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Long non-coding RNA HOTAIR promotes expression of ADAMTS-5 in human osteoarthritic articular chondrocytes

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Accumulating evidence indicated that inhibiting the expression of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-5 ameliorate cartilage degradation, suggesting ADAMTS-5 as an effective target for treating osteoarthritis (OA). A recent study has identified long noncoding RNA (lncRNA) HOTAIR and ADAMTS-5 as the most up-regulated lncRNA and the most upregulated gene, respectively, in human OA cartilage compared with normal cartilage. In the present study, we explored the regulatory effect of HOTAIR on the expression of ADAMTS-5 as well as the underlying mechanisms in human normal and OA articular chondrocytes. We found that human OA articular chondrocytes had significantly higher basal expression levels of HOTAIR and ADAMTS-5 than normal articular chondrocytes. Tumor necrosis factor (TNF)-α significantly enhanced the basal expression of HOTAIR and ADAMTS-5 in OA but not in normal articular chondrocytes. Lentiviral overexpression and knockdown of HOTAIR markedly increased and decreased the expression of ADAMTS-5, respectively, in OA but not in normal articular chondrocytes in the presence or absence of TNF-α. Neither overexpression/knockdown of HOTAIR nor TNF-α showed a significant effect on the ADAMTS-5 gene promoter in OA articular chondrocytes. Although HOTAIR showed no significant effect on the stability of ADAMTS-5 mRNA in normal articular chondrocytes, HOTAIR overexpression and knockdown respectively increased and decreased the ADAMTS-5 mRNA stability in OA articular chondrocytes. TNF-α enhanced the protective effect of HOTAIR on the ADAMTS-5 mRNA stability. In conclusion, this study provides the first evidence supporting that HOTAIR strongly promotes the expression of ADAMTS-5 by increasing its mRNA stability in human OA articular chondrocytes; this effect is enhanced by TNF-α. It adds new insights into the pathogenesis of OA and suggests that HOTAIR could be a new therapeutic target for ADAMTS-5 inhibition in human OA cartilage.

1. Introduction

Osteoarthritis (OA) is the most common chronic degenerative joint disease characterized by focal degeneration of the articular cartilage (Xue et al. 2013). Exposure to inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α stimulates chondrocyte responses that promote catabolism of type II collagen and proteoglycans (Goldring and Goldring 2007). The major feature of OA is the loss of the two most important components of cartilage extracellular matrix: Type II collagen and aggrecan (also known as chondroitin sulfate proteoglycan 1) (Wieland et al. 2005). Aggrecanases are a class of proteinases in the family of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), including ADAMTS 1, 4, 5, 8, 9, and 15 that have aggrecanase activity (Porter et al. 2005). Previous studies using transgenic mice reported that knockdown of aggrecanase-1 (ADAMTS-4), aggrecanase-2 (ADAMTS-5), or both attenuates the degradation of aggrecan in human cartilage stimulated by TNF-α (Glasson et al. 2005; Song et al. 2007; Stanton et al. 2005). A recent study has demonstrated a preferential role for ADAMTS-5 inhibition over ADAMTS-4 in the treatment of human OA cartilage (Larkin et al. 2015). Accumulating evidence indicated that inhibiting the expression of ADAMTS-5 ameliorates cartilage degradation, suggesting ADAMTS-5 as an effective target for OA treatment (Glasson et al. 2005; Stanton et al. 2005; Chu et al. 2013).

A novel class of noncoding RNAs, longer than 200 nucleotides and termed long noncoding RNAs (lncRNAs), has emerging as regulatory RNAs that play key roles in various cellular and physiological processes such as gene regulation and cell differentiation (Carninci et al. 2005; Kapranov et al. 2007). LncRNAs reportedly are dysregulated in a variety of human disorders including cancers, neurological disorders, and immunological disorders (Bhan and Mandal 2015). Recent studies have revealed that lncRNAs play key roles in the pathogenesis of OA (Liu et al. 2014; Su et al. 2015; Xing et al. 2014). HOX antisense intergenic RNA (HOTAIR), a 2.2 Kb lncRNA, was originally found to control tumor cell apoptosis, growth and metastasis in a variety of cancers (Bhan and Mandal 2015). A recent study has identified HOTAIR as the most upregulated lncRNA (over 21-fold) in human OA cartilage compared with normal cartilage, suggesting that differential expression of HOTAIR may be associated with the pathogenesis of OA (Xing et al. 2014). The same study also identified ADAMTS-5 as the most upregulated gene (over 15-fold) in OA cartilage compared with normal cartilage (Xing et al. 2014). In this study, we explored the regulatory effect of HOTAIR on the expression of ADAMTS-5 as well as the underlying mechanisms in human normal and OA articular chondrocytes.

