

RNAi-mediated knockdown of relaxin decreases *in vitro* proliferation and invasiveness of osteosarcoma MG-63 cells by inhibition of MMP-9

J.-F. MA, L. LIU¹, W.-J. YANG¹, L.N. ZANG², Y.-M. XI

Department of Spine, the Affiliated Hospital of Qingdao Medical College, Qingdao University, Qingdao, R.P. China

¹Department of Orthopedics, the Affiliated Hospital of Qingdao Medical College, Qingdao University, Qingdao, R.P. China

²Department of Internal medicine-cardiovascular, the Affiliated Hospital of Qingdao Medical College, Qingdao University, Qingdao, R.P. China

Abstract. – PURPOSE: The purpose of this study is to determine the role of relaxin knockdown by siRNA transfection in cellular growth and invasion of osteosarcoma MG-63 cells, and discusses the molecular mechanisms of this action.

MATERIALS AND METHODS: The expression of relaxin in MG-63 cell was examined by western blot or RT-PCR. To evaluate the biological role of relaxin, proliferation assay (MTT) and invasion assay (BD Matrigel™), apoptosis assay (TUNEL and ELISA) and cell cycle analysis (flow cytometer) were performed after silencing relaxin using siRNA. MMP-9 expressions were analyzed using RT-PCR, western blot and zymography after silencing relaxin.

RESULTS: Results showed that the downregulation of relaxin expression by siRNA in human osteosarcoma MG-63 cells significantly inhibited cell proliferation and invasion *in vitro*. Furthermore, relaxin knockdown led to cell arrest in the G1/G0 phase of the cell cycle, and eventual apoptosis enhancement in MG-63 cells. We provide evidence in our cell model that the relaxin siRNA down-regulated the expression of MMP-9 and the MMP-9 activity, suggesting that relaxin may promote the proliferation, invasion and metastasis of osteosarcoma cells by regulating the expression of MMP-9 and facilitating ECM degradation.

CONCLUSIONS: Therefore, siRNA-directed knockdown of relaxin may represent a viable clinical therapy for osteosarcoma.

Key Words:

Homocysteine (Hcy), Atherosclerosis (AS), Oxidative stress, Lutein.

SiRNA = small interfering RNA

MMP-9 = matrix metalloproteinase

RPMI 1640 = Roswell Park Memorial Institute 1640 medium

LGR7 = leucine-rich repeat-containing G protein-coupled receptor 7

LMNA = lamin A/C

dUTP = 2'-deoxyuridine, 5'-triphosphate

PBS = phosphate buffered saline

Introduction

Osteosarcoma is the most common osteogenic malignant tumor characterized by a high level of malignancy, relapse, metastasis and poor prognosis¹. Many patients are not cured by the current osteosarcoma therapy consisting of combination chemotherapy along with surgery and survival of patients remains dismal and novel treatment approaches are urgently needed².

Relaxin is a short circulating peptide hormone. Two highly homologous genes on human chromosome 9 encode relaxin-1 and relaxin-2 peptides with predicted 82% identity at amino acid level³⁻⁴. Since human relaxin-2 is the equivalent of relaxin-1 in non-primate species, both will be simply referred to as relaxin. Relaxin is mainly known as a reproductive hormone which is produced by the corpus luteum and/or placenta in many species. There are varying effects of relaxin on the cervix, mammary glands, nipples, pubic symphysis and uterus of different species³. Relaxin mediates various physiological processes of normal pregnancy and parturition, for example, in the relaxin knockout (KO) mouse⁵ and relaxin immunoneutralised rat⁶. There have been a number of studies showing the association of relaxin

Abbreviations

RT-PCR = reverse transcription polymerase chain reaction

MTT = (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)

with increased endometrial angiogenesis, thickening and bleeding⁷. Relaxin has also physiological roles in collagen biosynthesis inhibition and promotion of collagen breakdown in reproductive tissues but has also shown similar effects in non-reproductive tissues. This suggests that relaxin has potential as an effective treatment for fibrotic diseases. Fibrosis is the excessive accumulation of extracellular matrix components that includes collagens, glycoproteins such as fibronectin, and proteoglycans³. Fibrosis is manifested in organs such as the heart, lung, kidney and skin, where relaxin has been shown to act on to reduce the over-expression of collagen. It does so by inhibiting transforming growth factor (TGF)- β -stimulated collagen synthesis, at the same time increasing matrix metalloproteinase (MMP)-induced collagen degradation and decreasing the actions of the tissue inhibitors of MMPs (TIMPs).

