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Relaxin promotes *in vitro* tumour growth, invasion and angiogenesis of human Saos-2 osteosarcoma cells by AKT/VEGF pathway

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Abstract. – OBJECTIVES: In the present study, we determine the role of relaxin on cellular growth, invasion and angiogenesis of osteosarcoma Saos-2 cells *in vitro*, and discuss the molecular mechanisms of this action.

MATERIALS AND METHODS: Saos-2 cells were transfected with Akt1/2 siRNA or VEGF siRNA for 24 hours then treated with 10-100 ng/mL recombinant human relaxin-2 (rh-RLN) for 48 h. MTT, matrigel and bone marrow-derived endothelial cells (BMDECs) was used for cell proliferation, invasion and angiogenesis assay. Western blot was used for relaxin-2, pAKT and VEGF protein assay.

RESULTS: The results showed treatment with 10-100 ng/mL rh-RLN resulted in 18%, 48%, 107%, 212% increase in cell proliferation, respectively (vs control, *$p < 0.05$;$p < 0.01$), the relative invasive cells was 1.4;1.9;2.6;4.8 (control was defined to 1) (vs control, $^{\#}p < 0.01$; $^{\#\#}p < 0.001$) and the relative anglogenic branch points in Saos-2 cells was 1.04;1.36;1.69;2.10 (control was defined to 1.00) (vs control, *$p < 0.05$; **$p < 0.01$). Furthermore, treatment with rh-RLN exhibited a significant increase in the expression level of pAKT and VEGF proterin in dose-dependent manner. Saos-2 cells were transfected with AKT1/2 siRNA for 24 h. No significant increase of VEGF protein expression was shown after rh-RLN treatment.**

CONCLUSIONS: These results suggested that rh-RLN could promoted proliferation, invasion and angiogenesis by upregulation pAKT-dependent VEGF expression.

Key Words:
Osteosarcoma, Relaxin, Proliferation, Invasion, Angiogenesis.

Abbreviations

RLN = Relaxin
rh-RLN = Recombinant Human Relaxin
BMDEC = Bone Marrow-Derived Endothelial Cells
MMP = Matrix Metalloproteinase
AKT = Protein kinase B
VEGF = Vascular Endothelial Growth Factor
SIRNA = Small Interfering RNA
PI3K = Phosphoinositide 3-Kinase
mTORC1 = Mammalian Target of Rapamycin 1
HIF-1α = Hypoxia Inducible Factor-1α
Saos = Sarcoma Osteogenic;
RPMI 1640 medium = Roswell Park Memorial Institure- 1640 medium
DMEM = Dulbecco's Modified Eagle Medium
FBS = Fetal Bovine Serum; MTT (3-(4,5 Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide)
PBS = Phosphate Buffered Saline
HER2 = Human Epidermal Growth Factor Receptor 2

Introduction

The multifunctional heterodimeric peptide hormone relaxin (RLN), a member of the insulin-like superfamily, is an established endocrine factor in the cancer biology[1-4]. Upregulated in various human cancer tissues, RLN2 contributes to tumor cell proliferation, tissue invasion, and tumor angiogenesis[1-6]. Understanding the molecular mechanisms by which RLN2 enhances the tissue invasiveness of tumor cells in patients is of prognostic and therapeutic importance.

Relaxin binds predominantly to the 7-transmembrane G-protein-coupled receptor LGR7[7-9]. *In vitro* studies have linked relaxin expression with invasive behaviour of cancer cells[1,4,10-11]. With treatment with porcine relaxin, SK-BR3 and MCF-7 human breast cancer cells showed increased secretion of MMP-2, MMP-9 and MMP-7 causing increased migration through matrigel[11].

AKT is a "Master Regulator" that when activated by phosphorylation, modifies at least ten major regulatory proteins. It is important in initiation of many pathways in both normal and tumor cells. These play a central role in a variety of oncogenic processes including cell growth, proliferation, apoptotic cell death, motility, epithelial mesenchymal transition (EMT), angiogenesis and metastasis[12-13]. Studies have found VEGF pathway is a crit-

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ical signaling pathway in osteogenic sarcoma[14]. Further studies showed knockdown of VEGF by VEGF-siRNA inhibits growth and metastasis in osteosarcoma *in vivo* and *in vitro*[15-16].

