ANTICANCER RESEARCH *38*: 77-81 (2018)
doi:10.21873/anticanres.12194

The Long Noncoding RNA *OIP5-AS1* Is Involved in the Regulation of Cell Proliferation

MADOKA NAEMURA[1], MASAHIDE KUROKI[2], TOSHIYUKI TSUNODA[3,4], NAGISA ARIKAWA[1], YUUGA SAWATA[1], SENJI SHIRASAWA[3,4] and YOJIRO KOTAKE[1,2]

[1]*Graduate School of Humanity-Oriented Science and Engineering, and*
[2]*Department of Biological and Environmental Chemistry, Faculty of Humanity-Oriented Science and Engineering, Kindai University, Iizuka, Japan;*
[3]*Department of Cell Biology, Faculty of Medicine, and*
[4]*Central Research Institute for Advanced Molecular Medicine, Fukuoka University, Fukuoka, Japan*

Abstract. *Background/Aim: OPA-interacting protein 5 antisense transcript 1 (OIP5-AS1) is a long noncoding RNA located on human chromosome 15q15.1 and transcribed in the opposite direction to OIP5. Here, we report that OIP5-AS1 is involved in regulating cell proliferation. Materials and Methods: HeLa cells were transfected with OIP5-AS1-targeting siRNA oligonucleotides and anti-sense oligonucleotides. The cells were harvested 72 h after transfection and subjected to quantitative reverse transcription-polymerase chain reaction (qRT-PCR), and cell-cycle and apoptosis analysis. Results: OIP5-AS1 was expressed at a lower level in cells harbouring an oncogenic kirsten rat sarcoma viral oncogene homolog (K-RAS) mutation than in cells expressing wild-type K-RAS. Silencing OIP5-AS1 with siRNA oligonucleotides or anti-sense oligonucleotides reduced HeLa cell proliferation. Apoptosis and cell-cycle analysis showed that silencing OIP5-AS1 did not cause apoptosis, but did cause G_2/M phase cell-cycle arrest. Conclusion: These results suggest that OIP5-AS1 positively regulates cell proliferation by promoting G_2/M phase progression.*

Long noncoding RNAs (lncRNAs) are longer than 200 nucleotides and lack functional open reading frames. lncRNAs form a heterogeneous group whose members exhibit different mechanisms of action and are involved in multiple cellular functions, including transcriptional regulation, nuclear structure organization, and post-transcriptional processing (1-3). Recently, we reported that expression levels of multiple lncRNAs fluctuate in response to exogenous and endogenous expression of an oncogenic form of the small GTPase Ras (called oncogenic Ras) (4). Among the lncRNAs whose levels were reduced by oncogenic Ras, antisense noncoding RNA in the *INK4* locus (*ANRIL*) functions to promote cell proliferation (5-7). *ANRIL* is located in the cyclin-dependent kinase inhibitor 2A (*INK4*) locus, which encodes three tumour-suppressor proteins: the cyclin-dependent kinase inhibitors p15 and p16, and alternative reading frame (ARF), which stabilizes the tumour-suppressor protein p53. We and Yap *et al*. have reported that *ANRIL* binds to and recruits polycomb repression complex 1/2 to the *INK4* locus, thus repressing p15 and p16 transcription (5, 6, 8). Furthermore, we recently showed that *ANRIL* promotes human colorectal cancer cell proliferation in both two- and three-dimensional culture (9). Increased levels of *ANRIL* have been observed in several human cancer types, such as gastric (10), prostate (6) and ovarian (11) cancer, which suggests that *ANRIL* is involved in oncogenic transformation.

OPA-interacting protein 5 antisense transcript 1 (*OIP5-AS1*) is a lncRNA located on human chromosome 15q15.1 and evolutionarily conserved in vertebrates (12). Inhibiting cyrano, a zebrafish homolog of human *OIP5-AS1*, was shown to cause embryonic developmental defects (12). Recently, it was reported that silencing human *OIP5-AS1* in HeLa cells promoted cell proliferation, which suggests that OIP5-AS1 functions to inhibit cell proliferation (13). *OIP5-AS1* binds to HuR and prevents it from binding to and stabilizing protein-coding mRNAs such as cyclin A2, thus inhibiting cell proliferation. In this study, we investigated the role of *OIP5-AS1* in cell proliferation.

