Long non-coding RNA-GAS5 acts as a tumor suppressor in bladder transitional cell carcinoma via regulation of chemokine (C-C motif) ligand 1 expression

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Abstract. Long non-coding RNAs (lncRNAs) have important roles in diverse biological processes, including transcriptional regulation, cell growth and tumorigenesis. The present study aimed to investigate whether lncRNA-growth arrest-specific (GAS)5 regulated bladder cancer progression via regulation of chemokine (C-C) ligand (CCL)1 expression. The viability of BLX bladder cancer cells was detected using a Cell Counting kit-8 assay, and cell apoptosis was assessed by annexin V-propidium iodide double-staining. The expression levels of specific genes and proteins were analyzed by reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. In addition, cells were transfected with small interfering (si)RNAs or recombinant GAS5 in order to silence or overexpress GAS5, respectively. The results of the present study demonstrated that knockdown of GAS5 expression promoted bladder cancer cell proliferation, whereas overexpression of GAS5 suppressed cell proliferation. Furthermore, knockdown of GAS5 resulted in an increased percentage of cells in S and G2 phase, and a decreased percentage of cells in G1 phase. In addition, the present study performed a hierarchical cluster analysis of differentially expressed lncRNAs in bladder cancer cells and detected that CCL1 overexpression resulted in an upregulation of GAS5, which may improve the ability of cells to regulate a stress response *in vitro*. Furthermore, knockdown of GAS5 expression increased the mRNA and protein expression of CCL1 in bladder cancer cells. Gain-of-function and loss-of-function studies demonstrated that GAS5 was able to inhibit bladder cancer cell proliferation, at least in part, by suppressing the expression of CCL1. The results of the present study demonstrated that GAS5 was able to suppress bladder cancer cell proliferation, at least partially, by suppressing the expression of CCL1. The results of the present study may provide a basis for developing novel effective treatment strategies against bladder cancer.

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Introduction

Bladder cancer is a common malignancy with high rates of relapse, metastasis and mortality (1). An estimated 72,570 novel cases of bladder cancer were diagnosed, and ~15,210 patients succumbed to bladder cancer in the United States in 2013 (1). In China, bladder cancer represents 3.2% of all malignant tumors (2). Histologically, >95% of bladder tumors are derived from the urothelium. Bladder cancer can be classified as either non-muscle-invasive bladder carcinoma or muscle-invasive bladder carcinoma, and the majority of newly diagnosed patients present with non-muscle-invasive bladder carcinoma. In ~2/3 of patients with non-muscle-invasive bladder carcinoma, recurrence of the cancer is observed after initial management, and 5-30% of these cancers are transformed into muscle-invasive bladder carcinoma, for which the five-year survival rate is ~50% (3).

Eukaryotic genomes encode numerous long non-coding RNAs (lncRNAs), which are defined as endogenous cellular RNAs with >200 nucleotides that lack open reading frames of significant length (4). Within four years, the number of identified lncRNAs has increased to >8,000 (5). Although the function of the majority of lncRNAs is currently elusive, a rapidly increasing number of studies have provided accumulating evidence for their involvement in numerous biological processes, supporting the hypothesis that dysregulation of lncRNAs may be associated with cancer development, as well as invasion and metastasis of malignant cells (5-7).

In human urothelial carcinoma of the bladder, a gene microarray analysis of the lncRNA expression profile detected 1,122 differentially expressed (≥2-fold) lncRNAs; of these, 734 lncRNAs were upregulated and 388 were downregulated (8). Urothelial cancer associated-1 (9), linc-upregulated in bladder cancer 1 (10) and metastasis-associated lung adenocarcinoma transcript-1 (11,12) have been shown to be upregulated in invasive bladder cancer tissues. Conversely, maternally expressed gene-3 (13) and growth arrest-specific 5 (GAS5) (14) were found to be downregulated in bladder cancer tissues as compared with adjacent normal tissues. lncRNA-GAS5 has recently been identified to be involved in

the tumorigenesis of various types of cancer, including breast cancer (15), lung cancer (16), hepatocellular carcinoma (17), and renal cancer (4). Overexpression of GAS5 has been shown to reduce the rate of cell cycle progression, whereas downregulation of GAS5 inhibited apoptosis and accelerated cell cycle progression. Furthermore, overexpression of GAS5 has been reported to induce growth arrest and apoptosis, independent of other stimuli (14). To date, the underlying mechanisms of the effects of GAS5 on the regulation of cancer cell apoptosis have remained to be fully elucidated.

Although the effects of GAS5-induced tumor apoptosis have been studied in certain cancer types, the role of GAS5 in the apoptosis of bladder transitional cell lines and the possible involvement of chemokine (C-C) ligand 1 (CCL1) expression have largely remained elusive. Therefore, the aim of the present study was to investigate the involvement of GAS5 on the apoptosis of a bladder transitional cell line (BLX).

Materials and methods

Cell culture. The BLX human bladder cancer cells were obtained from the Cell Resource Center, Shanghai Institutes for Biological Sciences (Shanghai, China), and maintained in RPMI-1640 (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37˚C in a humidified incubator (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 5% CO_2 and 95% air. The medium was replaced every day.

Transfection. The small interfering (si)RNAs specific for human GAS5 and CCL1, and scramble siRNA were obtained from Dharmacon (Lafayette, CO, USA). The siRNA sequences were as follows: GAS5 forward, 5'-CACUCUGAGUGGGAC AAGCUCUUCA-3' and reverse, 5'-UGAAGAGCUUGUCCC ACUCAGAGUG-3'; CCL1 forward, 5'-GCUAUCAGUCCA CUGUGCUUGUGGU-3' and reverse, 5'-ACCACAAGCACA GUGGACUGAUAGC-3', and scramble forward. 5'-CACGAG UGGGUAACACUCGUCUUCA-3' and reverse, 5'-UGAAGA CGAGUGUUACCCACUCGUG-3'. The BLX cells were plated in 6-well plates (40, 000 cells/well). Following 24 h incubation, the cells were transfected with GAS-siRNA and CCL1-siRNA, respectively, using Lipofectamine 2000 (Invitrogen Life Technologies), according to the manufacturer's instructions. The final siRNA concentration was 100 nM. Untreated cells and scramble siRNA-transfected cells were used as a negative control (NC) and Mock group, respectively.