In neuroblastoma, attenuation of AKT2 impaired cell proliferation and anchorage-independent cell growth, and decreased the secretion of angiogenic factor VEGF *in vitro*. Furthermore, silencing AKT2 inhibited migration and invasion of neuroblastoma cells *in vitro* by VEGF inhibition[17]. Phosphorylated Akt and VEGF-A are also involved in angiogenesis of gastric adenocarcinoma, and Akt activation may contribute to angiogenesis via VEGF-A upregulation. The PI3K/Akt/VEGF signaling pathway may be involved in gastric adeno carcinoma[18].

In some sarcoma xenografts, inhibition of mTORC1 signaling may have direct effect on cell proliferation and survival, or an indirect effect *via* inhibition of HIF-1α, thus reducing tumor-elicited VEGF. Conversely, phosphoinositide 3-kinase/AKT signaling can induce tumor angiogenesis by regulating VEGF[19], which suggested that significant relation was found between AKT and VEGF. Cao et al[5] has recently found RLX controls the in-vitro invasive potential of human breast cancer cells through S100A4 dependent MMPs regulation. Liu[2]) has demonstrated that H2 relaxin (RLN2) facilitates castrate-resistant (CR) growth of prostate cancer (CaP) cells through PI3K/Akt/β-catenin-mediated activation of the androgen receptor (AR) pathway.

The aim of the present study was to investigate the effect of relaxin silencing by siRNA transfection on *in vitr*o tumour growth, invasion and angiogenesis of human Saos-2 osteosarcoma cells, and whether this effect is by AKT-dependent VEGF pathway.

Materials and Methods

Cell Lines and Reagents

The human osteosarcoma cell line Saos-2 were obtained from the ATCC (Rockville, MD, USA), and incubated in RPMI 1640 medium containing 10% fetal calf serum (FCS, Gibco, Carlsbad, CA, USA) 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. Recombinant Human Relaxin (25 ug) was from PeproTech, Shanghai, China. Akt1/2 siRNA, VEGF siRNA and scrambled control siRNA was acquired from Santa Cruz (Santa Cruz, CA, USA). All other reagents were obtained from Sigma-Aldrich Co, Saint Louis, MO, USA unless otherwise indicated.

siRNA Transfection

Saos-2 cells were grown in RPMI 1640 medium containing 10% fetal calf serum until 80% confluence. The Akt1/2 siRNA or VEGF siRNA or scrambled control siRNA transfections 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 24 h.

Cells Treatment

The Saos-2 cells or the Saos-2 cells transfected with Akt1/2 siRNA or VEGF siRNA or scrambled control siRNA for 24 h were rinsed with DMEM plus 0.1% FBS twice and were incubated in DMEM plus 0.1% FBS containing 10 to 100 ng/mL relaxin for 24 hours.

Cell Cytotoxicity (MTT) Assay

To assess the effect of relaxin on cell proliferation, cells treated above were seeded in 100 μL of serum-free medium at a density of 3.5×10^3 per well in 96-well plates. At 48 h, cell proliferation was measured with MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, China) and the absorbance was read at 450 nm on a Bio-Rad microplate reader (model 550; Bio-Rad Laboratories, Hercules, CA, USA). All measurements were done in quadruplicate, and the experiments were repeated thrice.

Chemoinvasion Assay

Following treatments, the cells were resuspended in fresh culture medium and incubated in chemoinvasion chamber containing polycarbonate filter coated with Matrigel (Chemicon International, Atlanta, GA, USA) for 24 h. In the upper chamber, 30,000 cells were seeded in fetal bovine serum-free culture media and the lower chamber contained culture media containing 10% FBS as a chemoattractant. The cells were allowed to migrate for 24 h, following which the chamber was washed with PBS and cells were visualized as per manufacturer's instruction. To quantitate the migratory cells, the invasion chamber was dipped in 10% acetic acid, and the resultant solution was spectrophotometrically read at 540 nm.

