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Relaxin enhances *in-vitro* invasiveness of breast cancer cell lines by upregulation of S100A4/MMPs signaling

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Abstract. – OBJECTIVES: Relaxin (RLX or RLN) levels are increased in cases of human breast cancer and has been shown to promote cancer cell migration in carcinoma cells of the breast; however, the cellular mechanisms of relaxin exposure in breast cancer cells are not fully understood. In human breast cancer cells, relaxin was shown to downregulate the metastasis-promoting protein S100A4, a highly significant prognostic factor for poor survival in breast cancer patients. RLX was also found to enhance *in-vitro* invasiveness of breast cancer cell lines by induction of matrix metalloproteinases (MMPs) expression. The aim of this study was to investigate the effects of relaxin on breast cancer cell invasion by S100A4 dependent MMPs pathway.

MATERIALS AND METHODS: The human breast cancer MDA-MB-231 cells were treated with 100-500 μg/L porcine RLX, or/and transfected with S100A4 siRNA (20 ng), or/and treated with MMPs inhibitor FN439 (0.3 nM).

RESULTS: We observed that incubation with porcine RLX increases *in-vitro* cell invasion and *in vitro* invasiveness. Enhanced invasiveness was accompanied by up-regulation of S100A4 and MMP-2 and MMP-9. The relaxin-induced increase in cell invasion was blocked almost when S100A4 expression was diminished using an S100A4 small interfering RNA knockdown approach or when MMPs was inhibited by MMPs inhibitor FN439. The relaxin-induced increase in MMP-2 and MMP-9 expression was blocked when S100A4 was inhibited by S100A4 siRNA transfection.

CONCLUSIONS: Our data demonstrate that the RLX controls the *in-vitro* invasive potential of human breast cancer cells through S100A4 dependent MMPs regulation.

Key Words:
Relaxin, S100A4, MMPs, Invasion, Breast cancer.

Introduction

The processes by which solid primary tumours, such as those of the breast, are able to disseminate and establish growth at a secondary site are still poorly understood. Tumour metastasis is complex, probably requiring both gain and loss of functions, enabling escape from the primary tumour, and growth at a secondary site. This has led to the search for metastasis-associated genes, which, unlike oncogenes, are unable to initiate tumour formation but are able to induce a metastatic phenotype in previously tumorigenic cells[1-2].

It has previously shown that the polypeptide hormone relaxin (RLX or RLN) is expressed exclusively in human breast cancer[3], and relaxin confers increased carcinoma cell growth, motility, adhesion and *in vitro* invasiveness in human breast cancer cells[4-6]. Furthermore, adenoviral-mediated delivery of prorelaxin 2 gene increases the invasiveness of canine breast cancer cells[7]. However, the cellular mechanisms of relaxin exposure in breast cancer cells are not fully understood. Previous studies found stimulation with RLX increases the invasiveness and migration of breast, endometrial, and thyroid adenocarcinoma cells *in vitro* accompanied by the up-regulation of matrix metalloproteinase activity and vascular endothelial growth factor expression, which are directly related to cancer progression[6,8-9].

The calcium-binding protein S100A4 promotes metastasis in several experimental animal models, and S100A4 protein expression is associated with patient outcome in a number of tumor types. S100A4 possesses a wide range of biological functions, such as regulation of angiogenesis, cell survival, motility, and invasion[10]. Elevated levels of the calcium-binding protein S100A4 are associated with poor patient survival in breast cancer patients and induce metastasis in rodent models[11-12]. Extracellular S100A4 is believed to aid in tumor metastasis by inducing the secretion

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of matrix metalloproteinases (MMPs)[13-15], promoting angiogenesis through the stimulation of endothelial cell motility. We have also reported that S100A4 promotes invasion and angiogenesis in breast cancer MDA-MB-231 cells by upregulating matrix metalloproteinase-13[16]. Significant relation was shown between relaxin and S100A4. In thyroid carcinoma cells, relaxin target protein S100A4 secreted by the thyroid carcinoma transfectants may not only enhance tumor cell motility but also promote xenograft angiogenesis as determined by the higher density of tumor microvessels and the angiogenic potential of S100A4 in *in vitro* tube formation assays[17]. In breast cancer cells, short-term exposure to relaxin for 24 hours increased cell motility in a relaxin receptor-dependent manner. This increase in cell motility was mediated by S100A4. Long-term exposure to relaxin secreted from stable transfectants reduced cell motility and *in vitro* invasiveness. Relaxin decreased cell proliferation and down-regulated cellular S100A4 levels in MDA-MB-231 and T47D breast cancer cells. Stable MDA/RLN2 transfectants produced smaller xenograft tumours containing reduced S100A4 protein levels *in vivo*[18]. It seems that RLX plays the different role in tumor progression *in vivo* and *in vitro*.

