Non-coding RNA Research 9 (2024) 720–731

Contents lists available at ScienceDirect

Non-coding RNA Research

journal homepage: www.keaipublishing.com/en/journals/non-coding-rna-research

Original Research Article

miR-218-5p, miR-124-3p and miR-23b-3p act synergistically to modulate the expression of NACC1, proliferation, and apoptosis in C-33A and CaSki cells

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ARTICLE INFO

Keywords:
miR-218-5p
miR-124-3p
miR-23b-3p
NACC1
Proliferation
Apoptosis

ABSTRACT

Background: In cervical cancer (CC), miR-218-5p, -124-3p, and −23b-3p act as tumor suppressors. These miRNAs have specific and common target genes that modulate apoptosis, proliferation, invasion, and migration; biological processes involved in cancer.
Methods: miR-218-5p, -124-3p, and −23b-3p mimics were transfected into C-33A and CaSki cells, and RT-qPCR was used to quantify the level of each miRNA and *NACC1*. Proliferation was assessed by BrdU and apoptosis by Annexin V/PI. In the TCGA and The Human Protein Atlas databases, the level of *NACC1* mRNA and protein (putative target of the three miRNAs) was analyzed in CC and normal tissue. The relationship of NACC1 with the overall survival in CC was analyzed in GEPIA2. *NACC1* mRNA and protein levels were higher in CC tissues compared with cervical tissue without injury.
Results: An increased expression of NACC1 was associated with lower overall survival in CC patients. The levels of miR-218-5p, -124-3p, and −23b-3p were lower, and NACC1 was higher in C-33A and CaSki cells compared to HaCaT cells. The increase of miR-218-5p, -124-3p, and −23b-3p induced a significant decrease in *NACC1* mRNA. The transfection of the three miRNAs together caused more drastic changes in the level of *NACC1*, in the proliferation, and in the apoptosis with respect to the individual transfections of each miRNA.
Conclusion: The results indicate that miR-218-5p, -124-3p, and −23b-3p act synergistically to decrease NACC1 expression and proliferation while promoting apoptosis in C-33A and CaSki cells. The levels of *NACC1*, miR-218-5p, -124-3p, and −23b-3p may be a potential prognostic indicator in CC.

1. Introduction

Worldwide, cervical cancer (CC) ranks fourth in frequency among women [1]. High-risk oncogenic human papillomaviruses (HR-HPV) are the etiological agents of CC, predominantly HPV-16 and HPV-18 [1,2]. Persistent HPV-16 infection is the most frequent among CC cases [3], but it is insufficient to promote malignant transformation. Immunological alterations, chromosomal aberrations, environmental factors, genetic mutations, and epigenetic alterations also play an important role in the initiation and progression of CC [1,2,4,5]. Despite advances in the study and understanding of the mechanisms behind this malignancy's evolution, survival rates remain unsatisfactory. In patients with CC undergoing treatment, the 5-year survival rate is 67% (National Cancer Institute-Surveillance, Epidemiology, and End Results Program. https://seer.cancer.gov/statfacts/html/cervix.html). Patients with stage I CC undergo conization and hysterectomy, while those in stages

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https://doi.org/10.1016/j.ncrna.2024.02.016
Received 18 October 2023; Received in revised form 13 February 2024; Accepted 22 February 2024
Available online 27 February 2024

