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Identification and Characterization of a Noncoding RNA at the Mouse Pcna Locus

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AK007836 encodes a noncoding RNA (ncRNA) consisting of 2 exons. Since AK007836 is located just upstream of *Pcna* and transcribed in the opposite direction to that of *Pcna*, we analyzed its expression pattern. Both ncRNA and *Pcna* expressions were detected in *in vitro* and *in vivo* cells, showing a positive correlation. A 177 bp region separating the first exons of *Pcna* and AK007836 has a bidirectional promoter activity. When the expression of ncRNA was reduced by siRNA, *Pcna* expression was also reduced in normal cells, but not in cancer cells. These results suggest that the ncRNA is divergently transcribed from the bidirectional promoter, positively regulating the neighboring protein-coding *Pcna* gene transcription, and this regulatory function is somehow disrupted in cancer cells.

INTRODUCTION

Proliferating cell nuclear antigen (PCNA) is an auxiliary protein of DNA polymerase delta with roles in DNA replication and repair, as well as cell cycle progression and cell differentiation (Maga and Hubscher, 2003). The transcript level of *PCNA* is growth regulated (Baserga, 1991; Jaskulski et al., 1988), and this regulation is under the control of both transcriptional and post-transcriptional mechanisms (Baserga, 1991; Chang et al., 1990). In an attempt to understand the regulatory mechanisms for PCNA gene expression, promoters of different lengths and introns have been intensively characterized, and the unique properties of the regulatory regions have been identified (Alder et al., 1992; Matsuoka et al., 1993; Ottavio et al., 1990; Pietrzkowski et al., 1991). In the case of human PCNA, the first and the fourth introns have negative regulatory effects on the expression of PCNA (Alder et al., 1992; Ottavio et al., 1990). Through Northern blot analysis, a 500-600 bp-long antisense PCNA transcript (PCNAAS) was discovered at the human PCNA locus, which later has been reported to be transcribed from a promoter located within the first intron with no coding capacity (Tommasi and Pfeifer, 1999). Since the antisense RNA base pairs with the first coding exon of PCNA, the function of PCNAAS has been proposed to induce silencing of PCNA in the G0/G1 phase, whereas binding of an S phase-specific transcription factor E2F at the position +583 within the first intron might play a key role in switching from negative to positive regulation (Tommasi and Pfeifer, 1999). Although there is a size discrepancy between the experimental and computational results, it is not surprising that the current version of UCSC human genome browser views 384-bp noncoding RNA (ncRNA), "PCNAAS" consisting of a single exon directly overlapping with the first exon of human PCNA gene.

In the early 1990s, the mouse Pcna gene was also cloned and its nucleotide sequence was analyzed (Yamaguchi et al., 1991). Its promoter is located within a 200 bp region just upstream of the transcription initiation site where several putative transcriptional regulatory elements are located. The negative regulatory region (NRR) was also identified in the mouse Pcna gene (Matsuoka et al., 1993). However, the location of mouse NRR (between nucleotides -1231 and -624) was different from that of human NRR. Furthermore, there was no obvious sequence similarity between these two species. Through *in silico* analysis, we found one antisense noncoding RNA (AK007836) transcribed from the 5′ upstream region of the mouse Pcna locus. Like NRR, the genomic location and the length of this noncoding antisense RNA (AK007836) were completely different from those of human PCNAAS, and even had one intron in it.

Therefore, we further examined in this study whether mouse ncRNA AK007836 would actually be expressed in normal murine tissues or in cell lines along with Pcna. Since the promoter of Pcna is located just upstream of AK007836, and the first exons of Pcna and AK007836 are only 177 bp apart, we tested the activity of the promoter (originally reported as a Pcna minimal promoter) in both orientations, proving that this promoter is bidirectional and is used for both Pcna and ncRNA expression. Finally, the potential function of ncRNA AK007836 was evaluated using siRNA-based gene knockdown in normal and cancer cells.