In vitro Angiogenesis Assay

The Saos-2 cells or the Saos-2 cells transfected with Akt1/2 siRNA or VEGF siRNA or scrambled control siRNA for 24 h were rinsed with DMEM plus 0.1% FBS twice and were incubated in DMEM plus 0.1% FBS containing 10 to 100 ng/mL relaxin for 24 hours. The conditioned medium was filtered off for future research. Bone marrow-derived endothelial cells (BMDECs) (4×10^4) were seeded onto eight-well chamber slides and the aforementioned conditioned medium was added. Cells were cultured for 72 h until capillary network formation was observed. The number of branch points and total number of branches per point were counted after H&E staining to quantify the degree of angiogenesis.

Western Blot Analysis

Following treatments, cells were harvested by trypsinization (trypsin 0.25% w/v, 1 mM ethylenediaminetetraacetic acid), washed with PBS, and lysed overnight at –20°C in a 400 uL lysis buffer [10 mmol/L Tris-HCl (pH 8.0), containing 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, and 1% Triton X-100] to confluent cells grown in 60 mm dishes. Twenty-five micrograms of protein were loaded on 4% to 20% Novex Tris-Glycine gradient denaturing polyacrylamide gels (Invitrogen) in a 1× SDS-PAGE buffer (1 g/L SDS, 3 g/L Tris base, and 14.4 g/L glycine). Proteins were transferred to polyvinylpyrrolidine difluoride membranes electrophoretically and incubated overnight at 4°C in Blotto [5% dry milk in 1×TBS (0.9% NaCl, 10 mmol/L Tris (pH 7.4), and 0.5% MgCl2)]. Membranes were incubated for 60 minutes at room temperature with anti-relaxin (1:100), pAkt (1:200) and VEGF (1:100) primary antibody in Blotto, followed by three 10-minute washes with Blotto. After washing with Blotto, the membrane was incubated with a 1:4,000 dilution of horseradish peroxidase-linked anti-mouse secondary antibodies. The immune complexes were detected using eletrochemiluminescence (ECL) (Amersham Biosciences, Piscataway, NJ, USA). Western blotting detection reagents. The membranes were stripped of bound antibody and reprobed with an anti-β-actin antibody to confirm equal loading of the samples.

Statistical Analysis

All measures were summarized as mean±SE. Associations of categorical variables were evaluated using the Fisher exact test. All tests were two-sided and conducted at the a = 0.05 significance level.

Results

Relaxin Promotes Saos-2 Cells Growth

To determine whether relaxin had an increased effect on proliferation with MTT assay. Saos-2 cells was treated with 10, 30, 50, 100 ng/mL recombinant human relaxin (rh-RLN) for 24 hours. The results showed treatment with 10, 30, 50, 100 ng/mL rh-RLN resulted in 18%, 48%, 107%, 212% increase in cell proliferation, respectively (vs control, $^{*}p < 0.05$; $^{**}p < 0.01$). The results also showed rh-RLN dose-dependently increased relaxin expression with maximum effect observed at a concentration of 100 ng/mL (Figure 1A).

Relaxin Promotes Saos-2 Cells Invasion

To address the role of relaxin in Saos-2 invasion, relaxin expression was achieved by treating Saos-2 cells with 10, 30, 50, 100 ng/mL rh-RLN for 24 h. Contrast to control cells, the relative invasive cells in Saos-2 cells treated 10, 30, 50, 100 ng/mL rh-RLN was 1.4, 1.9, 2.6, 4.8 (control was defined to 1) (vs control, $^{\#}p < 0.01$; $^{\#\#}p < 0.001$, Figure 1B). The results showed rh-RLN treatment showed dose-dependently increased in the invasive Saos-2 cells.

