Long Noncoding RNA ANROC on the INK4 Locus Functions to Suppress Cell Proliferation

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Abstract. Background/Aim: The INK4 locus encodes three important genes p15INK4B, p16INK4A, and ARF, which function to suppress oncogenesis, and a long noncoding RNA, ANROC, which, in contrast, functions to promote oncogenesis. Herein, we report a fifth genetic element on the INK4 locus, a long noncoding RNA with unknown function named associated negative regulation of cell proliferation (ANROC), which played a role in the suppression of cell proliferation. Materials and Methods: Following ANROC silencing in cells by siRNA, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and cell cycle analysis using flow cytometry were performed. Results: ANROC expression was decreased by oncogenic RAS signalling. ANROC knockdown enhanced HeLa cell proliferation and induced cyclin B1 mRNA, which promotes G2/M progression of the cell cycle. Furthermore, flow cytometric analysis revealed that ANROC knockdown increased the percentage of cells in the S and G2/M phases of the cell cycle. Conclusion: ANROC functions to suppress cell cycle progression by suppressing cyclin B1 expression, thus inhibiting cell proliferation.

The INK4 locus is located on human chromosome 9p21 and is mutated, deleted, and transcriptionally suppressed in a wide range of human cancers (1, 2). This locus encodes three tumour suppressor genes, the CDK inhibitors p16INK4A and p15INK4B, and the p53 stabilizing factor ARF, which are mainly regulated at the transcriptional level (3). Along with Bracken et al., we have reported that polycomb repression complex1 (PRC1) and 2 (PRC2) directly bind to and epigenetically suppress p16INK4A and p15INK4B transcription on the INK4 locus (4, 5). Furthermore, we and Yap et al. have revealed that an antisense noncoding RNA transcribed from the INK4 locus (ANRIL) binds to and recruits PRC1 and PRC2 to the INK4 locus, thus suppressing p16INK4A and p15INK4B transcription (6, 7). ANRIL is classified as a long noncoding RNA (lncRNA); these are defined by lengths exceeding 200 nucleotides. Recent studies have revealed that IncRNAs have various functions such as gene regulation, organization of nuclear architecture, and regulation of interacting proteins and RNAs, and are involved in normal physiology and disease (8). ANRIL also functions to suppress cellular senescence and to promote cancer cell proliferation, and thus is thought to be an oncogene (6, 7, 9-11).

Indeed, many studies of clinical cancer specimens have revealed that ANRIL is highly expressed in many types of human cancers such as gastric cancer, non-small cell lung cancer, hepatocellular carcinoma, ovarian cancer, and cervical cancer (12).

Cell proliferation is stringently modulated by the cell cycle, the progression of which is promoted by protein kinase complexes comprising cyclins and cyclin-dependent kinases (CDKs) and is negatively regulated by CDK inhibitors (CKIs) (13). Disruption of cell cycle regulation leads to oncogenic transformation (13). In this study, we identified another functional IncRNA transcribed from the INK4 locus named ANROC, and showed that it participates in the suppression of cell cycle progression via inhibiting cyclin B1, which leads to inhibition of cell proliferation.

Materials and Methods

Cell culture and small interfering RNA (siRNA). The Human cervical cancer cells HeLa were cultured as described previously (10). HeLa cells were infected with retroviruses carrying HRASG12V. Retrovirus generation and infection were performed as described previously (5). siRNAs were used to down-regulate ANROC expression in HeLa cells. ANROC siRNAs were synthesized by GeneDesign, Inc. (Osaka, Japan). The nucleotide sequence of ANROC siRNA was 5’-ACCGCAUUUCAUCGAUCU-3’ with 3’dTdT overhangs. A total
of 5×10⁵ HeLa cells were seeded and cultured for 24 h, and then siRNA transfection was performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. Cells transfected with siRNAs for 72 h were subjected to quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and cell cycle analysis.