II-IV receive radiotherapy and chemotherapy in addition to surgery [6]. Cisplatin, the drug of choice for treating CC, often leads to the development of chemoresistance. This issue has motivated the implementation of dual cisplatin-based therapy and other chemotherapeutic agents [7]. Despite the addition of one or more adjuvants to the treatment, survival rates do not improve (23.5% for cisplatin alone vs 19.3% for cisplatin + paclitaxel) [8]. The survival rate depends, in part, on early detection and the histological type of CC. Survival rates are lower in patients with adenocarcinoma/adenosquamous carcinoma (AC/ASC) compared to those with squamous cell epithelial carcinoma (SCC). Advanced stage and tumor size are factors that negatively affect patients' survival [9]. The late detection of CC cases results from various factors, including the diagnostic methods used, such as cytology and histopathology, which detect morphological changes in cells or tissues. As a result, the search for molecular biomarkers that increase diagnostic sensitivity and specificity has intensified. The expression levels of proteins $p16^{ink4a}$ and Ki-67 have been proposed as biomarkers for cervical carcinogenesis, whose evaluation improves the detection of low (LSIL) and high-grade (HSIL) squamous intraepithelial cases at risk of progressing to CC [10]. Immunohistochemistry (IHC) for $p16^{ink4a}$/Ki-67 has a sensitivity of 86% for LSIL and 88% for HSIL, and a specificity of 90.9% and 72.1% for HSIL/CC, respectively. However, it has limited utility in tumor diagnosis [11]. Moreover, the use of $p16^{ink4a}$/Ki-67 in routine diagnosis and CC screening programs is severely limited due to problems in interpreting staining patterns in IHC and the scarcity of trained cytologists/pathologists [12]. The current state of knowledge does not fully explain the molecular mechanisms involved in the progression of CC and the identification of useful molecular biomarkers for detecting CC *in situ* at risk of progressing to invasive cancer is necessary. For this purpose, it is explored whether profiles of microRNAs characteristic of carcinogenesis or progression can serve as diagnostic or prognostic biomarkers.

microRNAs have important regulatory effects on gene expression at the post-transcriptional level. Several miRNAs are abnormally expressed in different types of cancer and their role is essential in carcinogenesis and disease progression [13]. In CC, various oncogenes regulated by tumor-suppressing microRNAs are overexpressed, including those regulated by miR-218-5p, -124-3p and-23b-3p [5,14]. By regulating their target mRNAs, miR-218-5p, -124-3p y-23b-3p modulate apoptosis, proliferation, cell cycle, differentiation, invasion, migration, cell signaling, and other cellular functions [15,16]. Various studies on SCC (C-33A, CaSki, SiHa) and AC (HeLa) cell lines have shown that oncogenes modulated by miR-218-5p, -124-3p, and −23b-3p play a crucial role in regulating cellular processes involved in CC progression [17–19].

Our work group demonstrated that an increase in miR-23b-3p decreases the invasion and proliferation of CaSki HPV-16+ and C-33A HPV- cells [17]. Zhang et al., found low levels of miR-124-3p in CC tissues and in SiHa HPV-16+ cells, and HeLa HPV-18+ cells. They confirmed that overexpression of this miRNA decreases epithelial-mesenchymal transition (EMT), proliferation, migration, invasion, and promotes apoptosis [18]. Liu et al., reported low levels of miR-218-5p in cell lines and CC biopsies, discovering that miR-218-5p overexpression reduces migration, invasion, and proliferation of C-33A, HeLa, and SiHa cells [20]. This information indicates that miR-218-5p, -124-3p, and −23b-3p regulate common processes involved in CC metastasis. It remains to be confirmed whether these miRNAs regulate proliferation, invasion, and migration through common targets or, conversely, they add their effects by modulating specific mRNAs.

In a previous bioinformatics analysis, our research group found that miR-218-5p, -124-3p, and −23b-3p have 119 common target transcripts, and 24 of them regulate migration, apoptosis, and angiogenesis. The nucleus accumbens-associated protein 1 (NACC1) mRNA is among the 24 mRNAs regulated by those three miRNAs. NACC1 is a transcription factor, a member of the BTB/POZ family [21–23], that regulates epigenetic reprogramming and apoptosis. NACC1 is an oncoprotein that induces EMT, invasion, and migration and is overexpressed in uroepithelial carcinoma, retinoblastoma, ovarian cancer, nasopharyngeal carcinoma, and CC [23–28]. NACC1 activates the PI3K/Akt/mTOR signaling pathway, which promotes carcinogenesis and cancer progression. Alterations in the PI3K/Akt/mTOR pathway have been identified in 50% of tumors, making it the most activated pathway in human cancer. mTOR and Akt are the main effector proteins of the pathway; and Akt can exercise numerous functions through cross-interactions with various signaling pathways. Akt is involved in growth regulation, metabolic reprogramming, increased cell anabolism, proliferation, survival, migration, angiogenesis, invasion, and apoptosis of cancer cells. Alterations in Akt function are associated with carcinogenesis and/or cancer progression, and hyperactivation of the PI3K/Akt/mTOR pathway often supports the development of resistance to treatment [26,29,30]. In retinoblastoma WERI-Rb-1 cells, it was found that increased levels of miR-218-5p led to a reduction in NACC1 levels, correlating with a marked decrease in cell viability, colony formation, and increased apoptosis. The results suggest that miR-218-5p regulates the expression of *NACC1* [23].