Effect of Relaxin-2 on Angiogenesis in vitro

Contrast to control cells, the relative angiogenic branch points in Saos-2 cells treated 10, 30, 50, 100 ng/mL rh-RLN was 104, 136, 169, 210 (control was defined to 100) (vs control, $^{*}p < 0.05$; $^{\#\#}p < 0.01$, Figure 2). The results showed rh-RLN treatment showed dose-dependently increased in the relative anglogenic branch points in Saos-2 cells.

BMDECs treated with conditional medium from Saos-2 cells treated 10, 30, 50, 100 ng/mL rh-RLN or/and transfected with Akt1/2 siRNA or VEGF siRNA by *in vitro* angiogenesis assay. The conditioned medium of Saos-2 cells was collected after treatment following filtering of medium. BMDECs cells seeded in eight-chamber slides were cultured with the above medium for 48 h until the formation of capillary network was observed. In the end of the experiment, angiogenesis was assessed by H&E staining and photographed. Each bar represents mean±SE ($n = 3$); vs control, $^{*}p < 0.05$; $^{**}p < 0.01$.

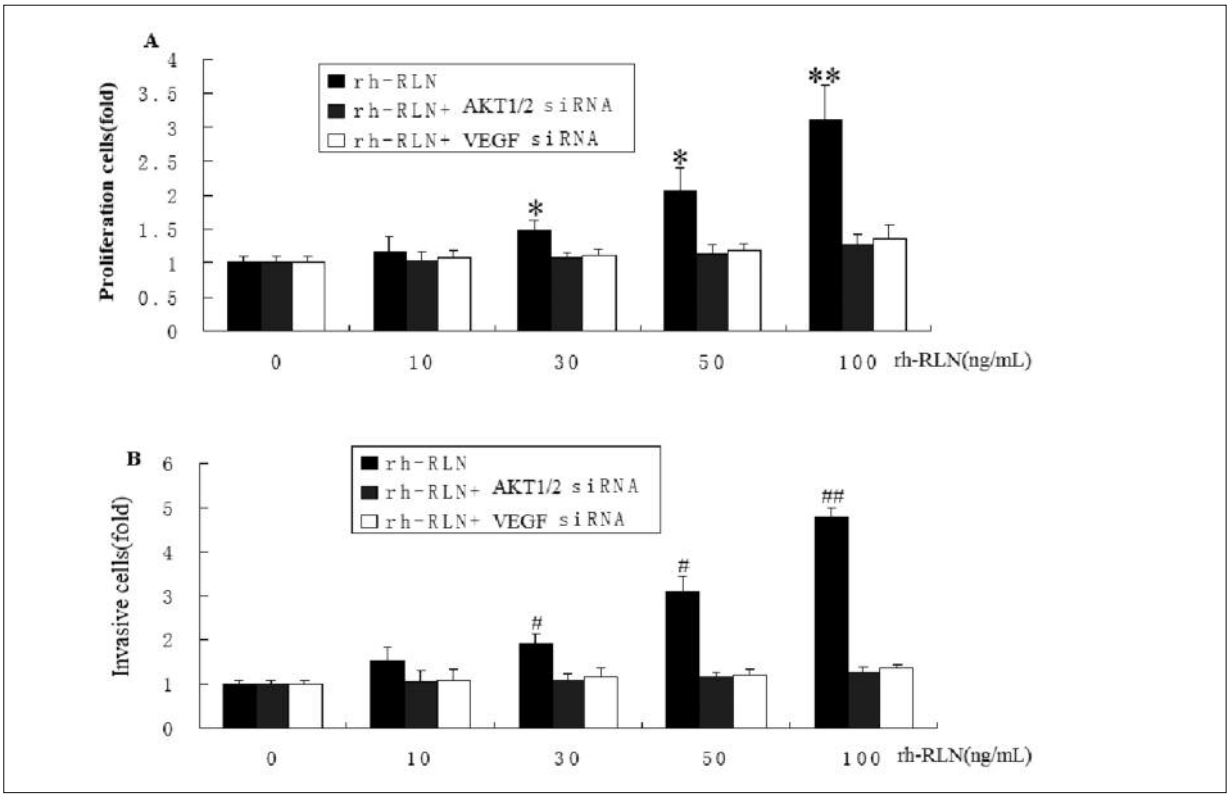