Most recently relaxin has been associated with cancer biology. A number of putative roles, including the modulation of tumor growth, neovascularization, metastasis and oncogenic progression, have been correlated to relaxin overexpression (8-11). In breast cancer, relaxin controls the *in vitro* invasive potential of human breast cancer cells through by induction of MMP expression (12), which was the S100A4 dependent (13).

The aim of this study was to investigate whether relaxin down-regulation is involved in reducing invasion, metastasis and growth in osteosarcoma cells *in vitro*.

Materials and Methods

Cell Lines and Reagents

The human osteosarcoma cell lines MG-63, U-2OS and 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 cm² culture flasks (Falcon, Mountain View, CA) until they had formed a confluent monolayer. Relaxin siRNA (h) was acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

siRNA Transfection

SiRNA (h) were acquired from Santa Cruz. By using a DharmaFECT transfection kit (Lafayette, CO, USA), cells were transfected with fluorescein-labeled siRNAs; i.e., nonsilencing siRNA

(50 nM), lamin A/C siRNA (50 nM), and relaxin siRNA (5-50 nM). Cells were harvested after 24-72 h and analyzed for expression of relaxin and lamin A/C.

Western Blot Analysis

Forty micrograms of protein was resolved over Tris-glycine, pH 8.6, 12% polyacrylamide gels for relaxin, LGR7, MMP-9 and lamin A/C and then transferred onto the nitrocellulose membranes. Expression levels of proteins were analyzed as according to the manufacturer's instruction.

Semiquantitative PCR

Total RNA was extracted from cells in different conditions using the RNeasy kit (Qiagen, Valencia, CA, USA). RT-PCR for LGR7, relaxin and MMP-9 gene expression was done using Taqman One-Step RT-PCR Master Mix Reagents kit (Applied Biosystems, Foster City, CA, USA) and the following primers and Taqman dual-labeled probe set: Relaxin: sense: 5'-TGCTGCGCTGGTTCTTGCC'-3', reverse: 5'-ACCGTGTGCTATCCTCAGTTCCT-3'. MMP-9 sense: 5'-TACCACCTCGAACTTTGACAGC-GA-3', reverse 5'-AAAGGCACAGTAGTGGC-CGTAGAA-3'. LGR7, sense 5'-TGTCATTTG-CATGCGACCTT-3' Reverse, 5'-AGTCG-GCACAGCAGAGAGAAA-3'; Expression of LGR7, Relaxin and MMP-9 mRNA was normalized by that of β -actin mRNA. Each point was done in quadruplicate and the means and SDs were calculated.

Relaxin Knockdown Promotes MG-63 Cell Apoptosis by ELISA Assay

The Cell Apoptosis ELISA Detection Kit (Roche, Palo Alto, CA, USA) was used to detect apoptosis in MG-63 cells after relaxin siRNA (10-50 μ M) transfection for 48 hours according to the manufacturer's protocol. Briefly, MG-63 cells were transfected with relaxin siRNA (10-50 μ M) for 48 hours. After treatment, the cytoplasmic histone DNA fragments from MG-63 cells were extracted and bound to immobilized anti-histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone DNA fragments. After addition of substrate for peroxidase, the spectrophotometric absorbance of the samples was determined using ULTRA Multifunctional Microplate Reader (Tecan Group Ltd, Männedorf,

Switzerland) at 405 nm.

Relaxin Knockdown Promotes MG-63 Cell Apoptosis by TUNEL assay

MG-63 cells was transfected with relaxin siRNA (10-50 μ M) for 48 hours. After treatment, the apoptosis was evaluated on the basis of the terminal transferase dUTP nick end labeling (TUNEL) assay according to the manufacturer's instructions. TUNEL-positive cells were colored using diaminobenzidine (DAB) as the chromogen, and counterstained with hematoxylin. The percentage of TUNEL-positive cells was assessed in five randomly selected fields each section. All assays were performed in quadruplicate.

Cell Cycle Analysis by Flow Cytometer

MG-63 cells (5×10^5) were seeded into each well of a 6-well culture plate and incubated overnight. Cells were then transfected with the indicated siRNAs. After an additional incubation for 48 h, cells were detached and fixed with 500 μ l of 70% ethanol at -20°C for 2 h. Subsequently, the cells were washed twice with PBS then stained with propidium iodide (50 μ g/ml propidium iodide and 100 μ g/ml RNase A in PBS) at 37°C for 30 min. Cell cycle analysis was performed on a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA).

Matrigel Invasion Assay

The invasiveness of osteosarcoma (OS) cells was tested after transfection (48 hs) as previously described. The cells ($1 \times 10^6/\text{mL}$) were added to the upper wells coated with Matrigel with serum-free medium containing 25 μ g/mL fibronectin as a chemoattractive agent in the lower wells. After a 48-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.