MATERIALS AND METHODS

Cell culture and tissue sample preparation

Murine C3H10T1/2 mesenchymal cells, NIH3T3 fibroblast cells, Lewis lung carcinoma (LLC) cells, and B16F10 melanoma cells

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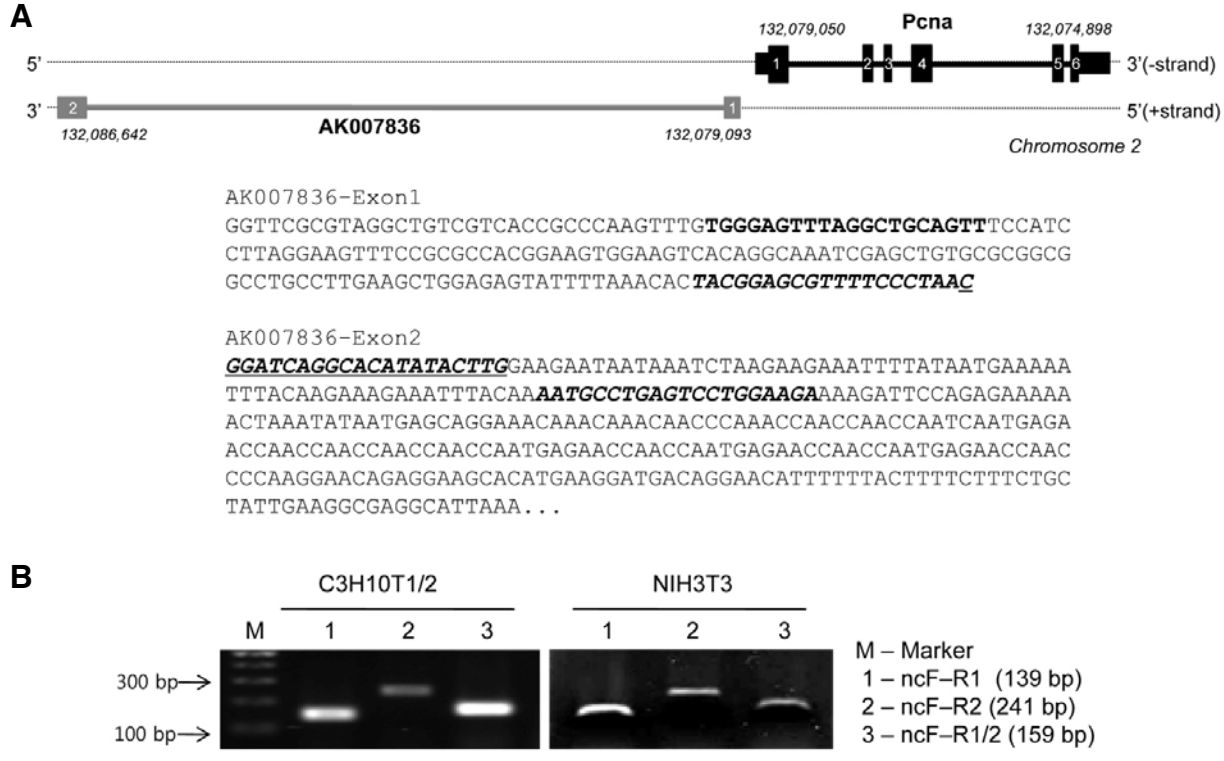

Fig. 1. Genomic organization and expression analysis of murine ncRNA AK007836. (A) AK007836 is located in the 5′-upstream region of a strand opposite to the *Pcna*-bearing strand. Exons for *Pcna* and AK007836 are marked in black and gray boxes, respectively, along with the exon numbers. The cDNA sequences (exon 1 and a part of exon 2) of the AK007836 transcript (ncRNA) and the primer sequences used for RT-PCR are shown below. One forward primer (ncF) and three reverse primers are written in bold and bold italic, respectively. Reverse primers R1 and R2 are located in exons 1 and 2, respectively. R1/2 spanning the spliced junction between exons 1 and 2 is underlined. (B) RT-PCR analysis of AK007836 in C3H10T1/2 and NIH3T3 cells. Total RNAs were isolated from two cell lines and used for RT-PCR. Combinations of a forward primer and three different reverse primers all detected AK007836 transcript with the expected length in which the intron is spliced out correctly.

