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RLN2 regulates *in vitro* invasion and viability of osteosarcoma MG-63 cells via S100A4/MMP-9 signal

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Abstract. – OBJECTIVE: Relaxin-2 (RLN2) increases cell migration, invasiveness and proliferation in vitro of osteosarcoma cells, but the molecular mechanisms of this action are still unknown. In the present study, we identified S100A4 /MMP-9 signaling as a major mediator of the actions of RLN2 in osteosarcoma cells *in vitro*.

MATERIALS AND METHODS: We have established stable transfectants of osteosarcoma MG-63 cells using small interfering RNA (siRNA) targeting RLN2. The stable transfectants (MG-63/RLN2 siRNA cells) were treated with 20 μM BB94 (a kind of MMP-9 activator) or 100 μM recombinant Human RLN2 (B-29/A-24) for 24 hs or transfected with S100A4 cDNA plasmid for 48 hrs or MMP-9 siRNA for 48 hrs then treated 100 μM recombinant Human RLN2 (B-29/A-24). Western blot assay was used to detect RLN2, S100A4 and MMP-9 expression. Matrigel invasion assay and wound healing assay was used to detect invasion *in vitro*. MTT was used to detect cell viability.

RESULTS: Knockdown of RLN2 using small interfering RNA decreases S100A4 and MMP-9 expression and inhibits invasion and cell viability *in vitro*. in MG-63 cells. Treatment with 100 μM recombinant Human RLN2 (B-29/A-24) for 24 hrs in MG-63/ RLN2 siRNA cells increases S100A4 and MMP-9 expression, and increases the invasion and cell viability in vitro in MG-63 cells. Transfection with S100A4 cDNA plasmid in MG-63/RLN2 siRNA cells for 48 hrs increases MMP-9 expression, and increase the invasion and cell viability of MG-63/RLN2 siRNA cells. Treatment with 20 μM BB94 (MMP-9 activator) for 24 hrs in MG-63/RLN2 siRNA cells increases MMP-9 expression, and increases the invasion and cell viability *in vitro*. in MG-63 cells.

CONCLUSIONS: Our results indicate that RLN2 regulats cell migration, invasiveness and proliferation of osteosarcoma cells *in vitro*, which may be mediated through S100A4/MMP-9 signaling.

Key Words:
Osteosarcoma, Invasion, Relaxin-2, S100A4, MMP-9.

Introduction

Osteosarcoma (OS) is one of the most common primary bone tumors in children and adolescents. It is most often localized in the metaphysis of the adolescent long bones[1]. Although the combination of modern surgery and systemic chemotherapy has improved OS treatment markedly, no substantial change in survival has been seen over the past 20 years[2]. For this reason, identifications of new molecular targets are of great importance.

H2 relaxin (RLN2) is a peptide hormone that is a member of the insulin-like superfamily. It has found to be overexpressed in hepatocellular carcinoma, prostate cancer and OS[3-6]. It promotes prostate cancer[4], stimulation with relaxin increased cell proliferation, invasiveness, and adhesion *in vitro*. The suppression of relaxin/LGR7 via short interfering RNAs decreased cell invasiveness and increased cell apoptosis. It breast cancer cell[7,8], RLX enhances *in vitro* invasiveness of breast cancer cell lines by induction of MMP expression. In human thyroid carcinoma cells[9], RLX acted as an autocrine/paracrine factor and significantly increased anchorage-independent growth and thyroid carcinoma cell motility and invasiveness through elastin matrices.

Our groups have demonstrated overexpression of RLN2 can induce tumor growth, invasion and angiogenesis of human Saos-2[10], and inhibition of RLN2 using siRNA blocks RLN2-mediated proliferation and invasiveness of OS MG-63

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cells[11]. However, the mechanism that RLN2 functions in detail is not clear.

