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LncRNA-ANRIL regulates CDKN2A to promote malignant proliferation of Kasumi-1 cells

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Abstract

Objective This study aimed to investigate the regulatory effects of long non-coding RNA-ANRIL on CDKN2A in the cell cycle of Kasumi-1 cells and elucidate the underlying molecular mechanisms.

Methods ANRIL and CDKN2A expression levels were quantified using RT-qPCR in peripheral blood samples from acute myeloid leukemia (AML) patients. CDKN2A knockdown efficiency was validated via RT-qPCR, and cell cycle distribution was analyzed using flow cytometry. Cell proliferation assays were conducted with CCK-8 following palbociclib treatment and CDKN2A downregulation. RNA immunoprecipitation (RIP) identified potential ANRIL-associated targets, while western blotting assessed the expression levels of GSK3β/β-catenin/cyclin D1 signaling components and related proteins.

Results ANRIL and CDKN2A were markedly overexpressed in AML patient samples. Knockdown of ANRIL and CDKN2A led to G1 phase arrest accompanied by reduced CDK2/4/6 and cyclin D1 protein levels, while ANRIL upregulation induced the opposite effect. Palbociclib treatment for 24 h and 48 h elevated the G1 phase cell population and suppressed CDK2/4/6 and cyclin D1 protein expression, demonstrating its ability to counteract ANRIL-driven cell cycle progression. Downregulation of ANRIL and CDKN2A also suppressed the GSK3β/β-catenin signaling pathway, reducing cyclin D1 expression, whereas ANRIL upregulation reactivated this axis. Co-transfection experiments showed that simultaneous cyclin D1 suppression and ANRIL overexpression attenuated ANRIL's stimulatory effects on cell cycle progression. RIP analysis confirmed a physical interaction between ANRIL and CDKN2A. Furthermore, CDKN2A downregulation inhibited cell proliferation and reversed GSK3β/β-catenin/cyclin D1 pathway activation mediated by ANRIL upregulation.

Conclusion ANRIL facilitates Kasumi-1 cell survival by modulating CDKN2A to activate the GSK3β/β-catenin/cyclin D1 signaling pathway.

Keywords AML-M2, ANRIL, CDKN2A, Cell cycle, Palbociclib

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Introduction

Acute myeloid leukemia (AML) is characterized by impaired maturation of myeloid cells in both bone marrow and peripheral blood, driven by uncontrolled proliferation of myeloid progenitor cells and inhibited differentiation, ultimately disrupting normal hematopoiesis [1]. Representing over 50% of leukemia cases, AML exhibits a higher incidence of the M2 and M5 subtypes [2]. Long non-coding RNAs (lncRNAs), defined as RNA molecules longer than 200 nucleotides without a distinct open reading frame, play multifaceted regulatory roles in cancer biology. ANRIL, located on chromosome 9p21, undergoes intricate regulation mediated by diverse mechanisms [3]. In lung, pharyngeal, and hepatocellular carcinomas, for example, its expression is modulated by oncogenic transcription factors such as c-MYC, SOX2, and SP1 [4–6]. In AML, ANRIL is activated via the adiponectin receptor 1 (AdipoR1)/AMP-activated protein kinase (AMPK)/sirtuin 1 (SIRT1) glucose metabolism axis, which enhances the survival of malignant cells [7]. ANRIL promotes proliferation, migration, and invasion of AML cells while suppressing apoptosis through its regulation of microRNA (miR)-34a, histone deacetylase 1 (HDAC1), and ASPP2 [8]. The ANRIL locus includes the CDKN2A gene, suggesting a regulatory interplay influencing AML progression [9], indicating a potential regulatory relationship impacting AML development. CDKN2A encodes two proteins, p16 and p14, each playing distinct roles in cell cycle regulation. p16 enforces G1 phase arrest by inhibiting cyclin-dependent kinases and preventing retinoblastoma protein phosphorylation, while p14 suppresses HDM2 activity, stabilizing p53 protein levels [10, 11]. CDKN2A expression has been linked to tumor cell proliferation, angiogenesis within the tumor microenvironment, and decreased chemotherapy sensitivity [12]. This research paper focuses specifically on p16. However, its precise role in AML remains poorly understood.

ANRIL expression has been shown to be elevated in non-M3 AML patients, particularly in the M2 subtype of Kasumi-1 cells compared to normal cells. Building upon established stable modulation of ANRIL expression in Kasumi-1 cells (down and upregulation of ANRIL expression), prior findings indicate that ANRIL downregulation may suppress cell proliferation and PCNA expression, induce apoptosis, and inhibit the activation of the PI3K/AKT signaling pathway. Conversely, ANRIL upregulation appears to enhance cell proliferation and PCNA expression, reduce apoptosis, and activate PI3K/AKT signaling [13, 14]. These observations suggest that ANRIL regulates proliferation and apoptosis in Kasumi-1 cells through modulation of the PI3K/AKT pathway. Collectively, the evidence indicates that ANRIL functions as an oncogenic regulator, modulating proliferation and survival of M2-type AML cells through the PI3K/AKT pathway. Furthermore, Akt activation influences several downstream effectors, such as glycogen synthase kinase 3 (GSK3), which promotes cell cycle progression and increases cell survival [15]. The β-catenin/cyclin D1 axis, downstream of GSK-3β, further governs cell proliferation and differentiation [15, 16]. Dysregulated cell division leading to uncontrolled proliferation remains a hallmark of cancer progression [17]. The eukaryotic cell cycle comprises G1, S, G2, and M phases, orchestrated by cyclin-dependent kinases (CDKs) and their regulatory subunits [15]. In early G1, CDK4 and CDK6 associate with D-type cyclins, resulting in their activation, while CDK2 subsequently interacts with these complexes to drive G1/S phase progression [18]. Notably, p39CDK2 functions as a meiosis-specific mediator by interacting with SUN1 to facilitate telomere clustering [19]. Targeting dysregulated cell division and proliferation remains a cornerstone of cancer therapy. Palbociclib, a selective cyclin-dependent kinase (CDK) 4/6 inhibitor, exhibits significant antitumor potential. Beyond its established role in breast cancer treatment, it also shows promising efficacy against AML [20].

The regulatory function of ANRIL in AML-M2 cell proliferation through the GSK-3β/β-catenin/cyclin D1 pathway remains insufficiently characterized. Expanding on prior work from our group, this study examined ANRIL expression in Kasumi-1 cells and its impact on malignant biological behavior by modulating the GSK-3β/β-catenin/cyclin D1 signaling axis via CDKN2A. The results provide foundational insights into AML pathogenesis and inform the development of targeted therapeutic strategies.

Results

LncRNA-ANRIL was highly expressed in AML

Analysis of ANRIL expression in bone marrow samples from AML patients and normal controls using the TCGA and GTEx databases revealed significantly elevated levels in AML patients (Fig. 1A). To validate these observations, RT-qPCR was performed on peripheral blood samples from clinical AML patients and healthy controls, confirming a substantial upregulation of ANRIL in AML samples (Fig. 1B). Collectively, ANRIL expression was markedly higher in AML.

ANRIL affects Kasumi-1 cells cycle progression through the GSK3β/β-catenin/cyclin D1 pathway

Based on prior evidence implicating ANRIL in cell proliferation [13, 14], further modulation of ANRIL expression in Kasumi-1 cells achieved an 80% knockdown efficiency and a 10-fold overexpression (Fig. 2A, B). This study sought to determine whether ANRIL's influence on proliferation was linked to cell cycle regulation.