were cultured in a Dulbecco's Modified Eagle's Medium (*DMEM,* WelGENE Inc., Korea) supplemented with 10% fetal bovine serum (FBS, WelGENE Inc.) and 100 μg/ml penicillin-streptomycin (WelGENE Inc.) at 37°C in 5% CO_2. B16BL6 melanoma cells were cultured in a MEM (WelGENE Inc) supplemented with MEM vitamin, 10% FBS, and 100 μg/ml penicillin-streptomycin. Mouse Embryonic Fibroblast (MEF) cells isolated from E13.5 mouse embryos were cultured in DMEM (10% FBS). To manipulate the cell cycle progression, NIH3T3 cells were first starved for 72 h in a DMEM medium without serum, and then subsequently stimulated with 10% FBS for 6, 12, 18, or 30 h as reported previously (Jaskulski et al., 1988; Ottavio et al., 1990; Zerler et al., 1987). To obtain mouse samples, four-month-old ICR male mice were sacrificed, and tissues from organs such as the brain, heart, kidney, liver, spleen, small intestine, and testis were dissected and immediately frozen in liquid nitrogen until use.

Total RNA isolation and analysis of gene expression using RT-PCR

Total RNAs were isolated from cultured cells or mouse tissues using Trizol reagent (Invitrogen, USA). cDNAs were synthesized using 0.5 μg of total RNAs through a reverse transcription reaction (ImProm-*II*TM Reverse Transcriptase*,* Pro-mega, USA). PCR amplification was performed under the following conditions: initial denaturation for 5 min at 94°C, and then 30-35 cycles of 94°C for 30 s (denaturation), 54°C for 20 s (annealing), and 72°C for 1 min (polymerization). The primer sequences were as follows: *Pcna,* 5′- TGC TCT GAG GTA CCT GAA CT-3′ (forward) and 5′- TGC TTC CTC ATC TTC AAT CT-3′ (reverse); AK007836, 5′-TGG GAG TTT AGG CTG CAG TT-3′ (nc-Forward, ncF), 5′-GTT AGG GAA AAC G CT CCG TA-3′ (nc-Reverse1, R1), 5′-CAA GTA TAT GTG CCT GAT CCG-3′ (nc-Reverse1/2, R1/2), and 5′-TCT TCC AGG ACT CAG GCA TT-3′ (nc-Reverse2, R2); the control β-actin, 5′-CAT GTT TGA GAC CTT CAA CAC CCC-3′ (forward) and 5′- GCC ATC TCC TGC TCG AAG TCT AG-3′ (reverse).

Construction of luciferase reporter plasmids

The minimal promoter in the 5′-flanking region of the *Pcna* gene was amplified by PCR using a forward primer (5′-CTA AGG ATG GAA ACT GCA GCC-3′) and a reverse primer (5′-AGG CCT ACA GCG ACA ACT AC-3′). The purified PCR products (289 bp) were ligated into the pGEM®-T Easy Vector (Promega) and then transformed into DH5α competent cells. Positive clones were selected through restriction enzyme analysis, and then they were subcloned into the pGL3-basic vector using the *Eco*RI site to produce a plasmid, pGL3-uPCNA. The sequence and orientation of the insert (ori+ or ori-) were confirmed by sequencing. We seeded 4×10^5 NIH3T3 cells/well into a 12-well plate and cultured them overnight until 90% confluent. The cells were transfected with pGL3 empty vector or pGL3-uPCNA

(ori+/-), along with pRL-TK vectors using Lipofectamine 2000 (Invitrogen). The cells were washed with PBS and lysed with passive lysis buffer (Promega) 24 h after transfection. Luciferase reporter assay was performed using the Dual-Luciferase® Reporter Assay System (Promega) and measured using the GLOMAX 20/20 luminometer (Promega).