Published data indicate that miR-218-5p, -124-3p, and −23b-3p regulate proliferation, invasion, and migration. However, it is unknown whether they potentiate their effects through the regulation of common targets. The central objective of this research was to analyze the effect of overexpression of miR-23b-3p, -124-3p, or -218-5p on the expression level of *NACC1*, proliferation, and apoptosis in CaSki and C-33A cells. Additionally, this work aimed to analyze the levels of 24 mRNAs bioinformatically predicted as common targets of miR-218-5p, -124-3p, and −23b-3p, in expression data obtained from CC tissues registered in the TCGA platform, and to verify the relationship between mRNA expression levels and patient survival.

2. Materials and methods

2.1. Analysis of the expression of probable target mRNAs in TCGA databases

Expression data were obtained from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx), the analysis was performed using the Gene Expression Profiling Interactive Analysis (GEPIA2) platform (24), where box graphs were generated to show the expression level of 24 genes. The expression data come from cervical squamous cell cancer (CESC) and normal cervical tissue. Images of NACC1 expression in tissue samples from CC and normal cervical tissues, stained by immunohistochemistry were obtained from the Human Protein Atlas (HPA). Images available at https://www.proteinatlas.org/ENSG00000160877-NACC1/pathology/cervical+cancer#img (accessed on 29 October 2022).

2.2. Survival analysis

Survival data from patients diagnosed with CC at various stages, available in TCGA, were analyzed to identify the association between *NACC1* expression and overall survival (OS). Results of *NACC1* expression were analyzed in samples of CESC and endocervical adenocarcinoma in patients with EMT, PI3K-AKT activation of the pathway, or hormonal alterations. The mean expression value served as the cut-off point. Tissues with *NACC1* levels above the cut-off value comprised the high-expression group while those with levels below the cut-off value were included in the low-expression group. Kaplan-Meier (KM) curves were built for high or low-expression groups of NACC1. The analysis was done in the platform GEPIA2. The hazard ratio (HR) and 95% confidence interval were calculated, and a p-value of <0.05 was considered statistically significant.

2.3. Cell culture

The cell lines C-33A (HPV-) y CaSki (HPV-16+) were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA), and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high in glucose (Invitrogen, Carlsbad, CA, USA). HaCaT cells, donated by The National Cancer Institute (INCan), were cultured in DMEM-F12 medium (Caisson, Smithfield, Washington, USA). Both DMEM and DMEM-F12 media were supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NE, USA). All cells were incubated at 37 °C with 5% CO_2.

2.4. Cell transfection

4×10^5 cells/well were grown in 6-well plates and were cultured to approximately 80% confluency before transfection. Then, cells were transfected with 100 nM of miRNAs mimics: hsa-miR-23b-3p (MIMAT0000418, assay ID MC10499); hsa-miR-124-3p (MIMAT0000422, assay ID MC10060); and/or hsa-miR-218-5p (MIMAT0000275, assay ID MC10328). The transfection was done with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. As a negative control, CaSki and C-33A cells were transfected with 100 nM of mirVana™ miRNA Mimic, Negative Control #1. All mimics were obtained from Ambion (Austin, TX, USA). The cells were incubated for 24 h, 48 h, 72 h, and 96 h, after transfection, to be used for subsequent experiments. Each experiment was performed in triplicate.

2.5. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA from HaCaT, C-33A, and CaSki cells was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The total RNA concentration was measured at an absorbance of 260/280 nm using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For the expression analysis of miR-218-5p, -124-3p, and −23b-3p, 5 ng of total RNA was reverse-transcribed using TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems, Vilnius, Lithuania) according to the manufacturer's protocols. Quantification of miR-23b-3p (Assay ID 245306 mat); miR-124-3p (Assay ID 003188 mat); and miR-218-5p (Assay ID 000521) was evaluated using the TaqMan microRNA assay kit (Applied Biosystems, Vilnius, Lithuania). The TaqMan RNA–to–CT 1-Step Kit (Applied Biosystems, Vilnius, Lithuania), was used to measure NACC1 mRNA (Assay ID Hs00369413_m1). Reactions were conducted on the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative expression levels of miR-23b-3p, -124-3p, -218-5p, and *NACC1* were evaluated using the $2^{-\Delta\Delta Ct}$ method. RNU6 (Assay ID 001973) or GAPDH (Assay ID Hs99999905_m1) were used as normalization controls. The experiment was performed in triplicate.