In this study, we show that S100A4 and MMP-2,9 production is significantly enhanced by relaxin in human breast cancer MDA-MB-231 cells. The invasion and metastasis-enhancing effect of relaxin is mediated by and dependent on S100A4 /MMP-2 and 9 in human breast cancer cells.

Materials and Methods

Cells Culture

The breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection (Rockville, MD, USA). The cell line was maintained and propagated in 1:1 DMEM:F12 media supplemented with 10% fetal bovine serum (FBS) and 0.1% gentamicin sulfate (Gemini Bio products, Sacramento, CA, USA). Cell media were purchased from Invitrogen (Hangzhou, China).

Small Interfering RNA (siRNA)

MDA-MB-231 cells at 80% confluency,were transfected with S100A4 siRNA from Santa Cruz(Shanghai China).The target sequence for the S100A4 siRNA was: AG CUU GAA CUU GUC ACC CTC-3'. Silencer Negative Control (NC) siRNA with no significant sequence similarity to human gene sequences was used as control. MDA-MB-231 cells were seeded in 12-well plate 24 hours before transfection. 100 nM of S100A4 siRNA and NC siRNA were transfected into cells following manufacturer's instruction. After 24-hour incubation, cells were treated with porcine relaxin for 24 hours as below**.**Then the cells were used in the invasion assay and m RNA and protein assay.

Relaxin Treatments

For incubations with recombinant human Relaxin2 (RLX), MDA-MB-231 cells were cultured in medium containing 1% fetal calf serum (FCS) for 24 h ,then treated with 100-500 ug/L RLX (Santa Cruz, Shanghai, China) in 10% FCS for 24 h. The cells were used in the invasion assay. Cells were harvested in Trizol for RNA extraction or in 2× reducing Laemmli buffer for protein extraction.

After MDA-MB-231 cells were transfected with S100A4 siRNA for 24 hours, the MDA-MB-231/S100A4 siRNA cells were treated with 100-500 μg/L RLX (Santa Cruz, Shanghai, China) in 10% FCS for 24 h. Then the cells were used in the invasion assay. Cells were harvested in Trizol for RNA extraction or in 2× reducing Laemmli buffer for protein extraction.

FN439 (MMP Inhibitor) Treatments.

MDA-MB-231 cells were cultured in medium containing 1% FCS for 24 h, after which the cells were treated with MMP inhibitor FN439 (MMP inhibitor I, Calbiochem, San Diego, CA, USA) and 100-500 ug/L RLX together for 24 hours. FN439 was used at 0.3 mM (final concentration) to provide nonspecific inhibition of MMP activity. Then the cells were used in the invasion assay. Cells were harvested in Trizol for RNA extraction or in 2× reducing Laemmli buffer for protein extraction.

Invasion Assay

Invasion was quantified using a modified Matrigel Boyden chamber assay. The BD BioCoat Matrigel invasion chamber (BD Bioscience, Bedford, MA, USA) was used according to the manufacturer's instructions. 2.5×10^4 cells in serum-free media were seeded onto Matrigel-coated filters. RLN was added at 100 or 500 ng/ml 2 hours after plating the cells. In the experiments where MMP activity was inhibited,

the non-selective MMP inhibitor FN439 was added together with RLN at 0.3 mM. In the experiments of S100A4 siRNA transfection, MDA-MB-231 cells were transfected with S100A4 siRNA for 24 hours, then the 2.5×10^4 cells/S100A4 siRNA in serum-free media were seeded onto Matrigel-coated filters. In the lower chambers, 5% FBS (fetal bovine serum) was added as a chemoattractant. After 24 h incubation, the filters were stained using the Diff-Quik-TM kit (BD Biosciences), and cells that had invaded through the filter were counted under magnification (randomly selected high-power fields). The counting was performed in three random fields for each sample, and mean values from three independent experiments were used.

Migration Assay

The ability of cells to migrate in response to RLN stimulation was assessed using the membrane invasion culture system (MICS) as described before[19]. Cell migration in response to the chemotactic effect of RLX was studied using the MICS chambers containing polycarbonate filter with 10 μm pores that had been soaked in 0.1% gelatin. Briefly, untreated tumor cells (5 x 10^4) were seeded in each upper well and media with RLN (100,500 ug/L) was added to the lower wells. RLX was added at 100 or 500 ng/ml 2 hours after plating the cells. In the experiments where MMP activity was inhibited, the non-selective MMP inhibitor FN439 was added together with RLN at 0.3 mM. In the experiments of S100A4 siRNA transfection, MDA-MB-231 cells were transfected with S100A4 siRNA for 24 hours, then the 5×10^4 cells/S100A4 siRNA cells were seeded in each upper well. Tumor cells were allowed to incubate at 37°C for six hours in Dulbecco's modified Eagle's medium (DMEM) with 5% FCS. Migration was calculated as the percentage of cells that had successfully migrated through the matrix-coated membrane to the lower wells compared to the total number of cells seeded into the upper wells. Each experiment was performed in triplicate.