To overexpress GAS5 and CCL1, the pcDNA-GAS5 and pcDNA-CCL1 plasmids were constructed by introducing a *Kpn*I-*Xho*I fragment containing GAS5 and CCL1 cDNA into the same sites in a pcDNA3.1 plasmid. The GAS5 and CCL1 genes were amplified from cDNA prepared from BLX cells by polymerase chain reaction (PCR) using the following forward and reverse primers: GAS5 forward, 5'-CACUCU GAGUGGGACAAGCUCUUCA-3' and reverse, 5'-UGA AGAGCUUGUCCCACUCAGAGUG-3'; and CCL1 forward, 5'-GCUAUCAGUCCACUGUGCUUGUGGU-3' and reverse, 5'-ACCACAAGCACAGUGGACUGAUAGC-3'. The empty pcDNA3.1 vector was used as a control. The BLX cells were transfected with pcDNA-GAS5 and pcDNA-CCL1 plasmids (2 μg/1.5x1,000,000 cells) respectively, using Lipofectamine 2000 (Invitrogen Life Technologies), according to the manufacturer's instructions.

Assessment of cell viability using Cell Counting kit (CCK)8. Bladder cancer cells were plated ($5.0x10^3$/well) and treated in 96-well plates (three wells per group) with various stimuli for 24, 48 or 72 h. Subsequently, 10 μl CCK8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to the cells, and the optical density value of the cells was measured at 450 nm using an ELX 808 Ultra microplate reader (BioTek Instruments, Inc., Winooski, VT, USA), according to the manufacturer's instructions.

Quantification of apoptosis by flow cytometry. Apoptosis was assessed using annexin V, a protein that binds to phosphatidylserine (PS) residues, which are exposed on the cell surface of apoptotic cells. The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). The BLX cells were plated ($5.0x10^5$/well, 1 ml) and treated in six-well plates (three wells per group) with various stimuli for 48 h. After treatment, the cells were washed twice with phosphate-buffered saline (pH 7.4), and re-suspended in staining buffer containing 10 μl propidium iodide (PI) and 5 μl annexin V-FITC Double-staining was performed at room temperature for 15 min in the dark prior to flow cytometric analysis. The cells were immediately analyzed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and CellQuest pro software version 5.2 (BD Biosciences, San Jose, CA, USA). Quantitative analysis of apoptotic cells was performed using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling method, which examines DNA-strand breaks during apoptosis, using the BD ApoAlert™ DNA Fragmentation Assay kit (cat. no. 630107; BD Biosciences). Briefly, the cells were trypsinized, fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After being washed, the cells were incubated with the reaction mixture for 60 min at 37˚C. The stained cells were then analyzed using a flow cytometer.

Reverse transcription-quantitative (RT-q)PCR. The bladder cancer cells were plated ($5.0x10^5$/well) in six-well plates (three wells per group) and after 24 h, cells were transfected with GAS5-siRNAs for 48 h. Total RNA was extracted from the bladder cancer cells using TRIzol® (Invitrogen Life Technologies), according to the manufacturer's instructions. Synthesis of cDNA was performed by RT with 2 μg total RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega Corp. Madison, WI, USA) with oligo dT (15) primers (Fermentas, Thermo Fisher Scientific), according to the manufacturer's instructions. qPCR was performed using a SYBR Green PCR kit (Toyobo Co., Ltd., Osaka, Japan) on an ABI 7300 Real Time PCR system (Applied Biosystems, Life Technologies, Thermo Fisher Scientific), according to the manufacturer's instructions of the kit. The PCR cycling conditions were as follows: 2 min polymerase activation at 95˚C, followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 55˚C for 60 sec,and extension at 72˚C for 20 sec.

The sequences of the primers were as follows: GAS5 forward, 5'-TTGCGATTCTGTTTTGTGCT-3' and reverse, 5'-GTG GGGTCCTCAGTGGG-3'; and β-actin, forward, 5'-ACAGGG GAGGTGATAGCATT-3' and reverse, 5'-GACCAAAAGCCT TCATACATCTC-3'. β-actin was used as an internal control to normalize the data and to determine the relative expression levels of the target genes. Following completion of the reaction, the amplification curve and melting curve were analyzed. Gene expression values were determined using the $2^{-\Delta\Delta CT}$ method (17).

Western blot analysis. For the whole cell extracts, the bladder cancer cells were washed with ice-cold phosphate-buffered saline. The cell pellets were homogenized in extraction buffer, containing 8 M urea, 10% glycerol, 10 mM Tris-HCl (pH 6.8), 1% SDS, 5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 10 μg/ml pepstatin and 10 μg/ml leupeptin]. Protein was extracted using NP-40 lysis buffer (cat. no. FNN0021; Thermo Fisher Scientific). Protein concentration was determined using a Pierce BCA protein assay (Pierce Biotechnology, Inc., Rockford, IL, USA). Subsequently, the samples were boiled for 5-10 min and centrifuged at 12,000 x g for 10 min in order to obtain the supernatant. Aliquots of 50 μg protein were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Following saturation with 5% (w/v) non-fat dry milk in Tris-buffered saline with 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with monoclonal rat anti-CCL1 (cat. no. sc-74092; Santa Cruz Biotechnology, Inc., Dallas, TX, USA.) at dilutions of 1: 1,000 at 4˚C overnight. After three washes with TBST, the membranes were incubated at 37˚C for 1 h with secondary antibodies conjugated to IRDye® 800 CW Infrared Dye (LI-COR Biosciences, Lincoln, NE, USA), including polyclonal donkey anti-goat IgG (cat. no. A-11056; Thermo Fisher Scientific) and polyclonal donkey anti-mouse IgG (cat. no. A-21202; Thermo Fisher Scientific) at dilutions of 1:10,000. The membranes were then washed three times with TBST. The blots were visualized using the Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE, USA). Signals were densitometrically assessed (Odyssey Application Software version 3.0; LI-COR Biosciences) and normalized to the β-actin signals, in order to correct for unequal loading, using a mouse monoclonal anti-β-actin antibody (cat. no. AP0060; 1:1,000; Bioworld Technology, Inc., St. Louis Park, MN, USA).