2. Investigations and results

2.1. HOTAIR increased ADAMTS-5 expression in human OA articular chondrocytes

As shown in Fig. 1, the basal expression level of HOTAIR in human OA articular chondrocytes was approximately 7.3-fold of that in human normal articular chondrocytes. Compared with the controls, lentiviral transduction of HOTAIR increased HOTAIR by 6.5-fold and 2.5-fold in normal and OA articular chondrocytes, respectively; while lentiviral transduction of HOTAIR-siRNA knocked down HOTAIR by approximately 82% and 76% in normal and OA articular chondrocytes, respectively (Fig. 1). TNF-α markedly enhanced the basal expression of HOTAIR by

approximately threefold in OA articular chondrocytes, whereas showing no significant effect on HOTAIR expression in normal articular chondrocytes (Fig. 1).

As shown in Fig. 2, the basal expression level of ADAMTS-5 mRNA in human OA articular chondrocytes was approximately threefold of that in human normal articular chondrocytes. Overexpression and knockdown of HOTAIR showed no significant effect on the ADAMTS-5 mRNA level in normal articular chondrocytes (Fig. 2). In OA articular chondrocytes, however, HOTAIR overexpression increased the ADAMTS-5 mRNA level by 2.7-fold, and HOTAIR knockdown decreased the ADAMTS-5 mRNA level

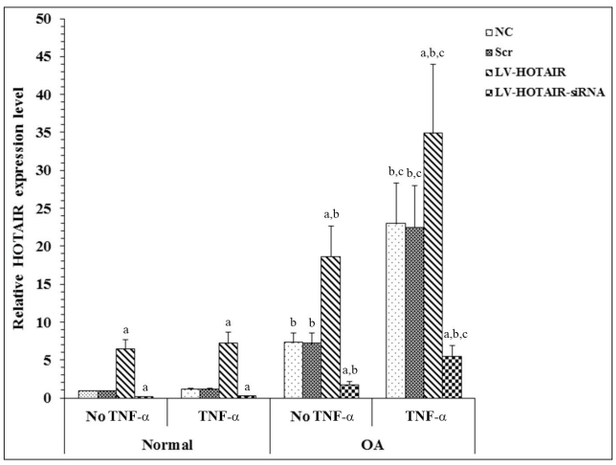

Fig. 1: Expression levels of HOTAIR in human normal and OA articular chondrocytes with or without overexpression or knockdown of HOTAIR in the presence and absence of TNF-α. Human normal and OA articular chondrocytes were transduced with human HOTAIR lentivirus (LV-HOTAIR) or human HOTAIR siRNA lentivirus (LV-HOTAIR-siRNA) to overexpress or knock down HOTAIR. Nontransduced cells (NC) and cells transduced with the scrambled siRNA lentivirus (Scr) were used as controls. The cells were cultured in the presence or absence of 60 ng/mL of TNF-α for 24 h. Then the expression of HOTAIR was measured with real-time quantitative RT-PCR and expressed as fold changes to that of NC in Normal/No TNF-α (designated as 1). [a]$p<0.05$ vs. NC and Scr; [b]$p<0.05$ vs. normal; [c]$p<0.05$ vs. OA/No TNF-α .