S100A4 is a ubiquitous small, calcium-binding protein that enables cell migration and invasion to increase cell motility[12]. Consequently, S100A4 expression is up-regulated during wound healing, neurite outgrowth, fibrosis, or neovascularization-all physiological processes that rely on increased cell motility. However, overexpression of S100A4 is often correlated with pathological conditions such as epithelial-mesenchymal transition, tumor outgrowth, and metastasis formation[13,14]. In human thyroid carcinoma cells, down-regulation of S100A4 by small interfering RNA decreased cell invasion, metastasis, and angiogenesis via reducion of VEGF and MMP-9 expression, and vice versa[15]. In hepatocarcinoma (HCC), S100A4 contributes to HCC metastasis by activation of NF-kB dependent MMP-9 expression[16]. In human renal cancer cells and squamous cell laryngeal cancer cells[17,18], S100A4 silencing by RNA interference significantly inhibited NF-kB and NF-kB-mediated MMP-2/9 activation and cellular migration, proliferation, and promoted apoptosis. S100A4 was also overexpressed in osteosarcoma[19]. Zhang et al[20] has reported that knockdown of S100A4 decreases tumorigenesis and metastasis in osteosarcoma cells by repression of matrix metalloproteinase-9. It is concluded that extracellular S100A4 inhibition is an attractive approach for the treatment of human cancer.

Radestock et al[21] has reported relaxin promotes growth of human thyroid carcinoma cell xenografts via S100A4 upregulation. However, in breast cancer, relaxin upregulates and reduces xenograft tumour growth of human MDA-MB-231 breast cancer cells[22]. In MDA-MB-231 human breast cancer cells *in vitro*, relaxin downregulates the calcium binding protein S100A4[23]. Our previous study[6] found overexpression of RLN2 and S100A4 was related to the prediction of metastasis potency and poor prognosis for osteosarcoma patients.

The aim of this study was to investigate the relaxin-2 expression in 36 cases of osteosarcoma tissues, and analyzed the association between clinical parameters of osteosarcoma and relaxin-2 expression. Moreover, we address the fundamental question of whether relaxin-2 down-regulation is involved in reducing invasion and growth in osteosarcoma cells *in vitro*.

In the present study, we provide evidence to support the hypothesis that RLN2 regulates invasiveness and growth of human OS through the S100A4/MMP-9 signal.

Materials and Methods

Cell Culture

Osteosarcoma cell line MG-63 was obtained from the ATCC (Shanghai, China), and incubated in RPMI 1640 medium containing 10% fetal calf serum (FCS, Gibco) and 1% antibiotics (P/S, penicillin 10.000 U/ml and streptomycin 10.000 mg/ml, in 75 cm^2 culture flasks (Falcon, Mountain View, CA, USA) until they had formed a confluent monolayer. RLN2 siRNA (h) and MMP-9 siRNA (h) was acquired from Santa Cruz.

siRNA/cDNA Transfection and BB94 Treatment

The human RLN2 siRNA and scrambled control siRNA (control siRNA) transfections (MG-63/siRNA) were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in Opti-MEM (Invitrogen) according to the manufacturer's protocol with a final siRNA concentration of 100 nM. The transfection reagent was removed after 12 h and the cells were harvested after 48 h. Then the MG-63/control siRNA and MG-63/RLN2 siRNA was selected with G418 (400 ug/mL) for 21 days to acquired the stable MG-63/control siRNA and MG-63/RLN2 siRNA clones.

To further investigate the relationship between RLN2 and S100A4,MMP-9 expression, stable MG-63/control siRNA and MG-63/RLN2 siRNA cells was treated with 20 μM BB94 (MMP-9 activitor) or 100 μM recombinant Human RLN2 (B-29/A-24) for 24 hs or transiently transfected with MMP-9 siRNA for 24 hs then treated with 100 μM recombinant Human RLN2 (B-29/A-24) for 24 hs. RLN2, S100A4 and MMP-9 expression was detected as below.

The pcDNA3.1-S100A4 cDNA plasmid (S100A4 cDNA) and its control pcDNA3.1 plasmid was kindly gifted by Dr. Jia[15]. To study whether RLN2-induced MMP-9 expression was by S100A4 signal, MG-63/siRNA cells was transfected with S100A4 cDNA and its control pcDNA3.1 plasmid for 48 h as described above.