2.6. Cell proliferation assay

The proliferation assay was performed using the Millipore© BrdU Cell Proliferation Assay kit (Sigma-Aldrich, Temecula, USA) following the manufacturer's protocols. Cells C-33A and CaSki were seeded in 96 well plates (1×10^4 cells/well) and incubated at 37 °C with 5% CO_2 for 24 h before transfection. The cells were transfected with miRNAs mimics and 20 μL of BrdU were added, diluted 1:500, to each well. At 24 h, 48 h, 72 h, and 96 h post-transfection, the immunostaining was performed, and proliferation was evaluated. Optical density (OD) was measured at 450 nm in a microplate spectrophotometer (Thermo ScientificTM MultiskanTM GO, Finland). Non-transfected and scramble-transfected cells served as negative controls. The experiment was performed in triplicate.

2.7. Cell apoptosis assay

Apoptosis in C-33A and CaSki cells was assessed by double-staining flow cytometry using propidium iodide (PI) and annexin V using Alexa Fluor 488 Annexin V/Dead Cell Apoptosis, for Flow Cytometry (Invitrogen. No. V13241 Eugene, Oregon, EE. UU.). The determination was made according to the protocol described by the manufacturer. Cells were plated in 6-well plates (4×10^5 cells/well) and harvested after 48 h, followed by washing with cold PBS twice. Then, the cells were re-suspended in binding buffer, reacted with 25 μL of the annexin-V conjugate (1 μg/mL) and PI (2 μg/mL), and incubated on ice for 15 min in the dark. Apoptosis rates were analyzed by flow cytometry using the Accuri ™ C6 flow cytometer BD system (BD, USA) and BD Accuri software C6 Software workspace. The experiment was performed in triplicate.

2.8. Statistical analysis

Differential analysis of TCGA and GTEx data was conducted using the one-way ANOVA test on the GEPIA2 platform, which calculates the differences in gene expression levels between normal and tumor tissues. Comparative analysis between the two experimental groups was performed using Student's t-test with GraphPad Prism software (version 8.0; GraphPad Software, Inc.). The obtained data were expressed as the mean ± standard deviation (SD). A p-value of <0.05 was considered statistically significant.

3. Results

3.1. Overexpression of NACC1 in CC biopsies

Bioinformatic analysis previously identified 24 mRNAs as targets of miR-218-5p, -124-3p and −23b-3p: *LHX4, NAA15, GABRB3, GSK3B, PRLR, RFFL, SERPINB10, CACUL1, NACC1, PURA, RUNX2, ABL2, GNA12, RAPGEF2, MITF, MARK1, PTPN11, JAG1, TCF4, CCDC6, FZD4, ZBTB16, SP1, AFF1* [31].

To verify the mRNA level of these 24 genes in CC tissues, the information recorded on the TCGA platform was examied. Data from 306 patients diagnosed with squamous cell cervical cancer and 13 samples of healthy cervical tissue were included (Supplementary Fig. 1). While *NAA15, GSK3B, RFFL, SERPINB10, CCDC6, MARK1, TCF4, and NACC1* showed higher expression levels in CC biopsies compared to healthy tissue (Fig. 1A–G), only *NACC1* expression was significantly higher in epithelium biopsies of CC, indicative of its role as an oncogene (Fig. 1H).

Additionally, we analyzed the levels of NAA15, GSK3B, RFFL, SERPINB10, CCDC6, MARK1, TCF4, and NACC1 in normal and CC cervical tissue samples, stained by immunohistochemistry (Fig. 2A–H). NACC1 expression was higher in CC, compared to normal tissue (Fig. 2H).