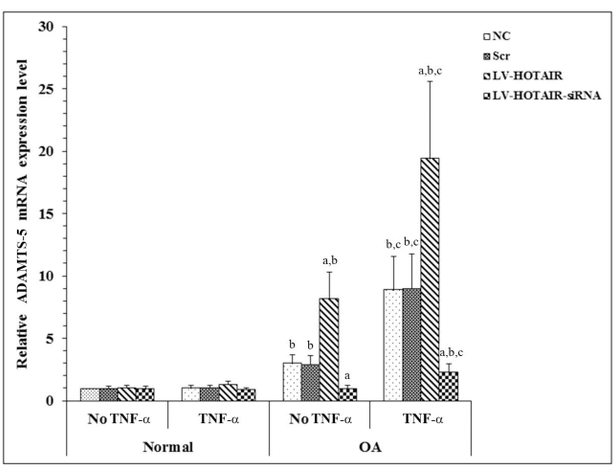

Fig. 2: Effect of HOTAIR on ADAMTS-5 mRNA levels in human normal and OA articular chondrocytes in the presence and absence of TNF-α. Human normal and OA articular chondrocytes were transduced with human HOTAIR lentivirus (LV-HOTAIR) or human HOTAIR siRNA lentivirus (LV-HOTAIR-siRNA) to overexpress or knock down HOTAIR. Nontransduced cells (NC) and cells transduced with the scrambled siRNA lentivirus (Scr) were used as controls. The cells were cultured in the presence or absence of 60 ng/mL of TNF-α for 24 h. Then the expression of ADAMTS-5 mRNA was measured with real-time quantitative RT-PCR. Relative quantification of the ADAMTS-5 mRNA level was normalized against that of GAPDH in the same sample and then expressed as fold changes to that of NC in Normal/No TNF-α (designated as 1). [a]$p<0.05$ vs. NC and Scr; [b]$p<0.05$ vs. normal; [c]$p<0.05$ vs. OA/No TNF-α .

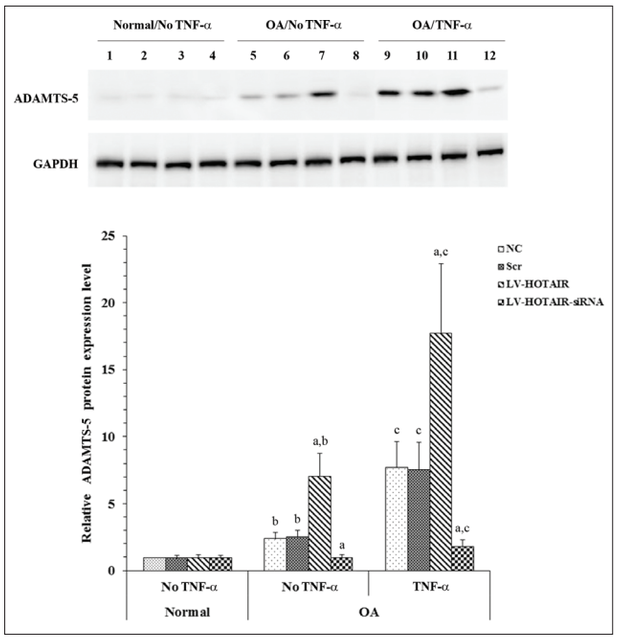

Fig. 3: Effect of HOTAIR on ADAMTS-5 protein levels in human normal and OA articular chondrocytes with or without overexpression or knockdown of HOTAIR in the presence and absence of TNF-α. Human normal and OA articular chondrocytes were transduced with human HOTAIR lentivirus (LV-HOTAIR) or human HOTAIR siRNA lentivirus (LV-HOTAIR-siRNA) to overexpress or knock down HOTAIR. Nontransduced cells (NC) and cells transduced with the scrambled siRNA lentivirus (Scr) were used as controls. The cells were cultured in the presence or absence of 60 ng/mL of TNF-α for 24 h. Then the expression of ADAMTS-5 protein was measured with Western blot analyses. The arrangement of test samples were as follows: *lanes 1-4*, Normal/No TNF-α; *lanes 5-8*, OA/No TNF-α; *lanes 9-12*, OA/TNF-α; *lanes 1, 5 and 9*, NC; *lanes 2, 6 and 10*, Scr; *lanes 3, 7 and 11*, LV-HOTAIR; *lanes 4, 8 and 12*, LV-HOTAIR-siRNA. GAPDH was used as a loading control. Density of the ADAMTS-5 blot was normalized against that of the GAPDH blot to obtain a relative blot density, which was expressed as fold changes to that of NC in Normal/No TNF-α (designated as 1). [a]$p<0.05$ vs. NC and Scr; [b]$p<0.05$ vs. normal; [c]$p<0.05$ vs. OA/No TNF-α.