qRT-PCR. Total RNA was extracted from cells using a RNeasy Plus kit (Qiagen, Hilden, Germany) and subjected to DNase treatment with a TURBO DNA-free Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. cDNA synthesis was performed with the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific). The synthesized cDNAs were amplified using a Quantitect SYBR Green PCR kit (Qiagen) with 200 nM primers. The nucleotide sequences of primers were as follows: ANROC 5'-CTGAGGCGCTGTAGCAGAAAA-3' and 5'-AGACTGAGACTGGCACCAGC-3', cyclin A1 5'-GACAAGCTCAAGTCACGACGACGCA-3' and 5'-ATGACCTCTTGACCAAGACTGGA-3', cyclin B1 5'-GACCTGTCAGGCTCTTCTGC-3' and 5'-GGTTATTGTTGGCTTGACTGTGCTG-3', cyclin D1 5'-GAGCTGCTGCAAATGGAACGTCGGG-3' and 5'-AAAGGGAAATGCGTTTGTCGGG-3', cyclin E2 5'-TGTGTCTCTGGATGTTGACTGCC-3' and 5'-CTCTATGTTCGACCACCTGACCCAGG-3'. The nucleotide sequences of primers for p16IK4A, p15INKB, and ARF have been described previously. Real-time PCR assays were performed using the Mx3000P Real-Time Q-PCR System (Agilent Technologies, Santa Clara, CA, USA).

Cell cycle analysis. A total of 1×10⁶ cells were fixed with 70% ethanol overnight at −20°C and stained using a Muse™ Cell Cycle Kit (Merck Millipore, Darmstadt, Germany) according to the manufacturer’s instructions. The stained cells were then analysed with a Muse Cell Analyzer (Merck Millipore).

Statistical analysis. All experiments were performed in triplicate and the data are shown as means and standard deviations. The results were analysed using a two-tailed t-test using Microsoft Office Excel. p-Values of <0.05 were considered to indicate statistically significant differences.

Results

We have previously shown that the lncRNA ANRIL transcribed from the INK4 locus, suppressed p16INK4A and p15INK4B transcription in a cis-acting manner (6). We also found another lncRNA with an unknown function (GebeBank ID: BX091351.1, C DNA divergent transcript) transcribed from the periphery of the INK4 locus by searching the expressed sequence tags (EST) database provided by the National Center for Biotechnology Information (NCBI; Figure 1A). In this study, we demonstrated that this lncRNA participates in the negative regulation of cell proliferation, and thus named it ANROC (associated negative regulation of
cell proliferation). The ANROC sequence comprises only one exon with a polyadenylation site, and the transcript length is 616 bases, but there is no significant open reading frame.

Initially, we analysed ANROC expression in W138 and TIG-3 human normal diploid foetal lung fibroblasts and in H1299 and ABC-1 human non-small cell lung cancer, HCT116 human colorectal cancer, MCF7 human breast cancer, and HeLa human cervical cancer cells. RT-PCR data revealed that ANROC was highly expressed in H1299 and HeLa cells (Figure 1B). We have previously shown that oncogenic RAS signalling affects the transcription of the INK4 locus (6). The forced expression of oncogenic HRASG12V induces p16INK4A and p15INK4B transcription and inversely suppresses ANRIL expression (6). We, therefore, examined whether ANROC expression is also affected by oncogenic RAS signalling. qRT-PCR data revealed that ANROC RNA levels were decreased by overexpressing oncogenic HRASG12V, which indicates that ANROC expression is suppressed by oncogenic RAS signalling (Figure 1C).

We next examined the biological function of ANROC in HeLa cells, in which ANROC is highly expressed. Silencing of ANROC resulted in the promotion of HeLa cell proliferation (Figure 2A and B). The same result was obtained with the human oral cancer cell line CAL27 (Figure 2C). These results suggested that ANROC functions to suppress cell proliferation.

Given that another lncRNA transcribed from the INK4 locus, ANRIL, suppresses p16INK4A and p15INK4B transcription (6), we examined whether ANROC also participates in the regulation of the INK4 locus. qRT-PCR data showed that ANROC knockdown caused an increase in p16INK4A, p15INK4B, and ARF mRNA levels, but did not affect ANRIL RNA levels (Figure 3A), which suggests that ANROC plays a role in the suppression of p16INK4A, p15INK4B, and ARF transcription. Even though the expression of p16INK4A, p15INK4B, and ARF was increased by ANROC knockdown, cell proliferation was unexpectedly promoted. We therefore examined whether ANROC regulates cyclins that promote cell proliferation. qRT-PCR data indicated that ANROC knockdown caused an increase in cyclin B1 mRNA levels (Figure 3B).