Zymography

Whole cell protein was extracted from samples of as described above. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gelatin zymography was performed as previously described[20]. Briefly, all protein extracts were adjusted to a concentration of 15 mg and mixed with SDS-PAGE sample buffer. Samples were fractionated in an 8% polyacrylamide gel containing gelatin (0.5 mg/mL) by electrophoresis at 100 V for 90 min. Gels were run in duplicate. To insure that equal amounts of protein were loaded in each lane, one gel was stained with Coomassie PhastGel Blue R for 2 h and destained with 7% acetic acid. The other gel was soaked in 0.25% Triton X-100 for 30 min at room temperature to remove the SDS, and incubated in digestion buffer (50 mM TriseHCl, pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$, 2 mM $ZnSO_4$) containing 5 mM phenylmethylsufonyl fluoride (PMSF) at 37°C overnight, to allow substrate digestion. The gels were then stained with 0.1% Coomassie PhastGel Blue R for 2 h, and destained with 7% acetic acid. Gelantinolytic activities produced clear bands of digested gelatin against a dark blue background of stained gelatin

Western Blotting

Whole cell extracts were prepared using cell lysis buffer (20 mM Tris, pH 7.5, 0.1% Triton X, 0.5% deoxycholate, 1 mM PMSF, 10 l g/ml aprotinin, 10l g/ml leupeptin) and cleared by centrifugation at 12,000 g, 4°C. Total protein concentration was measured using the bicinchoninic acid (BCA) assay kit (Sigma, St. Louis, MO, USA) with bovine serum albumin as a standard.Cell lysates containing 30 ug total protein were analyzed by immunoblotting. Anti-S100A4, Anti-MMP-2,9 and anti-MMP-13 antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA). Anti-actin antibodies were obtained from LabVision (Freemont, CA, USA). Chemoluminescent detection was performed in accordance with the manufacturer's instructions. Signal intensities were quantified using ImagePro Plus software version 4.0 and normalized to respective loading control as appropriate.

RNA Extraction and RT-PCR Assay

Total RNA was extracted from MDA-MB-231/RLX/FN439 cells using the TRIZOL (Gibco Invitrogen, Invitrogen China Limited/Applied Biosystems China Limited, Beijing, China) method. The amount of RNA obtained was determined by spectrophotometric measurements at 260 nm. The sample was incubated for 10 min at 70°C followed by addition of 4 ul of 5×transcription buffer, 2 μl of 0.1 M DTT (1,4-Dithio-DL-threitol), 1 ul of 10 mM dNTPs (d-nucleoside triphosphates), and 200 U of Superscript II (Gibco Invitrogen). The mixture was incubated for 60 min at 42°C. The final volume of the reaction

was 20 ul. For PCR amplification,different amounts of the synthesized cDNA (diluted 1:10 in water) were analyzed to evaluate the linearity of the reaction. Then polymerase reaction was carried out in a solution contained 1.5 mM Mg-Cl2, 200 uM of each nucleotide in PCR buffer, 10 pmol of the primers, and 0.25 U platinum Taq DNA polymerase (Gibco Invitrogen). Primers was as follows: 5'-primer: S100A4-5'-ACGTGTCTGAAGGAGCCATGGTGG-3,5'-CTATCCTGGCCACCTTCTTC-3' for S100A4; 5-TACCACCTCGAACTTTGACAGCGA-3, 5_-AAAGGCACAGTAGTGGCCGTAGAA-3, for MMP-9; 5-AGA TGC ATA TTC GGA CCC ACA-3 and 5-CCTCAT GTT TGT GCA GGA GAG-3 for MMP-2 and 5-CAA CTG GGA CGA CAT GGA GA-3 and 5-CAG GCA GCT CGT AGC TCT TC-3 for -actin.The amplification conditions were as follows: A denaturation step for 5 min at 95°C, followed by 50 sec at 95°C, 50 sec at 65°C and 45 sec at 72°C for 28 cycles, followed by 15 min at 72°C. The PCR products separated on 1.5% agarose/TAE gels were visualized by staining with ethidium bromide and semiquantified using an AlphaEase FC Version 4 analysis software (Alphalmager HP, Santa Clara, CA, USA; Alpha Innotech, San Leandro, CA, USA). The densitometric analysis of the data was normalized to the b-actin. Results were mean±SD from three separate experiments for each group.