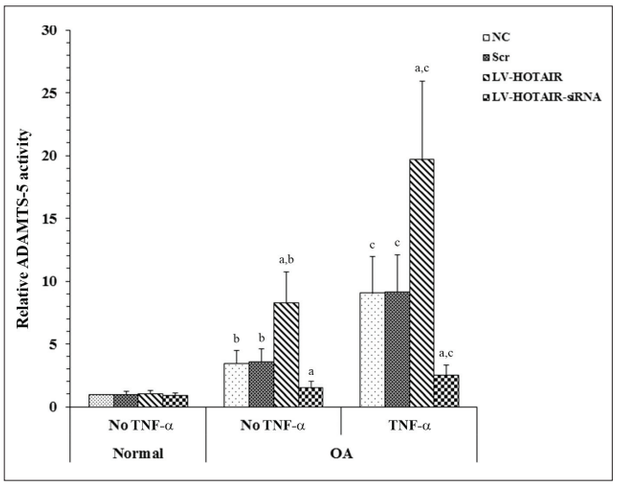

Fig. 4: Effect of HOTAIR on ADAMTS-5 activities of human normal and OA articular chondrocytes with or without overexpression or knockdown of HOTAIR in the presence and absence of TNF-α. Human normal and OA articular chondrocytes were transduced with human HOTAIR lentivirus (LV-HOTAIR) or human HOTAIR siRNA lentivirus (LV-HOTAIR-siRNA) to overexpress or knock down HOTAIR. Nontransduced cells (NC) and cells transduced with the scrambled siRNA lentivirus (Scr) were used as controls. The cells were cultured in the presence or absence of 60 ng/mL of TNF-α for 24 h. Then the proteolytic activity of ADAMTS-5 in the cell culture media were measured with an ELISA kit (Abnova) and expressed as fold changes to that of NC in Normal/No TNF-α (designated as 1). [a]$p<0.05$ vs. NC and Scr; [b]$p<0.05$ vs. Normal; [c]$p<0.05$ vs. OA/No TNF-α.

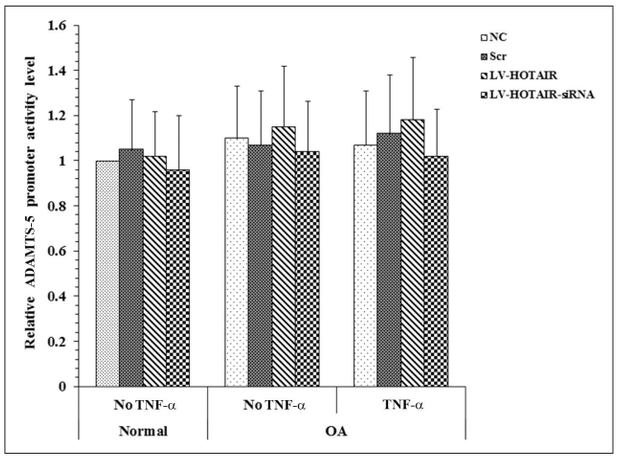

Fig. 5: Effect of HOTAIR on ADAMTS-5 gene promoter activities in human normal and OA articular chondrocytes with or without overexpression or knockdown of HOTAIR in the presence and absence of TNF-α. Human normal and OA articular chondrocytes were transduced with human HOTAIR lentivirus (LV-HOTAIR) or human HOTAIR siRNA lentivirus (LV-HOTAIR-siRNA) to overexpress or knock down HOTAIR. Nontransduced cells (NC) and cells transduced with the scrambled siRNA lentivirus (Scr) were used as controls. The cells were transfected with human ADAMTS-5 gene promoter/luciferase reporter plasmids (Switchgear Genomics) and then cultured in the presence or absence of 60 ng/mL of TNF-α for 24 h. Luciferase activities were expressed as fold changes to that of NC in Normal/No TNF-α (designated as 1). [a]$p<0.05$ vs. NC and Scr; [b]$p<0.05$ vs. Normal; [c]$p<0.05$ vs. OA/No TNF-α .

by 67%, bringing it down to the basal level in normal articular chondrocytes (Fig. 2). While TNF-α showed no significant effect on the ADAMTS-5 mRNA level in normal articular chondrocytes, it increased the basal ADAMTS-mRNA level by approximately threefold in OA articular chondrocytes, which was mostly abolished (~75%) by knocking down HOTAIR (Fig. 2).

Western blot analyses with an antibody targeting the amino terminal end of the prodomain of ADAMTS-5 (Cat. No. ab45042; Abcam) confirmed that while HOTAIR showed no significant effect on the ADAMTS-5 protein level in normal articular chondrocytes, HOTAIR overexpression and knockdown respectively increased and decreased the ADAMTS-5 protein level by 2.9-fold and by 59% in OA articular chondrocytes (Fig. 3). TNF-α increased the basal ADAMTS-5 protein level by 3.2-fold in OA articular chondrocytes, which was mostly abolished (~77%) by knocking down HOTAIR (Fig. 3). ELISA assays were next performed to measure the proteolytic activity of ADAMTS-5 in the cell culture media (Fig. 4). In agreement with the above changes in the expression of ADAMTS-5, HOTAIR overexpression and knockdown increased and decreased the ADAMTS-5 activity by approximately 3.5-fold and by 80% in OA articular chondrocytes, while showing no significant effect on the ADAMTS-5 activity in normal articular chondrocytes (Fig. 4). TNF-α increased the ADAMTS-5 activity by approximately 2.6-fold in OA articular chondrocytes, which was mostly abolished (~72%) by knocking down HOTAIR (Fig. 4).