siRNA transfection and real-time RT-PCR

AK007836 and non-specific control siRNA were designed and produced by Genolution (Seoul, Korea**)**. The target sequences for siRNA validation were 5′-GTC GTC ACC GCC CAA GTT T-3′ (si_AK007836 #1, si_AK#1), 5′-CGG ATC AGG CAC ATA TAC T-3′ (si_AK007836 #2, si_AK#2), and 5′-CCA ATG AGA ACC AAC CAA T-3′ (si_AK007836 #3, si_AK#3). Cultured cells, grown to 30% confluence, were transfected with 20 nM AK007836 siRNA or non-specific control siRNA using a G-Fectin reagent (Genolution) according to the manufacturer's instructions. Total RNAs were extracted using Trizol (Invitrogen) after 24 h of transfection. Real-time RT-PCR was performed using a One-step SYBR Primescript RT-PCR kit (Takara, Japan) and the SmartCycler system (Cepheid, USA). Primer sequences used for amplifications were as follows: AK007836, 5′-TGG GAG TTT AGG CTG CAG TT-3′ (forward) and 5′-CTG ATC CGT TAG GGA AAA CG-3′ (reverse); Pcna, 5′-AGG AGG CGG T AA CCA TAG AGA T-3′ (forward) and 3′-ACT CTA CAA CAA GGG GCA CAT C-3′ (reverse). For semi-quantitative RT-PCR analysis, Multi-Gauge V3.0 software (Fujifilm) was used.

RESULTS

AK007836 was originally reported as a cDNA isolated from the 10-day-old male mouse pancreas, with its size being 7550 bp containing 2 exons without any coding capacity (Fig. 1A). Here, we revealed the existence of this ncRNA transcript in two mouse fibroblast cell lines, C3H10T1/2 and NIH3T3, using RT-PCR (Fig. 1B). With a fixed forward primer (ncF), three different reverse primers (R1, R1/2, and R2) located in different exons successfully detected the expected size of the ncRNA transcript. In particular, the result from the reverse primers located in the second exon (R1/2 and -R2) proved that the intron was precisely spliced out of the primary transcript.

To examine the expression pattern of ncRNA AK007836 along with that of Pcna, cells were made quiescent and then re-stimulated to promote cell proliferation. Pcna mRNA was nearly undetectable in serum-starved cells, but the expression level increased following the serum stimulation showing a peak level at 18 h (Fig. 2A). Similarly, peak upregulation of ncRNA upon serum stimulation was observed 18 h after stimulation. When both Pcna and ncRNA AK007836 expression profiles were examined *in vivo* using various mouse tissues, the expression patterns were similar to those detected *in vitro*, showing a positive relationship between AK007836 expression and Pcna expression (Fig. 2B).

After examining the co-expression of Pcna and AK007836 in the mouse tissues as well as in the cultured cells, we tested whether these two genes lying head-to-head share a common bidirectional promoter, since the first exons of ncRNA AK00 7836 and Pcna are only 177 bp apart. To test whether this region, reportedly a Pcna minimal promoter (Yamaguchi et al., 1991), is also used for ncRNA expression, we cloned this region into the pGL3-basic vector in forward (pGL3-uPCNA ori+) and reverse (pGL3-uPCNA ori-) orientations (Fig. 3A). The transcriptional activity of this region was > 1,000-fold higher than that of the control vector in both orientations (Fig. 3B), demonstrating that the Pcna minimal promoter is functional in both directions with same strength and is probably used for ncRNA expression.