Bioinformatics analysis. The total RNA from the CCL1-none (DMSO) and CCL1 transfer (CCL1) groups were individually hybridized with gene chips of the mouse lncRNA microarray V2.0 (8x 60K; Arraystar). Briefly, RNA was purified from 1 μg total RNA following removal of rRNA. The RNA was then transcribed into fluorescent cRNA with the entire length of the transcripts, without 3' bias, using random primers. The labeled cRNAs were hybridized to the gene microarray. Finally, an Agilent Scanner (G2505B; Agilent Technologies, Inc., Santa Clara, CA, USA) was used to scan the arrays and the array results were analyzed using Agilent Feature Extraction software (version 10.7.3.1). The GeneSpring GX v11.5.1 software package (Agilent Technologies, Inc.) was used to analyze the subsequent data processing. Microarray hybridization was performed by Kangchen Biology Engineering Co., Ltd. (Shanghai, China). Differentially expressed lncRNAs of statistical significance were identified through Volcano Plot filtering. The threshold used to identify upregulated or downregulated RNAs was a fold-change >2.0 (P<0.05). The resulting differentially expressed genes were subjected to hierarchical clustering (Cluster 3.0) and TreeView analysis (Stanford University, Stanford, CA, USA).

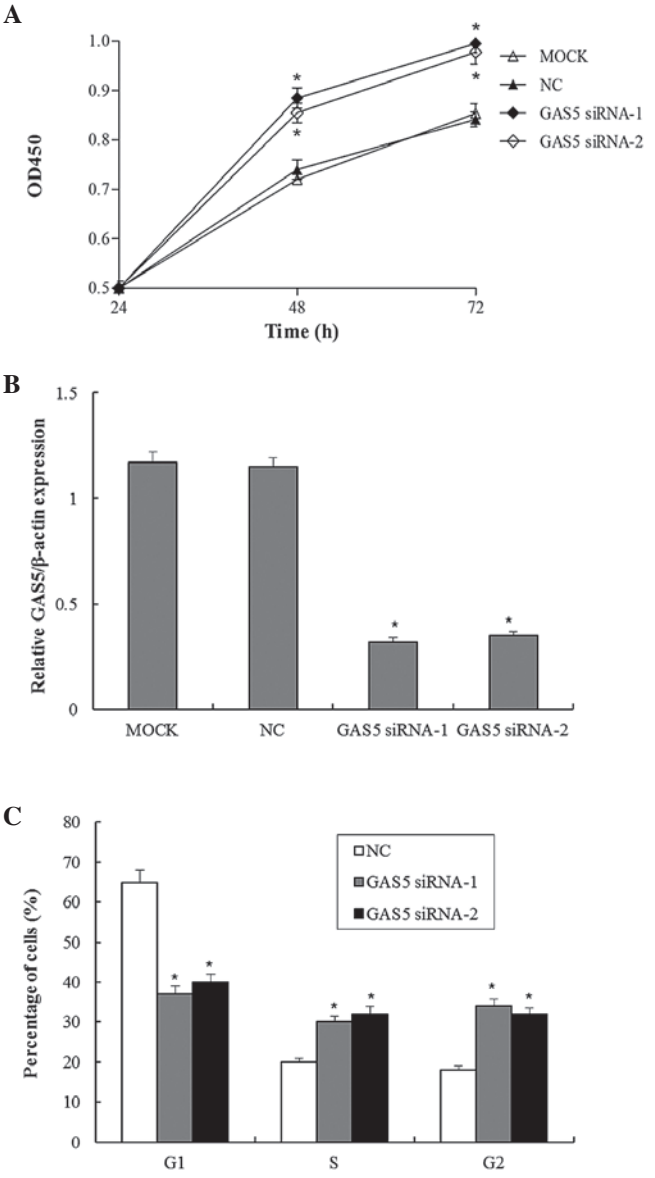

Figure 1. GAS5 inhibits bladder cancer cell proliferation. (A) Bladder cancer cells were transfected with GAS5-siRNA for 24, 48 or 72 h, and cell viability was examined using the Cell Counting kit-8 assay. (B) Bladder cancer cells were transfected with GAS5-siRNA, and the GAS5 expression levels were detected after 48 h of incubation by reverse transcription-quantitative polymerase chain reaction. (C) Bladder cancer cells were transfected with GAS5-siRNA, and following 48 h of incubation, the relative number of cells in each cell cycle phase was detected by propidium iodide staining and fluorescence-activated cell sorting analysis. Values are expressed as the mean ± standard error of the mean (n=3/group). *P<0.05, compared with the control. OD, optical density; NC, negative control; Mock, scramble siRNA-transfected group; GAS5, growth arrest specific 5; GAS5 siRNA-1 and GAS5 siRNA-2, transfected with GAS5-small interfering RNA.

