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Molecules and Cells

miR-372 Regulates Cell Cycle and Apoptosis of AGS Human Gastric Cancer Cell Line through Direct Regulation of LATS2

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Previously, we have reported tissue- and stage-specific expression of miR-372 in human embryonic stem cells and so far, not many reports speculate the function of this microRNA (miRNA). In this study, we screened various human cancer cell lines including gastric cancer cell lines and found first time that miR-372 is expressed only in AGS human gastric adenocarcinoma cell line. Inhibition of miR-372 using antisense miR-372 oligonucleotide (AS-miR-372) suppressed proliferation, arrested the cell cycle at G2/M phase, and increased apoptosis of AGS cells. Furthermore, AS-miR-372 treatment increased expression of LATS2, while over-expression of miR-372 decreased luciferase reporter activity driven by the 3′ untranslated region (3′ UTR) of LATS2 mRNA. Over-expression of LATS2 induced changes in AGS cells similar to those in AGS cells treated with AS-miR-372. Taken together, these findings demonstrate an oncogenic role for miR-372 in controlling cell growth, cell cycle, and apoptosis through down-regulation of a tumor suppressor gene, LATS2.

INTRODUCTION

MicroRNAs (miRNAs) are endogenous, noncoding, ~22 nt small RNAs which are involved in sequence-specific negative regulation of the stability and translation of target mRNAs (Lee et al., 1993). miRNAs exhibit cell- and tissue-specific expression (Lee and Ambros, 2001) and play important regulatory roles in cell cycle, apoptosis, and development (Bartel, 2004; Carleton et al., 2007; He and Hannon, 2004). It has been shown that miRNAs are aberrantly expressed or mutated in cancer, suggesting that they may play a role as a novel class of oncogenes or tumor suppressor genes (Bentwich et al., 2005; Zamore and Haley, 2005). The first evidence that miRNAs are involved in cancer came from the finding that miR-15 and miR-16 (chromosome 13q14) were deleted or missing in B cell chronic lymphocytic leukemia (CLL) cells (Cimmino et al., 2005). These miRNAs promote apoptosis by targeting mRNA of the anti-apoptotic BCL-2 gene and, thus, function as tumor suppressors. Let-7 is another miRNA with tumor-suppressing properties and its expression is reduced in human lung cancer associated with poor postoperative prognosis (Takamizawa et al., 2004). In contrast, the miR-17-92 cluster at chromosome 13q31.3 is overexpressed in lung cancers, and its introduction dramatically increases lung cancer cell growth (Hayashita et al., 2005). The oncogenic properties of miR-17-92 can be partly explained by its predicted targets including the tumor suppressor genes PTEN and RB2 (Lewis et al., 2003).

Gastric cancer ranks as a leading cause of cancer death in many parts of the world, including East Asia (Leung et al., 2008). miRNAs are reported to play important roles as tumor suppressors or oncogenes in gastric cancers (Saito et al., 2009). We previously reported that the miR-371-373 cluster is uniquely expressed in human embryonic stem cells (hESCs) (Suh et al., 2004). Recently, it was found that miR-372 and miR-373 act as oncogenes in the tumorigenesis of human testicular germ cell tumors (Tera-1 and 833KE cells), through direct inhibition of LATS2 expression (Voorhoeve et al., 2006). In addition, miR-373 was found to affect the esophageal cancer cells growth through inhibition of LATS2 expression (Lee et al., 2009). However, it is not clear whether miRNA 371-373 cluster affects the growth of other human cancer cells, especially in gastric cancer cells. In order to determine the roles of the miRNA 371-373 cluster in human gastric cancer cells, we screened the expression pattern of miRNA 372 in several human gastric cancer cell lines and found that it is expressed only in AGS cell line. The down-regulation of miR-372 restricted the cell cycle at G2/M phase and increased apoptosis in the AGS cells through increased LATS2 expression. LATS2 is a novel tumor suppressor gene and its overexpression causes G2/M arrest through inhibition of Cdc2 kinase activity and induces apoptosis in HeLa cells (Kamikubo et al., 2003). The LATS2 gene is located at chromosome 13q11-q12, where a loss of heterozygosity has been frequently observed in many primary

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cancers (Yabuta et al., 2000). Taken together, our results suggest that miR-372 supports growth and survival of the gastric cancer cells through down-regulation of the tumor-suppressor gene LATS2.