Figure 1. Relaxin promotes Saos-2 cells growth and invasion. A, The Saos-2 cells or the Saos-2 cells transfected with Akt1/2 siRNA or VEGF siRNA or scrambled control siRNA for 24 h, then incubated with 10 to 100 ng/mL relaxin for 24 hours. Proliferation was with MTT assay. Each bar represents mean±SE (n = 3); vs control, $^{*}p < 0.05$; $^{**}p < 0.01$. B, Histogram showing invasive capability of treated cells above. Each bar represents mean±SE (n = 3); vs control, $^{\#}p < 0.01$; $^{\#\#}p < 0.001$.

Relaxin Promotes AKT1/2 and VEGF Expression

Next we determined the effect of relaxin on the expression and activity of VEGF and AKT. Saos-2 cells treated with 10, 30, 50, 100 ng/mL rh-RLN for 24 h exhibited a significant increase in the expression level of pAKT (Figure 3) and VEGF proterin (Figure 3) in dose-dependent manner.

Relaxin Promotes VEGF Expression by Upregulation of pAKT

Although Saos-2 cells treated with 10, 30, 50, 100 ng/mL rh-RLN for 24 h exhibited a significant increase in the expression level of Akt1/2 siRNA and VEGF siRNA proterin, however when the Saos-2 cells was transfected with AKT1/2 siRNA to inhibit AKT1/2 for 24

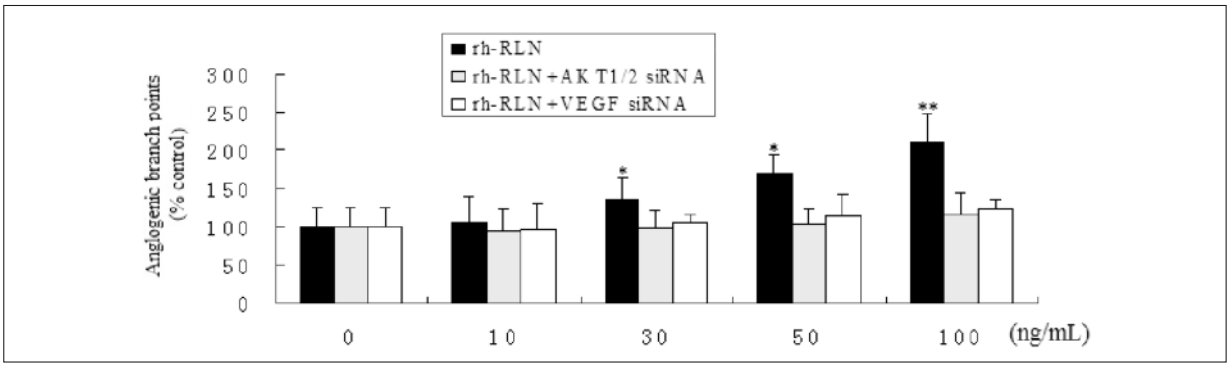

Figure 2. Effect of relaxin-2 with rh-RLN on angiogenesis.

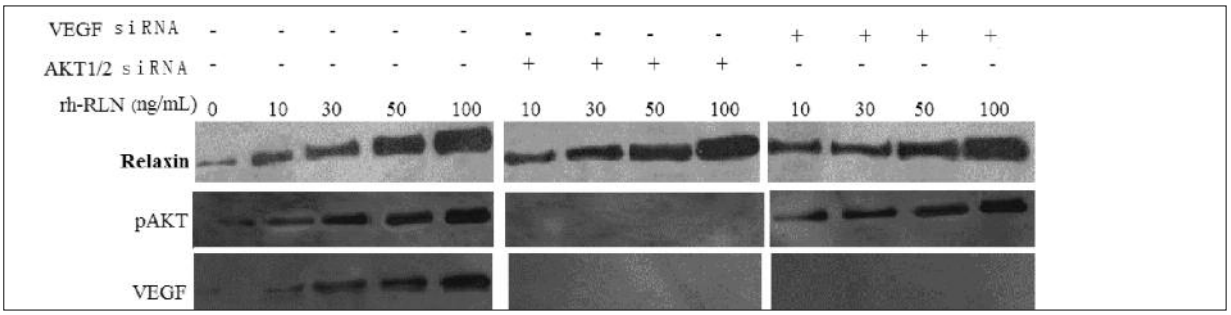

Figure 3. The effect of relaxin on AKT1/2 and VEGF expression in Saos-2 cells.