Statistical analysis. Values are expressed as the mean ± standard error of the mean for each group. All statistical analyses

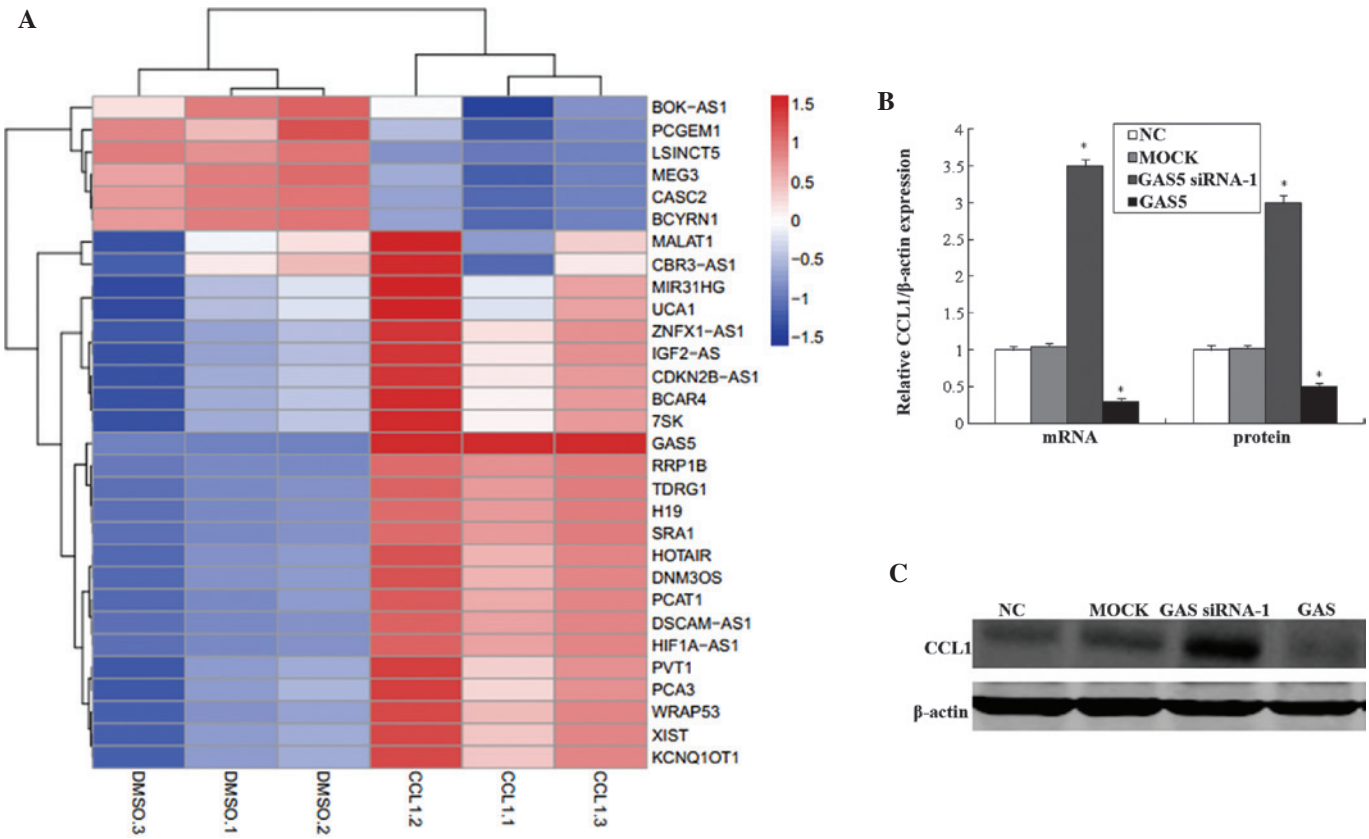

Figure 2. GAS5 regulates the expression of CCL1. (A) Hierarchical clustering of differentially expressed lncRNAs in CCL1-none groups (DMSO.1, DMSO.2 and DMSO.3) and CCL1-transfer groups (CCL1.1, CCL1.2 and CCL1.3). (B and C) Bladder cancer cells were transfected with GAS5-siRNA or pcDNA-GAS5, and the expression levels of CCL1 were examined following 48 h of incubation by reverse transcription-quantitative polymerase chain reaction and western blotting. Values are expressed as the mean ± standard error of the mean (n=3/group). *P<0.05, compared with the control. NC, negative control; Mock, scramble siRNA-transfected group; CCL1, chemokine (C-C) ligand 1; LncRNA, long non-coding RNA; GAS5, growth arrest-specific 5; siRNA, small interfering RNA;. DMSO, treated with dimethyl sulfoxide; CCL1, transfected with CCL1-siRNA group; GAS5 siRNA-1, transfected with GAS5-siRNA; GAS5, transfected with pcDNA-GAS5 plasmid vector.

were performed using GraphPad Prism version 4.0 (GraphPad Software, Inc., La Jolla, CA, USA). Inter-group differences were analyzed by one-way analysis of variance, followed by Tukey's multiple comparison test as a post-hoc test to compare the group means if overall P<0.05. P<0.05 was considered to indicate a statistically significant difference.

Results

GAS5 inhibits bladder cancer cell proliferation in vitro. To evaluate the potential role of GAS5 in regulating bladder cancer cell proliferation, BLX cells transfected with scramble-siRNA or GAS5-siRNAs were subjected to a CCK8 cell viability assay. The viability of the BLX cells transfected with GAS5-siRNA was significantly higher as compared with that in the untransfected group or the scramble-siRNA group, and GAS5 knockdown was able to increase the cell count by ~20% in the GAS5-siRNA-transfected groups (Fig. 1A). Furthermore, the expression levels of GAS5 were decreased in the GAS5-siRNA-transfected BLX cells, demonstrating the knockdown efficiency of the siRNAs (Fig. 1B). The present study further investigated whether GAS5 knockdown was able to induce cell apoptosis and influence cell cycle progression. Following knockdown of GAS5 expression, the cell cycle distribution was analyzed by flow cytometry. As compared with the untransfected group, GAS5 knockdown resulted in an increased percentage of cells in S and G2 phase, and a decreased percentage of cells in G1 phase (Fig. 1C). This result inversely demonstrated the tumor-suppressive function of GAS5, as cancer cells may be prevented from proliferating by G1 cell cycle arrest. All of these results indicated that down-regulation of GAS5 expression in bladder cancer contributed to bladder cancer cell proliferation.