Correspondence to: Yojiro Kotake, Ph.D., Department of Biological and Environmental Chemistry, Faculty of Humanity-Oriented Science and Engineering, Kindai University, 11-6 Kayanomori, Iizuka, Fukuoka 820-8555, Japan. Tel: +81 948225659 (Ext. 469), Fax: +81 94823053, e-mail: ykotake@fuk.kindai.ac.jp

Key Words: Long noncoding RNA, *OIP5-AS1*, cell proliferation, cell cycle.

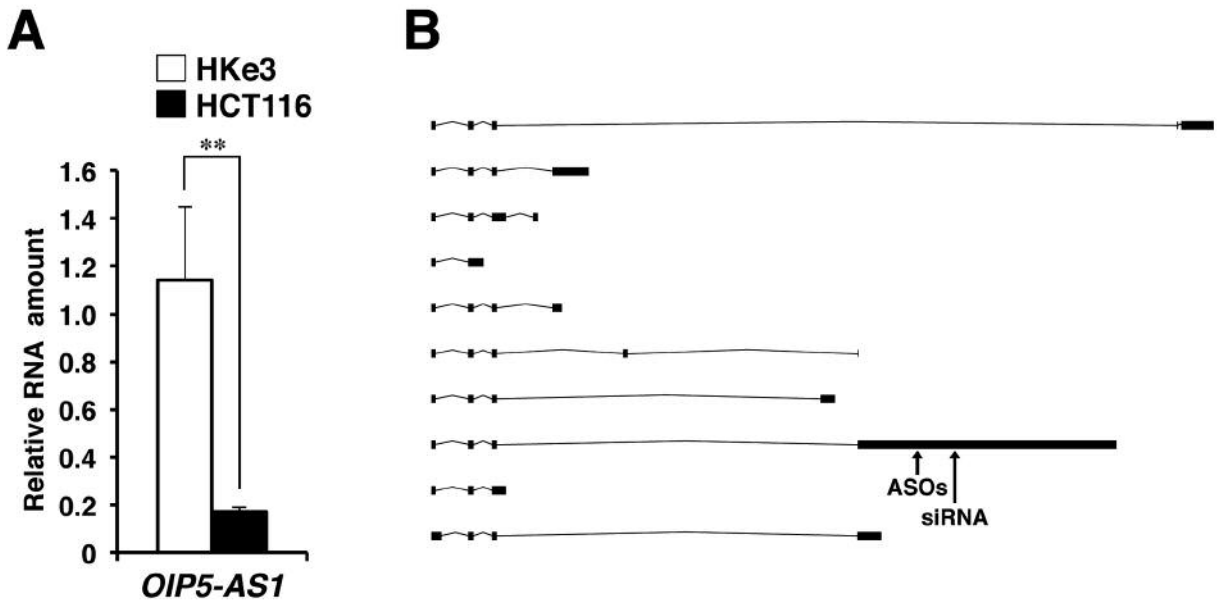

Figure 1. *Opa-interacting protein 5 antisense transcript (OIP5-AS) expression and splice variants. A: OIP5-AS1 expression levels in HKe3 and HCT116 cells were determined by quantitative reverse transcription polymerase chain reaction. The results are expressed relative to the corresponding values for HKe3 cells. Data are presented as the mean±standard deviation (n=3). Data were analyzed using two-tailed Student's t-tests. **Significantly different at p<0.01. B: Schematic representation of OIP5-AS1 splice variants based on data from the Ensembl genome browser. Arrows indicate the positions of small interfering (si)RNA and anti-sense oligonucleotides (ASOs) designed for OIP5-AS1 silencing.*

Materials and Methods

Cell culture. The HeLa human cervical cancer and HCT116 human colorectal cancer cell lines (American Type Culture Collection, Frederick, MD, USA) were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (Gibco, Grand Island, NY, USA). HKe3 cells were established from HCT116 cells with disrupted oncogenic K-RAS (14). The cells were cultured at 37˚C in an atmosphere containing 5% CO_2.

