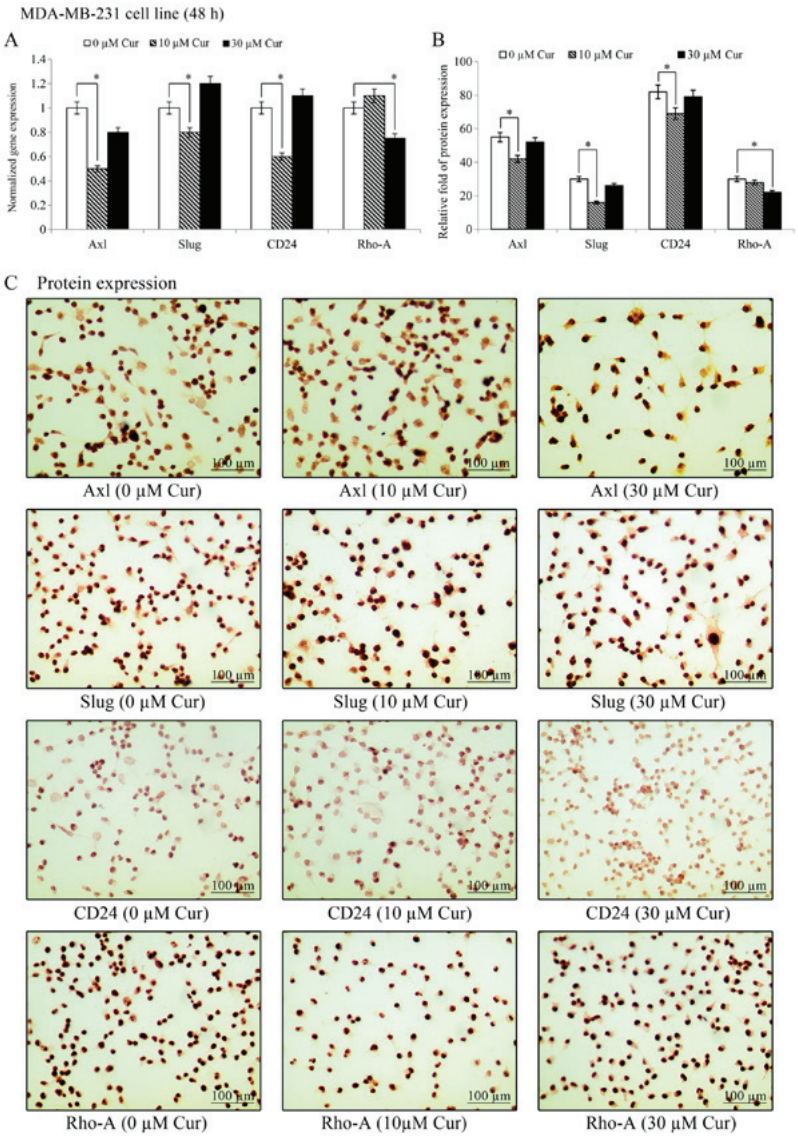

Figure 7. Graphs showing the effect of curcumin (Cur) (10 and 30 μM for 48 h) on *Axl*, *Slug*, *CD24* and *Rho-A* in the MDA-MB-231 cell line. (A) Normalized fold gene transcript levels examined by RT-qPCR and (B) relative protein expression (%) examined by immunocytochemistry in comparison to their own controls. Bars represent the means ± standard error of the mean (n=3; *P<0.05). (C) Representative images of the effects of Cur on protein expression levels.

to the level of the untreated control (scrambled + 0 μM Cur) when miR-34a was blocked and the cells were treated with curcumin (30 μM for 24 h). Representative images of the migratory capabilities following treatment with curcumin and miR-34a blockage in the MCF-10F cell line are shown in Fig. 6C. In addition, representative images of the invasive capabilities following treatment with curcumin and miR-34a blockage are shown in Fig. 6D.