GAS5 negatively regulates CCL1 expression in vitro. The present study investigated the possible mechanisms underlying the regulation of bladder cancer cell proliferation by lncRNAs. A hierarchical cluster analysis of differentially expressed lncRNAs in bladder cancer cells was performed, which identified that CCL1 is associated with lncRNA expression (Fig. 2A). Overexpression of CCL1 was associated with lncRNA-GAS5, the expression of which was highest among the 30 lncRNAs assessed *in vitro*. This indicated that the overexpression of CCL1 significantly upregulated the expression of GAS5. CCL1 was previously shown to promote M2 macrophage and T-helper cell type 2 polarization in tumors that subvert the immune system by establishing a microenvironment of immune cells and cytokines that suppress specific anti-tumor responses (18). To further validate the interaction between GAS5 and CCL1, gene knockdown and overexpression of GAS5 were implemented.

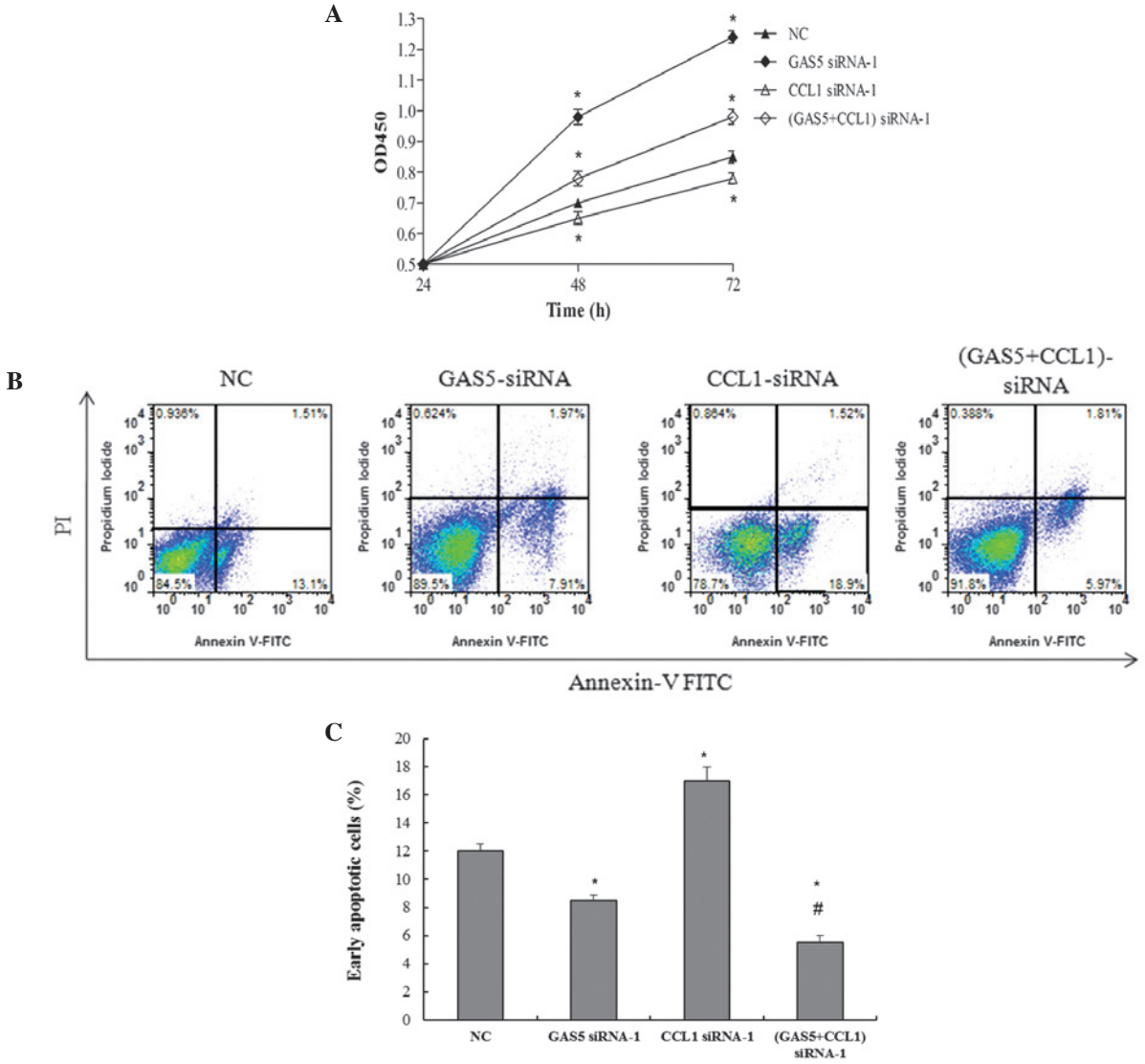

Figure 3. GAS5 inhibits bladder cancer cell proliferation, partly via regulaton of CCL1. (A) Bladder cancer cells were transfected with GAS5-siRNA and CCL1-siRNA, and cell viability was examined using the Cell Counting kit-8 assay. Apoptotic cells were detected using (B) flow cytometric analysis of annexin V/propidium iodide double staining and (C) quantitative analysis using a TUNEL assay. TUNEL-positive cells were examined using flow cytometry. Values are expressed as the mean ± standard error of the mean (n=3/group). *P<0.05, compared with the NC group; #P<0.05, compared with the GAS5-siRNA group. FITC, fluorescein isothiocyanate; OD, optical density; NC, negative control; CCL1, chemokine (C-C) ligand 1; siRNA, small interfering RNA; GAS5, growth arrest-specific 5; GAS5 siRNA-1, transfected with GAS5-siRNA; CCL1 siRNA-1, transfected with CCL1-siRNA; GAS5+CCL1 siRNA-1, co-transfected with GAS5-siRNA and CCL1-siRNA.

GAS5 knockdown markedly increased the mRNA and protein expression levels of CCL1. Conversely, overexpression of GAS5 decreased the mRNA and protein expression levels of CCL1 in bladder cancer cells (Fig. 2B and C).