Matrigel Invasion Assay

The invasiveness of MG-63 cells was tested after transfection. The cells (1×10^6/mL) were added to the upper wells coated with Matrigel in the lower wells. After a 24-h incubation period, cells that migrated through the filters into the lower chamber were counted by the number of cells on the lower side of the membrane in five random fields after staining with Hema-3 kit.

Wound Healing Assay

Cells were grown in DMEM with 10% FBS in six-well plates until 60-80% confluence. A straight "wound" was made by scratching with a plastic pipette tip. After culture in FBS-free medium for 24 h, cell migration was analyzed using light microscopy. The distance of the scratch at the same position was measured by using the Image-Pro-Plus software. The migration rate was calculated by the following formula: % Migration = (the length of initial wound – the length of wound after 18 h)/100/the length of initial wound. All experiments were repeated three times independently.

MTT Assay

Cell viability was measured using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Guangzhou, China). In brief, the cells were seeded in 96-well culture plates. The volume of culture medium was 100 ml. Ninety minutes before the end of the experiment, 10 ml of MTT solution [5 mg/ml in phosphate buffered saline (PBS)] was added to each well and incubated at 37°C for 90 min. The culture medium was removed and the purple crystals formed were dissolved in 150 ml of 0.1 N hydrochloric acid in isopropanol. The absorbance was measured in a microplate reader at 570 nm with a reference wavelength of 690 nm.

Western Blotting

Cells in various groups were washed with ice-cold PBS and whole cell extracts were prepared using cell lysis buffer and cleared by centrifugation at 12,000/g, 4°C. Total protein concentration was measured using the BCA assay kit with bovine serum albumin (BSA) as a standard. Equal amounts of protein were loaded and analyzed by immunoblotting. Enhanced chemiluminescence detection was performed in accordance with the manufacturer's instructions and normalized to that of β-actin. The primary antibody was as below: Anti-RLN2, anti-S100A4 and anti-MMP-9. Experiments were performed in triplicate and repeated three times.

Statistical Analysis

All experiments were processed in triplicate and carried out on three or more separate occasions. Data presented are means of the three or more independent experiments ± SE. Statistically significant differences were determined by Student's *t* test and were defined as $^{*}p<0.05$. All analyses were performed with SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA).

Results

Specific siRNA Inhibited RLN2 Expression in MG-63 cells

In order to investigate effect of inhibition RLN2 in the subsequent experiments, the RLN2 siRNA was used to suppress RLN2 expression in MG-63 cells. The result of western blot assays show that the RLN2 protein was significantly lower in RLN2 siRNA stably transfected MG-63 cells than in those infected by control siRNA (Figure 1).

To study the effect of RLN2 re-expression on OS cells, RLN2 siRNA stably transfected MG-63 cells (MG-63/RLN2 siRNA) were treated with 100 μM recombinant Human RLN2 (B-29/A-24) for 24 hs. The result of western blot assays show that the RLN2 protein was significantly restored (Figure 1).

RLN2 Regulates S100A4-Dependant MMP-9 Expression

In order to investigate the effect of inhibiting RLN2 on the activity of S100A4/MMP-9 signal-