Effects of curcumin on gene and protein expression levels in the MDA-MB-231 cell line. The effects of treatment with 10 and 30 μM curcumin on *Axl*, *Slug* and *CD24* gene expression on the MDA-MB-231 cell line are presented in Fig. 7. The results revealed that treatment with 10 μM curcumin significantly (P<0.05) decreased the gene transcript levels of *Axl*, *Slug* and *CD24*, while those of *Rho-A* remained unaffected. However, treatment with 30 μM curcumin decreased the *Rho-A* transcript levels, while those of the other genes were unaffected. As shown in Fig. 7B, treatment with 10 μM curcumin decreased the Axl, Slug, and CD24 protein expression levels in the MDA-MB-231 cell line; however, the levels of Rho-A were unaffected by this concentration. However, treatment with 30 μM curcumin decreased only Rho-A protein expression in comparison to its own control. Representative images of the effects of curcumin on protein expression levels in the MDA-MB-231 cell line are shown in Fig. 7C. The results revealed that treatment with 10 μM curcumin decreased Axl, Slug and CD24 protein expression. However, 30 μM curcumin decreased only Rho-A protein expression in comparison to its own control.

Effects of anti-miR-34a and the time of post-transfection on gene expression levels in the MDA-MB-231 cell line. The results of the miR-34a expression level in the MDA-MB-231 cell line in comparison to the untreated control and negative control (scrambled) are presented in Fig. 8A. The results indicated that miR-34a expression significantly decreased (P<0.05) in the anti-miR-34a-transfected cells compared to the

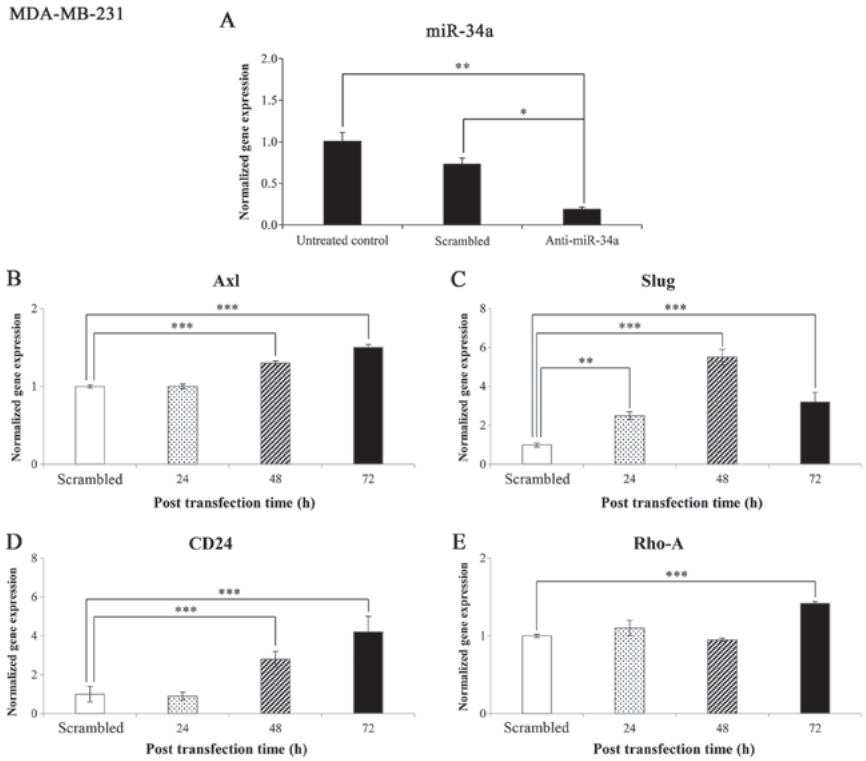

Figure 8. (A) Graph showing the fold change in miR-34a expression in the MDA-MB-231 cell line in comparison to the untreated cells and negative control (scrambled). (B-E) Graphs showing the effects of the time of post-transfection with Anti-miR-34a on (B) *Axl*, (C) *Slug*, (D) *CD24* and (E) *Rho-A* normalized gene expression after 24, 48 and 72 h in the same cell line. Bars in the figure indicate means ± standard error of the mean (n=3; *P<0.05, **P<0.005, ***P<0.001).