Statistical Analysis

All statistical analyses were performed using the SPSS10.0 software (SPSS Inc., Chicago, IL, USA). The results were presented as means±s.d. of three replicate assays.Differences between the groups were assessed by the Student's *t*-test or analysis of variance (ANOVA). $p < 0.05$ was considered to indicate statistical significance.

Results

Relaxin Promotes S100A4 Expression in a Does-Dependent Manner

To assess the kinetics of the relaxin (RLX) effect, MDA-MB-231 cells were exposed to 100-500 g/L RLX four 24 hours, and S100A4 expression was analyzed. S100A4 protein (Figure 1A) and S100A4 mRNA (Figure 1B) was increased in a dose-dependent manner.

Relaxin Promotes MMP-2, 9 Expression in a Does-Dependent Manner

Measurement of gelatinolytic activity by zymography yielded no detectable amounts of MMP-2 and MMP-9 in untreated MDA-MB-231 cells (Figure 2A) or the respective supernatant (Figure 2B). Incubation with 100-500 μg/L RLX four 24 hours led to a strong increase in gelatinolytic activity (Figure 2A) or supernatant (Figure 2B), no MMP-3,7 and MMP-13 was detected (data not shown). In accordance with the results obtained by zymography, measurement of mRNA expression by RT-PCR (reverse transcriptase-polymerase chain reaction) showed RLX-induced up-regulation of MMP-2, 9 in the MDA-MB-231 cells (Figure 2C).

Relaxin Promotes MMP-2, 9 Expression by S100A4 Dependant Signaling

MDA-MB-231 cells was transfected with S100A4 siRNA for 24 hours, then the MDA-MB-231/S100A4 siRNA cells were exposed to 100-500 μg/L RLX four 24 hours. The results

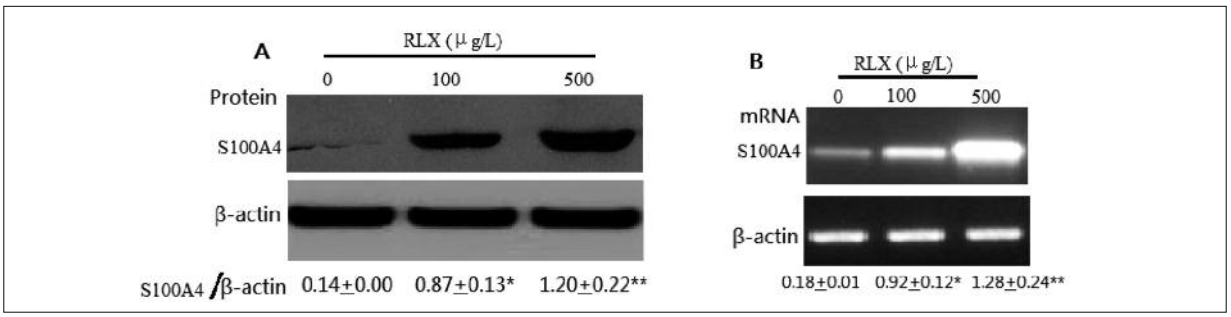

Figure 1. S100A4 expression upon incubation with RLX. MDA-MB-231 cells were incubated with 100-500 μg/L RLX for 24 hours. Western blot ***(A)*** and RT-PCR ***(B)*** assay for S100A4 protein and mRNA levels (vs control, *$p < 0.05$, **$p < 0.01$). Results from 3 independent experiments with SDs are shown. Statistical analysis was done by employing SPSS software and paired Student's t test. The value of $p < 0.05$ was considered statistically significant as indicated with a star (*).