Gelatin Zymography

Equal number of MG-63 cells was transiently transfected with relaxin-siRNA and control siRNA for 48 h. The conditioned media were collected, concentrated using Amicon filter (Merck Millipore, Darmstadt, Germany) as per manufacturer's protocol, and electrophoresed (40 μ g protein) under nonreducing conditions. The gelatinolytic activity of MMP-9 was determined with a zymography kit (Invitrogen, Carlsbad, CA, USA)

as per vendor's protocol.

MMP-9 ELISA Assay

Twenty-four hours posttransfection, the cell culture media were collected and used for quantifying MMP-9 levels by using an MMP-9-specific ELISA kit from Amersham Bioscience Ltd (London, UK) and following the vendor's protocol.

Statistical Analysis

All measures were summarized as mean \pm SE. Associations of categorical variables were evaluated using the Fisher exact test. All tests were two-sided and conducted at the $\alpha = 0.05$ significance level. All statistical analyses were done with the SPSS 11.0 software (SPSS Inc., Chicago, IL, USA).

Results

Expression of Relaxin in Osteosarcoma Cells

We analyzed the relaxin expression in the MG-63, U-2OS and Saos-2 cells by western blot and quantitative RT-PCR assay (Figure 1). There was a distinct difference in the expression of the relaxin genes. The highest level of relaxin gene expression was detected by RT-PCR and western blot in MG-63 cells, and the lowest level was detected in Saos-2 cells. MG-63 cell line was used in subsequent experiments.

siRNA Targeting Relaxin Knocks Down Relaxin Expression

As shown in Figure 2A, high (85%) transfection efficiency of siRNAs was observed in MG-63 cells. As determined by Western blot analysis, cells transfected with relaxin siRNA displayed a dose-dependent reduction in the expression levels of relaxin protein (Figure 2B). Nonsilencing siRNA did not exhibit any effect on protein levels of relaxin (Figure 2B). Similarly, cells transfected with lamin A/C siRNA (which is used as a positive control for siRNA transfection) significantly reduced the expression of lamin A/C protein (data not shown). Further, the suppression of relaxin by siRNA in cells was confirmed by RT-PCR analysis. Cells transfected with relaxin siRNA exhibited a significant reduction in mRNA level of relaxin (Figure 2C). These data confirmed the suppression effect of siRNA and established the effi-

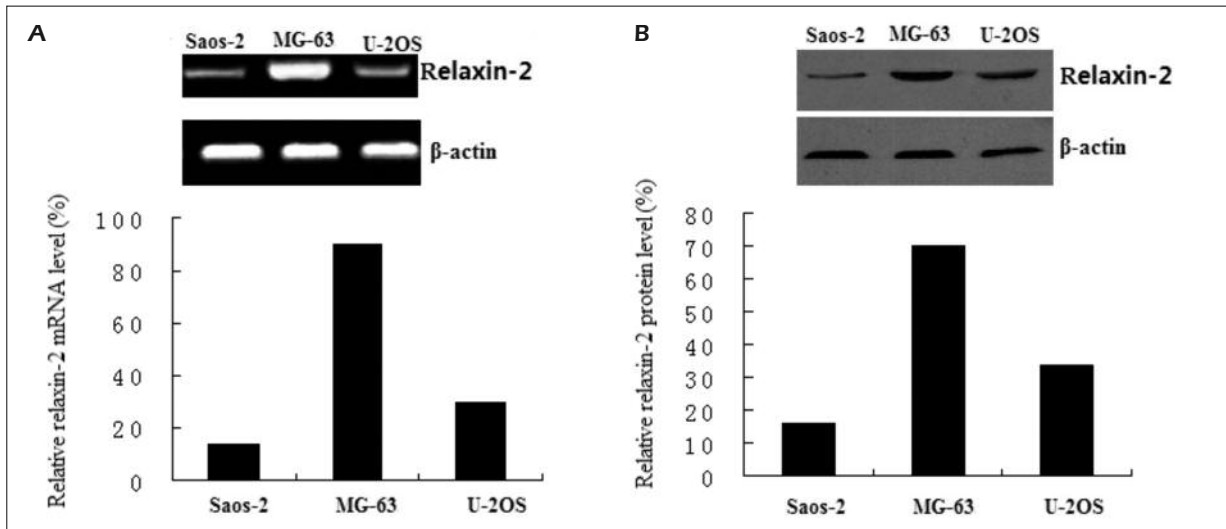


Figure 1. Protein and mRNA expression of relaxin in osteosarcoma cell lines. **A**, Semiquantitative RT-PCR analysis shows mRNA expression of relaxin in MG-63, U-2OS and Saos-2 cells. β -actin was used as an internal control. **B**, Western immunoblot analysis of relaxin protein expression in MG-63, U-2OS and Saos-2 cells. Blots were reprobred with β -actin antibody to analyze the equal loading of proteins.