Small interfering RNA (siRNA) and anti-sense oligonucleotide (ASO) transfection. siRNAs or ASOs were transfected into cells using Lipofectamine RNAiMAX (Invitrogen) or Lipofectamine 2000 (Invitrogen), respectively, according to the manufacturer's instructions. The nucleotide sequence of the *OIP5-AS1*-targeting siRNA was 5'-GCAGCAUGCUGUGUGCAAA-3' with 3' dTdT overhangs. The ASOs (Antisense LNA GapmeR) against *OIP5-AS1* were produced by Exiqon (Vedbaek, Denmark). The ASOs contained locked nucleic acid and phosphorothioate modifications. The nucleotide sequence of the ASOs against *OIP5-AS1* was 5'-GAAGTTGGTAGATTAC-3'.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA was extracted using an RNeasy Plus kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized using a SuperScript III First-Strand Synthesis System (Invitrogen). q-PCR was performed using SYBR green PCR master mix (Qiagen) with specific primer sets as follows: human *OIP5-AS1*: 5'-TGCACATACACAGGTTAGA ACAAG-3' and 5'-GAACCTAAACTTGGGTCTCTGG-3'; human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): 5'-GCAAA TTCCATGGCACCGT-3' and 5'-TCGCCCCACTTGATTTTGG-3'.

Apoptosis analysis. Cells were labelled with CellEvent Caspase-3/7 Green Detection Reagent (Invitrogen) according to the manufacturer's instructions, then treated with Hoechst 33342 (Invitrogen). The fluorescence of cells was detected and analysed using an IN Cell Analyzer 2200 (GE Healthcare, Little Chalfont, UK).

Cell-cycle analysis. Cells were fixed in 70% ethanol overnight and stained using a Muse Cell Cycle Kit (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions. The DNA content of the cells was analysed using a Muse Cell Analyzer and analysis software (Merck Millipore).

Statistical analysis. Data are presented as means and standard deviations. Statistical analyses were performed by two-tailed Student's *t*-test. *p*-Values of less than 0.05 were considered to be statistically significant.

Results

We previously reported that expression levels of multiple lncRNAs fluctuate in response to forced expression of oncogenic Ras (4). *OIP5-AS1* is one lncRNA affected by oncogenic Ras signalling. We examined the levels of *OIP5-AS1* in HCT116 and HKe3 cells. HCT116 cells harbour a