GAS5 inhibits bladder cancer cell proliferation by regulating CCL1. Knockdown of GAS5 expression increased BLX cell proliferation, and an association was detected between GAS5 and CCL1; therefore, it was hypothesized that the role of GAS5 in regulating bladder cancer cell proliferation was mediated by modulating CCL1 expression. As determined by CCK8 assay, GAS5-siRNA-enhanced BLX cell proliferation was partially suppressed by co-transfection with CCL1-siRNA (Fig. 3A). In addition, BLX cell proliferation was partially suppressed by CCL1 knockout compared with that in the control group. In addition, the present study investigated whether GAS5 was able to induce cell death via an apoptotic mechanism. Annexin V-PI double-staining was used for the detection of PS externalization, which is a hallmark of early apoptosis. In line with the results of the CCK-8 assay, the proportion of early-phase apoptotic cells following transfection with GAS5-siRNA was decreased, while it was markedly increased in the group transfected with CCL1-siRNA. The apoptotic rate following co-transfection was reduced as compared with that following GAS5-siRNA transfection alone (Fig. 3B and C). In the cells overexpressing GAS5, forced expression of CCL1 was able to partially restore cell proliferation (Fig. 4A). Consistent with the results of the CCK8 assay, the proportion of early-phase apoptotic cells was increased in GAS5 overexpression, and decreased with CCL1 overexpression. The apoptotic rate of co-transfection was decreased, compared with that transfected to overexpress GAS5 only (Fig. 4B and C). These results indicated that GAS5 may

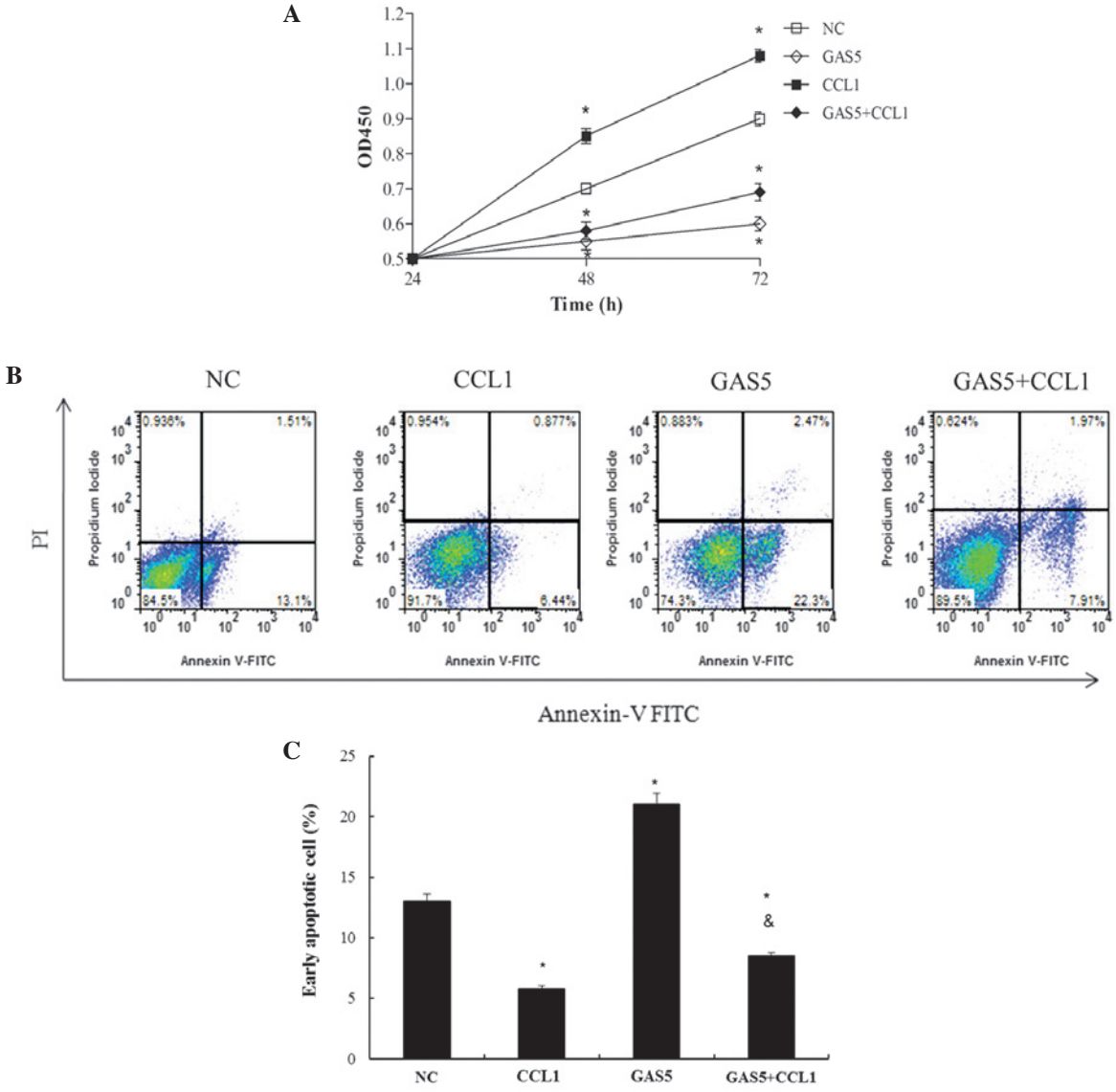

Figure 4. (A) Bladder cancer cells were transfected with GAS5- and/or CCL1-overexpression vectors and the number of cells/well was determined using the Cell Couting kit-8 assay (absorbance, 450 nm). (B) Apoptotic cells were detected by flow cytometric analysis of annexin V/propidium iodide double staining and (C) quantitative analysis using a TUNEL assay. TUNEL-positive cells were examined using flow cytometry.. Values are expressed as the mean ± standard error of the mean (n=3/group). *P<0.05, compared with the NC group; &P<0.05, compared with the GAS5 group. OD, optical density; FITC, fluorescein isothiocyanate; NC, negative control; CCL1, chemokine (C-C) ligand 1; GAS5, growth arrest-specific 5; GAS5, transfected with the pcDNA-GAS5 plasmid vector; CCL1, transfected with the pcDNA-CCL1 plasmid vector; GAS5+CCL1, co-transfected with the pcDNA-GAS5 and pcDNA-CCL1 plasmid vectors.

decrease bladder cancer progression, at least in part, by regulating CCL1 expression.