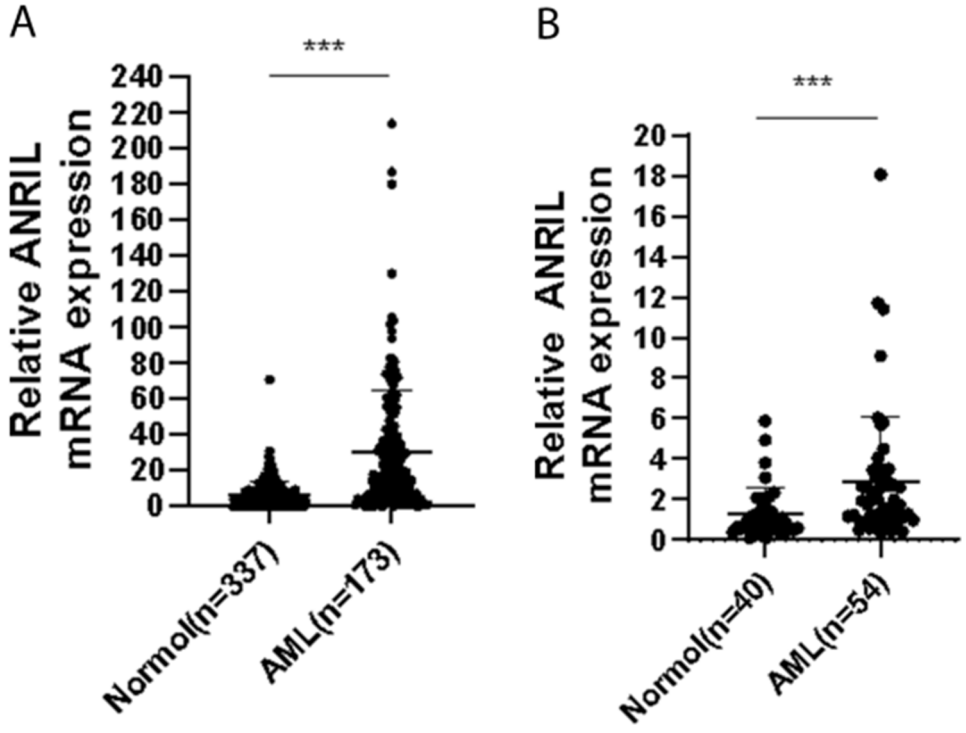

Fig. 1 Elevated expression of LncRNA-ANRIL in AML. **A**. ANRIL expression levels in bone marrow samples from AML patients and healthy controls were analyzed using TCGA and GTEx databases. **B**. RT-qPCR quantification of ANRIL mRNA expression in clinical peripheral blood samples. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Propidium iodide-based cell cycle analysis revealed that ANRIL knockdown led to an accumulation of cells in the G0/G1 phase, accompanied by a decrease in the S and G2/M phase populations (Fig. 2C, D). Conversely, ANRIL overexpression reduced the proportion of cells in the G0/G1 phase while increasing their distribution in the S and G2/M phases (Fig. 2E, F), supporting ANRIL's regulatory role in cell cycle progression. To investigate the mechanism by which ANRIL regulates cell cycle progression, RT-qPCR and Western blotting were employed to quantify the expression levels of CDK2, p-CDK2, CDK4, CDK6, and cyclin D1 proteins. ANRIL downregulation led to a marked decrease in these protein levels (Fig. 2G, I, J), whereas its upregulation resulted in a significant increase (Fig. 2H, K, L), indicating that ANRIL modulates cell cycle progression via CDKs and cyclin D1. GSK3β, a downstream effector of the PI3K/AKT pathway, is a serine/threonine kinase that interacts with key molecular targets, including the cell cycle regulator cyclin D1 and β-catenin [15]. The GSK3β/β-catenin/cyclin D1 axis is central to the regulation of cell proliferation and differentiation [15, 16]. To assess the role of this signaling pathway in ANRIL-driven cell cycle progression in Kasumi-1 cells, the expression levels of GSK3β and β-catenin were analyzed. RT-qPCR and Western blotting demonstrated that ANRIL downregulation significantly suppressed GSK3β and β-catenin expression (Fig. 2G, I, J), while its upregulation markedly enhanced their expression (Fig. 2H, K, L). To confirm ANRIL's effects on GSK3β/β-catenin/cyclin D1 pathway, co-transfection of siRNA cyclin D1 with pcDNA-ANRIL into Kasumi-1 cells was performed. Propidium iodide-based cell cycle analysis showed that co-transfection of siRNA cyclin D1 with pcDNA-ANRIL arrested cells in the G0/G1 phase, thereby reducing the proportion of cells in the S and G2/M phases (Fig. 2M, N). Collectively, these results indicate that ANRIL promotes Kasumi-1 cell cycle progression through the GSK3β/β-catenin/cyclin D1 pathway.

Palbociclib reverses the upregulation of ANRIL expression in Kasumi-1 cells

To investigate the role of CDK2/4/6 and cyclin D1 in Kasumi-1 cells, a replication experiment was performed using palbociclib, a small-molecule inhibitor that blocks cell cycle progression. It was hypothesized that palbociclib could mitigate the adverse effects of ANRIL upregulation through CDK inhibition. In order to evaluate this hypothesis, Kasumi-1 cells were treated with varying concentrations of palbociclib for 24 h to assess its impact on cell proliferation. GraphPad Prism 8 analysis determined an IC50 value of 15 μmol for palbociclib in Kasumi-1 cells, which was subsequently used as the working concentration (Fig. 3A). ANRIL-expressing