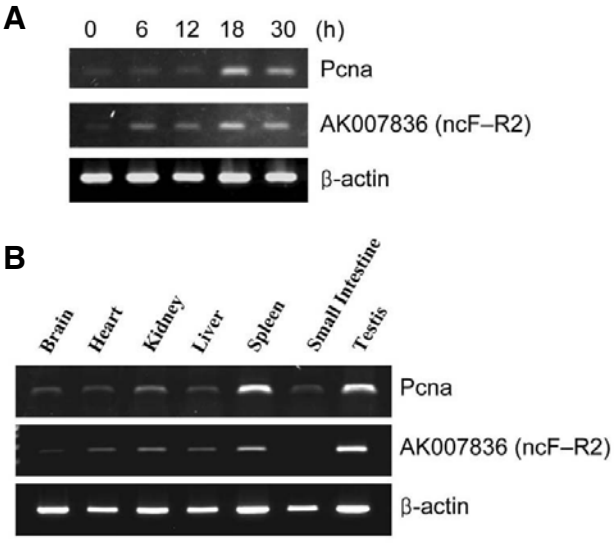

Fig. 2. Co-expression of *Pcna* and AK007836 *in vitro* and *in vivo*. (A) Expression pattern of *Pcna* and AK007836 in NIH3T3 cells stimulated with FBS. The cells were made quiescent (0 h) and then stimulated for the indicated times (in hours). This figure is representative of three independent experiments that all showed similar results. (B) Total RNAs were isolated from various mouse organs such as the brain, heart, kidney, liver, spleen, small intestine, and testis, and then were used for RT-PCR. All analyses were replicated with at least two independent source materials. (ncF-R2) indicates the primers ncF and R2 used to amplify AK007836.

Although the function of long ncRNAs is mostly unknown, some have been implicated to affect the expression of adjacent genes (Wilusz et al., 2009). To directly assess the function of ncRNA AK007836 on Pcna expression, we transfected the siRNA against AK007836 and then examined the expression of Pcna. NIH3T3 and B16F10 were used as representatives for normal and cancer cells, respectively. The siRNA sufficiently knocked down the expression of AK007836 in both NIH3T3 and B16F10 melanoma cells (72% and 62% reduction in NIH3T3 and B16F10, respectively) and showed a reduction of Pcna transcription in NIH3T3 cells. Interestingly, however, AK007836 inhibition did not affect the Pcna expression in the cancer cell line, B16F10 (Fig. 4A). To exclude the possibility of off-target effects of siRNA in cancer cells, we tested the effect of 3 different siRNAs for AK007836. Compared with the effect of siRNA #3, siRNAs #1 and #2 showed variable results to some extent depending on cell types and culture condition (Figs. 4B and 4C). Since siRNA #3 had a relatively high efficiency and reproducible results, we used siRNA #3 for further study. The additional sets of experiments using two normal (C3H10T1/2, and MEF) and two cancer cell lines (B16BL6 and LLC) showed that the difference observed before (Fig. 4A) was a general phenomenon (Fig. 4D), i.e., ncRNA AK007836 has a positive effect on Pcna expression in normal cells, which is somehow disrupted in cancer cells.

DISCUSSION

Most eukaryotic genomes (more than 90% of genomic DNA) have been reported to be transcribed as RNAs without any