Discussion

Non-coding RNAs are the predominant transcripts in the mammalian genome, exceeding the number of protein-coding genes. It is thought that alterations in the expression of small non-coding RNAs, particularly microRNAs, may contribute to the pathogenesis of bladder cancer (19,20). A novel class of non-coding RNAs, known as lncRNAs (>200 nucleotides), which are endogenous RNA transcripts with reduced or absent protein-coding potential, have been reported to be abundantly transcribed (1). Previous studies have demonstrated the importance of lncRNAs in the tumorigenesis and metastasis of malignant cells (5,6). Downregulation of the lncRNA ncRuPAR has been shown to contribute to tumor inhibition in colorectal cancer (21), and the lncRNA TARID directs demethylation and activation of the tumor suppressor gene TCF21 via GADD45A (22).

GAS5 is a recently identified lncRNA, which is associated with cell cycle regulation (14). GAS5 has a crucial role in normal growth arrest in T-cell lines as well as non-transformed lymphocytes (23). In addition, GAS5 knockdown is associated with renal cell carcinoma (RCC) carcinogenesis and progression, whereas overexpression of GAS5 is able to act as a tumor suppressor in RCC (4). Recent clinical studies demonstrated that GAS5 is downregulated in the majority of patients with hepatocellular carcinoma and bladder cancer (14,17). However, there are few reports regarding the interaction between GAS5 and bladder cancer. The present study performed a hierarchical cluster analysis of the differentially expressed lncRNAs in the bladder

cancer cell and identified CCL1 as being associated with GAS5 expression. Based on these findings, the present study aimed to determine whether the expression of CCL1 was regulated by GAS5 in bladder cancer cell proliferation. Gain-of-function and loss-of-function studies demonstrated that GAS5 regulated cell cycle progression and inhibited bladder cancer cell proliferation, partially via regulation of CCL1 expression.

The CCL1/chemokine (C-C motif) receptor (CCR)8 axis is involved in leukemia as well as lymphoma. It has been demonstrated *in vitro* that the CCL1/CCR8 autocrine loop protects lymphoma and T-cell leukemia cells from apoptosis (24). In the present study, overexpression of CCL1 induced GAS5 upregulation, which may enhanced the ability of the cells to regulate a stress response *in vitro*. It has been indicated that inflammatory cytokines and microbial products markedly induce the expression of CCL1 (25). In humans, CCL1 has been detected in the lymph node lymphatic sinuses, but not in the peripheral lymphatics. The CCL1 receptor CCR8 is highly expressed in human malignant melanoma. Tumor cell migration to lymphatic endothelial cells has been shown to be inhibited by suppression of either CCR8 or CCL1 *in vitro*; furthermore, recombinant CCL1 promoted the migration of $CCR8^+$ tumor cells (24). In a murine model, suppression of CCR8 by a soluble antagonist or short hairpin RNA significantly decreased lymph node metastasis. In addition, inhibition of CCR8 caused the arrest of tumor cells in the collecting lymphatic vessels at the junction with the lymph node subcapsular sinus (24). Of note, the $CCR8^+$ myeloid cell subset is increased in patients with cancer. A previous study demonstrated that the expression of CCL1 was elevated in tumors, and the presence of CCR8+ myeloid cells was increased in the peripheral blood and cancer tissues, thus suggesting that the CCL1/CCR8 axis is involved in cancer-associated inflammation and may have a role in immune evasion. GAS5 has been reported to prevent glucocorticoid receptor localization to cognate genes, regulating various immune response genes, including II-8, CXCL10, CCL1 and CSF1 (26). In the present study, hierarchical cluster analysis indicated that overexpression of CCL1 was associated with lncRNA-GAS5. These results suggested a correlation between GAS5 and CCL1. However, the association between GAS5 and CCL1 requires further validation through a luciferase reporter assay to confirm whether GAS5 can regulate the CCL1 promoter.

In conclusion, the present study demonstrated that GAS5 silencing promoted bladder cancer proliferation and suppressed cell apoptosis. In addition, GAS5 knockdown resulted in an increased percentage of cells in the S and G2 phases, and a decreased percentage of cells in the G1 phase. Hierarchical cluster analysis revealed that CCL1 overexpression resulted in an upregulation of GAS5. Furthermore, knockdown of GAS5 increased the mRNA and protein expression levels of CCL1 in the bladder cancer cells, induced BLX cell proliferation and inhibited cancer cell apoptosis. The overexpression of GAS5 suppressed the cell proliferation and enhanced the apoptotic rate of the cancer cells. In addition, BLX cell proliferation was partially suppressed by CCL1 silencing, whereas CCL1 overexpression resulted in significant cell proliferation. The results demonstrated that GAS5 suppressed bladder cancer cell proliferation, at least partially, by suppressing the expression of CCL1. These findings provide a basis for the development of novel effective therapeutic strategies for the treatment of bladder cancer.