Taken together, these findings indicated that HOTAIR could potently promote the expression/activity of ADAMTS-5 in human OA articular chondrocytes; the effect was strongly enhanced by TNF-α.

2.2. HOTAIR increased ADAMTS-5 mRNA stability in human OA articular chondrocytes

To examine whether HOTAIR could trans-activate the ADAMTS-5 gene promoter in human articular chondrocytes, we transfected a human ADAMTS-5 gene promoter/luciferase reporter into human normal and OA articular chondrocytes. As shown in Fig. 5, luciferase reporter assays revealed that neither overexpression/knockdown of HOTAIR nor TNF-α showed significant effect on the ADAMTS-5 gene promoter in OA articular chondrocytes, suggesting that HOTAIR and TNF-α did not promote the expression of ADAMTS-5 in human OA articular chondrocytes at the gene promoter/transcription level.

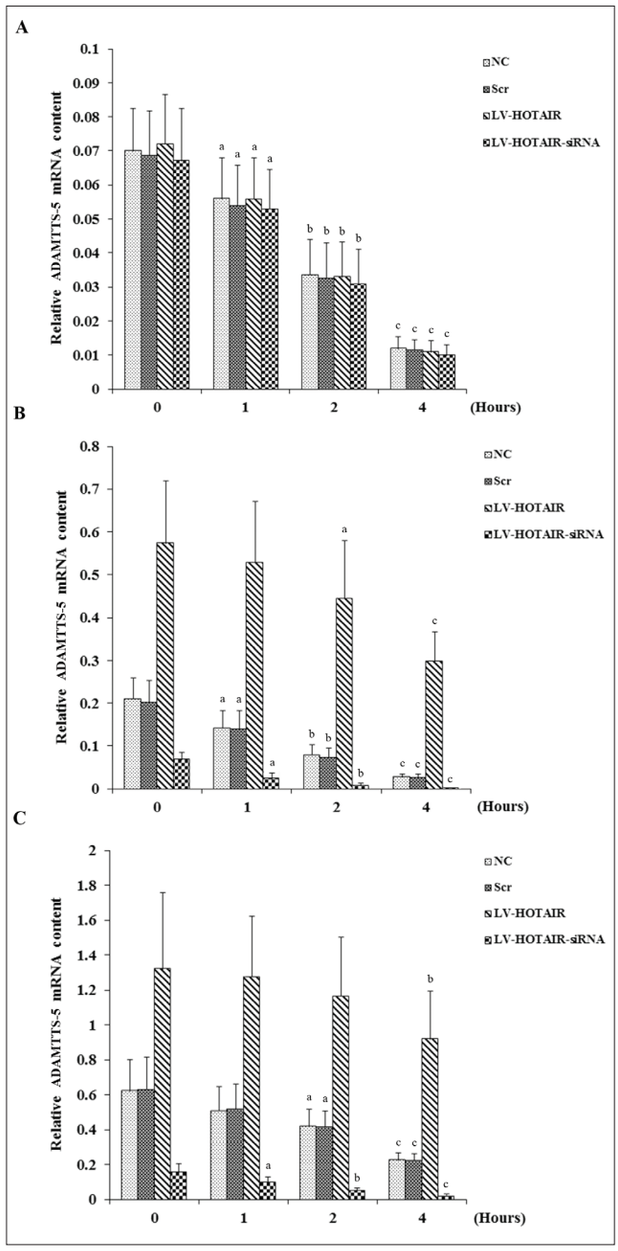

Fig. 6: Effect of HOTAIR on ADAMTS-5 mRNA stability in human normal and OA articular chondrocytes with or without overexpression or knockdown of HOTAIR in the presence and absence of TNF-α. Human normal and OA articular chondrocytes were transduced with human HOTAIR lentivirus (LV-HOTAIR) or human HOTAIR siRNA lentivirus (LV-HOTAIR-siRNA) to overexpress or knock down HOTAIR. Nontransduced cells (NC) and cells transduced with the scrambled siRNA lentivirus (Scr) were used as controls. The cells were cultured in the presence or absence of 60 ng/mL of TNF-α for 24 h. Then the cells were pre-treated with transcription inhibitor actinomycin D (1 mg/mL) for 30 min and then cultured in media containing actinomycin D (1 mg/mL) for 1, 2 and 4 h. The mRNA levels of ADAMTS-5 were determined with real-time quantitative RT-PCR at 1, 2 and 4 h of actinomycin D treatment. Relative quantification of the ADAMTS-5 mRNA level was normalized against that of GAPDH in the same sample. The arrangement of experimental groups was as follows: (*A*) normal articular chondrocytes without TNF-α treatment; (*B*) OA articular chondrocytes without TNF-α treatment; (*C*) OA articular chondrocytes with TNF-α treatment. [a]$p<0.05$ vs. 0 h; [b]$p<0.05$ vs. 1 h; [c]$p<0.05$ vs. 2 hours.

