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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 $p15^{INK4B}$, $p16^{INK4A}$, and ARF, which function to suppress oncogenesis, and a long noncoding RNA, ANRIL, 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 G_2/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 $p16^{INK4A}$ and $p15^{INK4B}$, 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 $p16^{INK4A}$ and $p15^{INK4B}$ 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 $p16^{INK4A}$ and $p15^{INK4B}$ 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 lncRNAs 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 lncRNA 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 $HRAS^{G12V}$. 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'-ACCGCAUUUCAUCGAUCUU-3' with 3'dTdT overhangs. A total

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Key Words: INK4 locus, long noncoding RNA, cell cycle, cyclin B1, CDK inhibitor.

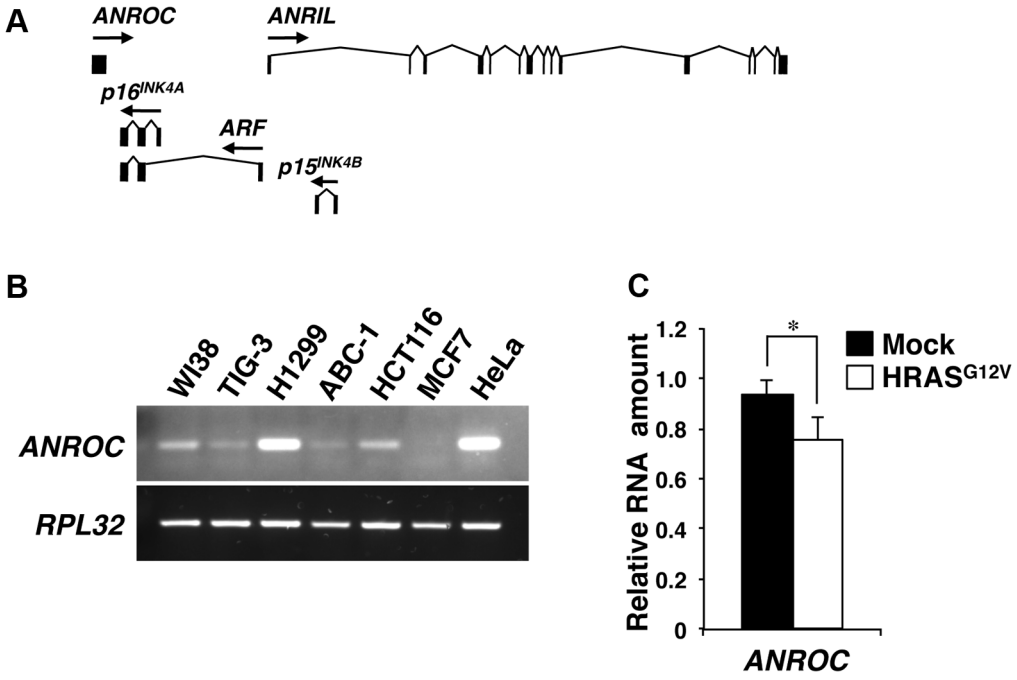

Figure 1. *Analysis of ANROC expression. A: A schematic diagram of ANROC, ANRIL, p16INK4A, p15INK4B, and ARF. The arrow heads indicate the direction of transcription. The black boxes show the exons of each gene. B: ANROC expression in the indicated human cell lines was detected by RT-PCR. Ribosomal protein L32 (RPL32) expression was detected as an internal control. C: HeLa cells were infected with retroviruses carrying HRAS^{G12V} or mock vector. The expression levels of ANROC were assessed by qRT-PCR. The results are shown as relative values based on the values of HeLa cells/mock. *p<0.05.*

of 5×10^5 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'-CTGAGGCCTGGTGAGCAAAA-3' and 5'-AGACTCGAGACT GGCACATC-3', *cyclin A1* 5'-GCACACTCAAGTCAGACCTGCA-3' and 5'-ATCACATCTGTGCCAAGACTGGA-3', *cyclin B1* 5'-GACCTGTGTCAGGCTTTCTCTG-3' and 5'-GGTATTTTGGTC TGACTGCTTGC-3', *cyclin D1* 5'-GAGCTGCTGCAAATG GAACTG-3' and 5'-AAAGAAAGTGCGTTGTGCGG-3', *cyclin E1* 5'-TGTGTCCTGGATGTTGACTGCC-3' and 5'-CTCTA TGTCGCACCACTGATACC-3'. The nucleotide sequences of primers for *p15^{INK4B}*, *p16^{INK4A}*, *ARF*, *GAPDH* (9), and *ANRIL* (10) 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 1×10^6 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 *p16^{INK4A}* and *p15^{INK4B}* transcription in a *cis*-acting manner (6). We also found another lncRNA with an unknown function (GebeBank ID: BX091351.1, *CDKN2A* 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