3.2. NACC1 overexpression correlates with lower overall survival in patients with CC who present EMT, activation of the PI3K-AKT pathway, or hormonal abnormalities

NACC1 expression data were subjected to survival analysis using the Kaplan-Meier (KM) estimator. Survival analysis revealed that the NACC1 level was not related to OS in 292 patients with CC at different stages of disease progression (HR = 0.93, P = 0.742), (Fig. 3A). In contrast, elevated NACC1 levels were associated to lower OS (HR = 2.6, P = 0.049) in CC patients, particularly those exhibiting EMT, PI3K-AKT pathway activation, or hormonal abnormalities (Fig. 3B).

3.3. NACC1 mRNA is a potential target of miR-218-5p, -124-3p, and −23b-3p

To evaluate whether miR-218-5p, -124-3p, and −23b-3p regulate *NACC1* expression, a bioinformatics analysis was performed to locate

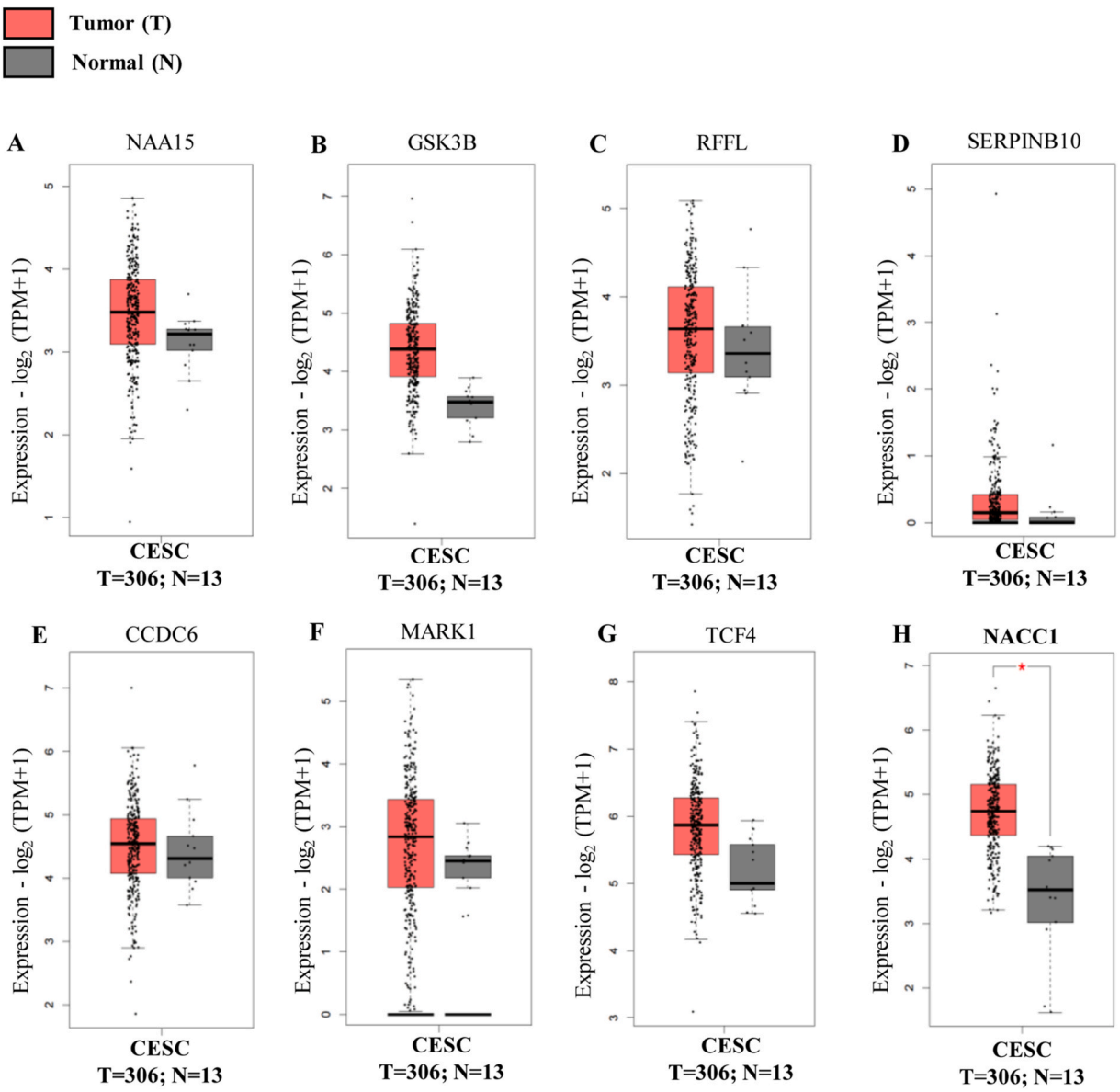

Fig. 1. Expression of genes predicted to be targeted by miR-218-5p, -124-3p, and −23b-3p in CC tissues. (A–H) Levels of NAA15, GSK3B, RFFL, SERPINB10, CCDC6, MARK1, TCF4, NACC1 were analyzed. The expression data from 306 CC tissue samples and 13 normal cervical epithelial tissues recorded in TCGA were assessed. The GEPIA2 platform was used to process the data. $P < 0.05$. *$P < 0.05$.

recognition elements for the three miRNAs (MREs) in the 3′-untranslated regions (3′-UTR) of *NACC1* mRNA. Using TargetScanHuman 8.0 platform, *NACC1* was found to contain two MREs for miR-218-5p, one for miR-23b-3p, and one for miR-124-3p (Fig. 4A). Subsequently, the levels of each miRNA and *NACC1* in HaCaT, C-33A and CaSki cells were determined. The expression levels of miR-23b-3p, -124-3p, and -218-5p, were significantly lower in C-33A and CaSki cells compared to non-tumor HaCaT cells (Fig. 4B). In contrast, *NACC1* transcript levels were significantly higher in C-33A and CaSki cells than in HaCaT (Fig. 4C).