We next examined the effect of HOTAIR on the stability of ADAMTS-5 mRNA in human articular chondrocytes. As shown in Fig. 6*A*, in normal articular chondrocytes, HOTAIR showed no significant effect on the stability of ADAMTS-5 mRNA; the ADAMTS-5

mRNA level was similar in all experimental groups over time (Fig. 6*A*). In OA articular chondrocytes, however, HOTAIR overexpression and knockdown respectively increased and decreased the ADAMTS-5 mRNA stability: The ADAMTS-5 mRNA levels as compared to the control level (0 hour of actinomycin D treatment; designated as 100%) at 1, 2 and 4 hours were approximately 67%, 38% and 13% in the controls, 91%, 76% and 52% in cells with HOTAIR overexpression, and 39%, 13% and 4% in cells with HOTAIR knockdown, respectively (Fig. 6*B*). As shown in Fig. 6*C*, TNF-α enhanced the protective effect of HOTAIR on the ADAMTS-5 mRNA stability: The ADAMTS-5 mRNA levels as compared to the control level (0 hour of actinomycin D treatment; designated as 100%) at 1, 2 and 4 hours were approximately 82%, 66% and 36% in the controls, 96%, 88% and 70% in cells with HOTAIR overexpression, and 63%, 33% and 12% in cells with HOTAIR knockdown, respectively.

3. Discussion

A key feature of OA is cartilage breakdown mediated by complex interactions between cytokines (e.g. TNF-α) and proteases (e.g. matrix metalloproteinases) (Porter et al. 2005). Proteolysis of the major constituents of the cartilage matrix is achieved not only by the classical matrix metalloproteinase family, but also by the relatively new aggrecanase/ADAMTS family of metalloproteinase (Xue et al. 2013). It has been shown that inhibiting the expression of ADAMTS-5 ameliorates cartilage degradation, suggesting ADAMTS-5 as an effective target for OA treatment (Glasson et al. 2005; Stanton et al. 2005; Chu et al. 2013). A recent study has identified HOTAIR and ADAMTS-5 as the most upregulated lncRNA and the most upregulated gene, respectively, in human OA cartilage compared with normal cartilage (Xing et al. 2014). In this study, we have demonstrated that HOTAIR promotes the expression of ADAMTS-5 in human OA articular chondrocytes.

We used human OA articular chondrocytes as a cell model, with human normal articular chondrocytes as a control. The OA articular chondrocytes were stimulated with TNF-α, which is an established key mediator for cartilage degradation (Xue et al. 2013). It has been reported that TNF-α promotes expression of cytokines in chondrocytes, and its presence is correlated with a loss of aggrecan (Xue et al. 2013). TNF-α is produced by many cell types including chondrocytes in response to inflammation, infection, and other environmental stresses (Moos et al. 2000; Xue et al. 2013); its receptor TNFR1 is upregulated in OA chondrocytes (Westacott et al. 1994). We noted that in parallel to a higher basal expression level of HOTAIR, OA articular chondrocytes also had a higher basal expression level of ADAMTS-5 than normal articular chondrocytes; overexpression and knockdown of HOTAIR markedly increased and decreased the expression/activity of ADAMTS-5, respectively, in OA but not normal articular chondrocytes. The findings suggest that HOTAIR is critical for the elevated expression of ADAMTS-5, which is functionally manifested by its elevated proteolytic activities, in OA articular chondrocytes; nevertheless, the presence of other factor(s) in OA articular chondrocytes is required for HOTAIR to take effect. The collaborating factors could be cytokines upregulated in OA articular chondrocytes, since the majority of cytokines known to be involved in cartilage metabolism, such as IL-1β and TNF-α, are synthesized by the chondrocytes themselves and upregulated in OA cartilage (Moos et al. 2000). This is supported by our finding that exogenous TNF-α enhanced the promoting effect of HOTAIR on the expression of ADAMTS-5 in OA articular chondrocytes; at least part of the effect was attributable to TNFα-induced expression of HOTAIR. However, as overexpression of HOTAIR in the presence of TNF-α did not increase the expression of ADAMTS-5 in normal articular chondrocytes, other cytokines/factors in addition to TNF-α should also be required for HOTAIR to promote ADAMTS-5 expression in OA articular chondrocytes. It is noteworthy that knockdown of HOTAIR abolished the elevated expression of ADAMTS-5 in OA articular chondrocytes in the presence or absence of TNF-α, suggesting that HOTAIR is critical for the elevated expression of ADAMTS-5 in OA articular chondrocytes. Therefore, HOTAIR could be a new therapeutic target for ADAMTS-5 inhibition, which reportedly is preferential over ADAMTS-4 inhibition in treatment of human OA cartilage (Larkin et al. 2015).