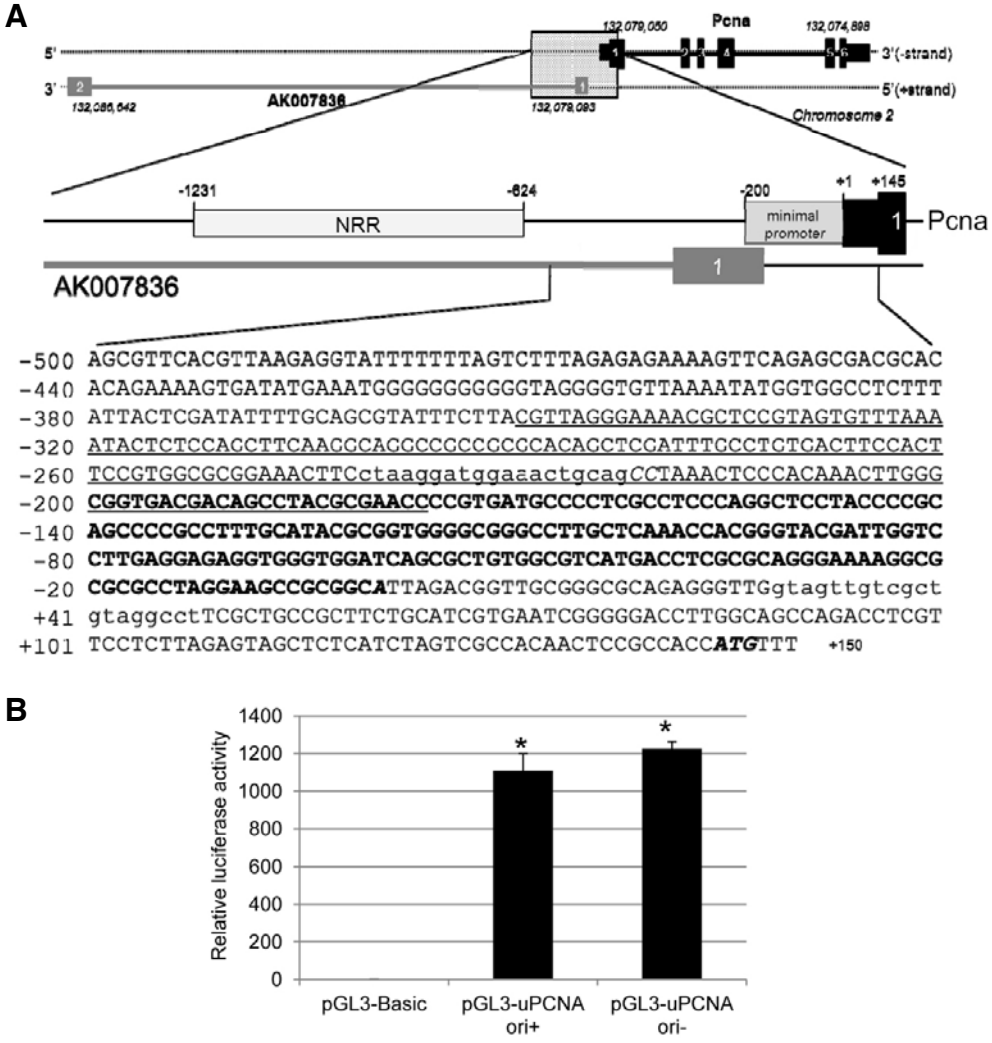

Fig. 3. Bidirectional promoter analysis. (A) Nucleotide sequence of the 5′ flanking region of mouse *Pcna*. Genomic organization of *Pcna* and that of ncRNA AK007836 are drawn above as in Fig. 1. The upstream region of *Pcna* (the dotted box) containing the first exon and a part of the first intron of AK007836 are enlarged below. The previously reported minimal promoter region (-200) and negative regulatory region (NRR, -1231~-624) (Matsuoka et al., 1993) are indicated. The nucleotide sequences from -500 to +150 are presented below. The first exon of AK007836 is underlined. A minimal promoter for *Pcna* is written in bold, and the primer sequences used for the amplification of the minimal promoter region are written in small letters. The transcription start site (+1) and the ATG initiation codon of *Pcna* are marked in bold italic. (B) NIH3T3 cells were transfected with the pGL3-basic plasmid or the pGL3 plasmid containing the minimal promoter of *Pcna* in the right direction (pGL3-uPCNA ori+) or in the reverse orientation (pGL3-uPCNA ori-), along with the *Renilla* luciferase plasmid as a transfection control. The luciferase activity was measured 24 h after transfection. The ratio of firefly luciferase activity to *Renilla* luciferase activity was expressed as relative luciferase activity. Four independent experiments were performed. The values are expressed as mean ± SD. *P < 0.001 as compared with pGL3-basic.

coding capacity (Costa, 2005; 2010). Although various functions of non-coding RNAs (ncRNAs) have been implicated in many biological systems, most of them remain to be proven. It is therefore imperative to discover novel ncRNAs and further characterize the mode of action for better understanding of their biological meaning.

In the upstream region of Pcna genomic locus, a noncoding RNA has been reported both in humans and mice. The genomic location and the action mechanism of a human noncoding RNA, PCNAAS, were defined previously (Tommasi and Pfeifer, 1999). However, there have been no reports so far of the identification and characterization of a noncoding RNA at the mouse Pcna locus.