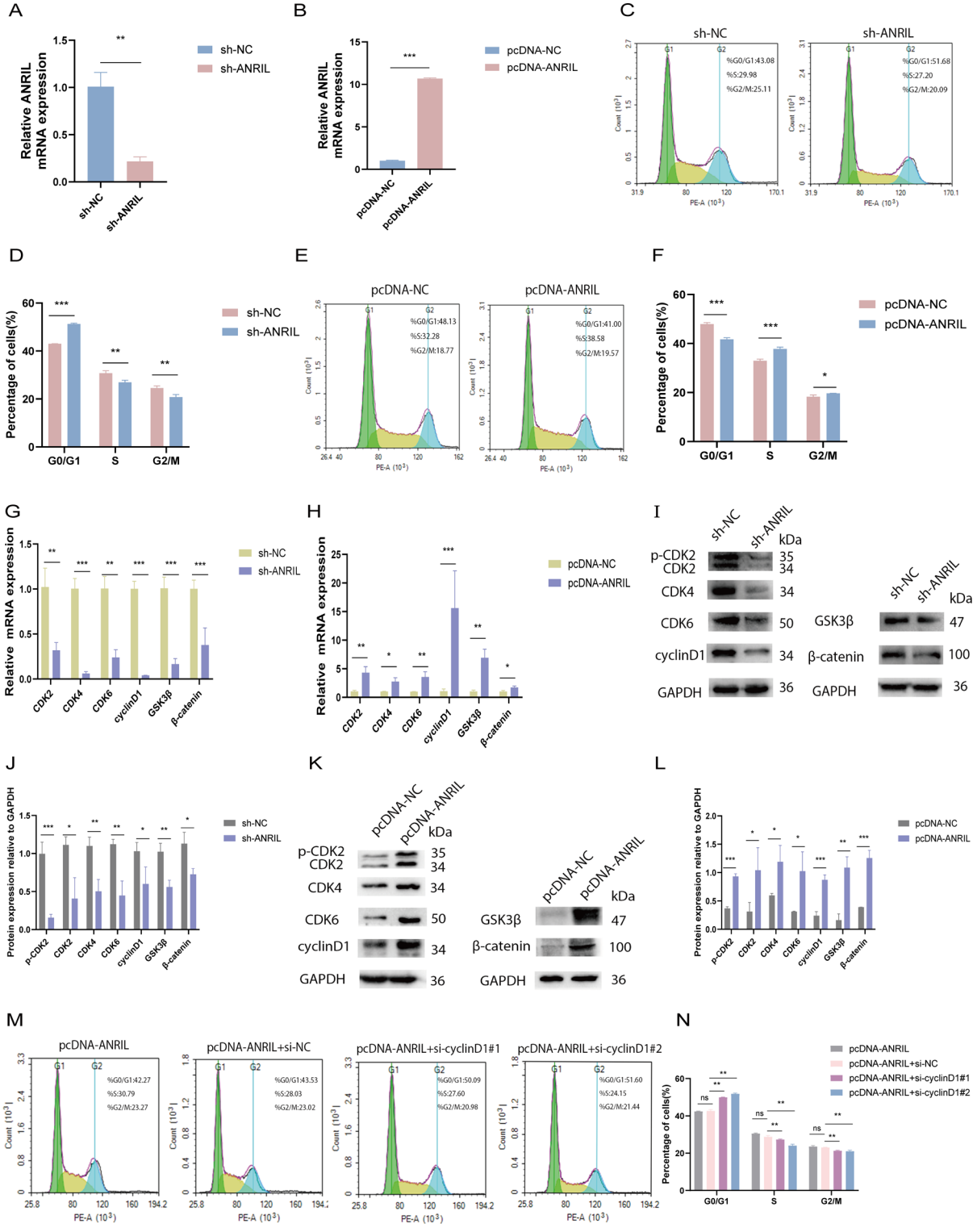

Fig. 2 The ANRIL-regulated GSK3β/β-catenin/cyclin D1 signaling pathway modulated Kasumi-1 cell cycle progression. **A**, **B**. Validation of ANRIL down-regulation and upregulation efficiency. **C**, **D**, **E**, **F**. Flow cytometry analysis demonstrating cell cycle distribution under ANRIL upregulation and down-regulation. **G**, **H**. RT-qPCR analysis quantifying mRNA levels of CDK2, CDK4, CDK6, cyclin D1, GSK3β, and β-catenin following ANRIL modulation. **I**, **J**, **K**, **L**. Western blotting evaluation of ANRIL expression effects on protein levels of CDK2, p-CDK2, CDK4, CDK6, cyclin D1, GSK3β, and β-catenin. **M**, **N**. Flow cytometry analysis demonstrating cell cycle distribution following the co-transfection of ANRIL overexpression and cyclin D1 suppression. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

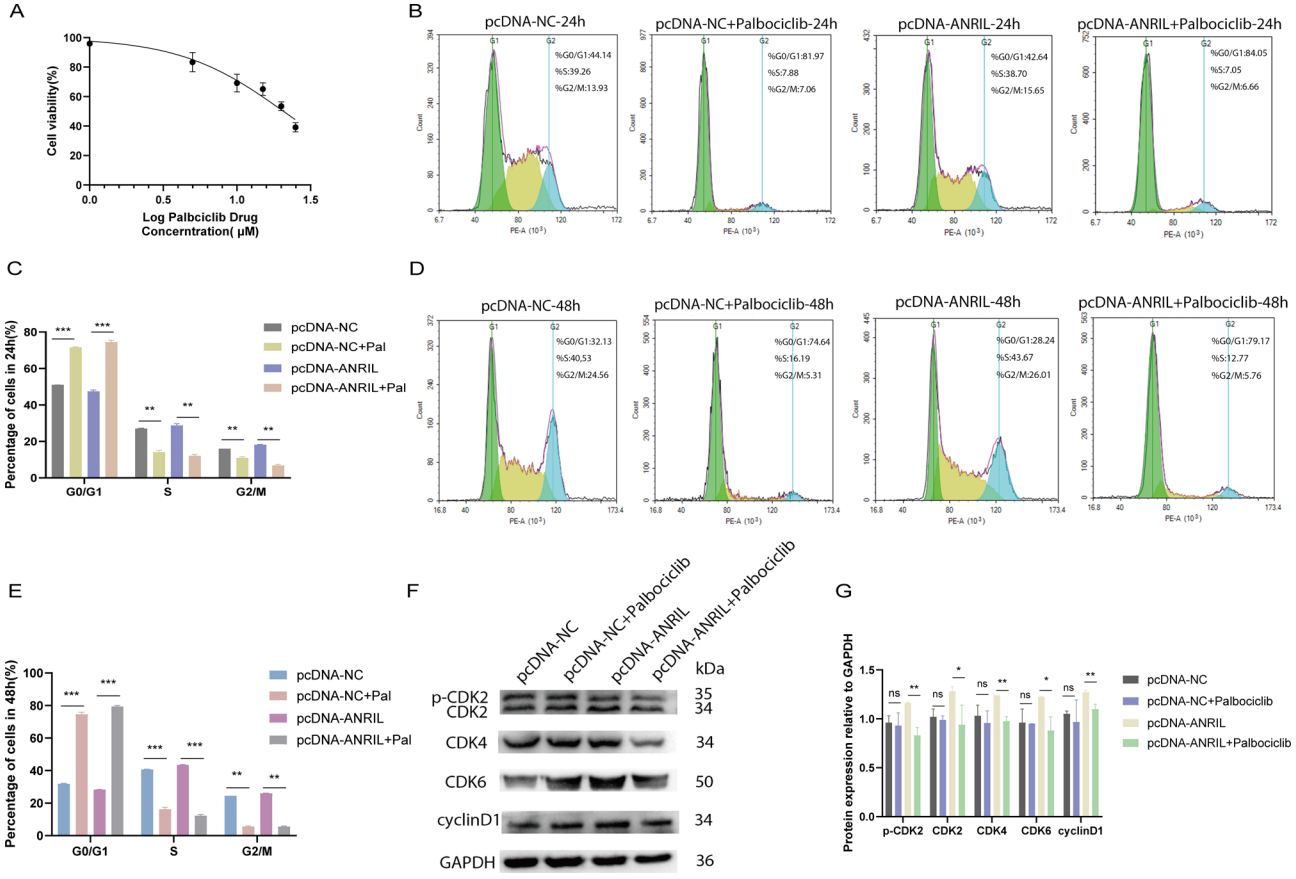

Fig. 3 Impact of ANRIL upregulation reversal by Palbociclib on Kasumi-1 cells. **A**. CCK-8 assay determines the optimal Palbociclib concentration by treating Kasumi-1 cells with 0, 1, 5, 10, 15, 20, and 25 μmol for 24 h. **B**, **C**. Flow cytometry reveals cell cycle distribution following treatment with 15 μmol Palbociclib for 24 h. **D**, **E**. Flow cytometry reveals cell cycle distribution following treatment with 15 μmol Palbociclib for 48 h. **F**, **G**. Western blotting analysis assesses CDK2, p-CDK2, CDK4, CDK6, and cyclin D1 protein expression after palbociclib treatment. ns $P>0.05$, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

Kasumi-1 cells were subjected to cell cycle analysis via propidium iodide staining following 24 h and 48 h of palbociclib exposure. Results indicated that ANRIL upregulation promoted cell cycle progression in untreated conditions, while treatment with 15 μmol palbociclib led to cell cycle arrest at the G0/G1 phase, accompanied by a reduction in S and G2/M phases (Fig. 3B, C, D, E). Western blotting analysis revealed a decrease in CDK4, CDK6, and cyclin D1 protein levels after palbociclib treatment (Fig. 3F, G). Furthermore, CDK2 protein expression analysis demonstrated a reduction in both CDK2 and phosphorylated CDK2 (p-CDK2) levels, implicating CDK2 activity as dependent on CDK4/6 signaling (Fig. 3F, G). These findings indicate that palbociclib reverses ANRIL upregulation-induced cell cycle alterations by inhibiting CDK2/4/6 and cyclin D1 activity.