3.4. *NACC1 mRNA level is decreased in C-33A and CaSki cells overexpressing miR-218-5p, -124-3p and −23b-3p*

To assess the effect of overexpression of one or all three miRNAs on the level of *NACC1* mRNA, C-33A and CaSki cells were transiently transfected with miR-23b-3p, miR-124-3p, and miR-218-5p mimics or with scrambled (negative control). The levels of the three miRNAs were significantly increased in both cell lines (Fig. 5A–F), resulting in a significant decrease in *NACC1* mRNA levels. The increased levels of the three miRNAs correspond to lower *NACC1* levels in CaSki and C-33A cells (Fig. 5G–H).

3.5. *Overexpression of miR-218-5p, -124-3p, and −23b-3p reduces the proliferation in C-33A and CaSki cells*

Given NACC1's role in modulating proliferation and survival [28] and, considering that overexpression of miR-218-5p, -124-3p, −23b-3p, or all three miRNAs together decreased *NACC1*mRNA levels, CaSki, and C-33A cells were transfected with each miRNA mimic or with all three to assess cell proliferation. The results showed that overexpression of miR-218-5p, -124-3p, or −23b-3p decreases the proliferation of C-33A and CaSki cells (Fig. 6A and F) compared to the negative control. Proliferation rates were lower in both cell lines when overexpressing miR-218-5p + miR-124-3p + miR-23b-3p (Fig. 6E and J) compared to transfections with individual mimics (Fig. 6B–D and 6G-I). The increases levels of miR-218-5p, -124-3p, or −23b-3p negatively affected the