Showing no significant effect on the ADAMTS-5 gene promoter activity, HOTAIR markedly increased the stability of ADAMTS-5 mRNA in OA articular chondrocytes; the effect was enhanced by TNF-α. This provides a mechanistic explanation for the promoting effect of HOTAIR and TNF-α on the expression of ADAMTS-5 in human OA articular chondrocytes. How HOTAIR increases the ADAMTS-7 mRNA stability in OA articular chondrocytes is still unclear. LncRNAs are localized in the nucleus or cytoplasm and involve in regulating gene expression through epigenetic mechanisms including chromatin remodeling, regulation of splicing, and by acting as sponges for microRNAs (Hu et al. 2012; Mercer and Mattick 2013). Are any of these mechanisms involved in the regulatory effect of HOTAIR on the ADAMTS-5 mRNA stability? This, and the aforementioned potential involvement of multiple cytokines in the promoting effect of HOTAIR on ADAMTS-5 expression in human OA articular chondrocytes, will be explored in our future studies.

HOTAIR was originally found to control tumor cell apoptosis, growth and metastasis in a variety of cancers (Bhan and Mandal 2015). Recent studies have suggested that ADAMTS-5 plays a critical role in tumor progression in laryngeal and colorectal cancers (Filou et al. 2013, 2015). Therefore, it will also be intriguing to investigate the potential role of HOTAIR/ADAMTS-5 signaling in tumor progression and metastasis in the field of cancer research.

In conclusion, this study provides the first evidence supporting that HOTAIR strongly promotes the expression/activity of ADAMTS-5 by increasing its mRNA stability in human OA articular chondrocytes; this effect is enhanced by TNF-α. It adds new insights into the pathogenesis of OA and suggests that HOTAIR could be a new therapeutic target for ADAMTS-5 inhibition in human OA cartilage.

4. Experimental

4.1. Chondrocyte culture and treatments

Primary human normal (Cat. No. 402-05a) and OA (Cat. No. 402OA-05a) articular chondrocytes and chondrocyte growth medium (cat. no. 411-500) were purchased from Cell Applications Inc. (San Diego, CA, USA). The cells were grown in chondrocyte growth medium (Cell Applications Inc.) supplemented with 5% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) and 100 U/mL penicillin-streptomycin (Sigma-Aldrich, Beijing, China) in an incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The cells were cultured in the presence or absence of 60 ng/mL of TNF-α (Sigma-Aldrich) for 24 h.

4.2. Lentiviral transduction

Human HOTAIR lentiviral vector (Cat. No. 183370; Applied Biological Materials Inc., Richmond, BC, Canada) was transfected with the packaging vectors psPAX2 and pMD2.G into 293T cells by calcium chloride to produce the lentivirus to transduce human normal and OA articular chondrocytes. Human HOTAIR siRNA lentivirus (Cat. No. iV009919) and scrambled siRNA lentivirus (Cat. No. LVP015) were purchased from Applied Biological Materials Inc. and directly used to transduce human normal and OA articular chondrocytes. Six hours after lentiviral transduction, cells were subject to subsequent experiments.