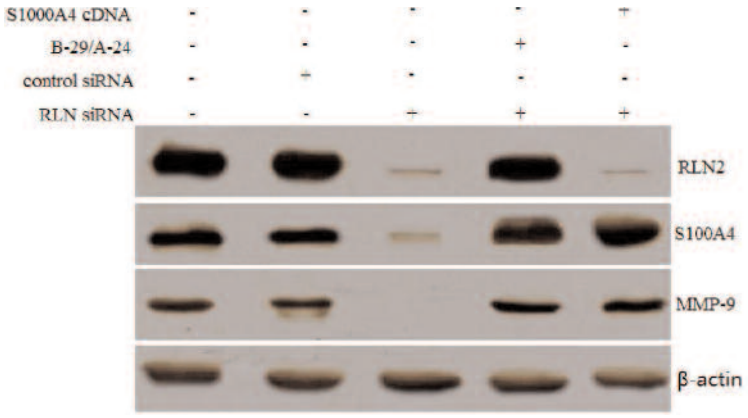

Figure 1. Expression of RLN2 in OS cells following different treatment. The expression of RLN2, S100A4 and MMP-9 protein was measured by western blot in MG-63 cells with specific siRNA transfection or/and treated with 100 μM recombinant Human RLN2 (B-29/A-24) for 24 hs or transfected with S100A4 cDNA for 48 h.

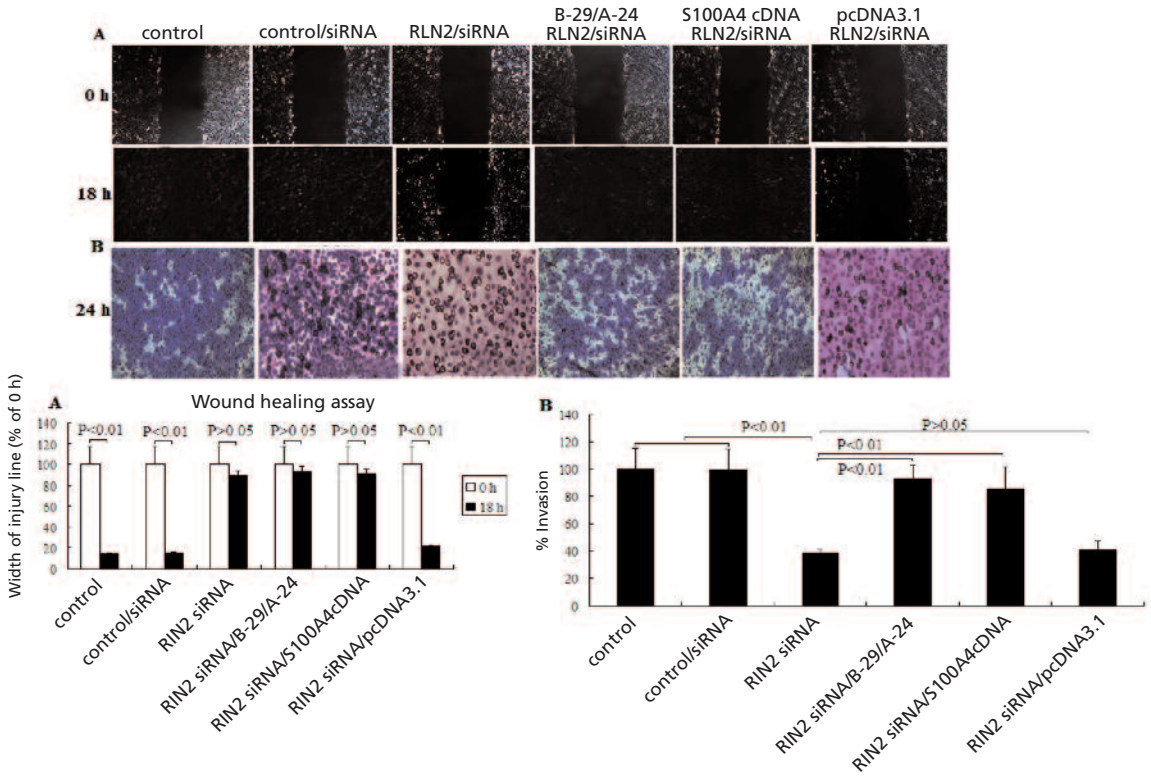

Figure 2. Effect of RLN2 on invasion and migration of MG-63 cells in vitro. ***A,*** Invasion assays were performed using wound healing assay. ***B,*** Invasion assays were performed using transwell culture chambers (original magnification×100).

ing pathway in OS cells, the S100A4 and MMP-9 was detected by western blot assay in the MG-63/RLN2 siRNA cells. Results revealed that S100A4 and MMP-9 protein expression was significantly inhibited compared to the control siRNA (Figure 1).