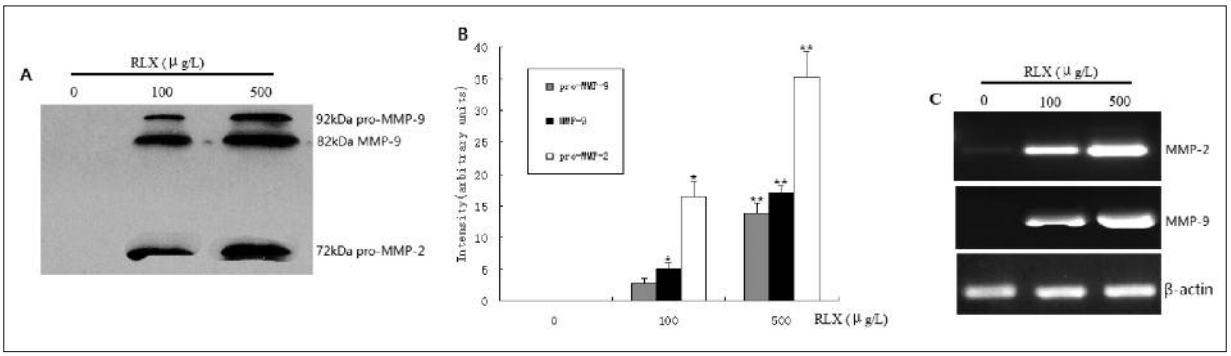

Figure 2. MMP-9 and 2 expression upon incubation with RLX. MDA-MB-231 cells were incubated with 100-500 μg/L RLX for 24 hours. ***A,*** Gelatin zymography of MDA-MB-231 cells incubated for 24 hours. ***B,*** Quantification of MMP-2 and 9 protein expression in gelatin zymograms of supernatants from MDA-MB-231 cells. ***C,*** MMP-9 mRNA and 2 mRNA expression upon incubation with RLX. Results from 3 independent experiments with SDs are shown. Statistical analysis was done by employing SPSS software and paired Student's t test. The value of $I < 0.05$ was considered statistically significant as indicated with a star (*), $p < 0.01$ (**) (vs control).

showed measurement of gelatinolytic activity by zymography yielded less detectable amounts of MMP-2 and MMP-9 in MDA-MB-231 cells transfected with S100A4 siRNA and treated with 100-500 μg/L RLX (Figure 3A). A strong decrease in gelatinolytic activity in the supernatant (Figure 3B) followed by decreased S00A4 expression (Figure 3C). In accordance with the results obtained by zymography, measurement of mRNA expression by RT-PCR

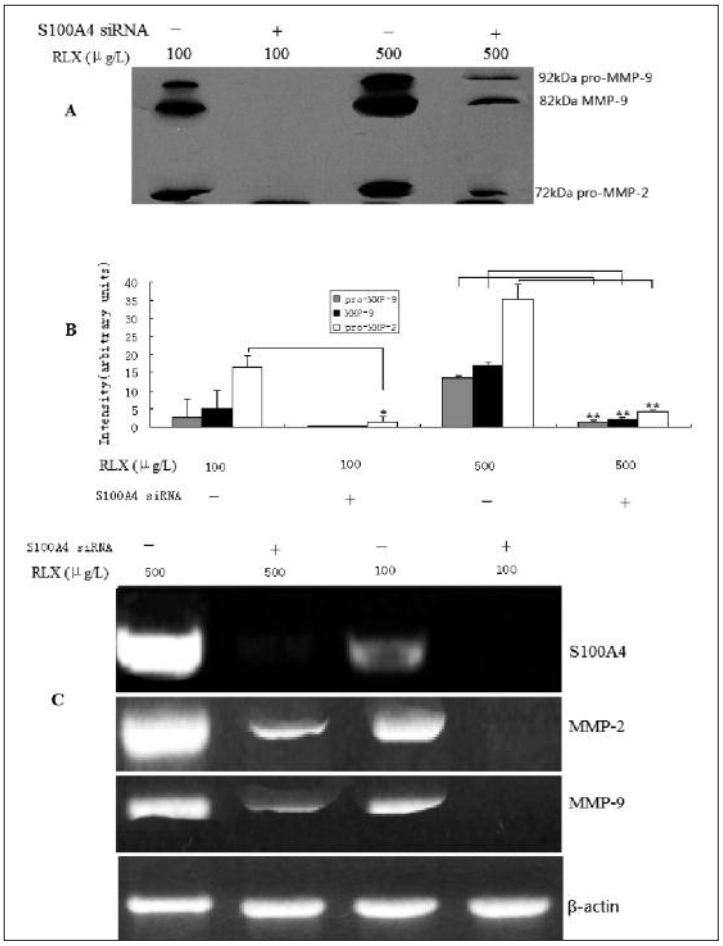

Figure 3. Relaxin promotes MMP-2, 9 expression by S100A4 dependent signaling. ***A,*** MDA-MB-231 cells were incubated with 100-500 μg/L RLX/S100A4 siRNA for 24 hours. Gelatin zymography of MDA-MB-231 cells was shown. ***B,*** Quantification of MMP-2 and 9 protein expression in gelatin zymograms of supernatants from MDA-MB-231 cells. ***C,*** MMP-9 mRNA and 2 mRNA expression upon incubation with RLX/S100A4 siRNA. Results from 3 independent experiments with SDs are shown. Statistical analysis was done by employing SPSS software and paired Student's t test. The value of $p < 0.05$ was considered statistically significant as indicated with a star (*). $p < 0.01$ (**) (vs control).

showed significant down-regulation of MMP-2, 9 (Figure 3C) in the MDA-MB-231 cells treated with RLX.