MATERIALS AND METHODS

Cell lines and cultures

Four human gastric adenocarcinoma cell lines (SNU-1, SNU-638, SNU-719, and AGS) were maintained in RPMI 1640 (Life Technologies) and a normal human gastric cell line (Hs 677.sT; ATCC CRL-7407) cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum. All cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

miRNA-oligonucleotides and transfection

FITC-labeled, mature antisense oligodeoxynucleotides specifically against miR-372 (AS-miR-372) and scrambled oligodeoxynucleotides (scramble) were purchased from Samchully Pharm. Co., LTD. and FITC conjugated to monitor transfection efficiency by fluorescence microscopy. Transfection was performed by RNAiMAX (Invitrogen) using miRNA oligos in OpTi-MEM I (GIBCO) according to the manufacturer's instructions.

Plasmid construction and transfection

Full-length human LATS2 cDNA was cloned by RT-PCR of RNA from the SNU-1 cell line using a forward primer 5′-GGATCCATGAGGCCAAAGACT-3′ and a reverse primer 5′-CTCGAGCTACACGTACACAGG-3′ and subcloned into the pCMV-Tag 2B vector (Stratagene). AGS cells were transfected with the pCMV-LATS2 construct using Lipofectamine (GibcoBRL). This construct was not contained 5′ or 3′ UTR in LATS2 mRNA which thus could not be regulated by miR-372.

Luciferase assay

To generate the luciferase reporter constructs psiCHECK2-LATS2 3′UTR WT and psiCHECK2-LATS2 3′UTR MUT, complimentary oligonucleotides containing the wild type 3′UTR of human LATS2 or point mutations in miR-372 target sites of the 3′UTR of human LATS2, respectively, were annealed. Oligonucleotide sequences were as follows: LATS2-WT UP, 5′-TCGAGGAAAATTTAGTACAGTTTAGAAAGAGCACTTATTTTGTT-3′; LATS2-WT DOWN, 5′-GGCCGCAACAAAATAAGTGCTCTTTCTAAACTGTACTAAAATTTTC-3′; LATS2 MUT-UP, 5′-TCGAGGAAAATTTAGTACAGTTTAGAAAGCATTCGATTTTTGTT-3′; LATS2-MUT DOWN, 5′-GGCCGCAACAAAAATCGAATGCTTTCTAAACTGTACTAAAATTTTC-3′. Annealed oligonucleotides were ligated into the *Xho*I/*Not*I site of the psiCHECK2 renilla/firefly dual-luciferase expression vector (Promega). AGS cells were transiently co-transfected with psiCHECK2-LATS2 3′UTR and miR-372 using Lipofectamine. Firefly and Renilla luciferase activities were measured consecutively with the Dual-LuciferaseR Reporter Assay System (Promega) using the Wallac Victor 1420 Multilabel Counter (EG&G Wallac).

Northern blot analysis

Total RNA (80 μg) from each cell line was loaded on a 15% denaturing polyacrylamide gel. The resolved RNA was transferred to a Zeta-Probe GT blotting membrane (Bio-Rad) and probed with miR-372 (5′-ACGCTCAAATGTCGCAGCACTTT-3′) labeled at the 5′ end with ^{32}P-γ-ATP. Prehybridization and hybridization were carried out using Express Hyb Hybridization Solution (Clontech) according to the manufacturer's instructions.

Real-time reverse transcription PCR

Total RNA was reverse transcribed using Retroscript (Ambion) and the level of LATS2 expression was determined by real-time PCR using the SYBR Green PCR Master Mix (Perkin-Elmer, Applied Biosystems), (PCR primers: LATS2-F, 5′-AAGAGCTACTCGCCATACGCCTTT-3′; LATS2-R, 5′-AGCTTTGGCCATTTCTTGCTCCAG-3′). In order to determine the expression level of miR-372, cDNA was synthesized using QuantiMir (SBI) according to the manufacturer's instructions. For further quantitation of miR-372 expression, single tube TaqMan miRNA assays (Applied Biosytems) were used. All RT reactions, including no-template controls and RT primer minus controls, were run using the stem-loop RT miR-372 primer in a GeneAmp PCR 9700 Thermocycler (ABI 7300 Real-Time PCR System, Applied Biosystems).