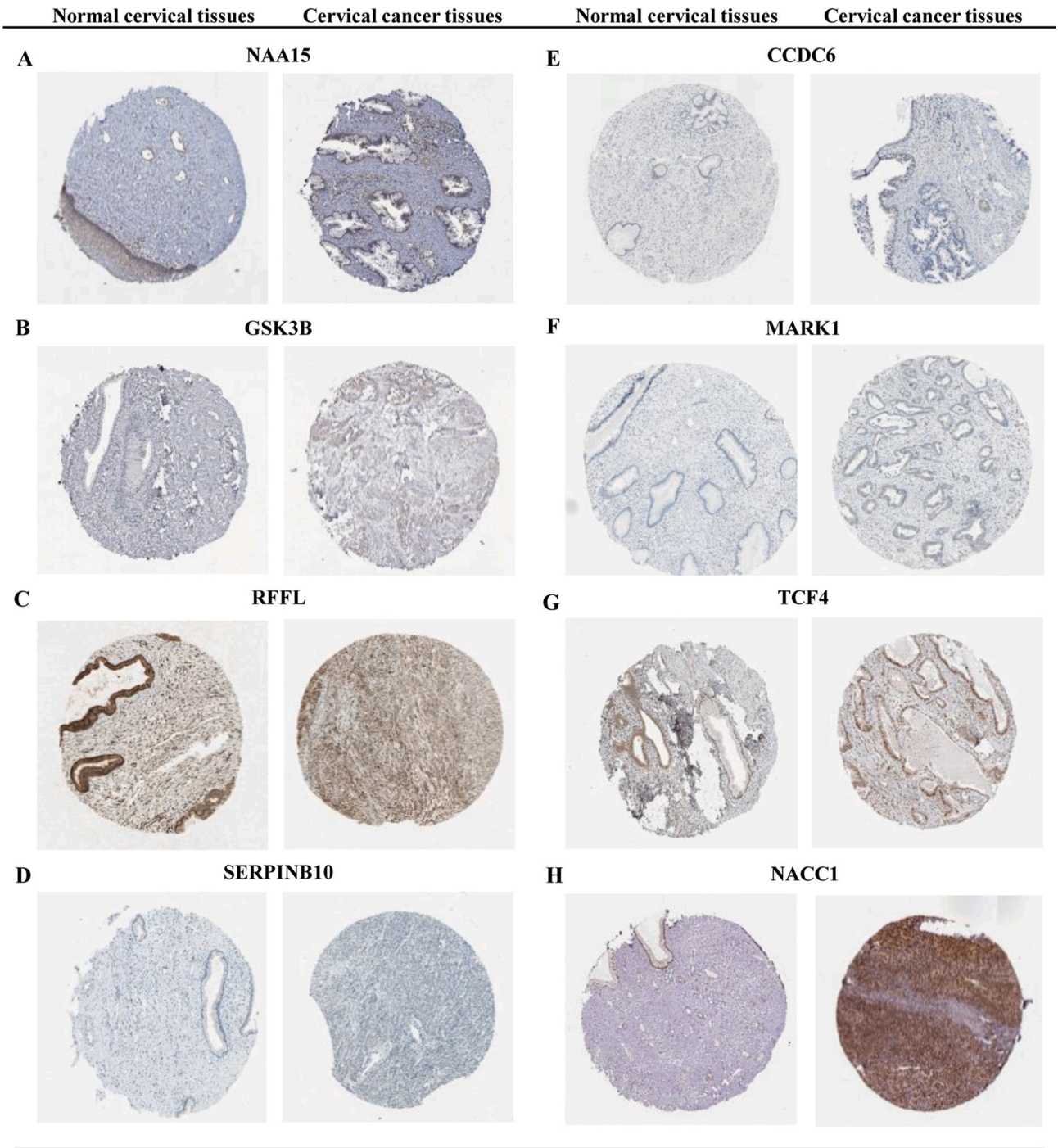

Fig. 2. Expression of NAA15, GSK3B, RFFL, SERPINB10, CCDC6, MARK1, TCF4, and NACC by immunohistochemistry in the normal cervical epithelium and CC tissue (A–H). Images available at https://www.proteinatlas.org, accessed 29 October 2022.

proliferation of C-33A and CaSki cells.

3.6. Overexpression of miR-218-5p, -124-3p, and −23b-3p promotes apoptosis of C-33A and CaSki cells

In addition to high proliferation, tumor cells exhibit a low rate of apoptosis [32], and it has been described that NACC1 modulates both processes. To assess the effect of overexpressing miR-218-5p, -124-3p, and −23b-3p on apoptosis, C-33A and CaSki cells were transfected with each miRNA mimic or with the three combined, and apoptosis rates were quantified using flow cytometry. The results demonstrate that an elevation of miR-218-5p, -124-3p, or −23b-3p significantly increased apoptosis in C-33A and CaSki cells (Fig. 7A and B) in contrast to negative control. An increased level of miR-218-5p + miR-124-3p + miR-23b-3p promoted the highest level of apoptosis in both cell lines. These results confirm that miR-218-5p, -124-3p, and −23b-3p positively modulate apoptosis.