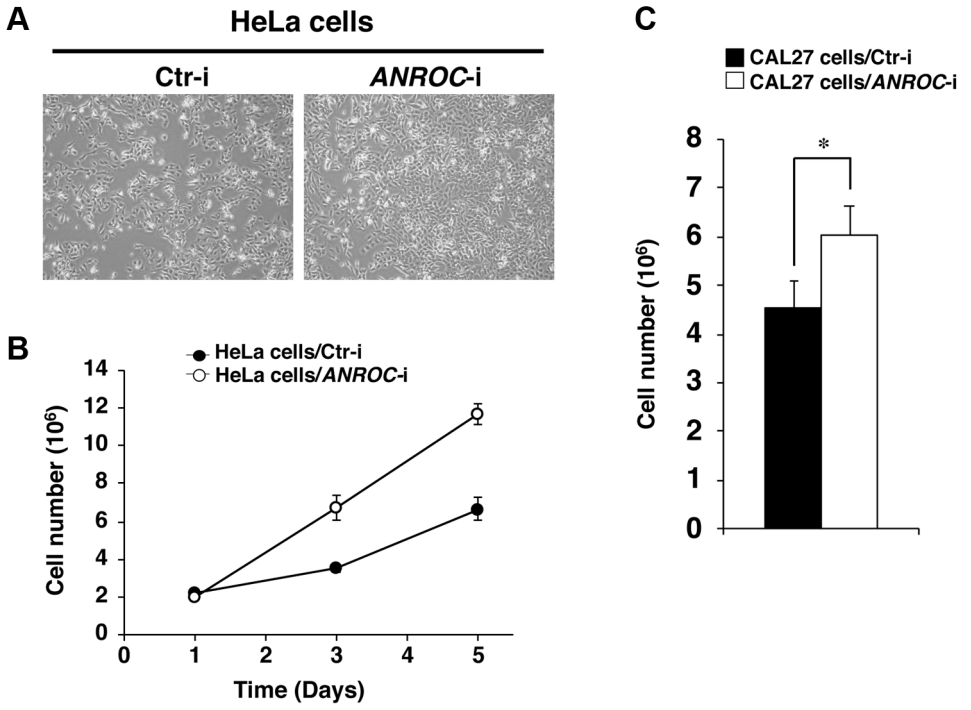

Figure 2. *Silencing of ANROC promotes HeLa cell proliferation. A: Phase-contrast images of HeLa cells transfected with control siRNA (Ctr-i) or siRNA against ANROC (ANROC-i) for 72 h. B: Proliferation curves of HeLa cells/Ctr-i and /ANROC-i. Viable cells were counted by staining with trypan blue. The 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.*

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 WI38 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 $HRAS^{G12V}$ induces $p16^{INK4A}$ and $p15^{INK4B}$ 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 $HRAS^{G12V}$, 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 $p16^{INK4A}$ and $p15^{INK4B}$ 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 $p16^{INK4A}$, $p15^{INK4B}$, and *ARF* mRNA levels, but did not affect *ANRIL* RNA levels (Figure 3A), which suggests that *ANROC* plays a role in the suppression of $p16^{INK4A}$, $p15^{INK4B}$, and *ARF* transcription. Even though the expression of $p16^{INK4A}$, $p15^{INK4B}$, 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 G_1 phase and increased the number

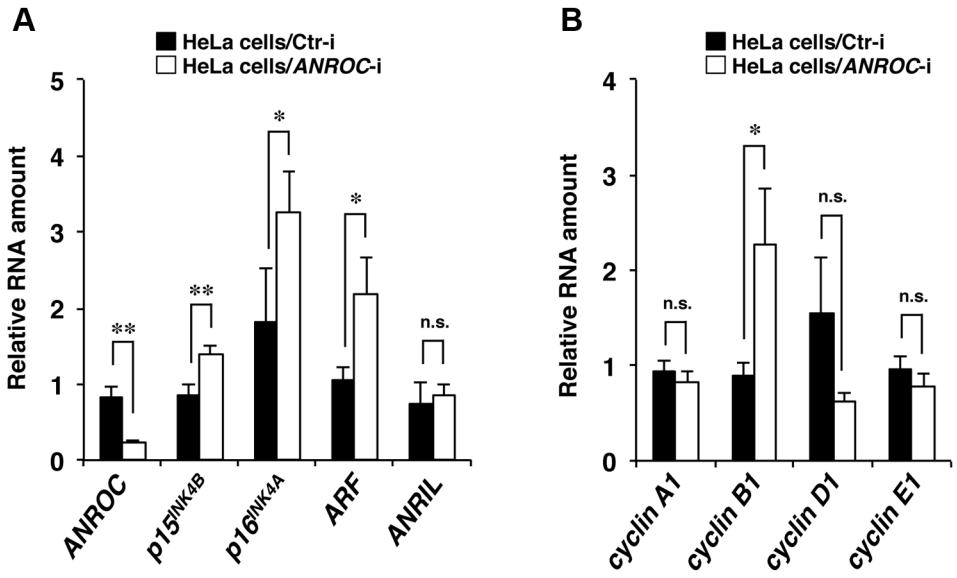

Figure 3. *Silencing of ANROC increases the mRNA expression levels of $p16^{INK4A}$, $p15^{INK4B}$, ARF, and cyclin B1. The mRNA levels of ANROC, $p16^{INK4A}$, $p15^{INK4B}$, ARF, ANRIL (A) and cyclins (B) were measured by qRT-PCR. The results are shown as relative values based on the values of HeLa cells transfected with control siRNA (Ctr-i). *p<0.05, **p<0.01, n.s., not significant.*

of cells in the S and G_2/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 $p16^{INK4A}$ and $p15^{INK4B}$, 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 $p16^{INK4A}$ and $p15^{INK4B}$ on the *INK4* locus in a *cis*-acting manner (6, 7). In this study, we also showed that silencing