Western blot analysis

Cells were lysed in PRO-PREP lysis buffer (iNtRON BIOTECHNOLOGY). Proteins were resolved by SDS-PAGE, transferred onto PVDF transfer membranes (PIERCE), and probed with appropriate dilutions of anti-human LATS2 polyclonal antibody (sc-23065, Santa Cruz Biotechnology), anti-cyclin B monoclonal antibody (610219, BD Biosciences), anti-Cdc2 monoclonal antibody (610037, BD Biosciences), anti-Bcl-2 monoclonal antibody (#2872, Cell Signaling), or anti-β-actin monoclonal antibody (Sigma). Immunoreactivity was detected using the ECL detection system (PIERCE).

Cell cycle analysis

Cells were harvested and washed twice in PBS (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4 $7H_20$; 1.4 mM KH_2PO_4; pH 7.2) at room temperature and resuspended at 2×10^6 cells/ml in PBS. For propidium iodide staining, washed cells were fixed in pure ethanol at -20°C overnight and the rest of the steps were performed based on the manufacture's procedure (Molecular Probes Inc.). Cells were washed twice in PBS, resuspended in FACS buffer (PBS, 0.2% BSA and 1% sodium azide) and analyzed using a FACS flow cytometer (Becton Dickinson, Inc.).

Annexin V staining

Annexin V staining was performed using an Annexin-V-FLUOS staining kit (Roche Molecular Biochemicals). Briefly, cells were washed twice with PBS (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4 $7H_20$; 1.4 mM KH_2PO_4; pH 7.2) and resuspended in binding buffer (0.01 M HEPES, pH 7.4; 0.14 M NaCl; 2.5 mM $CaCl_2$) containing Annexin V and propidium iodide. Fluorescence intensity was measured using a FACS flow cytometer (Becton Dickinson, Inc.).

Caspase-3/7 activity assay

Caspase-3/7 activity was measured using the ApoONE Homogeneous Caspase-3/7 Assay Kit (Promega) according to the manufacturer's instructions. Caspase activity was determined by fluorometric detection of the hydrolyzed product from the substrate (rhodamine-labeled DEVD peptide) using a microplate spectrofluorometer (EG&G Wallac).

RESULTS

miR-372 regulates cell growth through prevention of apoptosis and mediation of cell cycle progress at G2/M phase

We first analyzed global miRNA expression in three human gastric cancer cell lines such as AGS, SNU-1, and SNU-638 using *Genopal™* miRNA microarray (Supplementary Fig. 1).

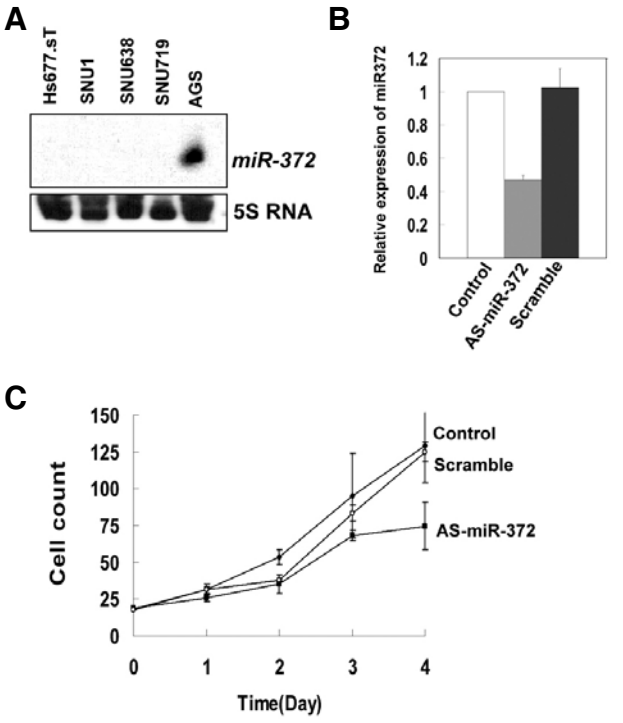

Fig. 1. miR-372 is required for the growth of AGS human gastric adenocarcinoma cells. (A) Northern blot analysis of miR-372 expression in four gastric cancer cell lines and a normal gastric cell line (Hs677.sT). Blots were probed for expression of miR-372 and 5S RNA as a loading control. (B) The suppressive effect of AS-miR-372 treatment on the miR-372 expression. AGS cells were transiently transfected with 100 nM of AS-miR-372 or scramble. Untreated cells were used as a control. miR-372 expression was determined by TaqMan real-time PCR and normalized to U6 RNA expression in the same sample. (C) Cell growth was monitored by enumerating on a hematocytometer at indicated times. The data represent the mean ± SD of three different experiments. All oligonucleotides used in experiments were modified with 2′-O-methyl and conjugated with FITC to assess transfection efficiency (FITC-conjugated-miRNA antisense-2′-O-methyl oligonucleotide). Scramble was used as negative control.