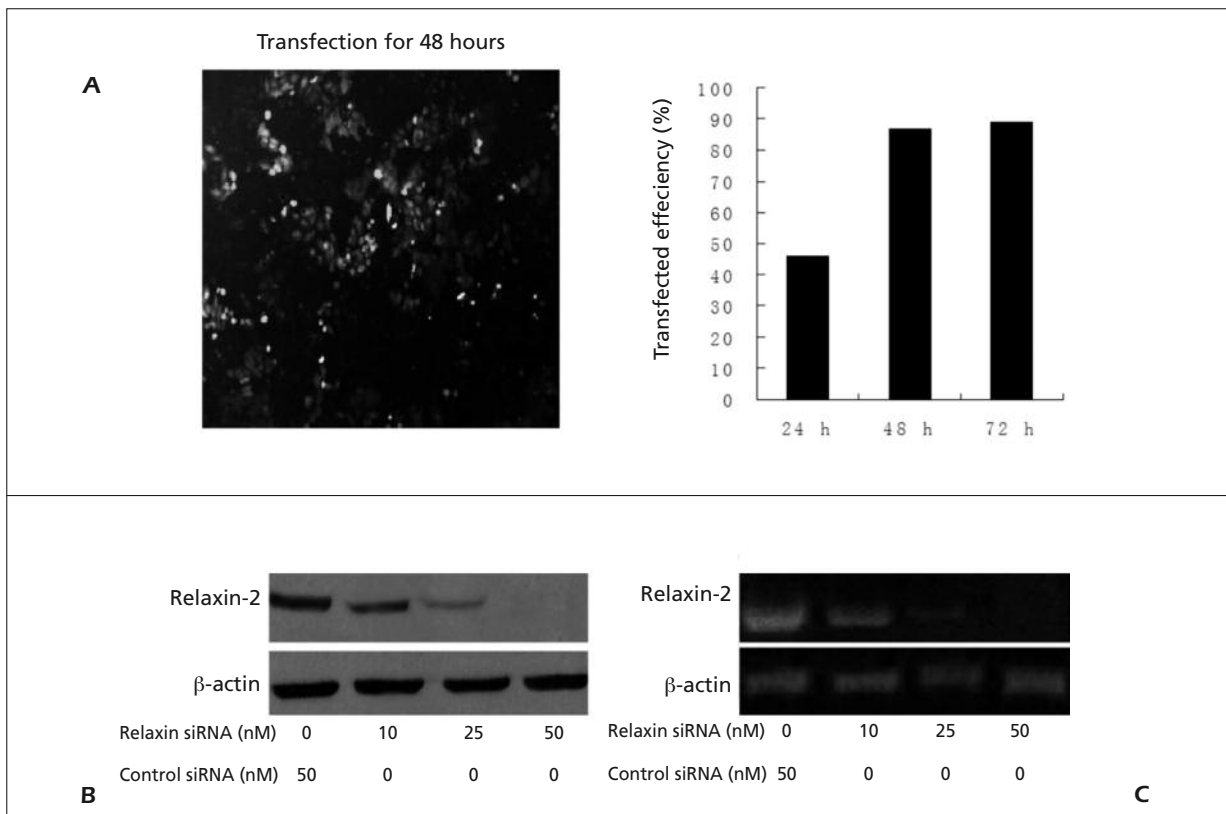


Figure 2. Effect of relaxin knockdown on mRNA and protein expressions of MG-63 cells. **A**, Transfected efficiency was observed by fluorescence microscopy in siRNA control transfected MG-63 cells. **B**, Western immunoblot analysis of relaxin expression in MG-63 cells transfected with increasing concentration of relaxin siRNA. Blots were reprobred with β -actin antibody to analyze the equal loading of proteins. **C**, Semiquantitative RT-PCR analysis shows mRNA expression of relaxin in cells transfected with increasing concentration of relaxin siRNA. Control siRNA was used as a control in parallel. β -actin was used as an internal control.