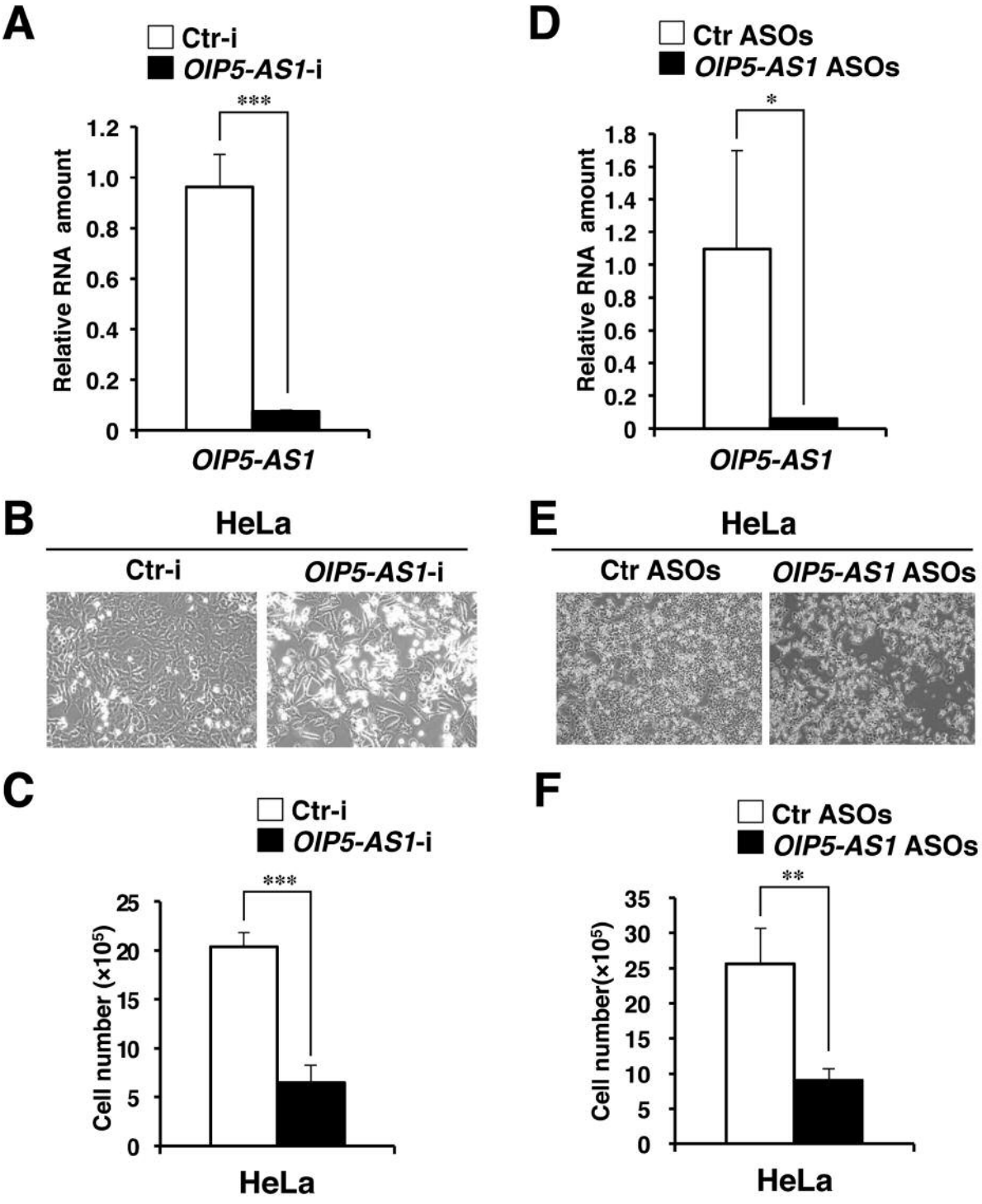

Figure 2. *Depletion of opa-interacting protein 5 antisense transcript 1 (OIP5-AS1) by small interfering (si)RNAs or anti-sense oligonucleotides (ASOs) inhibits HeLa cell proliferation. A: HeLa cells (2×10^5) were seeded and incubated overnight, and then transfected with control (Ctr-i) and OIP5-AS1-targeting (OIP5-AS1-i) siRNA. At 72 h after transfection, cells were harvested and subjected to quantitative reverse transcription PCR to determine OIP5-AS1 expression levels. The results are expressed relative to the corresponding values for HeLa cells transfected with control siRNA. B: HeLa cells transfected with siRNA were observed by phase-contrast microscopy at 72 h after transfection and counted by trypan blue staining (C). D: HeLa cells (5×10^5) were seeded and incubated overnight, and then transfected with control (Ctr) and OIP5-AS1-targeting ASOs. Quantitative reverse transcription PCR was performed as in (A). Imaging (E) and counting (F) of cells were performed as in (B) and (C). Data are presented as mean±standard deviation (n=3) (A, C, D, F). Data were analysed using two-tailed Student's t-tests. Significantly different at: *p<0.05, **p<0.01, ***p<0.001.*