We found the first time that miR-371-373 cluster was expressed in AGS cells. We carried out Northern blot analysis to confirm the expression of miRNA-371-373 cluster in four human gastric cancer cell lines (SNU-1, SNU-638, SNU-719, and AGS) and one normal human gastric cell line (Hs677.sT). The result showed that, among miRNA 371-373 cluster, expression of miR-372 was strongly elevated in AGS cells (Fig. 1A and Supplementary Fig. 2). To study the effects of miR-372 suppression on the growth, AGS cells were transfected with AS-miR-372 and growth was determined by a direct counting method. AS-miR-372 treatment reduced miR-372 expression 2-fold compared with untreated and scramble-treated controls (Fig. 1B). The growth of AS-miR-372-treated cells was reduced to ~47% that of untreated and scramble-treated cells (Fig. 1C). These results suggest that miR-372 is required for the growth of AGS cells.

Since AS-miR-372 treatment significantly decreased the growth of AGS cells (Fig. 1C), cell cycle analysis of AS-miR-372-treated AGS cells was performed by flow cytometry analysis of DNA content. Interestingly, the percentage of cells in G2/M phase of AS-miR-372-treated AGS cells increased to 52.75% compared with those of untreated (37.18%) and scramble-treated (40.63%) AGS cells (Fig. 2A). To further analyze the G_2/M transition, expression levels of Cyclin B and Cdc2, which are universal cell cycle regulators implicated in the G_2/M transition, were examined (Doree and Galas, 1994). Expression of these two genes was significantly reduced by AS-miR-372 treatment (Fig. 2B). These results indicate that miR-372 plays an important role in cell cycle progress at G2/M phase.

Cell cycle arrest can promote apoptotic cell death (Bartek and Lukas, 2006). In order to determine clearly whether suppression of miR-372 can induce apoptosis, AS-miR-372-treated AGS cells were labeled with Annexin V and analyzed by flow cytometry. Compared with those of untreated and scramble-treated AGS control cells, apoptosis was significantly increased in AS-miR-372-treated AGS cells (Fig. 2C). In addition, we measured the caspase-3/7 activity and found that AS-miR-372 treatment induced a ~1.3-fold increase in caspase-3/7 activity in AGS cells compared to controls (Fig. 2D). AS-miR-372 treatment also reduced expression of Bcl-2, an anti-apoptotic protein, to 45% of untreated control (Fig. 2B) and ICAD to 30% of untreated control (Supplementary Fig. 3). Together, these results indicate that miR-372 supports cell growth through prevention of apoptosis and mediation of cell cycle progress at G2/M phase.

miR-372 regulates LATS2 expression level in AGS cells

miR-372 has been reported to support proliferation of testicular germ cell tumors through direct inhibition of the expression of tumor suppressor LATS2 (Voorhoeve et al., 2006). Thus, it is possible that, in AGS cells, miR-372 supports cell growth through inhibition of the LATS2 expression. To test this possibility, expression levels of *LATS2* mRNA in AGS cells after AS-miR-372 treatment were quantitated by real time PCR. Down-regulation of miR-372 by treatment with AS-miR-372 caused a ~1.2-fold increase in *LATS2* mRNA expression (Fig. 3A). However, treatment with scrambled oligonucleotide did not change *LATS2* mRNA expression. LATS2 protein expression was also increased by treatment with AS-miR-372 (Fig. 3B and 3C). These results indicate that miR-372 expression is associated with a decrease in LATS2 expression in AGS cells.

To determine whether down regulation of LATS2 expression by miR-372 is mediated through a LATS2 3′UTR, a luciferase reporter gene linked to the LATS2 3′UTR in the psiCHECK2 vector, psiCHECK2-LATS2 3′UTR, was used. A construct containing a mutant form of the LATS2 3′UTR, psiCHECK2-LATS2-MUT 3′UTR, and the empty vector psiCHECK2 were used as controls. When AGS cells were transfected with miR-372, the luciferase expression from the wild type LATS2 3′UTR was reduced to 50% of that of psiCHECK2 control vector. However, miR-372 treatment did not affect luciferase expression from the mutant form of the LATS2 3′UTR (Fig. 3D). Collectively, these results indicate that elevated expression of miR-372 contributed to a decrease in LATS2 level through destabilization of LATS2 mRNA.