Here, we demonstrate that AK007836 containing one intron and locating near the mouse Pcna locus is expressed from the opposite strand of Pcna as an ncRNA *in vivo* as well as *in vitro* (Figs. 1 and 2). The expression of a correctly spliced form of ncRNA AK007836 is induced by serum stimulation along with its neighboring protein-coding gene, Pcna, probably sharing a common bidirectional promoter (Figs. 2 and 3). In humans, ncRNA PCNAAS has also been reported to be expressed from the opposite strand of PCNA. Since PCNAAS overlaps with the PCNA gene, PCNAAS can hybridize with a PCNA mRNA. Interestingly enough, a minimal human PCNA promoter (-395 to -1) has also been reported to be bidirectional (Rizzo et al., 1990). Since the transcription start site of PCNAAS is 416 nu-

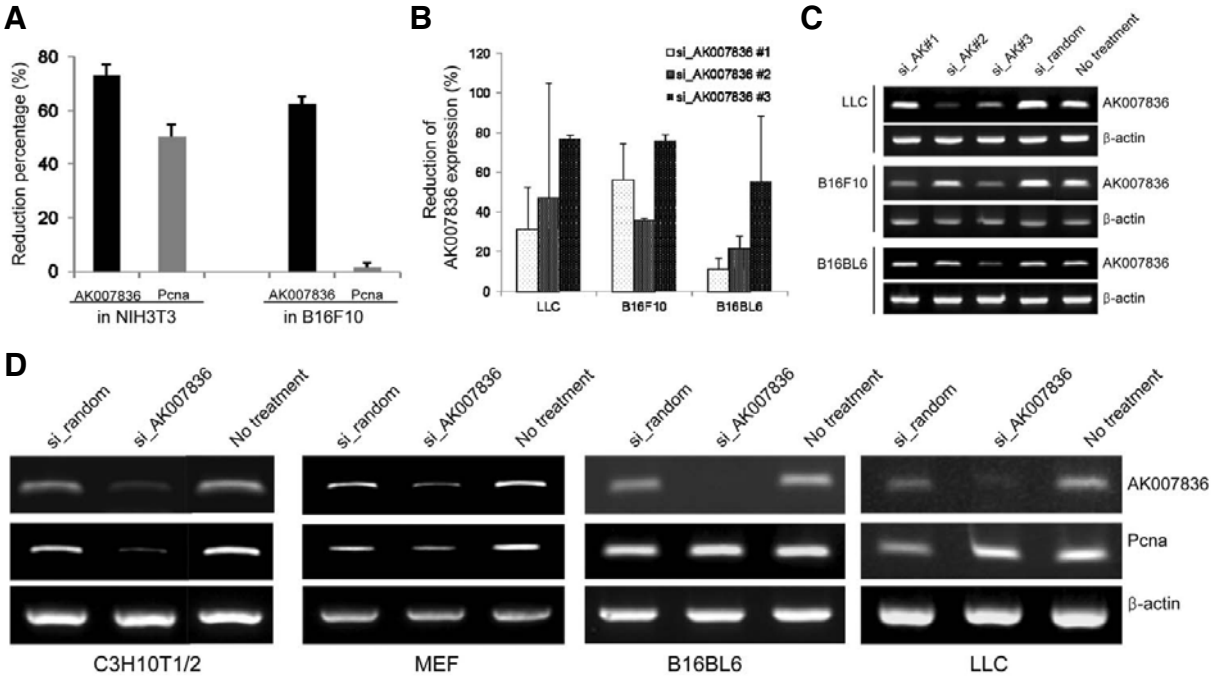

Fig. 4. Effects of AK007836 knockdown on *Pcna* expression. (A) AK007836 siRNA or non-specific control siRNA was transfected into NIH3T3 and B16F10 cells at a final concentration of 20 nM. After 24 h, total RNA was extracted and used for real-time RT-PCR. The β-actin was used to normalize the gene expression level in each sample. The reduction percentage was calculated relative to the values from control siRNA-treated samples. Each experiment was repeated three times using cells prepared separately at different times. (B) Three siRNAs targeting different genomic sequences were designed and tested for the efficiency of AK007837 inhibition in three different cancer cell lines, LLC, B16F10, and B16BL6. The siRNAs were transfected at a final concentration of 20 nM and incubated for 24 h. RT-PCR results were semi-quantitatively analyzed. (C) A representative gel image for (B). (D) To determine whether the reduction of *Pcna* expression by AK007836 knockdown is specifically abrogated in cancer cells, two normal (C3H10 T1/2 and MEF) and two cancer (B16BL6 and LLC) cell lines were tested using si_AK007836 #3. Data are representative of three independent experiments.