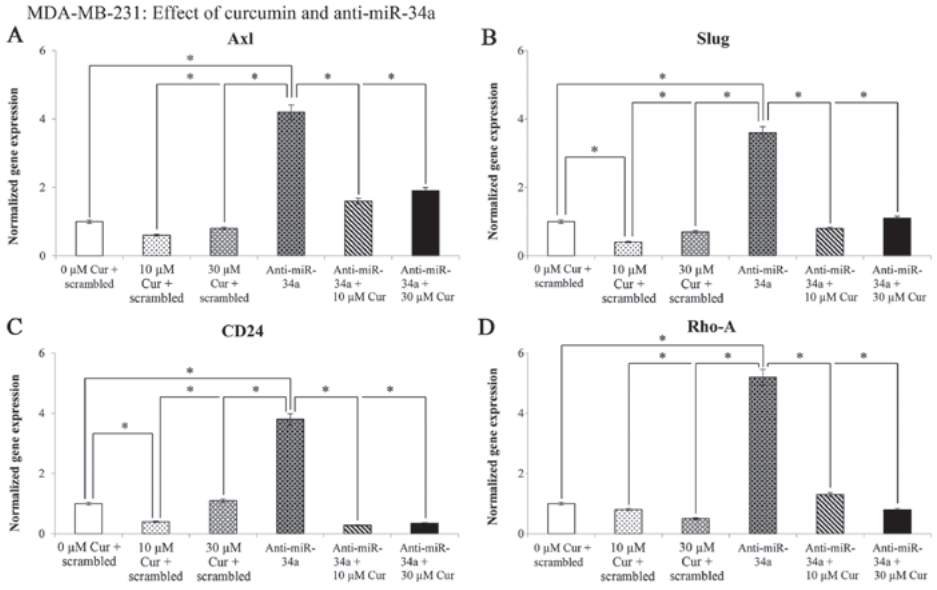

Figure 9. Graphs showing the effects of curcumin (Cur) (10 and 30 μM for 48 h) on (A) *Axl*, (B) *Slug*, (C) *CD24* and (D) *Rho-A* normalized gene expression, and transfection with anti-miR-34a analyzed by RT-qPCR. Bars represent the means ± standard error of the mean (n=3; *P<0.05).

cells transfected with the scramble control and the untreated cells.

The normalized gene expression levels of genes that code for proteins involved in EMT in the MDA-MB-231 cell line following transfection with anti-miR-34a are shown in Fig. 8B-E. miR-34a knockdown significantly (P<0.001) increased *Axl* expression after 48 and 72 h of transfection (28 and 50%, respectively) (Fig. 8B). miR-34a knockdown also significantly increased *Slug* gene expression after 24, 48 and 72 h of transfection (155, 452, 226%, respectively; 24 h, P<0.005; 48 and 72 h, P<0.001) (Fig. 8C). Following miR-34a knockdown, a significant (P<0.001) increase was also observed in *CD24* gene expression after 48 h and after 72 h of transfection (179 and 325%, respectively) (Fig. 8D). miR-34a knockdown also significantly (P<0.001) increased *Rho-A* gene expression (43%) after 72 h of transfection (Fig. 8E).