Given that ANROC knockdown increased cyclin B1 expression, which promotes transition to the G2/M of the cell cycle, we next examined whether ANROC participates in the regulation of the cell cycle. Cell cycle analysis showed that ANROC knockdown significantly decreased the proportion of cells in the G1 phase and increased the number

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Figure 2. Silencing of ANROC promotes HeLa cell proliferation. A: Phase-contrast images of HeLa cells transfected with control siRNA (Ctr-i) or siRNA against ANROC (ANROC-i) for 72 h. B: Proliferation curves of HeLa cells/Ctr-i and ANROC-i. Viable cells were counted by staining with trypan blue. The x-axis indicates days since transfection of siRNA. C: CAL27 cells transfected with siRNA for 72 h were counted by trypan blue staining. *p<0.05.
of cells in the S and G2/M phase (Figure 4A and B). Taken together, these data suggest that ANROC functions to suppress cell cycle progression via suppressing cyclin B1 expression.

Discussion

To date, many studies have revealed the biological importance of the INK4 locus, especially the function of the coding genes p16INK4A and p15INK4B, and the stabilizing factor of p53 tumour suppressor ARF, as well as the long noncoding RNA ANRIL (1, 12). In this study, we identified a fifth functional genetic element of the INK4 locus, ANROC, and revealed that it negatively regulates cell proliferation. We observed that ANROC expression is suppressed by oncogenic RAS signalling, which indicates that ANROC antagonizes the function of the oncogenic RAS signalling that causes hyperproliferation. In support of this, ANROC knockdown promoted the proliferation of HeLa and CAL27 cells, which suggests that ANROC functions to suppress cell proliferation. However, the molecular mechanism by which oncogenic RAS signalling suppresses ANROC expression is unclear and is an important issue that warrants further study.

We and Yap et al. have previously reported that the long noncoding RNA ANRIL suppresses the transcription of p16INK4A and p15INK4B on the INK4 locus in a cis-acting manner (6, 7). In this study, we also showed that silencing

Figure 3. Silencing of ANROC increases the mRNA expression levels of p16INK4A, p15INK4B, ARF, and cyclin B1. The mRNA levels of ANROC, p16INK4A, p15INK4B, ARF, ANRIL (A) and cyclins (B) were measured by qRT-PCR. The results are shown as relative values based on the values of HeLa cells transfected with control siRNA (Ctrl-i). *p<0.05, **p<0.01, n.s., not significant.

Figure 4. Silencing of ANROC results in an increase in the number of cells in the S and G2/M phases and a decrease in the number of cells in the G1 phase. A: Histogram of cell cycle analysis of HeLa cells transfected with control siRNA (Ctrl-i) or siRNA against ANROC (ANROC-i) for 72 h. The x-axes show the fluorescence intensity of propidium iodide (DNA content index). The y-axes show cell number. B: The percentages of HeLa cells transfected with siRNA in the G1, S, and G2/M phase. ***p<0.001.
of ANROC increased p16\textsuperscript{INK4A}, p15\textsuperscript{INK4B}, and ARF expression, which implies that ANROC negatively regulates the transcription of the INK4 locus as well as ANRIL. Even though these negative regulators of cell proliferation were increased, silencing of ANROC promoted cell proliferation, which indicates that ANROC plays a role in the negative regulation of cell proliferation in a trans-acting manner. In support of this, ANROC knockdown increased cyclin B1 expression, promoting G2/M progression. We also showed that silencing of ANROC resulted in a decrease of cells in the G1 phase and an increase of cells in the S and G2/M phases. Taking together these results, ANROC is proposed to act as a negative regulator of cell cycle progression via suppressing cyclin B1 expression, which leads to the inhibition of cell proliferation (Figure 5). Recent studies have shown that many IncRNAs function to regulate gene expression by associating with proteins such as polycomb proteins and transcription factors and miRNAs (8). The identification of factors associated with ANROC will be required to elucidate its mechanism of action.

The deletion, mutation, and transcriptional suppression of the INK4 locus is frequently observed in a wide range of human cancers (1). Thus, this locus is thought to be important in preventing oncogenesis. ANROC may also function as a tumour suppressor in addition to p16\textsuperscript{INK4A} and p15\textsuperscript{INK4B} on the INK4 locus. Clarification of the role of ANROC in oncogenesis is an important and requires further study.

Conflicts of Interest

The Authors have no conflicts of interest directly relevant to the contents of this article.

Authors’ Contributions

Conceptualization and design, Y.K. and T.T.; supervision, Y.K.; materials, Y.K. and T.T.; data collection, Y.K. and T.T.; analysis, Y.K. and T.T.; and writing, Y.K.

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