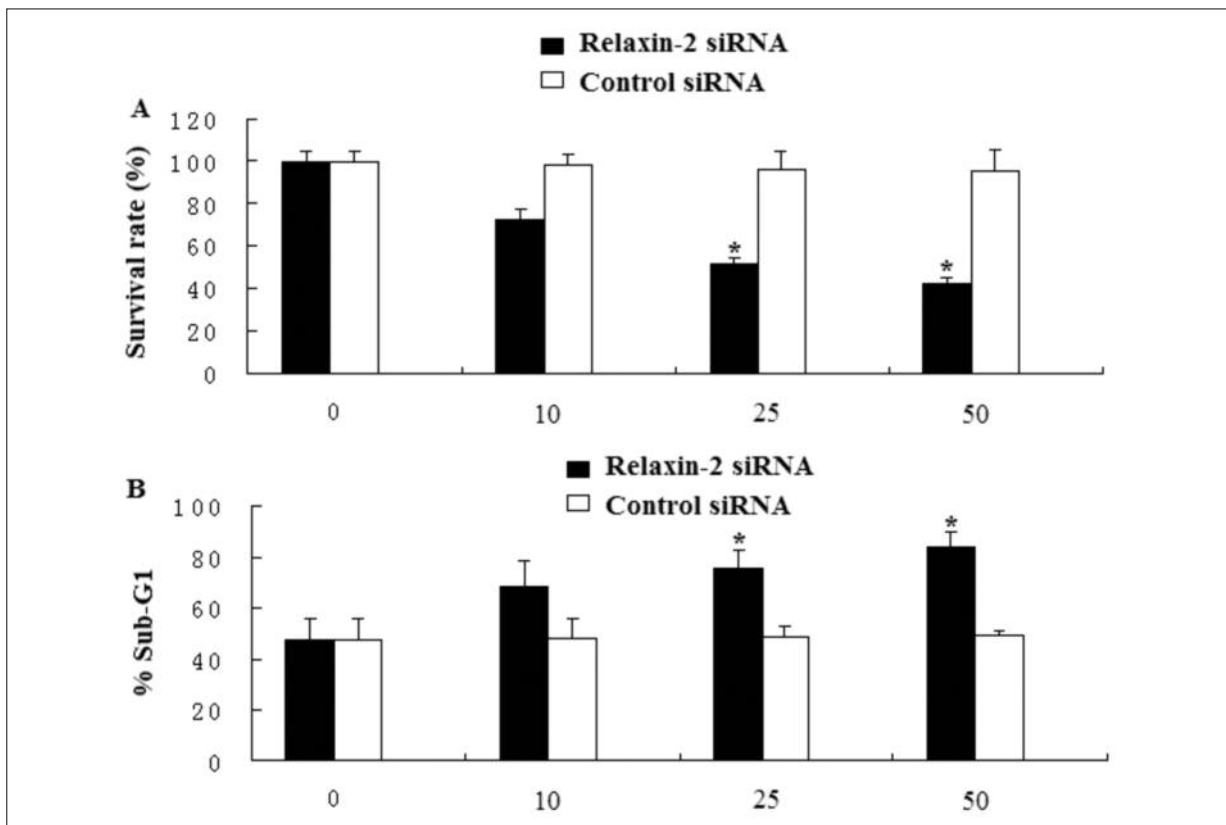


Figure 3. RNAi-mediated downregulation of Relaxin reduces MG-63 cell proliferation. **A**, Cell proliferation was assessed at the indicated times by MTT assays. Data are from three independent experiments. **B**, Cell cycle analysis of MG-63 was carried out by flow cytometry after 48-h transfection of Relaxin siRNA or siRNA control. * $p < 0.05$, compared to the control group.

ciency of siRNA transfection.

Relaxin Knockdown Inhibits MG-63 Cell Growth

To determine whether relaxin siRNA had an inhibitory effect on proliferation with MTT assay. Figure 3A showed that the survival rate for relaxin knockdown (10-50 nM relaxin siRNA) cells were significantly lower than those for control cells for 2 days of incubation. Furthermore, the cell cycle distribution of control and transfected cells was analyzed by flow cytometry. As shown in Figure 3B, there was a significant increase in the percentage of cells in G1 phase after transfection with relaxin siRNA. This result indicated that siRNA arrested the cells in G0 and G1 phases, delayed the progression of cell cycle and inhibited cell proliferation.

Relaxin Knockdown Promotes MG-63 Cell Apoptosis

We observed induction of apoptosis in MG-63 cells transfected with relaxin siRNA (10-50 uM)