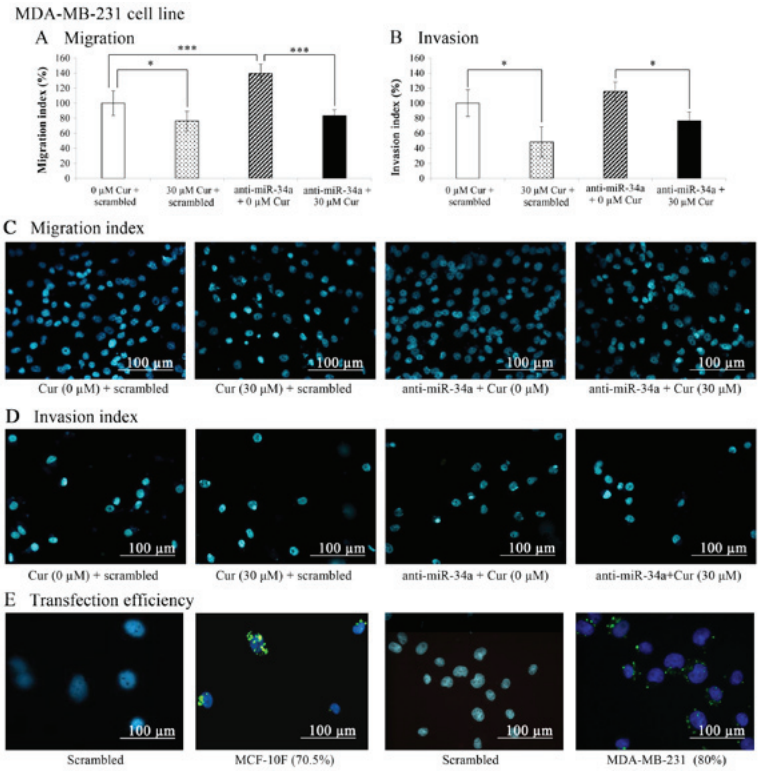

Figure 10. Graphs showing the effect of curcumin (Cur) (30 μM for 48 h) and anti-miR-34a on (A) migration and (B) invasion in the MDA-MB-231 cell line. Bars represent the means ± standard error of migration (%) and invasion (%) of MDA-MB-231, Cur, anti-miR-34a, and Cur plus anti-miR-34a (n=3; *P<0.05, ***P<0.001). Representative images of (C) migration index and (D) invasion index. (E) Transfection efficiency in the MCF-10F and MDA-MB-231 cell lines.

The effects of treatment with 10 and 30 μM curcumin on *Axl*, *Slug*, *CD24* and *Rho-A* gene expression are presented in Fig. 9. The results revealed a significant (P<0.05) decrease in *Axl* (39%), *Slug* (57%), *CD24* (62%) and *Rho-A* (53%) gene expression following transfection with the negative control (scramble) in comparison with the untreated control cells (0 μM cur + scrambled). Transfection with anti-miR-34a increased the expression levels of the *Axl*, *Slug*, *CD24* and *Rho-A* genes (304, 260, 282 and 430%, respectively). However, following treatment with curcumin and miR-34a knockdown, a significant (P<0.05) decrease was observed in the levels of the examined genes.

Effects of the blockade of miR-34a on the migratory and invasive capabilities of the MDA-MB-231 cell line. The results of the migration and invasion assays carried out in a Boyden chamber, as indicated in the graphs depicted in Fig. 10A and B revealed that curcumin (30 μM for 48 h) significantly (P<0.05) decreased the migration (23.7%) and invasion (51.6%) of the MDA-MB-231 cell line in comparison with the untreated control cells (0 μM cur + scrambled). On the other hand, when the cells were transfected with anti-miR-34a (Fig. 10A), cell migration significantly (P<0.001) increased in comparison with the untreated control (0 μM cur + scrambled). Curcumin plus anti-miR-34a significantly (P<0.001) decreased the migration of the MDA-MB-231 cell line in comparison with the cells treated with anti-miR-34a alone. The percentage of migrated treated cells treated with curcumin was lower than that of the untreated control following transfection with anti-miR-34a. Representative images of the migratory capabilities of the MDA-MB-231 cell line following treatment with curcumin and miR-34a blockade are shown in Fig. 10C.

Curcumin also significantly (P<0.05) decreased (39.1%) cell invasion. The invasive capabilities of the cells were significantly (P<0.05) decreased in the cells transfected with anti-miR-34a and treated with curcumin compared with the cells treated with anti-miR-34a alone. Curcumin significantly (P<0.05) decreased cell invasion to the level of the control (0 μM cur + scrambled) when miR-34a was blocked and the cells were treated with curcumin. Representative images of the invasive capabilities of the MDA-MB-231 cell line following treatment with curcumin and the miR-34a blockade are shown in Fig. 10D.