RLX-induced cell Migration and Invasion was Inhibited by S100A4 Downregulation

It has previously showed that RLX was significantly correlates with metastasis formation in breast cancer cells.Moreover, S100A4 drives metastasis formation by increasing cell migration and invasion.Therefore, we next examined RLX-induced cell motility by S100A4. Exposure of MDA-MB-231 cells to 100-500 μg/L RLX four 24 hours significantly increased the number of migrated cells to 140%-180% of that for vector treated MDA-MB-231 cells (Figure 2A). To determine the biological function of S100A4 for the migration and invasion of MDA-MB-231 cells, we performed specific siRNA knockdown of S100A4 in the MDA-MB-231 cells followed by RLX treatment and migration and invasion assays. Expression of the S100A4 monomeric band in MDA-MB-231 was reduced to almost undetectable levels 24 hours after siRNA transfection when compared with nonsilencing siRNA controls (Figure 4A). Next, we investigated the migration and invasion of these S100A4 siRNA treated MDA-MB-231 cells in the presence and absence of RLX. MDA-MB-231/S100A4 siRNA cells showed no significant increase in cell migration upon RLX treatment. Cell invasion of RLX-treated MDA-MB-231 cells was increased to 180%-250% of that for the vector-treated control cells (Figure 4B). In contrast, cell invasion of MDA-MB-231/S100A4 siRNA cells was not significantly changed upon RLX treatment. In sum-