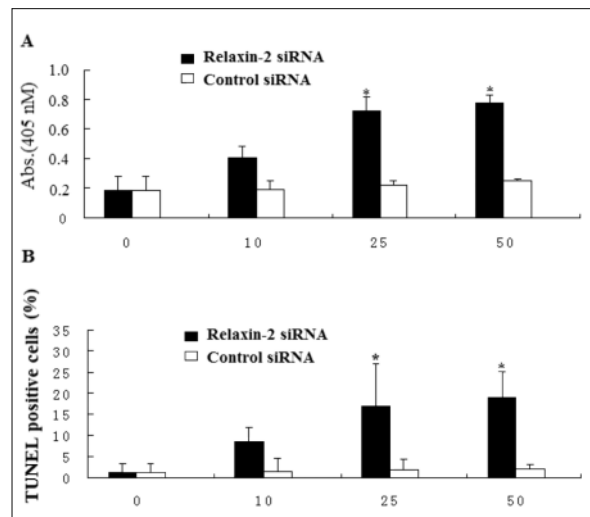


Figure 4. Evaluation of cell apoptosis by ELISA and TUNEL technique in MG-63 cells. Cells were transfected with relaxin/control siRNA (10-50 uM) for 48 hours, and analyzed for apoptotic cell death by ELISA (**A**) and TUNEL staining (**B**). Increased apoptotic response was evident in the relaxin siRNA group of the MG-63 cells relative to siRNA control groups. * $p < 0.05$, statistical significance.

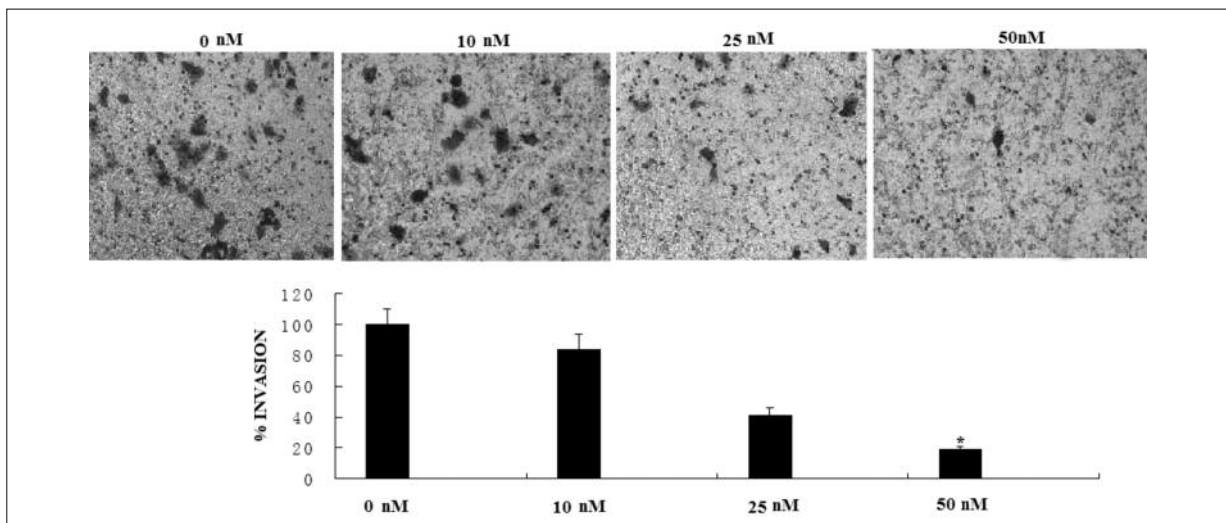


Figure 5. Relaxin silencing inhibits invasion of human MG-63 cells in Matrigel-coated invasion chambers. Cells were seeded in the upper chamber in medium supplemented with 25% FCS. Where indicated, cells were transfected with relaxin siRNA. After 48 h, cells migrated in the lower chamber were stained and counted. In the lower chamber, medium supplemented with 10% FCS was used as chemoattractant. Invasion of each untreated cell line was set to 100. Results are reported as percent migration \pm SD compared with untreated cells. Experiments were carried out twice in triplicate.

for 48 hours. Relaxin siRNA transfection induced much more apoptosis in the MG-63 cell lines as shown by histone DNA ELISA (Figure 4A) as well as TUNEL analysis (Figure 4B). These results are consistent with cell growth inhibition studies by MTT, suggesting that the loss of viable cells by relaxin siRNA transfection is partly due to the induction of an apoptotic cell death mechanism.

Relaxin Knockdown Inhibits MG-63 Cells Invasion

To address the role of relaxin in MG-63 invasion, knockdown of relaxin was achieved by transfecting MG-63 cells with relaxin siRNA compared with scrambled siRNA where no effect on relaxin expression was observed (Figure 5). Because we had hypothesized that relaxin is involved in MG-63 invasion, and the fact that MG-63 cells represent advanced metastatic cancer, we selected this particular cell line for our studies. Relaxin siRNA dose-dependently decreased relaxin expression with maximum effect observed at a concentration of 50 nM at 48 hours posttransfection (Figure 1). To further show the effect of Relaxin knockdown, MG-63 cells were subjected to invasion assay. Relaxin knockdown cells showed only a marginal invasion through the extracellular matrix compared with cells transfected with nonspecific siRNA (Figure 5).