In addition, transfection conditions were standardized by determining the transfection efficiency using a negative control (scrambled) that was previously marked by using fluorescence. As shown in Fig. 10E, the transfection efficiency was 70.5% in the MCF-10F and 80% in the MDA-MB-231 cell line in comparison with their respective controls.

Discussion

Studies have reported that curcumin possesses antioxidant, anti-proliferative, anti-migratory and apoptotic effects in several types of human cancers (32,33,35). We have previously demonstrated such effects in breast cell lines and it has been corroborated by other authors in different cancers (34,35,37,41). The present study demonstrated that curcumin acted upon *Axl*, *Slug*, *CD24*, genes implicated in EMT, as well as on *Rho-A* in a non-malignant MCF-10F and a malignant MDA-MB-231

cell line. As regards the fact that the MDA-MB-231 cells were more sensitive to low concentrations of curcumin, whereas at 30 μM the effect on gene expression was lost probably due to the negative ER and ERB2 receptors that render the cells to behave differently in comparison with a non-malignant cell line, as the MCF-10F cell line.

Curcumin also affected miRNAs as a regulator of genes implicated in EMT and *Rho-A*, affecting migration and invasion independent of their ER, PgR and HER2 receptors. Curcumin decreased *Axl*, *Slug*, *CD24* and *Rho-A* gene and protein expression in the MCF-10F cell line when compared to the untreated control cells. It has been previously demonstrated that *Slug* and *Axl* induce EMT and regulate several aspects of this process (42,43). These results are in agreement with the downregulation of *Axl* reversing EMT in mesenchymal normal human mammary epithelial cells that regulated breast cancer stem cell renewal (44,45).

In a previous study, we examined the effects of curcumin on breast carcinogenesis in an established *in vitro* experimental model of breast cancer (termed the Alpha-model) (46); we examined the effects of curcumin on EMT in breast cancer cells transformed by low concentrations of alpha particles and estrogen. This *in vitro* experimental model was developed by the exposure of the MCF-10F immortalized breast epithelial cell line to low radiation concentrations of high linear energy transfer (LET) α-particles (150 keV/μm) followed by culture in the presence of 17β-estradiol (estrogen). Three difference cell lines were used, namely the MCF-10F (normal), the Alpha5 (pre-tumorigenic) and the Tumor2 cells derived from Alpha5 injected into the nude mice. It has been demonstrated (36) that curcumin influences changes in the levels of genes associated with EMT and which are situated at the core of several signaling pathways known to mediate the transition and such results indicated that curcumin decreased the protein expression levels of N-cadherin, β-catenin, Slug, AXL, Twist1, Vimentin and Fibronectin, independently of the positivity of the markers in these cell lines. Curcumin was also shown to decrease the migratory and invasive capabilities of the cells compared with their respective controls. Thus, it was concluded that curcumin was able to prevent or attenuate cancer progression through the disruption of the EMT processes (36), i.e., by affecting the gene expression E-cadherin and other genes in the MDA-MB-231 cells. In another study, it was demonstrated that curcumin decreased the expression of levels of genes (such as *E-cadherin, N-cadherin, ZEB2, Twist1, Slug, AXL, vimentin, STAT-3,* and *fibronectin*) involved in the EMT process in the Tumor2 cells when compared to the respective control. In addition, it was demonstrated that curcumin altered the levels of the *p53* and *caveolin-1* genes, as well as those of the apoptotic genes, *caspase-3* and *caspase-8*, as well *cyclin D1* and *NF-κB*. These changes in expression levels led to a decrease in the migratory and invasive capabilities of such a cell line (38). This suggests that curcumin may affect the apoptosis and metastatic properties of malignant cells, exerting antitumor effects on breast cancer cells transformed by low concentrations of α-particles and estrogen *in vitro* (38).