References

1. Wang L, Fu D, Qiu Y, Xing X, Xu F, Han C, Xu X, Wei Z, Zhang Z, Ge J, *et al*: Genome-wide screening and identification of long noncoding RNAs and their interaction with protein coding RNAs in bladder urothelial cell carcinoma. Cancer Lett 349: 77-86, 2014.
2. Yang L, Parkin DM, Li LD, Chen YD and Bray F: Estimation and projection of the national profile of cancer mortality in China: 1991-2005. Br J Cancer 90: 2157-2166, 2004.
3. Tong ZT, Wei JH, Zhang JX, Liang CZ, Liao B, Lu J, Fan S, Chen ZH, Zhang F, Ma HH, *et al*: AIB1 predicts bladder cancer outcome and promotes bladder cancer cell proliferation through AKT and E2F1. Br J Cancer 108: 1470-1479, 2013.
4. Qiao HP, Gao WS, Huo JX and Yang ZS: Long non-coding RNA GAS5 functions as a tumor suppressor in renal cell carcinoma. Asian Pac J Cancer Prev 14: 1077-1082, 2013.
5. Martens-Uzunova ES, Böttcher R, Croce CM, Jenster G, Visakorpi T and Calin GA: Long noncoding RNA in prostate, bladde, and kidney cancer. Eur Urol 65: 1140-1151, 2014.
6. Gibb EA, Brown CJ and Lam WL: The functional role of long non-coding RNA in human carcinomas. Mol Cancer 10: 38, 2011.
7. Gutschner T and Diederichs S: The hallmarks of cancer: A long non-coding RNA point of view. RNA Biol 9: 703-719, 2012.
8. Luo H, Zhao X, Wan X, Huang S and Wu D: Gene microarray analysis of the lncRNA expression profile in human urothelial carcinoma of the bladder. Int J Clin Exp Med 7: 1244-1254, 2014.
9. Wang F, Li X, Xie X, Zhao L and Chen W: UCA1, a non-protein-coding RNA up-regulated in bladder carcinoma and embryo, influencing cell growth and promoting invasion. FEBS Lett 582: 1919-1927, 2008.
10. He W, Cai Q, Sun F, Zhong G, Wang P, Liu H, Luo J, Yu H, Huang J and Lin T: linc-UBC1 physically associates with polycomb repressive complex 2 (PRC2) and acts as a negative prognostic factor for lymph node metastasis and survival in bladder cancer. Biochim Biophys Acta 1832: 1528-1537, 2013.
11. Han Y, Liu Y, Zhang H, Wang T, Diao R, Jiang Z, Gui Y and Cai Z: Hsa-miR-125b suppresses bladder cancer development by down-regulating oncogene SIRT7 and oncogenic long non-coding RNA MALAT1. FEBS Lett 587: 3875-3882, 2013.
12. Han Y, Liu Y, Zhang H, Wang T, Diao R, Jiang Z, Gui Y and Cai Z: Hsa-miR-125b suppresses bladder cancer development by down-regulating oncogene SIRT7 and oncogenic long noncoding RNA MALAT1. FEBS Lett pii: S0014-S5793, 2013.
13. Ying L, Huang Y, Chen H, Wang Y, Xia L, Chen Y, Liu Y and Qiu F: Downregulated MEG3 activates autophagy and increases cell proliferation in bladder cancer. Mol Biosyst 9: 407-411, 2013.
14. Liu Z, Wang W, Jiang J, Bao E, Xu D, Zeng Y, Tao L and Qiu J: Downregulation of GAS5 promotes bladder cancer cell proliferation, partly by regulating CDK6. PLoS One 8: e73991, 2013.
15. Pickard MR and Williams GT: Regulation of apoptosis by long non-coding RNA GAS5 in breast cancer cells: Implications for chemotherapy. Breast Cancer Res Treat 145: 359-370, 2014.
16. Shi X, Sun M, Liu H, Yao Y, Kong R, Chen F and Song Y: A critical role for the long non-coding RNA GAS5 in proliferation and apoptosis in non-small-cell lung cancer. Mol Carcinog 54 (Suppl 1): E1-E12, 2015.
17. Tu ZQ, Li RJ, Mei JZ and Li XH: Down-regulation of long non-coding RNA GAS5 is associated with the prognosis of hepatocellular carcinoma. Int J Clin Exp Pathol 7: 4303-4309, 2014.
18. Eruslanov E, Stoffs T, Kim WJ, Daurkin I, Gilbert SM, Su LM, Vieweg J, Daaka Y and Kusmartsev S: Expansion of CCR8(+) inflammatory myeloid cells in cancer patients with urothelial and renal carcinomas. Clin Cancer Res 19: 1670-1680, 2013.
19. Drayton RM, Peter S, Myers K, Miah S, Dudziec E, Bryant HE and Catto JW: MicroRNA-99a and 100 mediated upregulation of FOXA1 in bladder cancer. Oncotarget 5: 6375-6386, 2014.
20. Drayton RM, Dudziec E, Peter S, Bertz S, Hartmann A, Bryant HE and Catto JW: Reduced expression of miRNA-27a modulates cisplatin resistance in bladder cancer by targeting the cystine/glutamate exchanger SLC7A11. Clin Cancer Res 20: 1990-2000, 2014.

21. Yan B, Gu W, Yang Z, Gu Z, Yue X, Gu Q and Liu L: Downregulation of a long noncoding RNA-ncRuPAR contributes to tumor inhibition in colorectal cancer. Tumour Biol 35: 11329-11335, 2014.
22. Arab K, Park YJ, Lindroth AM, Schäfer A, Oakes C, Weichenhan D, Lukanova A, Lundin E, Risch A, Meister M, *et al*: Long noncoding RNA TARID directs demethylation and activation of the tumor suppressor TCF21 via GADD45A. Mol Cell 55: 604-614, 2014.
23. Mourtada-Maarabouni M, Hedge VL, Kirkham L, Farzaneh F and Williams GT: Growth arrest in human T-cells is controlled by the non-coding RNA growth-arrest-specific transcript 5 (GAS5). J Cell Sci 121: 939-946, 2008.
24. Das S, Sarrou E, Podgrabinska S, Cassella M, Mungamuri SK, Feirt N, Gordon R, Nagi CS, Wang Y, Entenberg D, *et al*: Tumor cell entry into the lymph node is controlled by CCL1 chemokine expressed by lymph node lymphatic sinuses. J Exp Med 210: 1509-1528, 2013.
25. Gombert M, Dieu-Nosjean MC, Winterberg F, Bünemann E, Kubitza RC, Da Cunha L, Haahtela A, Lehtimäki S, Müller A, Rieker J, *et al*: CCL1-CCR8 interactions: An axis mediating the recruitment of T cells and Langerhans-type dendritic cells to sites of atopic skin inflammation. J Immunol 174: 5082-5091, 2005.
26. Yu AD, Wang ZC and Morris KV: Long noncoding RNAs: a potent source of regulation in immunity and disease. Immunol Cell Biol 93: 277-283, 2015.