cleotides downstream of PCNA, this bidirectional promoter does not seem to be used by RNA polymerase (pol) II for both PCNA and PCNAAS expression, considering that the RNA pol II promoter is located upstream of the gene. However, we cannot rule out the possibility of PCNAAS expression by RNA pol III whose promoter is located in the downstream of the gene; a minimal bidirectional PCNA promoter could have been shared by RNA pol II for PCNA and Pol III for PCNAAS. Since PCNAAS has only one exon as most genes transcribed by RNA pol III do, further investigation will be need to clearly the possibility of PCNAAS expression by RNA pol III. In mice, the ~200-bp 5′ upstream region of Pcna we tested in this study was originally identified as an active minimal promoter for Pcna through a transient CAT-reporter analysis (Yamaguchi et al., 1991). When we analyzed this promoter region by cloning it into the luciferase reporter vector, it turned out to be bidirectional (Fig. 3). Unlike human PCNA, the mouse promoter is located in between ncRNA AK007836 and Pcna separated by 177 bp. Thus, it is probable that this bidirectional promoter is shared by both Pcna and ncRNA expressing in opposite directions, which seemed to be common in higher vertebrate genes (Engström et al., 2006; Osato et al., 2007; Trinklein et al., 2004).

Since ncRNA AK007836 does not have any complementarity to that of Pcna, unlike human PCNAAS whose function is silencing PCNA, we compared the expression profiles of Pcna and ncRNA. Unexpectedly, a positive correlation was found (Fig. 2). When the expression level of ncRNA was reduced by siRNA, the Pcna expression also decreased (Fig. 4), indicating that ncRNA somehow acts positively on Pcna transcription. Although genetic or epigenetic differences between normal and cancer cells might cause a different susceptibility to RNAi, the results in cancerous cells (B16F10, B16BL6 and LLC) was different, still suggesting that a regulatory system mediated by ncRNA is somehow disrupted in cancer cells (Fig. 4) Although the precise mechanism by which AK007836 activates neighboring coding gene expression is still unknown, it might be an example of a class of long ncRNAs having a positive effect on gene expression (Feng et al., 2006; Lanz et al., 1999; Ørom et al., 2010; Shamovsky et al., 2006).

Accumulating evidence indicate that a variety of promoter associated ncRNAs such as promoter-associated long RNAs (PALRs), promoter-associated short RNAs (PASRs), and promoter upstream transcripts (PROMTs), etc., are involved in the protein coding gene transcription (suppression or activation) through epigenetic modification in addition to transcriptional interferences (Carninci, 2010; Costa, 2010; Han et al., 2007; Morris et al., 2008; Preker et al., 2008). The bidirectional promoter identified in this paper is located in a CpG island (68% GC in 200 bp Pcna minimal promoter). Recently, a GC rich promoter was reported to be associated with tiny RNAs mapping within -60 to +120 nt of transcription start sites (TSSs) in both invertebrates and vertebrates (Taft et al., 2009). And tiny TSS-associated RNAs have been reported to help the promoter regions to maintain protein coding genes in an active state (Seila et al., 2008). Although we are not sure whether tiny RNAs were expressed around the GC rich promoter region of

Pcna, we cannot rule out the possibility that ncRNA AK007836 could act along with these tiny RNAs expressed in both directions, if there are any, influencing Pcna expression.

It is now becoming clear that ncRNAs are key regulatory molecules in cells that regulate the expression of genes in close genomic proximity or act in trans on remote targets *via* a variety of mechanisms (An and Song, 2011; Ponting et al., 2009). Considering that PCNA/ Pcna has a critical role in cell proliferation and tumor progression, it is necessary to understand its function, regulation, and evolutionary origin, as well as regulatory elements that affect PCNA/Pcna gene expression. Since numerous ncRNAs have been shown to be misregulated in various diseases including cancer (Osato et al., 2007), our effort to define the biological property of ncRNA AK007836 needs to be followed with further analysis of the role of ncRNA in the regulation of PCNA/Pcna.

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