LncRNA-ANRIL positively regulates CDKN2A expression

To elucidate the downstream regulatory network governed by ANRIL and its mechanistic role in the cell cycle, bioinformatics tools, including the RNAINTER website (http://rnainter.org/), were utilized to predict potential downstream targets interacting with ANRIL (Fig. 4A). RT-qPCR and Western blotting analyses subsequently assessed CDKN2A expression following ANRIL modulation. The results indicated that ANRIL upregulation increased CDKN2A expression, while its downregulation reduced CDKN2A levels (Fig. 4B, C, D, E). Further analysis using RT-qPCR examined CDKN2A expression in peripheral blood samples from healthy individuals and AML patients, revealing significantly elevated CDKN2A expression in AML patients compared to healthy controls (Fig. 4F). UCSC database analysis confirmed the upregulation of CDKN2A across 32 tumor types, including AML [21], corroborating its heightened expression in patients with AML. These findings suggest a potential regulatory relationship between ANRIL and CDKN2A. To further explore this interaction, the amino acid sequence of CDKN2A targeted by ANRIL was identified using RNAINTER and the PDB database (https://www.rcsb.org/) (Fig. 4G). RNA immunoprecipitation assays validated a direct physical interaction between ANRIL and CDKN2A (Fig. 4H). Collectively, the findings

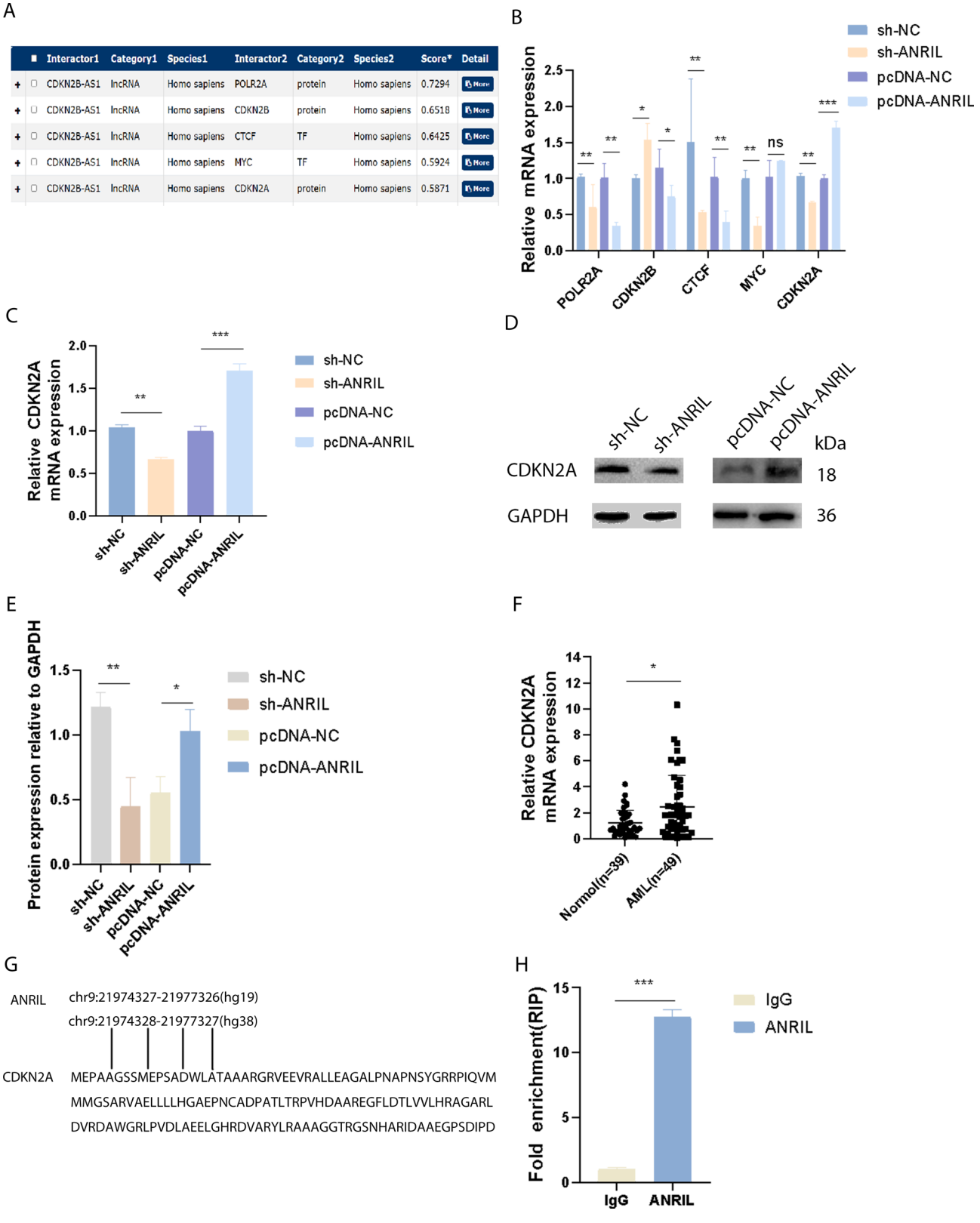

Fig. 4 LncRNA-ANRIL upregulated CDKN2A expression. **A**. Downstream targets interacting with ANRIL were predicted using the RNAINTER database. **B-F**. RT-qPCR and Western blotting analyses quantify CDKN2A expression levels in Kasumi-1 cells and clinical peripheral blood samples. **G**. Predicted binding site map of ANRIL and CDKN2A. **H**. RIP assay verifies the direct interaction between ANRIL and CDKN2A in Kasumi-1 cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

demonstrate that ANRIL positively regulates CDKN2A expression.