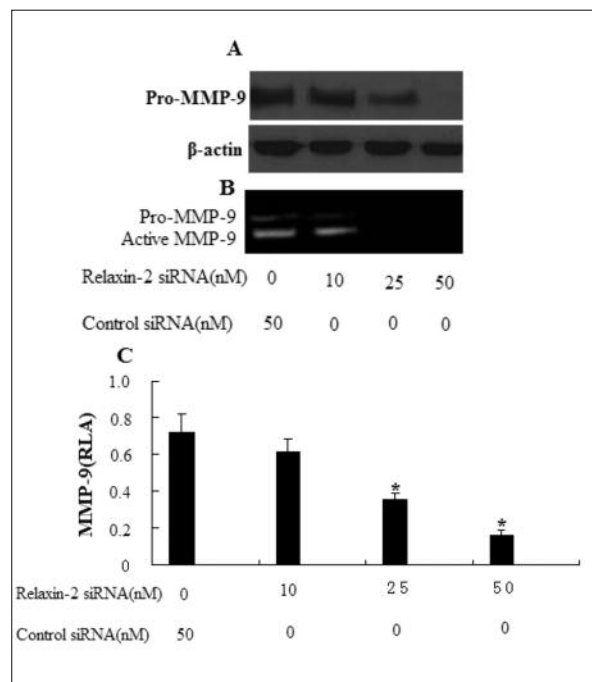


Figure 6. Effect of relaxin knockdown in MG-63 cells on the expression of MMP-9 gene. **A**, Western blot analysis to evaluate pro-MMP9 protein expression in relaxin knockdown cells. Blot was reprobbed with β -actin antibody to analyze the equal loading of proteins. **B**, Gelatin zymogram showing activity of pro-MMP-9 and active MMP-9 in control siRNA and relaxin siRNA transfected MG-63 cells. **C**, Quantification of MMP-9 secretion using MMP-9-specific ELISA done in the culture media of MG-63 cells transfected with either control or relaxin siRNA. Columns, mean of quadruple experiments. bars, SD * $p < 0.01$.

Relaxin knockdown (50 nM at 48 hours) caused 80% decrease in cell invasion, suggesting an essential role of Relaxin in conferring invasive properties to MG-63 cells.

Relaxin Knockdown Decreases MMP-9 Expression

Relaxin knocked down cells were subjected to western blot and RT-PCR analysis. We observed a decrease in pro-MMP-9 protein levels (Figure 6A), suggesting that relaxin may be involved in MMP-9 activation either by enhancing its expression or by stabilizing the protein. Gelatin zymography was done to assess MMP-9 activity in cultured medium from relaxin knockdown cells and we observed a significant decrease in MMP-9 activity (Figure 6B). To further strengthen our findings, MMP-9-specific ELISA was done to quantify secreted MMP-9 protein levels. Results indicated a consistent decrease in MMP-9 secretion compared with scrambled control (Figure 6C), further emphasizing the role of relaxin in regulation of MMP-9.

Discussion

The polypeptide hormone relaxin is increased in human carcinoma and is associated with increased migratory capacity of carcinoma cells of the breast^{10,12}, prostate⁹, and thyroid¹⁴. Furthermore, relaxin enhances cell motility and matrix invasion of human carcinoma cells and this action requires functional MMPs signaling¹²⁻¹³. Relaxin is also a potent stimulator of osteoclastogenesis from hematopoietic precursors and regulates the activity of mature osteoclasts¹⁵. However, whether relaxin inhibition could suppress cellular growth and invasion of osteosarcoma cells and the molecular mechanisms of this action is not fully understood.

To investigate the potential of relaxin as an effective therapeutic target for osteosarcoma gene therapy, we employed RNA interference to knockdown the endogenous relaxin expression in osteosarcoma cells, which showed that relaxin downregulation could inhibit the proliferation capacity of osteosarcoma cells. Moreover, relaxin downregulation could induce apoptosis enhancement in osteosarcoma cells. The results indicate that transfection might, to a large degree, inhibit proliferation by inducing apoptosis in osteosarcoma cells *in vitro*.

Several investigators have suggested that the

relaxin gene product may be of importance for the metastatic capacity of cancer cells, but the mechanisms by which relaxin enhances the metastatic potential are largely unknown. In the present study, we have investigated whether relaxin is involved in the metastatic process by regulating important events such as tumor cell attachment and invasion. We found that human osteosarcoma cells transfected with a siRNA against relaxin, displayed reduced *in vitro* invasive properties and alterations in the expression levels of MMP-9. To our knowledge, this report is the first indicating that altered relaxin expression induces changes in the expression of MMP-9 in the human osteosarcoma cells.