4.3. Real-time quantitative RT-PCR and measurement of ADAMTS-5 mRNA stability

Total RNA was isolated using miRNeasy kits (Qiagen, Beijing, China) according to the manufacturer's protocol. For measuring the expression level of HOTAIR, 1000 ng of total RNA was reverse transcribed using MuIV reverse transcriptase (Life Technologies) and random hexamer primers (Thermo Scientific, Waltham, MA) in a 20-μL reaction. cDNA was used as template for quantitative real-time RT-PCR using Fast SYBR Green (Applied Biosystems, Forster City, CA, USA) and an Applied Biosystems StepOnePlus machine. Human GAPDH mRNA was used for normalization. The primers used are as follows: for HOTAIR, 5′-GCAGTGGAATGGAACGGATT-3′ (forward) and 5′-CGTGGCATTTCTGGTCTTGTA-3′ (reverse); for ADAMTS-5, 5′-GCAACGTCAAGGCTCCTCTT -3′ (forward) and 5′-CTCCACAAATCTACTCAGTGAAGCA-3′ (reverse); for GAPDH, 5′-GACTCATGACCACAGTCCATGC-3′ (forward) and 5′-AGAGGCAGGGATGATGTTCTG-3′ (reverse). For measurement of the ADAMTS-5 mRNA stability, cells were cultured in the presence or absence of 60 ng/mL of TNF-α (Sigma-Aldrich) for 24 h. Then the cells were pre-treated with transcription inhibitor actinomycin D (1 mg/mL) (Sigma-Aldrich) for 30 min and then cultured in media containing actinomycin D (1 mg/mL) for 1, 2 and 4 h. The mRNA levels of ADAMTS-5 were determined with real-time quantitative RT-PCR at 1, 2 and 4 hours of actinomycin D treatment. Relative quantification of the ADAMTS-5 mRNA level was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and normalized against that of GAPDH in the same sample.

4.4. Western blot analysis

Whole cell lysates were extracted by incubating the cells with lysis buffer (50 mM Tris/HCl pH 7.2, 150 mM NaCl, l% (v/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.01% (v/v) aprotinin, 4 µg/ml pepstatin A, 10 µg/ml leupeptin and 1 mM phenylmethanesulfonyl fluoride; all purchased from Sigma-Aldrich) on ice for 30 min and removing cell debris by centrifugation at 2000 *g* for 15 min at 4 °C. Equal amount of proteins for each sample were separated by 10% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride microporous membrane (Millipore, Hong Kong, China). The membranes were blocked with 5% skim milk powder in TBS-T for 2 hours and incubated for 1 h with a 1:500 dilution of rabbit anti-human ADAMTS-5 polyclonal antibody (Cat. No. ab45042; Abcam, Shanghai, China) or a 1:1000 dilution of mouse anti-human GAPDH monoclonal antibody (Cat. No. sc-32233; Santa Cruz Biotechnology, Dallas, TX, USA), and then washed and revealed using bovine anti-rabbit (Cat. No. sc-2370; Santa Cruz Biotechnology) or anti-mouse (Cat. No. sc-2371; Santa Cruz Biotechnology) secondary antibody (1:5000, 1 hour). Peroxidase was revealed with an ECL kit (GE Healthcare, Shanghai, China). Three independent experiments were performed.

4.5. ADAMTS-5 activity assay

The ADAMTS-5 activity was assayed in the cell culture media using an ELISA-based Aggrecanase Activity Assay kit (Cat. No. KA1497; Abnova, Taipei, Taiwan) following the manufacturer's protocol. In brief, enzyme activity was assessed by proteolysis of a recombinant fragment of human aggrecan interglobular domain (provided with this kit) incubated with standard aggrecanase (in this case, recombinant human truncated ADAMTS-5; Cat. No. CC1034; Millipore) and the test samples. This proteolytic cleavage releases an ARGSVIL-peptide, which is then quantified by ELISA at 450 nm in a microtiter plate spectrophotometer (Cat. No. 168-1150; Bio-Rad Laboratories, Beijing, China). By the time of the assays, viable cells were counted using trypan blue and a hemocytomter as previously described (Doyle et al. 1995). The measured ADAMTS-5 concentration in the assay was then normalized against the number of viable cells (per 10^4 viable cells) to exclude the effect of TNF-α on chondrocyte proliferation and survival.

4.6. Luciferase reporter assay

Cells were transfected with a commercially available human ADAMTS-5 promoter/luciferase reporter (Cat. No. S705424; SwitchGear Genomics, Shanghai, China) using Lipofectamine 2000 transfection reagent (Life Technologies) for 6 h and then treated with or without TNF-α (60 ng/mL) (Sigma-Aldrich) for 24 h. Luciferase assays were performed with the LightSwitch Luciferase Assay Kit (Cat. No. LS010; SwitchGear Genomics) according to the manufacturer's protocol. Plasmid PRL-CMV (Promega; Madison, WI, USA) encoding *Renilla reniformis* luciferase (at one fifth molar ratio to the reporter plasmid) was co-transfected with the reporter plasmid in each transfection as an internal control for data normalization.

4.7. Statistical analysis

Statistical analyses were performed with SPSS for Windows 19.0 (IBM, Chicago, IL, USA). All continuous variable values were expressed as Mean±SD. Comparisons of means among multiple groups were performed with one-way ANOVA followed by *post hoc* pairwise comparisons using Tukey's tests. $p<0.05$ was considered statistically significant in this study.

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Conflicts of interes: None declared.

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