Downregulation of CDKN2A expression inhibits cell proliferation and cell cycle progression through the GSK 3β/β-catenin/cyclin D1 pathway and reverses ANRIL activation

To determine whether ANRIL regulates cell cycle progression by modulating CDKN2A expression, Kasumi-1 cells with stable CDKN2A downregulation were generated alongside control cells. RT-qPCR analysis confirmed an 80% knockdown efficiency (Fig. 5A), validating the establishment of CDKN2A-silenced strains. CCK-8 assays revealed that CDKN2A downregulation significantly suppressed Kasumi-1 cell proliferation (Fig. 5B). Propidium iodide-based cell cycle analysis showed that CDKN2A knockdown arrested cells in the G0/G1 phase, thereby reducing the proportion of cells in the S and G2/M phases (Fig. 5C, D). Western blotting further demonstrated decreased expression of CDK2, phosphorylated CDK2 (p-CDK2), CDK4, CDK6, cyclin D1, GSK3β, and β-catenin upon CDKN2A downregulation (Fig. 5E, F), implicating the GSK3β/β-catenin/cyclin D1 axis in this regulatory mechanism. To confirm ANRIL's effects on CDKN2A, co-transfection of sh-NC-CDKN2A or sh-CDKN2A with pcDNA-ANRIL into Kasumi-1 cells was performed, achieving a knockdown efficiency of 70% as verified by RT-qPCR (Fig. 5G). Subsequent Western blotting indicated that co-transfection of sh-CDKN2A and pcDNA-ANRIL significantly reduced protein levels of CDK2, p-CDK2, CDK4, CDK6, cyclin D1, GSK3β, and β-catenin (Fig. 5H, I). These results suggest that

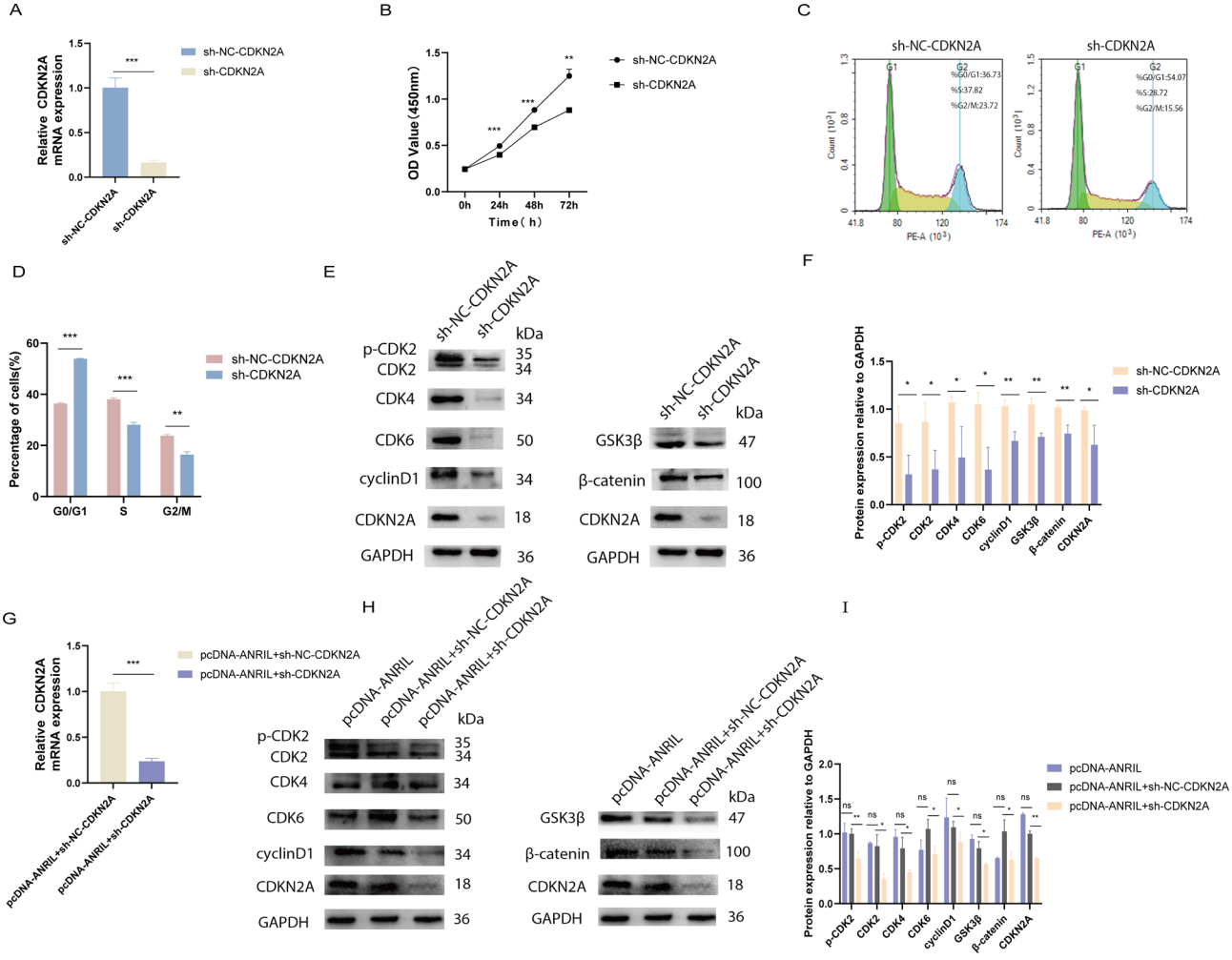

Fig. 5 CDKN2A downregulation suppressed cell proliferation and disrupted cell cycle progression via the GSK3β/β-catenin/cyclin D1 signaling pathway. **A**. RT-qPCR analysis confirmed the knockdown efficiency of CDKN2A. **B**. CCK-8 assay evaluated cell proliferation rates post-knockdown. **C**, **D**. Flow cytometry revealed cell cycle alterations following CDKN2A silencing. **E**, **F**. Western blotting analysis demonstrated the expression levels of CDK2, p-CDK2, CDK4, CDK6, cyclin D1, GSK3β, and β-catenin upon CDKN2A downregulation. **G**. RT-qPCR validation of CDKN2A knockdown efficiency post co-transfection with sh-CDKN2A and pcDNA-ANRIL. **H**, **I**. Western blotting assessed CDK2, p-CDK2, CDK4, CDK6, cyclin D1, GSK3β, and β-catenin protein expression following co-transfection of sh-CDKN2A and pcDNA-ANRIL. ns $P>0.05$, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

CDKN2A downregulation suppresses ANRIL-mediated malignant proliferation of Kasumi-1 cells. In conclusion, reduced CDKN2A expression inhibits Kasumi-1 cell proliferation and cell cycle progression via the GSK3β/β-catenin/cyclin D1 pathway, effectively counteracting ANRIL-mediated pathway activation.

Discussion

AML predominantly presents as a myeloid malignancy, characterized by the presence of malignant cells in peripheral blood or extramedullary infiltrates [22, 23]. AML with partial differentiation (AML-M2), a common subtype, primarily affects individuals aged 65–75 and is associated with low complete remission rates and poor prognosis. Effective therapeutic target identification remains critical for improving patient outcomes in AML-M2.

In this study, ANRIL was found to be significantly upregulated in AML, promoting disease progression by driving cell cycle activation. This effect is mediated through its interaction with CDKN2A, which subsequently activates the GSK3β/β-catenin/cyclin D1 signaling cascade. These results identify ANRIL as a novel oncogenic factor implicated in AML progression.