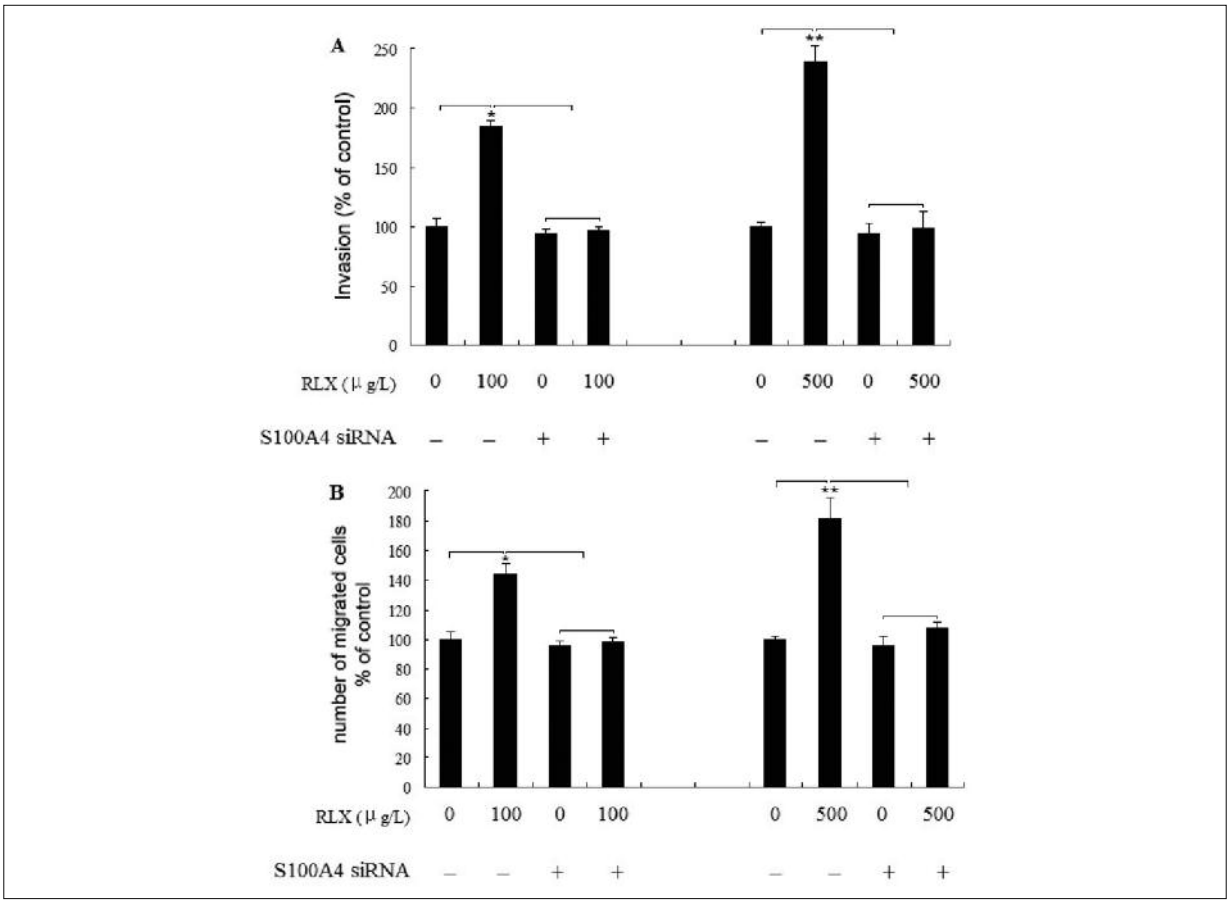

Figure 4. RLX-induced cell migration and invasion was inhibited by S100A4 downregulation. RLX-induced cell motility is inhibited by S100A4 siRNA transfection. Cell migration was determined with Boyden chamber assay. Cell invasion was measured with a Matrigel-covered Boyden chamber assay. ***A,*** RLX treatment promoted cell invasion, and cell invasion is inhibited in S100A4 siRNA-transfected MDA-MB-231 cells cells. ***B,*** RLX treatment promoted cell migration,and cell migration is inhibited in S100A4 siRNA-transfected MDA-MB-231 cells. Data represent mean ± SE (n = 5). Statistical significance was analyzed with Student's *t* test.

mary, RLX displayed an promigratory and invasive effect in MDA-MB-231 cells, and this effect was overcome by the exogenous downexpression of S100A4.

RLX-induced cell Migration and Invasion Was Inhibited by S100A4 Dependent MMPs inhibition

It has shown RLN stimulates S100A4 dependent MMP activation. Based on the results, we first used the MMP inhibitor FN 439 to determine if inhibition of MMPs would abrogate RLN-mediated effects on the invasive potential of MDA-MB-231 cells. At concentrations of 0.3 mM, FN 439 causes a broad nonspecific inhibition of MMPs function. RLN-induced invasion of MDA-MB-231 cells was dependent on the activity of MMPs.18 In the presence of FN439 (0.3 mM), RLN-induced invasion and metastasis was reduced to almost control levels (Figure 5A and B).

Discussion

Our *in vitro* results indicated that RLX stimulation enhances the invasive potential of MDA-MB-231 cells. RLX significantly augments migration of MDA-MB-231 cells using a MICS assay system. This effect was evident with RLX concentrations ranging from 100-500 ng/ml. Similarly, Wyatt et al[21] demonstrated that bronchial epithelial cells migrate in response to porcine RLX in a wounding assay. RLX also led to increased invasiveness of both HEC-1B and KLE cells through an artificial basement membrane. Binder et al[6] recently showed a similar effect of RLX in SK-BR3 human breast cancer cell line. Our *in vitro* results also showed RLX increased invasiveness of MDA-MB-231 cells responded to RLX in a dose-dependent matter.

Though RLX expression was increased in aggressive metastatic disease, and that the stimulation of breast cancer cells with RLX accelerated their invasiveness and growth. However, the cellular mechanisms of relaxin exposure in breast cancer cells are not fully understood.

There is growing evidence that RLX is known to regulate the expression of a variety of genes, including collagens and MMP-1[22], vascular endothelial growth factor[23] and cyclooxygenase-2[24]. RLX has been shown to enhance *in vitro* invasiveness of breast cancer cells by up-regulation[6] of MMP-2, -7, -9, -13 and -14.

Intensive research has demonstrated the central role of S100A4 in the process of cancer metastasis, which qualifies S100A4 as a potentially promising target for therapeutic intervention against metastasis[10,14,16,25-27]. Most of the work concerning S100A4 has concentrated on the elucidation of the mechanism by which this molecule drives metastasis.