LATS2 overexpression induces cell growth, cell cycle, and apoptosis of AGS cells

LATS2 induces cell cycle and apoptosis in cancer cells. We generated LATS2 expression plasmid (pCMV-LATS2, not containing 5′ and 3′ UTR; to prevent interfere effect of endogenous miR-372 effect on *LATS2*), and transfected to AGS cells and then analyzed cellular mechanisms such as cell growth, cycle and apoptosis. In this experiment, LATS2 expression could independently regulate cell cycle and apoptosis in AGS cells

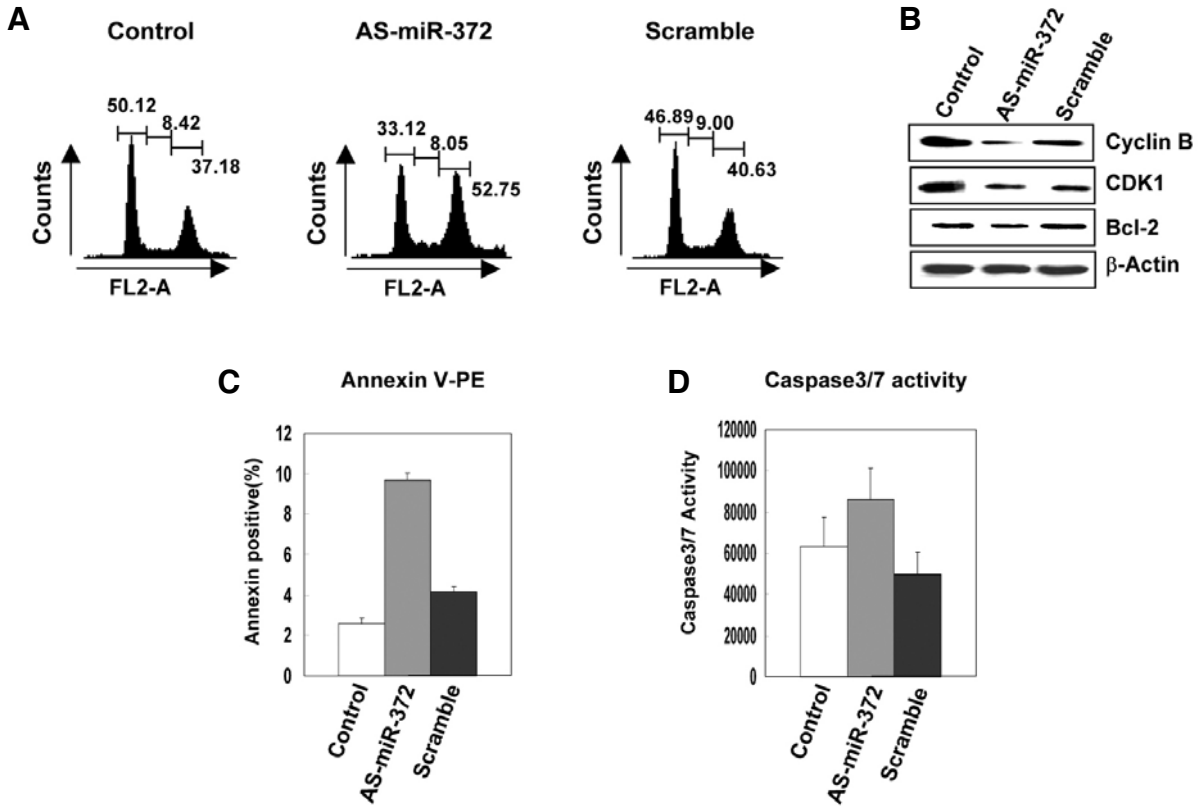

Fig. 2. AS-miR-372 treatment results in cell cycle arrest at G2/M phase and apoptosis of AGS cells. AGS cells were transiently transfected for 24 h as described in the Fig. 1B legend. (A) Cells were stained with propidium iodide (PI) and subjected to FACS analysis. (B) Expression levels of Cyclin B, Cdc2, and Bcl-2 were determined by Western blot. β-actin was used as a loading control. (C) Cells were stained with Annexin V and subjected to FACS analysis. (D) Caspase-3/7 activity was determined by the caspase-Glo 3/7 luminescent assay. The data represent the mean ± SD of three different experiments.