LncRNAs are increasingly recognized as dysregulated factors in various cancers, disrupting downstream gene expression and cellular homeostasis, thereby contributing to malignant transformation [24]. ANRIL is consistently overexpressed in multiple malignancies, including colon cancer [25], breast cancer [26], acute lymphoblastic leukemia (ALL) [27], and AML [8]. Its expression levels have been linked to tumor size, lymph node metastasis (LNM), TNM staging, poor prognosis in AML, and pathways associated with glucose metabolism [28, 29, 7]. Functioning as an oncogene, ANRIL acts as a competing endogenous RNA (ceRNA) by sponging microRNAs, leading to the suppression of tumor-suppressive microRNAs. For example, ANRIL promotes cellular proliferation via the miR-34a/ASPP2, miR-203a/CDK2, and miR-141-3p/CCND1/2 pathways in AML, hepatocellular carcinoma, and brain cancer, respectively [8, 30, 31]. Additionally, ANRIL regulates gene expression by modulating key signaling pathways. In oral squamous cell carcinoma, it enhances cellular proliferation through the activation of the TGF-β1/Smad signaling pathway [32]. In AML, ANRIL has been identified as a regulator of glucose metabolism by activating AdipoR1 and its downstream effectors, AMPK and SIRT1, which influence cell survival [7]. Evidence indicates that ANRIL contributes to the progression of AML, as its overexpression is consistently observed in the peripheral blood of AML patients. Notably, downregulation of ANRIL induces G1 phase cell cycle arrest, inhibiting cell cycle progression, while its upregulation promotes cell cycle advancement, accelerating transition to subsequent phases. Initial research also demonstrated significant overexpression of ANRIL in peripheral blood mononuclear cells (PBMCs) from AML patients, where ANRIL downregulation may suppress cell proliferation and PCNA expression, induce apoptosis, ANRIL upregulation appears to enhance cell proliferation and PCNA expression, reduce apoptosis. These results are consistent with prior studies [13, 14], suggesting that ANRIL exerts oncogenic effects in AML by modulating cell cycle dynamics and promoting cellular proliferation.

The precise mechanism through which ANRIL influences AML progression via cell cycle regulation remains to be fully elucidated. To address this, the expression of key cell cycle-associated proteins was analyzed. Dysregulated cell proliferation in hematologic malignancies is frequently driven by aberrant activity of cyclin-dependent kinases (CDKs) and their regulatory proteins. For example, hyperactivated cyclin D3-CDK6 suppresses glycolysis by inhibiting PFK1 and PKM2 in acute lymphoblastic leukemia (T-ALL) cells, thereby promoting proliferation and drug resistance [33]. As most cell cycle dysregulation-related diseases occur during the G1 phase, the G1/S phase transition is recognized as a critical point in cell cycle progression [34]. Regulatory proteins such as cyclin D1 and CDKs, including CDK2, CDK4, and CDK6, are fundamental for advancing the cell cycle from G1 to S phase [35, 36]. To further clarify ANRIL's role, its influence on the expression of CDK2, CDK4, CDK6, and cyclin D1 was evaluated. Results showed that ANRIL overexpression upregulated CDK2, CDK4, CDK6, and cyclin D1, while ANRIL knockdown reduced their expression. CDK4 and CDK6 act as initiators of G1 phase progression, while cyclin D1, a subtype of cyclin, forms regulatory complexes with CDK4 and CDK6. CDK2, active in late G1, also contributes to G1/S transition. Together, these CDKs, along with cyclin D1, orchestrate progression from the G1 to S phase, indicating that ANRIL promotes this transition by modulating their expression and activity. Furthermore, accumulating evidence highlights CDK4/6 as promising therapeutic targets in cancer treatment. Palbociclib, a CDK4/6 inhibitor, presents a promising therapeutic option for AML. Resistance to treatment is frequently observed in AML patients, however, palbociclib exhibits efficacy against resistant mutations [37]. Its therapeutic potential is amplified in combination with other agents. For instance, palbociclib combined with dexamethasone has demonstrated effectiveness in treating ALL [38]. Similarly, synergistic effects have been reported when palbociclib is paired with AURORA kinase inhibitors (danusertib and CCT137690), leading to significant inhibition of cell growth [39]. In AML, palbociclib synergizes with ATRA to promote cell differentiation and suppress AML cell

proliferation [40]. Additionally, the combination of palbociclib with venetoclax and azacitidine enhances their antitumor activity against AML [20]. Beyond its role as a CDK4/6 inhibitor, palbociclib also suppresses CDK2 activity in AML. Studies reveal that while CDK4/6 inhibition occurs during the early phase, this leads to a downstream reduction in CDK2 expression and kinase activity, indicating that CDK2 activation depends on prior CDK4/6 activity [41]. In the present study, palbociclib administration induced G1-phase cell cycle arrest by inhibiting CDK2, CDK4, CDK6, and cyclin D1 expression, effectively halting cell cycle progression. This inhibition of G1-phase transition ultimately suppressed tumor cell proliferation, highlighting palbociclib's anti-tumor efficacy. Consequently, palbociclib has been shown to impede cell cycle progression at both the 24 h and 48 h, suggesting that it impedes tumor cells from advancing into the cell cycle during the G1 phase, thereby suppressing their proliferation. CDK2, a cyclin-dependent kinase essential for cell cycle progression, exhibited stable CDK2 and p-CDK2 protein expression, suggesting a potential antitumor role for p-CDK2. Palbociclib, an effective anti-proliferative agent, has demonstrated the capacity to improve tolerance to high-dose cytarabine (Ara-C) therapy in elderly AML patients [42]. Optimal sequencing and timing of palbociclib administration in combination with other agents are critical to enhancing its anti-tumor efficacy [43]. However, resistance mechanisms and adverse effects remain significant concerns. Elevated CCNE1 and CDKN2D mRNA levels have been implicated in resistance to palbociclib [44]. Additionally, palbociclib treatment may cause bone marrow suppression, presenting as neutropenia, anemia, and thrombocytopenia, alongside gastrointestinal toxicities [45].

ANRIL modulates cyclin D1 expression, a key regulator of the G1-to-S phase transition in various cancers, thereby driving cell cycle progression [46]. GSK-3β also regulates cyclin D1 and β-catenin [15]. In AML, suppression of UCA1 expression inhibits cellular proliferation and induces apoptosis by disrupting the GSK3β/β-catenin signaling pathway [47]. Previous studies suggest that ANRIL influences Kasumi-1 cell proliferation through the PI3K/AKT signaling pathway. Since GSK3β functions as a downstream component of the PI3K/AKT axis, ANRIL may regulate the Kasumi-1 phenotype via the GSK3β/β-catenin pathway [13, 14]. The current findings demonstrate that ANRIL activates the GSK3β/β-catenin axis, leading to elevated cyclin D1 expression and subsequent acceleration of cell cycle progression. Conversely, co-transfection that leads to the ANRIL overexpression and cyclin D1 suppressionmay counteract the promoting influence of increased ANRIL levels on cell cycle progression. These results indicate that ANRIL promotes cell cycle progression through activation of the GSK3β/β-catenin/cyclin D1 pathway.