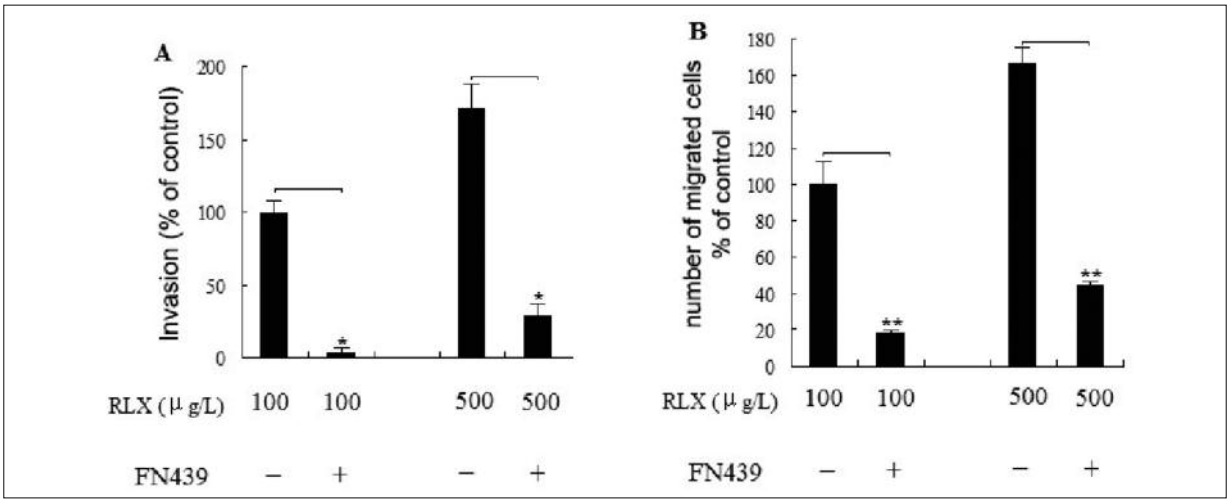

Figure 5. RLX-induced cell migration and invasion was inhibited by MMPs inhibition. RLX-induced cell motility is inhibited by FN439 (0.3 mM) treatment. Cell migration was determined with Boyden chamber assay. Cell invasion was measured with a Matrigel-covered Boyden chamber assay. ***A,*** RLX treatment promoted cell invasion,and cell invasion is inhibited in FN439 (0.3 mM) treated MDA-MB-231 cells cells. ***B,*** RLX treatment promoted cell migration,and cell migration is inhibited in FN439 (0.3 mM) treated MDA-MB-231 cells cells. Data represent mean ± SE (n = 5). Statistical significance was analyzed with Student's t test.

Recently, it has showed S100A4 may mediate some of the tumor-promoting effects of RLX in human thyroid carcinoma on motility and matrix invasion[28]. S100A4 knockdown by siRNA interference reduced cell motility in FTC133 transfectants and exogenous relaxin was unable to increase cell motility in S100A4 siRNA-treated FTC133 cells.

Our *in vitro* results indicated treatment MDA-MB-231 cells with 100-500 μg/L RLX promoted invasion and followed by increased S100A4 mRNA and protein. S100A4 siRNA knockdown in MDA-MB-231 cells prevented RLX-induced invasion increase, suggesting S100A4 to act downstream of RLX signaling to increase cell motility and invasion. Recently, it has showed S100A4 to be down-regulated in MDA-MB-231-RLX transfectants *in vivo*[18], which indicated that RLX played different role in regulate S100A4 in different tumor microenvironment. However, in human thyroid carcinoma cells, RLX markedly upregulated S100A4 gene activity and protein levels[28], suggesting cell type-specific effects of RLX on S100A4 regulation.

An essential step for tumor cell migration through host tissue stroma is the partial degradation of ECM. Our data provide some insight into the mechanism of RLX action in breast cancer MDA-MB-231 cells. Addition of the nonspecific MMP inhibitor decreased the RLX-induced changes in migration and invasiveness in MDA-MB-231 cell lines. High doses of MMP inhibitor FN-439 as used in this study provide a broad, nonspecific inhibition of various MMPs. We and others have previously reported that two of these proteases, MMP-2 and MMP-9, play a crucial role in metastasis of breast cancer[29-30]. Notably, RLX stimulation resulted in significantly higher levels of active MMP-2 and MMP-9 in MDA-MB-231 cells compared to vehicle. Thus, MMP-2 and MMP-9 could be the potential downstream targets of RLX stimulation in breast cancer cells.

RLX has been shown to enhance *in vitro* invasiveness of breast cancer cells by upregulation of MMPs[6], short-term exposure to relaxin promotes motility and *in vitro*-invasion and long-term exposure to relaxin confers growth inhibitory and anti-invasive properties in oestrogen-independent tumours *in vivo*[18,31]. Otherwise, S100A4 is determinant of cancer cell invasiveness and other traits of cancer progression, which are located upstream of matrix metalloproteinases (MMPs) in cell signaling pathways[32].We suggested that significant relationship was found among RLX, S100A4 and MMPs. Here we report the RLX as an promoter of S100A4 transcription in breast cancer cells. We show that RLX treatment promotes a constitutively active MMP-2 and MMP-9, inhibiting S100A4 expression and leading to restricted RLX-induced cell migration and invasion and decreased MMP-2 and MMP-9 activation *in vitro*. Therefore, we think RLX promotes invasion in *in-vitro* breast cancer cells through S100A4-MMPs pathways.

Conclusions

Our study reports RLX as a novel promoter of S100A4 activity, which leads to increased MMP-2 and 9 expression, by which induced RLX-induced cell motility and metastasis. Because metastasis is the major cause of breast cancer death, there is an urgent need for antimetastatic treatment. RLX, as a mediator of this disease progression, provides a promising therapeutic target. We provide evidence that targeting RLX expression restricts S100A4-induced cell migration and invasion *in vitro*.

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