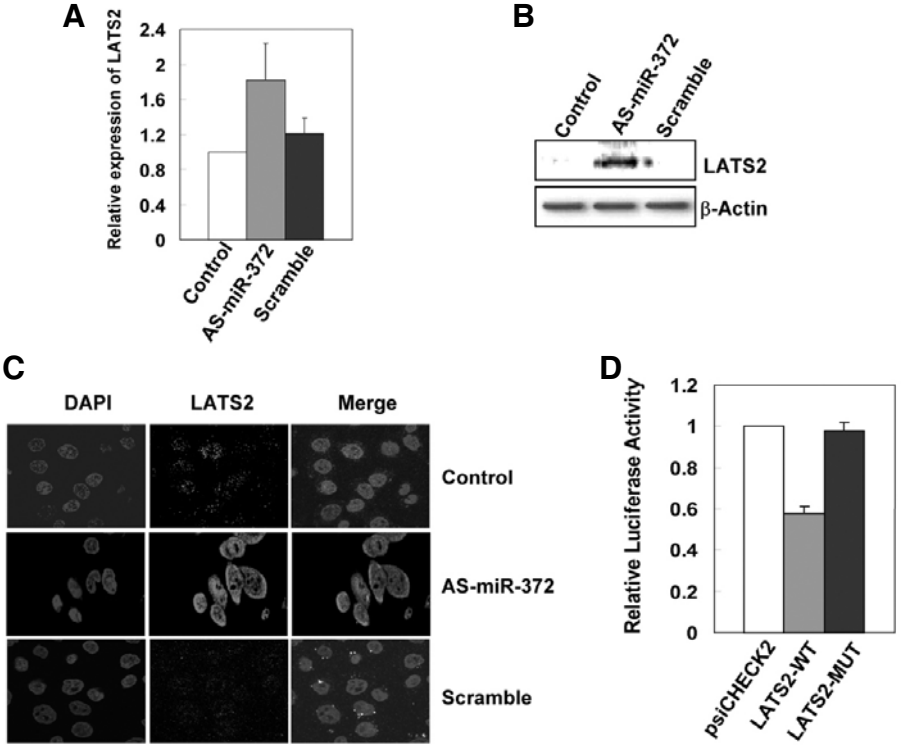

Fig. 3. AS-miR-372 treatment increases expression of LATS2 in AGS cells. AGS cells were transiently transfected for 24 h as described in the Fig. 1B legend. (A) LATS2 expression was determined by real-time RT-PCR. (B) LATS2 expression was determined by Western blot. (C) Immunostaining using an anti-LATS2 monoclonal antibody reveals that the AS-miR-372 transfected cells express higher levels of the LATS2 protein than the other two control cells. Scale bar is 50 μm. (D) AGS cells were transfected with renilla luciferase constructs containing the wild-type (WT) or mutant (MUT) target site of the LATS2 3′-UTR for 24 h. The empty vector, psiCHECK2, was used as a negative control. The luciferase values of psiCHECK2 control were set to 1.