LncRNAs regulate cell proliferation and survival by modulating the expression of genes encoding key cell cycle proteins, such as cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors [48]. CDKN2A, located at the same locus as ANRIL, encodes the cell cycle regulatory protein p16, implicating ANRIL in the regulation of cell proliferation and apoptosis [10]. The CDKN2A gene frequently undergoes inactivation or mutation in various cancers, including melanoma, pancreatic, and liver cancers, with such alterations being associated with tumor staging in hepatocellular carcinoma [49–51]. Additionally, CDKN2A has been shown to be co-expressed with CDK4, CDK6, and cyclin D1 [21]. In the current study, CDKN2A expression was elevated in the peripheral blood of AML patients, and ANRIL was identified as a positive regulator of CDKN2A expression. Downregulation of CDKN2A suppressed cell proliferation and cell cycle progression, highlighting its role in ANRIL-mediated regulation of Kasumi-1 cell growth. Further analysis demonstrated that CDKN2A downregulation can inhibit activation of the GSK3β/β-catenin/cyclin D1 signaling pathway. In Kasumi-1 cell lines overexpressing ANRIL, silencing CDKN2A reversed ANRIL-induced changes in cell cycle-related proteins and disrupted the GSK3β/β-catenin/cyclin D1 pathway. These results suggest that ANRIL regulates cell cycle progression through CDKN2A. CDKN2A encodes two proteins, p16 and p14, which are central to the regulation of cell proliferation [10, 11]. Notably, p16 shows increased expression in cervical and breast cancers [52, 53]. Studies have revealed a positive correlation between ANRIL expression and the p16-CDKN2A gene cluster in most tumors [54]. Upregulation of p16 may result directly from alterations in its interaction with pRb or indirectly via the p53 signaling pathway [55, 56]. Analysis of 713 cell lines and tissues expressing ANRIL showed significantly higher p16 mRNA levels compared to 298 cell lines and tissues lacking ANRIL expression. Furthermore, ANRIL transcriptional activity was more pronounced in p16 unmethylated cell lines than in p16 methylated ones, suggesting that p16 DNA methylation may inhibit ANRIL transcription [57]. Nonetheless, limitations remain. First, the exact mechanism by which ANRIL engages the GSK3β/β-catenin/cyclin D1 pathway requires further elucidation. Second, The sustained impact of palbociclib on the GSK 3β/β-catenin/cyclin D1 signaling pathway. Finally, the oncogenic role of ANRIL has been confirmed only in vitro, highlighting the need for in vivo validation in future research.

This study comprehensively investigates the regulatory role of ANRIL in cell cycle progression via the GSK-3β/β-catenin/cyclin D1 pathway, focusing on four

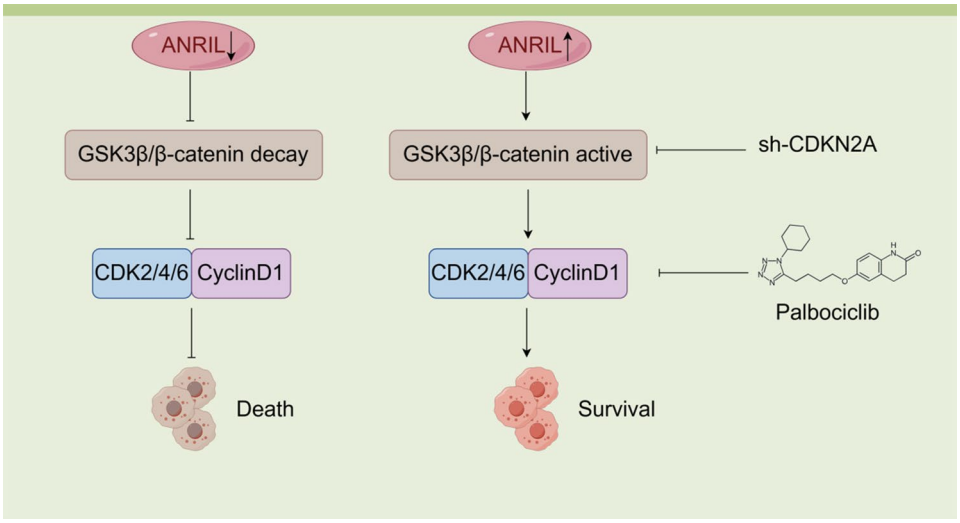

Fig. 6 Study Design of ANRIL's Promotion of Malignant Progression in Kasumi-1 Cells

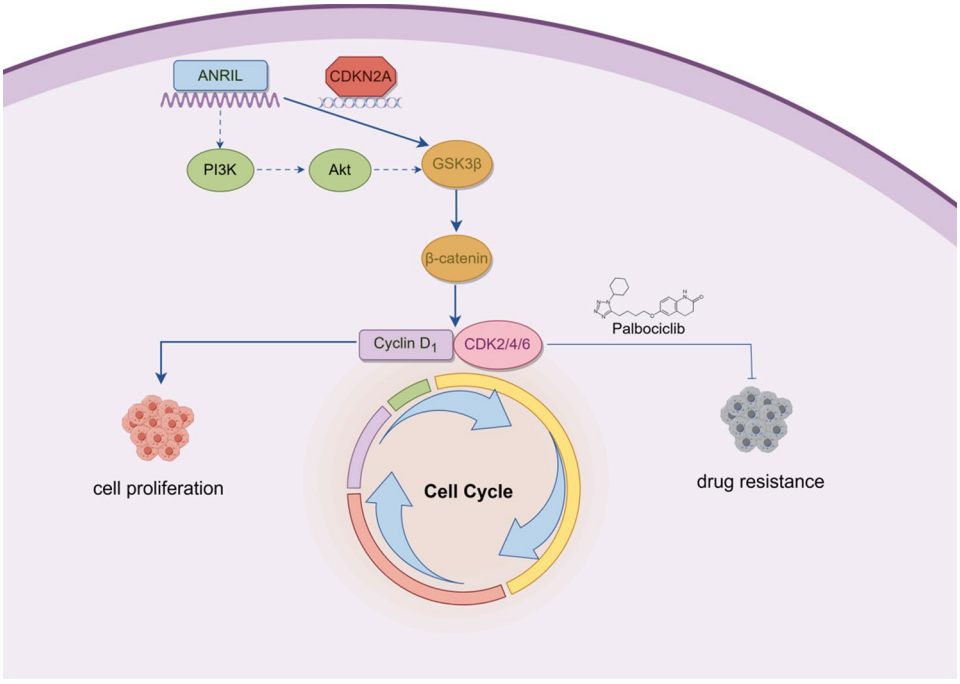

Fig. 7 Schematic representation depicting ANRIL-mediated regulation of cell cycle progression in Kasumi-1 cells

key mechanisms. First, suppression of ANRIL expression inhibits the GSK-3β/β-catenin pathway, leading to reduced expression of cell cycle-related proteins CDK2/4/6 and cyclin D1, which promotes apoptosis in cancer cells. Second, elevated ANRIL expression activates the GSK-3β/β-catenin pathway, consequently upregulating CDK2/4/6 and cyclin D1, thereby driving cancer cell proliferation. Third, ANRIL directly interacts with CDKN2A, and the downregulation of CDKN2A further suppresses the GSK-3β/β-catenin pathway, reducing CDK2/4/6 and cyclin D1 levels and enhancing apoptosis. Finally, palbociclib effectively inhibits cancer cell cycle progression by targeting CDK2/4/6 and cyclin D1 expression (Fig. 6).

Conclusion

ANRIL was found to be significantly upregulated in AML, with its expression influencing cell cycle progression. Suppression of ANRIL expression resulted in cell cycle arrest, whereas its upregulation promoted cell cycle progression, potentially through the positive regulation of CDKN2A and activation of the PI3K/AKT signaling pathway (Fig. 7). This evidence highlights ANRIL as a promising molecular target for AML-M2 therapy.