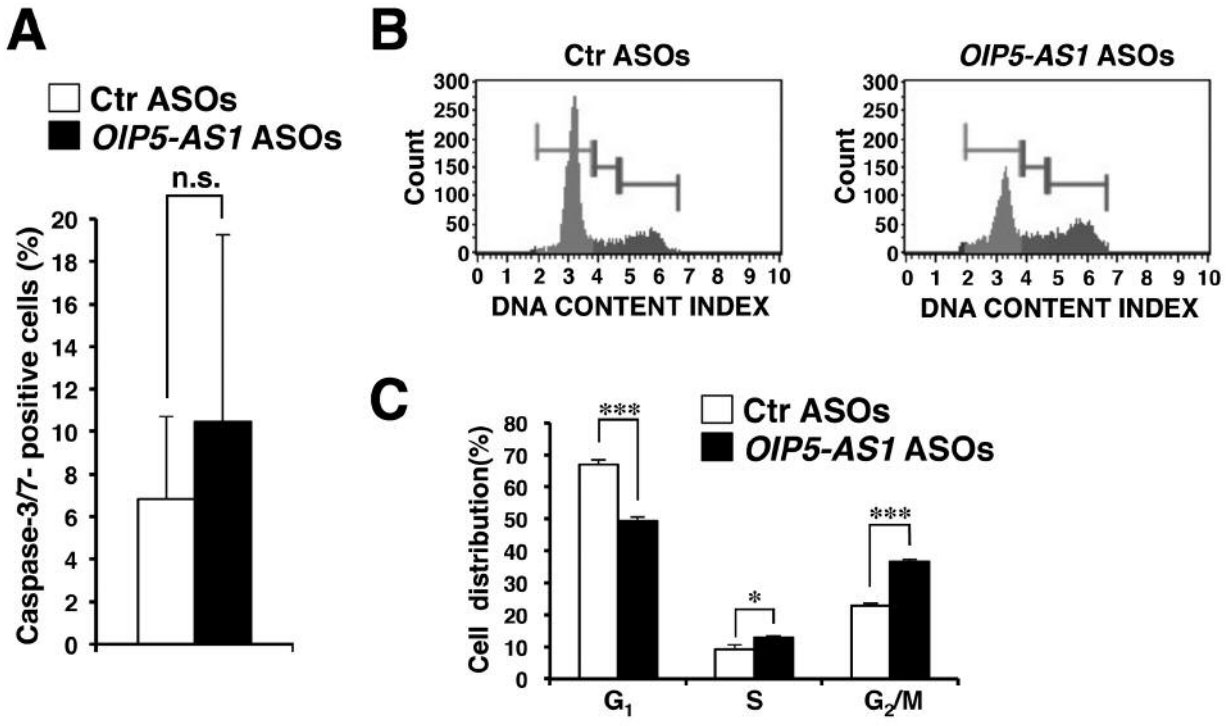

Figure 3. *Silencing opa-interacting protein 5 antisense transcript 1 (OIP5-AS1) does not cause apoptosis, but does cause cell-cycle arrest in G_2/M phase. A: At 72 h after transfection with anti-sense oligonucleotides (ASOs), cells were subjected to apoptosis analysis. The percentage of cells positive for active caspase-3 and -7 was determined by CellEvent Caspase-3/7 Green Detection. Data are presented as the mean±standard deviation (n=3). B: At 72 h after transfection with ASOs, cells were harvested and subjected to cell cycle analysis. C: The percentage of cells in each cell cycle phase. Data are presented as the mean±standard deviation (n=3). Data were analysed using two-tailed Student's t-tests. Significantly different at: *p<0.05, ***p<0.001; n.s., not significantly different.*

heterozygous activating *K-RAS* mutation (G13D); HKe3 cells were established by disrupting the mutated *K-RAS* of HCT116 cells (14). qRT-PCR showed that *OIP5-AS1* is expressed at a low level in HCT116 cells compared with HKe3 cells (Figure 1A), which indicates that oncogenic Ras signaling reduces *OIP5-AS1* expression. We next elucidated the biological function of *OIP5-AS1* in HeLa cells. *OIP5-AS1* has several splicing variants (Figure 1B). We depleted expression of the major *OIP5-AS1* transcriptional product using two different methods. We designed siRNAs for RNA interference-mediated degradation of *OIP5-AS1* and ASOs for RNaseH-mediated degradation of *OIP5-AS1* (Figure 1B). Transfection of siRNAs significantly reduced OIP5-AS1 expression (Figure 2A). Depleting *OIP5-AS1* using siRNAs reduced the number of HeLa cells (Figure 2B and C). Depleting *OIP5-AS1* using ASOs confirmed these results: transfection of ASOs, like siRNA transfection, reduced *OIP5-AS1* expression (Figure 2D) and repressed HeLa cell proliferation (Figure 2E and F). These results suggest that *OIP5-AS1* positively regulates HeLa cell proliferation. This is in contrast to the findings of a recent study by another group that showed that siRNA-mediated depletion of *OIP5-AS1* promoted HeLa cell proliferation, which suggested that *OIP5-AS1* functions to reduce cell proliferation (13). The reason for these conflicting results is not clear. The discrepancy might derive from the different target regions of the siRNAs or ASOs used to reduce the *OIP5-AS1* level.