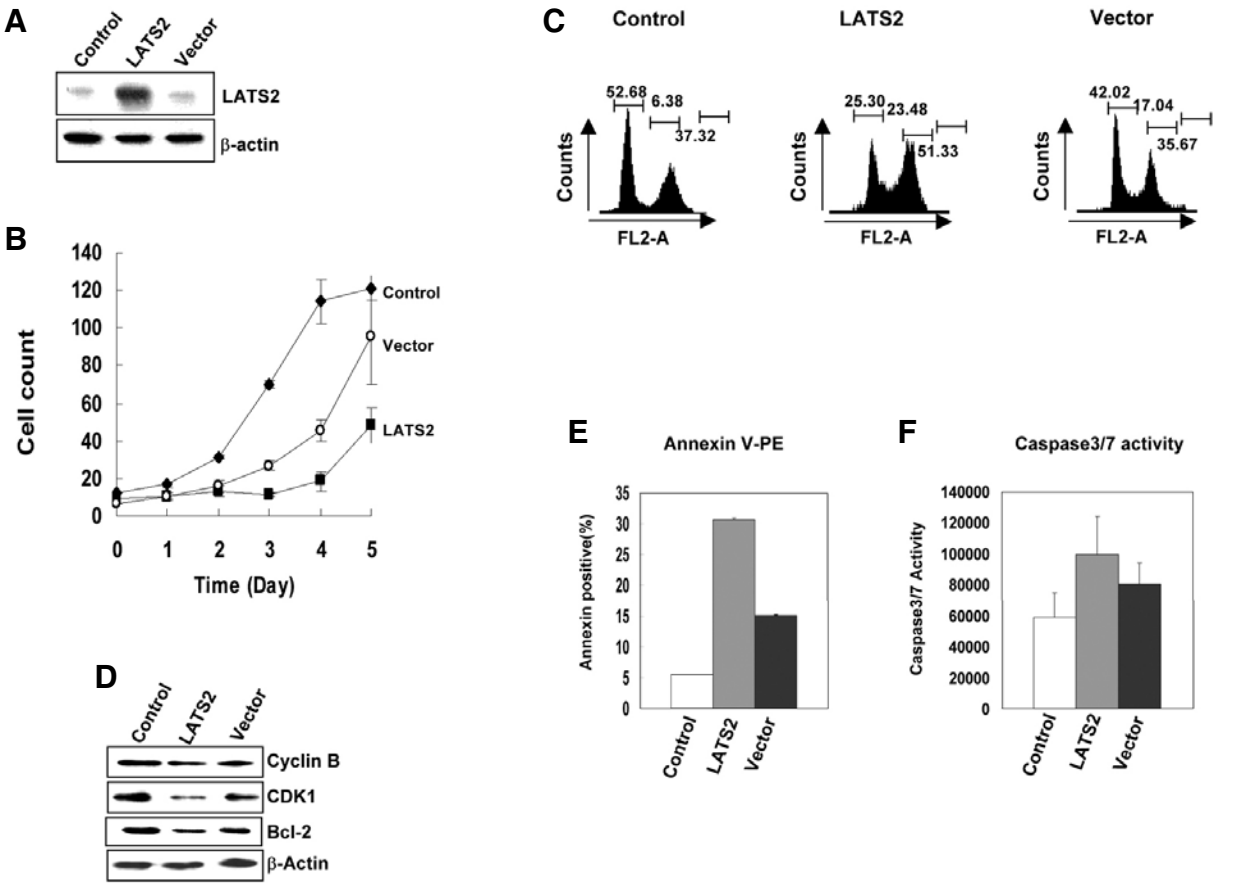

Fig. 4. Effects of LATS2 overexpression on the growth, cell cycle, and apoptosis of AGS cells. AGS cells were transiently transfected with pCMV-LATS2 or pCMV-Tag empty vector. The pCMV-LATS2 vector was not contained 5′ and 3′UTR of LATS2, and thus miR-372 could not regulate LATS2 expression. Untreated cells were used as a control. (A) At 24 h after transfection, LATS2 expression was measured by Western blot. (B) Cell growth was monitored by hematocytometer counts at the indicated times after transfection. (C) The 24 h after transfection, cells were stained with propidium iodide (PI) and subjected to FACS analysis. (D) The expression levels of Cyclin B, Cdc2, and Bcl-2 were determined by Western blot. (E) Cells were stained with Annexin V and subjected to FACS analysis. (F) Caspase-3/7 activity was determined by the caspase-Glo 3/7 luminescent assay. The data represent the mean ± SD of three different experiments.

without interference of miR-372. Overexpression of LATS2 (Fig. 4A) suppressed growth (Fig. 4B), arrested the cell cycle at G2/M phase (Fig. 4C), decreased expression of Cyclin B, Cdc2, Bcl-2 (Fig. 4D), ICAD (Supplementary Fig. 4), and increased apoptosis of AGS cells (Figs. 4E and 4F). The overall effect of LATS2 overexpression was comparable to that of AS-miR-372 treatment, suggesting that LATS2 is a key mediator of miR-372 effects on growth, cell cycle, and apoptosis of AGS cells.