h, no significant increase of VEGF protein expression was shown after rh-RLN treatment (Figure 3).

Relaxin Promotes Saos-2 Cells Growth, Invasion and Angiogenesis by pAKT Dependent VEGF Signal

Saos-2 cells treated with 10, 30, 50, 100 ng/mL rh-RLN for 24 h exhibited a significant increase in proliferation, invasion and angiogenesis ability. However, when the Saos-2 cells was transfected with AKT1/2 siRNA or VEGF siRNA for 24 h, no significant increase of proliferation (Figure 1A), invasion (Figure 1B) and angiogenesis ability (Figure 2) was shown after rh-RLN treatment.

Discussion

The polypeptide hormone relaxin is increased in human carcinoma and is associated with increased migratory capacity of carcinoma cells of the breast[11], prostate[4], and thyroid[6,10]. However, whether relaxin could promote cellular growth, invasion and angiogenesis of osteosarcoma cells and the molecular mechanisms of this action is not fully understood. The major new finding of this work is that the pregnancy hormone, relaxin, promotes *in vitro* tumour growth, invasion and angiogenesis in human Saos-2 osteosarcoma cells. In this study, we demonstrate for the first time that relaxin signals through AKT pathway to promote the VEGF signaling.

Tumor metastasis can be initiated as well as sustained by the activation of AKT pathways. AKT activity is much greater in high grade mammary tumors, and AKT produced more multi-focal tumors from HER2-positive cells[21]. Relaxin also increases phosphorylation of AKT in prostate cancer cells and activates the androgen receptor, and siRNA against AKT blocked cell migration, indicating that AKT activation is necessary for metastasis of these cultured cells[22]. Many reports have demonstrated that the phosphatidylinositol-3 kinase (PI3K)/AKT pathway is also a potent survival signal[23,24].

We show here that a 48 hour exposure to rh-RLN dose-dependently increased cell invasion and proliferation in Saos-2 cells. These data confirm previous findings by other Authors who described relaxin as an enhancer of cancer cell migration and proliferation employing the human breast cancer cell lines MCF-7 and SKBr3[11], human prostate cancer cells PC3[7], human thyroid carcinoma cells[10]. Our findings also clearly show that a 48 hour exposure to rh-RLN dose-dependently increased formation of capillary network. However, the mechanisms by which relaxin enhances the proliferation and metastatic potential are largely unknown.

In the present study, we show that a 48 hour exposure to rh-RLN dose-dependently increased the phosphorylation of AKT (pAKT) and VEGF expression in Saos-2 cells. When the Saos-2 cells was transfected with AKT1/2 siRNA to inhibit pAKT, no significant increase of VEGF expression was shown after rh-RLN treatment. Furthermore, when the Saos-2 cells was transfected with AKT1/2 siRNA or VEGF siRNA to inhibit pAKT or VEGF, no significant increase of proliferation, invasion and angiogenesis ability was shown after rh-RLN treatment. We, then, concluded that relaxin promotes *in vitro* tumour growth, invasion and angiogenesis of human Saos-2 osteosarcoma cells by AKT/VEGF pathway.

Conclusions

The *in vitro* up-regulation of pAKT/VEGF identifies a new and potentially clinically rele-

vant property of relaxin in human osteosarcoma. Further studies are ongoing to identify the signalling pathways involved in the relaxin-induced regulation of pAKT/VEGF.

Competing Interest

The Authors declare that they have no competing interests.

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