We next investigated the effects of *OIP5-AS1* depletion on apoptosis and the cell cycle. *OIP5-AS1* depletion did not affect the number of cells staining positively for caspase-3/7 activity (Figure 3A), which suggests that *OIP5-AS1* is not involved in regulating apoptosis. Cell-cycle analysis demonstrated that OIP5-AS1 depletion reduced the proportion of cells in the G_1 phase and markedly increased the proportion of cells in the G_2/M phase (Figure 3B and C). These results suggest that *OIP5-AS1* positively regulates cell proliferation by promoting G_2/M phase progression.

Discussion

In this study, we showed that *OIP5-AS1* positively regulates HeLa cell proliferation. We showed that *OIP5-AS1* is highly expressed in HCT116 cells harbouring an activating *K-RAS*

mutation compared to HKe3 cells established by disrupting the mutated *K-RAS* of HCT116 cells (14), which suggests that *K-RAS* signaling represses *OIP5-AS1* expression. We previously reported that Ras signalling repressed the expression of *ANRIL*, a lncRNA that promotes cell proliferation by repressing the cyclin-dependent kinase inhibitor p15 (5). Ras signaling controls cell fate determination *via* processes such as cell proliferation, cell survival, apoptosis and cell-cycle arrest by regulating many downstream effector molecules (15). *OIP5-AS1* and *ANRIL* may have an important role in determination of cell fate as downstream effectors of Ras signalling.

We demonstrated that depleting *OIP5-AS1* using siRNAs or ASOs repressed HeLa cell proliferation, which supports the findings of a previous report that showed that inhibiting cyrano, a zebrafish homolog of human *OIP5-AS1*, causes embryonic developmental defects (12). Recently, another group reported that *OIP5-AS1* functions to repress HeLa proliferation by binding to Hu Antigen R (HuR) and preventing it from binding to and stabilizing protein-coding mRNAs such as cyclin A2 (13). This is in contrast to our findings. This discrepancy may result from the different *OIP5-AS1* regions targeted by the siRNAs or ASOs used to deplete *OIP5-AS1*. Supporting this, a recent study reported that the genes affected by the silencing of ANRIL exon 1 and 19 differ (16). Like *OIP5-AS1*, *ANRIL* has several splice variants (17). It might be that each splice variant of *ANRIL* and *OIP5-AS1* has distinct target genes or functions.

Furthermore, we showed that depleting *OIP5-AS1* caused cell-cycle arrest in G_2/M phase. This finding suggests that *OIP5-AS* positively regulates cell proliferation by promoting G_2/M phase progression. However, the molecular mechanism by which *OIP5-AS1* promotes G_2/M phase progression and the role of *OIP5-AS1* in the Ras signalling pathway are yet to be determined and are important issues that require further investigation.

Acknowledgements

The Authors thank the members of the Kotake Laboratory for their technical assistance and helpful discussions. This work was supported by JSPS KAKENHI grant number 17K07184 (to YK) and the Naito Foundation (to YK). The Authors thank Ruth Tunn, Ph.D., from Edanz Group (www.edanzediting.com/ac) for editing a draft of this article.