The miR-34 family was originally cloned and characterized in 2007 as a p53 target gene (47,48). Soon thereafter, it became evident this miRNA played a main role as a master regulator of tumor suppression. Notably, the overexpression of miR-34 directly and indirectly suppresses several oncogenes, resulting in an increase in cancer cell death (including cancer stem cells), and in the inhibition of metastasis. Moreover, the expression of miR-34 is known to be deregulated in several types of human cancers (49). In 2013, a miR-34 mimic became the first miRNA to reach phase 1 clinical trials (50).

Others (31) have reported that the regulation of miRNAs by natural agents is a novel new strategy for the treatment of cancer. In the study by Tarasov *et al* (2007), the differential regulation of miRNAs by p53 revealed by massively parallel sequencing revealed miR-34a as a target of p53 that induces apoptosis and G1-arrest. Thus, miR-34a suppressed transcription factors implicated in EMT, thus inhibiting metastasis (51). It has also been demonstrated that miR-34 and SNAIL form a double-negative feedback loop to regulate EMT (52). Axl targeted by miR-34a is well known and its expression is significantly associated with metastatic cancer of an advanced clinical stage. Of note, Axl is associated with a poor prognosis in different types of cancer (53).

In this study, the effects of curcumin on the expression of EMT-associated genes in MCF-the 10F cell line were examined following the knockdown miR-34a. The transfection conditions were standardized by determining the transfection efficiency using a negative control (scrambled) by using fluorescence methods in the MCF-10F and in MDA-MB-231 cell lines in comparison with the control. The results revealed that curcumin decreased *Axl*, *Slug*, *CD24*, and *Rho-A* gene expressions after transfection with negative control (scrambled). Transfection with anti-miR-34a increased the expression of these genes. However, the cells in which miR-34a was knocked down were also treated with curcumin, a decrease in the expression of these genes was observed in comparison to the untreated cells. The knockdown of miR-34a expression in the MDA-MB-231 cell line increased *Axl*, *Slug*, *CD24* and *Rho-A* gene expression. However, when curcumin was added to the cells in which miR-34a was knocked down, there was a decrease in anti-miR-34a plus curcumin-treated group in comparison to the untreated cells. The effects of anti-miR-34a transfection were validated by using the Anti-miR™ negative control. This is a random commercial molecule which has been extensively tested in human cell lines by us and others. In addition, it has been widely demonstrated that this control does not alter known functions of miRNAs and mRNAs (54-57). In this study, the results suggested that this scrambled sequence did not affect the expression of miR-34a or that of EMT-related genes evaluated at 24 and 48 h post-transfection in the MCF-10F and MDA-MB-231 cell lines. Although other exposure times were analyzed on EMT-related genes without using this scrambled group, the lack of this control did not alter our general conclusions as the main focus of this study was on the rapid-response phenotypic changes related to EMT, such as cell migration and invasion. Therefore, only short-time exposures to curcumin and anti-miR-34a were evaluated. However, this rationale could be an important limitation if these results are extrapolated to a different context, such as biomedical applications. In this scenario, robust *in vivo* assays are warranted to validate the anti-miR-34a administration mechanisms and the integrity of curcumin over time. Currently, nanotechnology offers novel materials that guarantee high efficiency in the supply of therapeutic agents; for example,

gold nanostructures, dextran nanobubbles or nanoemulsions are emerging tools that improve aqueous solubility and the supply of curcumin to the tissue of interest (58-61). Similarly, RNA nanotechnology to administer anti-miRNA is already being validated in cancer (62). Therefore of this study may provide a novel strategy with which to combat breast cancer if nanomaterials are implemented in improving delivery over time and the specificity of therapeutic agents.

Previously, we determined the potential effects of curcumin on EMT in relation to migration and invasion and compared the Tumor2 and MDA-MB-231 cell lines (36,38). Curcumin inhibited the migration and invasive capabilities through EMT in the breast cancer cells. It has also been found that curcumin inhibits the proliferation and invasion of different types of cancer (41). In the present study, curcumin decreased cell migration and invasion in the control-transfected MCF-10F and MDA-MB-231 cell lines in comparison with the untreated cells. When these cells were transfected with anti-miR-34a, cell migration increased.

miRNAs are known as important targets for curcumin (63,64). These molecules can affect numerous cellular and molecular events (65-70), since it has been found that curcumin can affect the chemoresistance of cancer cells by changing the expression of certain miRNAs. Thus, curcumin functions as a master regulator of the genome by modulating the expression of thousands of genes, simultaneously controlling multiple signaling pathways (43,71). The therapeutic use of miRNAs has been considered for all these reasons. However, further studies are warranted using additional cell lines.