DISCUSSION

We have previously shown that miR-372 is expressed in human ESCs (Suh et al., 2004). Although its cellular function was not fully determined; however, it was possible to predict that miR-372 functions as a regulator in the growth and/or development of cells (Card et al., 2008; Kim et al., 2009; Lee et al., 2008). In this study, we found the first time that miR-372 supports the proliferation of a human gastric cancer cell line, AGS. This conclusion was established by results showing that down-regulation of miR-372 expression by AS-miR-372 suppressed the growth of AGS cells, arrested the cell cycle at G2/M phase and increased apoptosis. These findings are in agreement with a previous report demonstrating that miR-372 has oncogenic properties (Voorhoeve et al., 2006).

miRNAs are a class of small noncoding RNAs that play an important role in regulating gene expression in various living systems (Mertens-Talcott et al., 2007). Recent evidence indicates that miRNAs can function as tumor suppressors and oncogenes and play a crucial role in the initiation and progression of human cancer (Cho, 2007) including gastric cancer (Saito et al., 2009). In addition, expression profiles of miRNAs have been found to be correlated with cancer pathogenesis and useful in diagnosis and prognosis of cancers (Calin and Croce, 2006; Liu et al., 2004; Lu et al., 2005; Wu et al., 2007). Identification of cancer-specific miRNAs and their targets is critical for understanding their role in tumorigenesis (Calin et al., 2004; He et al., 2005; Iorio et al., 2005; Liu et al., 2004; O'Donnell et al., 2005; Volinia et al., 2006). Previously, miR-372/3 has been reported to support the growth of testicular germ cell tumors (Voorhoeve et al., 2006) and esophageal cancer cells (Lee et al., 2009). In this study, we found that miR-372 also supports the growth of gastric cancer cell line, AGS.

In order to understand the mechanism by which miR-372 supports the growth of AGS cells, it is crucial to identify its target genes. Voorhoeve and colleagues (Voorhoeve et al., 2006) identified the tumor suppressor LATS2 as a target gene of miR-

372 in human testicular germ cell tumors. Our results indicate that LATS2 is also a functional target of miR-372 in gastric cancer cells: (a) suppression of miR-372 increases LATS2 expression in AGS cells; (b) the expression of a luciferase reporter containing LATS2 3′UTR is inhibited when miR-372 is overexpressed. It is possible that there may be other target genes of miR-372 beside the LATS2 and, in fact, Voorhoeve et al. (2006) identified two more miR-372 predicted target genes: FYCO1 and Suv39-H1. Even though we did not analyze the other miR-372 target genes, our data and previous report suggest that LATS2 is the key mediator of oncogenic activity of miR-372: (a) as shown in our data the overall effect of LATS2 overexpression is comparable to that of AS-miR-372 treatment: (b) overexpression of miR-372 shows similar effects on cell death and growth as inhibition of LATS2 expression does (Voorhoeve et al., 2006). It is not yet clear how tumor suppressor LATS2 exerts its effects on cells. However, previous reports suggest that LATS2 overexpression results in cell cycle arrest at the G2/M phase via inhibition of Cdc2-Cyclin B kinase activity (Kamikubo et al., 2003), cell cycle arrest at G1/S phase via inhibition of E3 ubiquitin ligase activity of Mdm2 (Li et al., 2003), and apoptosis via down-regulation of Bcl-2 and Bcl-xL (Ke et al., 2004). In AGS cells, overexpression of LATS2 by either AS-miR-372 or LATS2 cDNA transfection decreased the expression of Cdc2/Cyclin B, and Bcl-2. Even though we did not determine the mechanisms responsible, it is highly possible that these changes lead to cell cycle arrest at G2/M phase and apoptosis of AGS cells.

Down-regulation of tumor suppressor LATS2 expression has been reported to provide an environment favorable for proliferation of cancer cells (Lee et al., 2009; Matsui et al., 2009). Our results suggest that miR-372 plays an important role in the down-regulation of LATS2 expression in gastric cancer cells. In the overexpression of LATS2, AGS cells were inhibited of cell growth and triggered to apoptosis when LATS2 expressions were independently regulated by miR-372. At present, we do not know why miR-372 expression was not detected in the other four gastric cell lines. They may have other mechanisms to down-regulate the expression of LATS2. In many primary cancers, a loss of heterozygosity has been frequently observed in a chromosomal region where the LATS2 gene resides (Yabuta et al., 2000). Further investigation is required to demonstrate whether there are mutational changes in the LATS2 genomic DNA of these four cell lines and to identify any other mechanisms regulating the expression of LATS2.

In conclusion, we have shown that miR-372 is expressed in human gastric cancer AGS cells and its expression supports growth of these cells through down-regulation of tumor suppressor LATS2. These findings suggested that miR-372 expression may play a crucial role in the tumorigenesis of gastric cancer through suppression of LATS2 expression.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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