To study the effect of RLN2 re-expression on S100A4 and MMP-9 in MG-63 cells, RLN2 siRNA stably transfected MG-63 cells (MG-63/RLN2 siRNA) were treated with 100 μM recombinant Human RLN2 (B-29/A-24) for 24 hs. The result of western blot assays show that the S100A4 and MMP-9 expression was significantly increased (Figure 1).

Although S100A4 and MMP-9 expression was significantly inhibited in the MG-63/RLN2 siRNA cells, MMP-9 expression was significantly increased when MG-63/RLN2 siRNA cells was transfected with S100A4 cDNA (Figure 1).

Effect of RLN2 Signaling on Cell Migration and Invasion of OS cells

To study the effect of RLN2 signaling on the cell migration, wound-healing assays were used via allowing the cells to move to the scar region for 18 h. We observed that inhibition of RLN2 expression by RLN2 siRNA in MG-63 cells suppressed wound closure compared with its respective vector-transfected cells (Figure 3A). However, treated with 20 mM BB94 increased the wound closure compared with RLN2 siRNA-transfected cells (data not shown).

RLN2 re-expression treated with 100 μM recombinant Human RLN2 (B-29/A-24) stimulated the wound closure in the MG-63/RLN2 siRNA cells (Figure 3A). However, treatment with 100 μM recombinant Human RLN2 (B-29/A-24) did not stimulated the wound closure in the MG-63 /RLN2 siRNA cells when transfected with MMP-9 siRNA in the MG-63 /RLN2 siRNA cells (data not shown).

Although RLN2 siRNA suppressed wound closure in MG-63 cells, MG-63/RLN2 siRNA cells transfected with S100A4 cDNA was also significantly stimulated the wound closure in the MG-63 /RLN2 siRNA cells compared with its respective vector-transfected cells (Figure 2A).

Invasion assays were performed using matrigel invasion assay. After 24 h of incubation, invading cells were counted. We observed that invasion as-

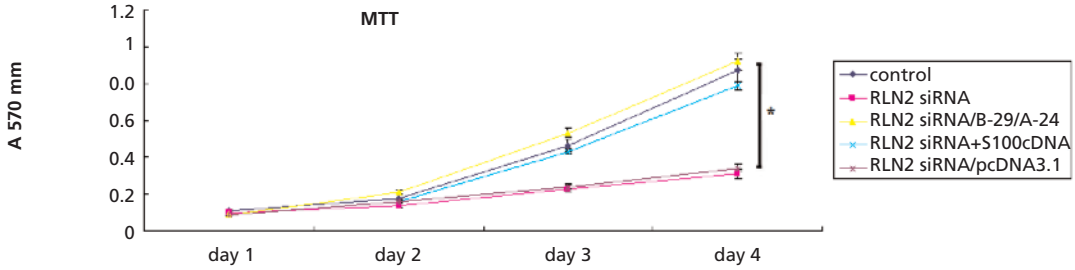

Figure 3. Effect of RLN2 signaling on cell viability in vitro. $^{*}p < 0.05$.

say has the same results as wound-healing assays (Figure 2B). These results demonstrated that RLN2 is correlated with cell migration and invasion potential of OS cells, which via S100A4/ MMP-9 signaling.

Effect of RLN2 Signaling on Cell Proliferation in vitro

We observed that inhibition of RLN2 expression by RLN2 siRNA in MG-63 cells suppressed cell viability of MG-63 cells as evidenced by MTT assay on Days 1-4 compared to control siRNA/MG-63 cells. However, RLN2 re-expression treated with 100 μM recombinant Human RLN2 (B-29/A-24) promoted cell viability of MG-63/ RLN2 siRNA cells (Figure 3).