Attachment to basement membrane components is important in the metastatic process. In the present study, we could demonstrate that cells with reduced expression of relaxin had altered the capacity to attach to Matrigel compared with control cells. All of the relaxin siRNA transfected clones showed significantly lower invasion than the control cells. This finding suggests that relaxin also affects the capacity of tumor cells to degrade basement membrane components.

Conclusions

By using a human osteosarcoma cell line transfected with relaxin siRNA, we obtained data suggesting that the relaxin gene product may be involved in promoting tumor proliferation, invasion and metastasis via deregulating MMP-9 expression. The results are encouraging for further work on the exact mechanisms through which these effects are mediated.

Acknowledgements

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Competing Interests

The Authors declare that they have no competing interests.

References

- 1) MA X, YANG Y, WANG Y, AN G, LV G. Small interfering RNA-directed knockdown of S100A4 decreases

- es proliferation and invasiveness of osteosarcoma cells. *Cancer Lett* 2010; 299: 171-181.
- 2) SICLARI VA, QIN L. Targeting the osteosarcoma cancer stem cell. *J Orthop Surg Res* 2010; 5: 78.
 - 3) SHERWOOD OD. Relaxin's physiological roles and other diverse actions. *Endocr Rev* 2004; 25: 205-234.
 - 4) BATHGATE RA, IVELL R, SANBORN BM. International Union of Pharmacology LVII: recommendations for the nomenclature of receptors for relaxin family peptides. *Pharmacol Rev* 2006; 58: 7-31.
 - 5) ZHAO L, ROCHE PJ, GUNNERSEN JM, HAMMOND VE, TREGEAR GW, WINTOUR EM, BECK F. Mice without a functional relaxin gene are unable to deliver milk to their pups. *Endocrinology* 1999; 140: 445-453.
 - 6) GUICO-LAMM ML, SHERWOOD OD. Monoclonal antibodies specific for rat relaxin. II. Passive immunization with monoclonal antibodies throughout the second half of pregnancy disrupts birth in intact rats. *Endocrinology* 1988; 123: 2479-2485.
 - 7) GOLDSMITH LT, WEISS G, PALEJWALA, S PLANT TM, WOJTCZUK A, LAMBERT WC, AMMUR N, HELLER D, SKURNICK JH, EDWARDS D, COLE DM. Relaxin regulation of endometrial structure and function in the rhesus monkey. *Proc Natl Acad Sci U S A* 2004; 101: 4685-4689.
 - 8) SILVERTOWN JD, SYMES JC, NESCHADIM A, NONAKA T, KAO JCH, SUMMERLEE AJ, MEDIN JA. Analog of H2 relaxin exhibits antagonistic properties and impairs prostate tumor growth. *FASEB J* 2007; 21: 754-765.
 - 9) SILVERTOWN JD, NG J, SATO T, SUMMERLEE AJ, MEDIN JA. H2 relaxin overexpression increases in vivo prostate xenograft tumor growth and angiogenesis. *Int J Cancer* 2006; 118: 62-73.
 - 10) SILVERTOWN JD, GEDDES BJ, SUMMERLEE AJ. Adenovirus mediated expression of human prorelaxin promotes the invasive potential of canine mammary cancer cells. *Endocrinology* 2003; 144: 3683-3691.
 - 11) FENG S, AGOULNIK IU, BOGATCHEVA NV, KAMAT AA, KWABI-ADDO B, LI R, AYALA G, ITTMANN MM, AGOULNIK AI. Relaxin promotes prostate cancer progression. *Clin Cancer Res* 2007; 13: 1695-1702.
 - 12) BINDER C, HAGEMANN T, HUSEN B, SCHULZ M, EINSPIANIER A. Relaxin enhances in-vitro invasiveness of breast cancer cell lines by up-regulation of matrix metalloproteases. *Mol Hum Reprod* 2002; 8: 789-796.
 - 13) CAO WH, LIU HM, LIU X, LI JG, LIANG J, LIU M, NIU ZH. Relaxin enhances in-vitro invasiveness of breast cancer cell lines by upregulation of S100A4/MMPs signaling. *Eur Rev Med Pharmacol Sci* 2013; 17: 609-617.
 - 14) HOMBACH-KLONISCH S, BIALEK J, TROJANOWICZ B. Relaxin enhances the oncogenic potential of human thyroid carcinoma cells. *Am J Pathol* 2006; 169: 617-632.
 - 15) FERLIN A, PEPE A, FACCIOLLI A, GIANESELLO L, FORESTA C. Relaxin stimulates osteoclast differentiation and activation. *Bone* 2010; 46: 504-513.