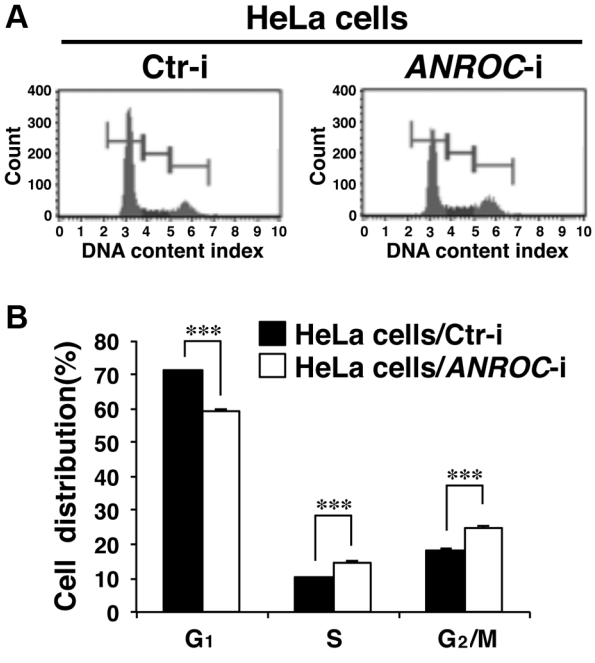

Figure 4. *Silencing of ANROC results in an increase in the number of cells in the S and G_2/M phases and a decrease in the number of cells in the G_1 phase. A: Histogram of cell cycle analysis of HeLa cells transfected with control siRNA (Ctr-i) or siRNA against ANROC (ANROC-i) for 72 h. The x-axes 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 G_2/M phase. ***p<0.001.*

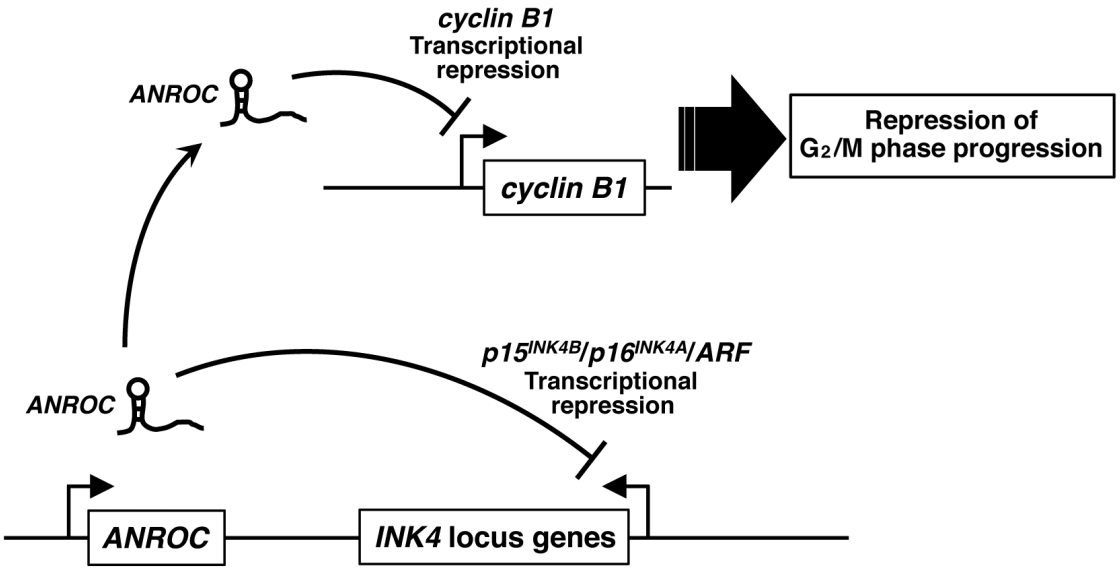

Figure 5. *Model of ANROC function. ANROC transcribed from the INK4 locus suppresses the expression of $p16^{INK4A}$, $p15^{INK4B}$, ARF, and cyclin B1 in cis- and trans-acting manners. ANROC suppresses the progression of the cell cycle, leading to the inhibition of cell proliferation.*

of *ANROC* increased $p16^{INK4A}$, $p15^{INK4B}$, 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 G_2/M progression. We also showed that silencing of *ANROC* resulted in a decrease of cells in the G_1 phase and an increase of cells in the S and G_2/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 lncRNAs 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 $p16^{INK4A}$ and $p15^{INK4B}$ 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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