References

1 Mercer TR, Dinger ME and Mattick JS: Long non-coding RNAs: insights into functions. Nat Rev Genet *10*: 155-159, 2009.
2 Kitagawa M, Kotake Y and Ohhata T: Long non-coding RNAs involved in cancer development and cell fate determination. Curr Drug Targets *13*: 1616-1621, 2012.
3 Wilusz JE, Sunwoo H and Spector DL: Long noncoding RNAs: functional surprises from the RNA world. Genes Dev *23*: 1494-1504, 2009.
4 Kotake Y, Naemura M, Kitagawa K, Niida H, Tsunoda T, Shirasawa S and Kitagawa M: Oncogenic Ras influences the expression of multiple lncRNAs. Cytotechnology *68*: 1591-1596, 2016.
5 Kotake Y, Nakagawa T, Kitagawa K, Suzuki S, Liu N, Kitagawa M and Xiong Y: Long non-coding RNA *ANRIL* is required for the PRC2 recruitment to and silencing of *p15(INK4B)* tumor-suppressor gene. Oncogene *30*: 1956-1962, 2011.
6 Yap KL, Li S, Munoz-Cabello AM, Raguz S, Zeng L, Mujtaba S, Gil J, Walsh MJ and Zhou MM: Molecular interplay of the noncoding RNA *ANRIL* and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of *INK4a*. Mol Cell *38*: 662-674, 2010.
7 Naemura M, Murasaki C, Inoue Y, Okamoto H and Kotake Y: Long noncoding RNA *ANRIL* regulates proliferation of non-small cell lung cancer and cervical cancer cells. Anticancer Res *35*: 5377-5382, 2015.
8 Kotake Y, Naemura M, Murasaki C, Inoue Y and Okamoto H: Transcriptional regulation of the *p16* tumor-suppressor gene. Anticancer Res *35*: 4397-4401, 2015.
9 Naemura M, Tsunoda T, Inoue Y, Okamoto H, Shirasawa S and Kotake Y: *ANRIL* regulates the proliferation of human colorectal cancer cells in both two- and three-dimensional culture. Mol Cell Biochem *412*: 141-146, 2016.
10 Zhang EB, Kong R, Yin DD, You LH, Sun M, Han L, Xu TP, Xia R, Yang JS, De W and Chen J: Long noncoding RNA *ANRIL* indicates a poor prognosis of gastric cancer and promotes tumor growth by epigenetically silencing of *miR-99a*/*miR-449a*. Oncotarget *5*: 2276-2292, 2014.
11 Qiu JJ, Wang Y, Liu YL, Zhang Y, Ding JX and Hua KQ: The long non-coding RNA *ANRIL* promotes proliferation and cell cycle progression and inhibits apoptosis and senescence in epithelial ovarian cancer. Oncotarget *7*: 32478-32492, 2016.
12 Ulitsky I, Shkumatava A, Jan CH, Sive H and Bartel DP: Conserved function of lncRNAs in vertebrate embryonic development despite rapid sequence evolution. Cell *147*: 1537-1550, 2011.
13 Kim J, Abdelmohsen K, Yang X, De S, Grammatikakis I, Noh JH and Gorospe M: LncRNA *OIP5-AS1*/cyrano sponges RNA-binding protein HuR. Nucleic Acids Res *44*: 2378-2392, 2016.
14 Shirasawa S, Furuse M, Yokoyama N and Sasazuki T: Altered growth of human colon cancer cell lines disrupted at activated Ki-ras. Science *260*: 85-88, 1993.
15 Karnoub AE and Weinberg RA: Ras oncogenes: split personalities. Nat Rev Mol Cell Biol *9*: 517-531, 2008.
16 Congrains A, Kamide K, Katsuya T, Yasuda O, Oguro R, Yamamoto K, Ohishi M and Rakugi H: CVD-associated non-coding RNA, *ANRIL*, modulates expression of atherogenic pathways in VSMC. Biochem Biophys Res Commun *419*: 612-616, 2012.
17 Burd CE, Jeck WR, Liu Y, Sanoff HK, Wang Z and Sharpless NE: Expression of linear and novel circular forms of an INK4/ARF-associated non-coding RNA correlates with atherosclerosis risk. PLoS Genet *6*: e1001233, 2010.

Received September 30, 2017
Revised November 14, 2017
Accepted November 15, 2017