Materials and methods

Reagents and antibodies

The Kasumi-1 cell line was procured from the Cell Bank of the Chinese Academy of Sciences. RPMI 1640 medium was supplied by Gibco (USA), and serum was obtained from Biological Industries (Israel). PCR primers were purchased from Shanghai Biotechnology, while RNA extraction reagents were provided by Thermo Fisher Scientific (USA). Reverse transcription and RT-qPCR reagents were sourced from TaKaRa (Japan). CCK-8 assay kits were acquired from Dalian Meilun Biotechnology, and flow cytometry reagents for cell cycle analysis were obtained from Hangzhou Lianke Biotechnology. Primary antibodies against GAPDH, cyclin D1, β-catenin, and CDKN2A were supplied by Wuhan Sanying Biotechnology, while antibodies targeting CDK2, CDK4, CDK6, and GSK3β were provided by Shenyang Wanlei Biotechnology. ECL chemiluminescence reagents were purchased from Millipore (USA), and the CDKN2A gene plasmid DNA was obtained from Guangzhou Aiji Biotechnology. The PCR instrument used in this study was manufactured by Roche (Switzerland).

Clinical samples

The study was approved by the Medical Ethics Committee of the Affiliated Hospital of Guizhou Medical University (approval number: 2016(65)), and informed consent was obtained from all participating patients. Peripheral blood samples were obtained from 54 patients with confirmed AML, with diagnosis established using the French-American-British (FAB) classification system. A control cohort consisted of peripheral blood samples collected from 40 healthy individuals undergoing routine medical examinations.

Cell culture

Kasumi-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco) under controlled humidity at 37 °C with 5% CO_2. The medium was refreshed, and cells were passaged every two days. The study design incorporated the following experimental groups: ANRIL knockdown (sh-ANRIL) and its corresponding control (sh-NC), ANRIL overexpression (pcDNA-ANRIL) and its control (pcDNA-NC), a drug-treated group combining ANRIL overexpression with Pabociclib (pcDNA-ANRIL + Pabociclib) and its control (pcDNA-NC + Pabociclib), CDKN2A knockdown (sh-CDKN2A) and its control (sh-NC-CDKN2A), co-transfection of ANRIL overexpression with cyclin D1 siRNA (pcDNA-ANRIL + si-cyclin D1), and co-transfection of ANRIL overexpression with CDKN2A knockdown (pcDNA-ANRIL + sh-CDKN2A).

RNA extraction and RT-qPCR

The total RNA was extracted using Trizol reagent and subsequently reverse-transcribed into cDNA with the TaKaRa kit. Quantitative analysis of target gene expression was conducted through SYBR-based RT-qPCR, with ACTB serving as the internal control. Gene expression levels were calculated using the 2−ΔΔCT method. The primer sequences used for RT-qPCR were as follows: ACTB (F: 5′-GCGTGACATTAAGGAGAAGC-3′, R: 5′-CCACGTCACACTTCATGATGG-3′) and ANRIL (F: 5′-ATAAGCCTCATTCTGATTCAACAGC-3′, R: 5′-AGC AGTACTGACTCGGGAAAG-3′).

Cell proliferation assay

Transfected Kasumi-1 cells (2 × 10^4 cells/well) were seeded into 96-well culture plates. To assess cell proliferation, 10 μL of CCK-8 reagent was added per well, followed by a 2-hour incubation. Absorbance at 450 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader at 24, 48, and 72 h. After each time point, an additional 2-hour incubation with CCK-8 reagent was performed. The resulting absorbance data were used to generate a proliferation curve.

Flow cytometry analysis

Cellular groups were harvested and centrifuged before fixation in 70% pre-cooled ethanol, followed by overnight incubation at 4 °C in an ice bath. The next day, the samples were washed with pre-cooled PBS, and the cell pellets were resuspended in RNase A. Propidium iodide staining was performed for 30 min under dark conditions. Cell cycle analysis was carried out using flow cytometry, with data processed via NovoExpress software.

Western blotting

Cells from each group were lysed using a RIPA: PMSF mixture at a 100:1 ratio, followed by high-speed centrifugation at 12,000 × g for 20 min at 4 °C to obtain the supernatant. The proteins were denatured by heating in a metal bath, and their concentrations were quantified using the BCA Protein Detection Kit. Equal amounts of protein were separated via 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 120 V and subsequently transferred onto PVDF membranes under a current of 260 mA for 90 min. The membranes were blocked with skimmed milk for 2 h at room temperature, incubated overnight at 4 °C with the primary antibody, and then probed with an enzyme-conjugated secondary antibody for 1 h. Protein signals were visualized using ECL chemiluminescence, enabling the detection of proteins separated during electrophoresis.

RNA immunoprecipitation (RIP) assay

Kasumi-1 cells were harvested for nucleoprotein extraction, which was resuspended in RIP buffer and divided into three groups for co-precipitation with IgG and CDKN2A antibodies. Supernatants obtained through centrifugation were incubated at 4 °C for two hours with IgG and human anti-CDKN2A antibodies. Protein A was subsequently added, followed by another hour of incubation at 4 °C. After centrifugation, the samples were washed three times with RIP buffer and once with PBS before bead resuspension in Trizol. ANRIL and CDKN2A expression levels were then measured via RT-qPCR, and their relative abundances were quantified.

Plasmid construction and transfection

The plasmid was constructed by Guangzhou Aiji Biotechnology, and experiments were conducted using the CDKN2A target sequence (CCGGGCTCTCTGAGAAACCTCGGGAAACTCGAGTTTCCCGAGGTTTCTCAGAGCTTTTTTTGAATT), which corresponds to the vector pLKO.1-U6-EF1a-copGFP-T2A-puro. Lentiviral expression vectors, including psPAX2 and pMD.2G, along with control plasmids, were transfected into 293T cells to produce lentiviral particles. The resulting viral supernatants were collected and used to infect Kasumi-1 cells in the presence of 5 μg/mL polybrene for 48 h. Infections were evaluated at 24, 48, and 72 h. Stable transfectants were subsequently generated through selection with 5 μg/mL puromycin.

Statistical analysis

SPSS version 20.0, GraphPad Prism version 8, and Figdraw were used for statistical analysis and visualization. Each experiment was independently repeated three times. Data distribution was assessed using normality and lognormality tests. For two-group comparisons, the unpaired two-tailed Student's t-test was applied to data with a normal distribution, while the Mann–Whitney U test was employed for non-normally distributed data. Quantitative analysis of Western blotting results was conducted using ImageJ software. Statistical significance was defined as a p-value < 0.05. All experimental procedures adhered to the relevant guidelines and regulations.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13008-025-00144-2.

Supplementary Material 1

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Author contributions

Jianxia Xu: Data curation, Formal analysis, Software, Validation, Writing – original draft, Writing – review & editing. Jingxin Zhang: original draft, Formal analysis, Data curation, Writing – original draft,. Chengsi Zhang: Writing – original draft, Formal analysis, Data curation. Huali Hu: Writing – original draft, Formal analysis, Data curation. Siqi Wang: Writing – review & editing, Methodology, Formal analysis. Fahua Deng: Writing – review & editing, Methodology, Formal analysis. Wu Zhou: Writing – review & editing, Writing – original draft,, Formal analysis. Yuancheng Liu: Writing – review & editing, Formal analysis, Data curation. Chenglong Hu: Writing – review & editing, Formal analysis, Data curation. Hai Huang: Conceptualization, Writing – review & editing. Sixi Wei: Conceptualization, Funding acquisition, Writing – review & editing.

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Data availability

Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate

Ethics approval was obtained from the Medical Ethics Committee of the Affiliated Hospital of Guizhou Medical University and the informed consent of the patients. Approval No. 65 of 2016, dated 3.3.2016.

Consent for publication

The manuscript has received approval for publication from all listed authors.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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