The results of this study indicated that curcumin decreased the migration of the MDA-MB-231 cell line. When these cells were transfected with anti-miR-34a, cell migration increased. However, curcumin decreased the migration of the MDA-MB-231 cell line transfected with anti-miR-34a in comparison with the control untreated cells. The percentage of migrated treated cells with curcumin was lower than the control following transfection with anti-miR-34a. This suggests that additional mechanisms may be involved in mediating the observed effects of curcumin on the invasive and migratory abilities of these cells.

An increasing number of preclinical studies support the hypothesis that curcumin may be a promising anticancer drug. *In vitro* studies require higher amounts of chemical compounds to analyze different physiological effects on cells; thus, the 10 and 30 μM concentrations of curcumin were used in this study. However, the poor bioavailability has limited the efficacy of curcumin in clinical trials, and plasma curcumin levels remain low (ng/ml) despite patients taking gram doses of curcumin, which is insufficient to yield the anticancer benefits of curcumin. This issue has been resolved by the development of highly bioavailable forms of curcumin (THERACURMIN®), and higher plasma curcumin levels can now be achieved without increased toxicity in patients. At the present time, some researchers are testing this substance in nanoparticles to improve the bioavailability (72).

Chemotherapy is known as one of the effective therapeutic approaches for the treatment of a wide variety of cancers (73,74). Recently, the regulation of miRNAs by natural, nontoxic chemopreventive agents, including curcumin has been described. Over the past decade, research on curcumin for its chemoprophylactic and anti-inflammatory properties has been increasing. Fadus *et al* (2017) reviewed that trials on curcumin trials are mainly focused on colorectal cancer, hepatocellular carcinoma and several other tissues (75). The authors indicated that there were at least 12 active clinical trials of curcumin in the USA, Israel and Hong Kong. As of July 2012, there have been observations from 67 clinical trials that have been published, with another 35 clinical trials which were in progress at that time (75).

In conclusion, the present study demonstrated that the *Axl*, *Slug* and *CD24* genes were implicated in EMT. *Rho-A* was also found to be involved in the migration and invasion of the MCF-10F and MDA-MB-231 cell lines. Curcumin affected the expression levels of genes involved in EMT and invasion by controlling miR-34a expression in the breast cell lines. Curcumin acted upon miRNAs as a regulator of genes implicated in EMT, as well as on *Rho-A*, affecting the migration and invasion of the non-malignant MCF-10F and malignant MDA-MB-231 breast cancer cell lines, independent of their ER, PgR and HER2 receptor status; these cell lines are both negative for such receptors.

Therefore, it is demonstrated that natural agents have the ability to inhibit cancer progression, increase drug sensitivity, reverse EMT and prevent metastasis through the modulation of miRNAs. These findings may provide a novel therapeutic approach for cancer treatment, particularly when used in combination with conventional therapeutics (31). Furthermore, to the best of our knowledge, this the first study to examine the effects of curcumin on *Rho-A* and genes involved in EMT, such as *Axl*, *Slug* and *CD24*. It is demonstrated that curcumin prevents the migration and invasion of breast cells by targeting miR-34a as a regulator of the above-mentioned genes.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

MG and GMC conceived and designed the study. MG, UK, FA, JPM, TCB and GMC performed the experiments. MG and GMC wrote the manuscript. GMC, UK, and FA, JPM and TCB edited and reviewed the manuscript. All authors have read and approved the manuscript and agree to be accountable

for all aspects of the research in ensuring that the accuracy or integrity of any part of the work is appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Consent for publication

The authors declare that they have no competing interests.

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