Although treatment with 100 μM recombinant Human RLN2 (B-29/A-24) increases cell viability in the MG-63/RLN2 siRNA cells, treatment with 100 μM recombinant Human RLN2 (B-29/A-24) did not increase cell viability in the MG-63/RLN2 siRNA cells when transfected with MMP-9 siRNA in the MG-63 /RLN2 siRNA cells (data not shown).

Although inhibition of RLN2 expression suppresses cell viability of MG-63 cells, MG-63/RLN2 siRNA cells transfected with S100A4 cDNA observes significant increase of MMP-9 expression and cell viability in MG-63/RLN2 siRNA cells (Figure 3). These results demonstrated that RLN2 is correlated with cell proliferation of OS cells, which is via S100A4/MMP-9 signaling.

Discussion

H2-relaxin (RNL2) is increased in human carcinoma and is associated with increased migratory capacity of carcinoma cells of the prostate (4) and thyroid[24]. We showed that RLN2 promotes growth and enhances cell matrix invasion of human OS cells[5]. However, the cellular mechanisms mediating this increase in invasion and growth are not fully understood.

In the present study, we observed that stable transfectant clones of MG-63 with RLN2 siRNA displayed a marked down- regulation of S100A4 protein when compared with mock siRNA transfectants or untransfected MG-63. MG-63/RLN2 siRNA cells responded to treatment with recombinant human RLN2 (B-29/A-24) with an increase in S100A4 protein. This report demonstrates that RNL2 positively regulates S100A4 expression in human OS MG-63 cells. In this study, we show that knockdown of RNL2 inhibited invasiveness and cell viability of OS cells and that its overexpression reverses this effect. However, S100A4 overexpression increased invasiveness and cell viability in RLN2 siRNA transfectants, suggesting that S100A4 to act downstream of RLN2 signaling to increase invasiveness and cell viability. These data provide evidence that the RNL2 gene controls invasiveness and cell viability of human OS cells under *in vitro* conditions through the transcriptional regulation of S100A4.

S100A4 was identified as a novel target molecule of RLN2. However, how RLN2/S100A4 actions is not clear. Degradation of ECM is required by tumor cells to invade distant tissues, and recently metastatic effects of RNL2 have been linked with ECM destruction in some cells[11,25-27]. MMP-9 is a proteolytic enzyme which is linked with ECM destruction, and can degrade the components of basement membranes, especially type IV collagen. It plays a crucial role in the invasion and metastasis of malignant tumors, including OS. In the present study, we observed RLN2 siRNA transfectants observed decrease in S100A4 and MMP-9 pro-

duction. RLN2 siRNA transfectants transfected with S100A4 cDNA increased the MMP-9 production. RLN2 siRNA transfectants treated with human recombinant RLN2 (B-29/A-24) observed increase in S100A4 and MMP-9 production. This report demonstrates that RNL2 regulates S100A4/MMP-9 signal in human OS cells.

Although RLN2 siRNA transfectants observed decrease invasiveness and cell viability of human OS cells *in vitro*, followed by decreased MMP-9 product, RLN2 siRNA transfectants treated with BB94 (a kind of MMP-9 activator) observed increase in invasiveness and cell viability of human OS MG-63 cells *in vitro*. Although MG-63/RLN2 siRNA cells treated with recombinant human RLN2 (B-29/A-24) observed increase in invasiveness and cell viability of human OS MG-63 cells *in vitro*, followed by increased S100A4 and MMP-9 product, transfection with MMP-9 siRNA in recombinant human RLN2 treated MG-63/RLN2 siRNA cells observed decrease in invasiveness and cell viability of human MG-63 cells *in vitro*. This report demonstrates that RNL2 regulates invasiveness and cell viability of human OS MG-63 cells via S100A4/MMP-9 signal.

Conclusions

We identified S100A4/MMP-9 signaling as novel target for RLN2 in human OS MG-63 cells. RLN2 mediated the invasiveness and cell viability-enhancing effects in OS MG-63 cells, and knowdown of RLN2 may reverses the effect. Further studies are needed to clarify the role and mechanisms of RLN2 *in vivo*.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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