4. Discussion

miRNAs play an important role in carcinogenesis and metastasis by regulating the translation of multiple target genes [14,15,17,33–35].

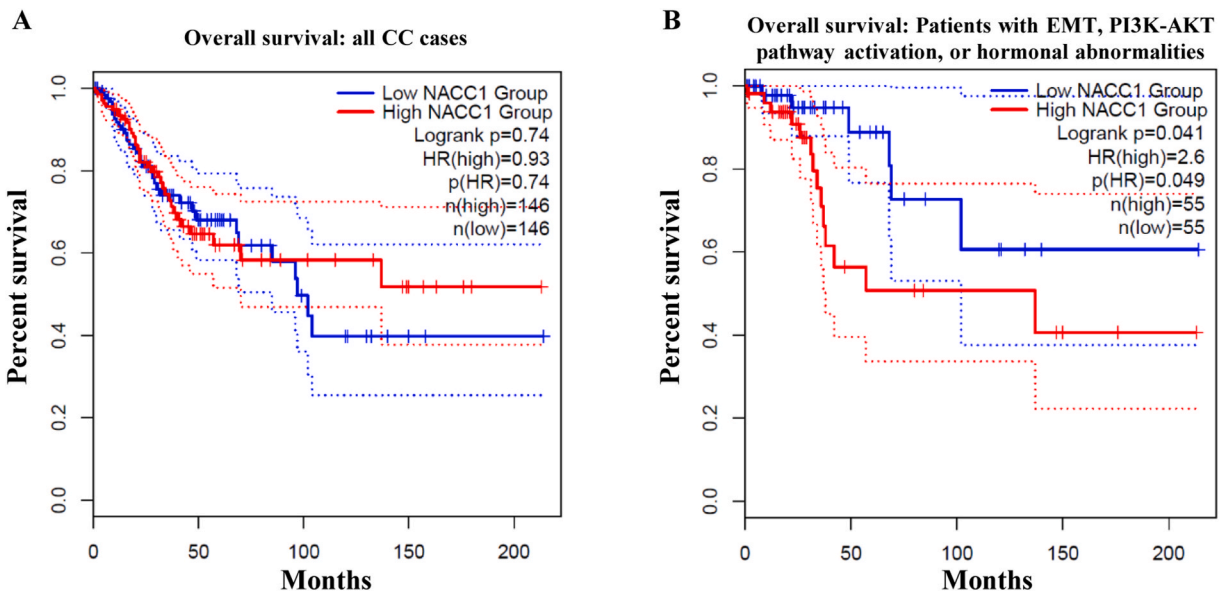

Fig. 3. Relationship between the NACC1 expression level and overall survival of patients with CC at different stages of evolution. Kaplan-Meier survival curve. The red line implies the high expression group and the black line represents the low expression group of NACC1. (A) In patients with CC at different stages of evolution, the NACC1 expression level is not related to OS. (B) High level of NACC1 is significantly related to lower OS in CC patients who present EMT, activation of the PI3K-AKT pathway, or hormonal disturbances.

Research on the function of deregulated miRNAs in CC has shown their participation in tumor progression, through the regulation of signaling pathways and cellular processes characteristic of cancer [36]. miR-218-5p, -124-3p and −23b-3p are underexpressed in tissues and in cell lines of CC. Exogenous overexpression of miR-23b-3p in SiHa cells is related to an increase in apoptosis. In CaSki cells, it leads to decreased migration, invasion, and proliferation, and in C-33A cells, it reduces proliferation and invasion. The increase in the level of miR-124-3p decreases invasion and proliferation while promoting apoptosis in SiHa and HeLa cells. Conversely, high levels of miR-218-5p reduce proliferation, invasion, and migration, while promoting apoptosis in HeLa cells [17,37–39].

Since miR-218-5p, -124-3p, and −23b-3p regulate proliferation, migration, invasion, and apoptosis in CC cell lines, each miRNA regulates the translation of several mRNAs and different miRNAs can regulate the same mRNA [40]. We investigated whether these three miRNAs regulate common mRNAs. Considering that bioinformatic analysis predicted that 24 genes may be targets of the three miRNAs [31], we analyzed their mRNA levels recorded in TCGA. We found that, of the 24 likely targets, only NACC1 mRNA and protein were significantly increased in CC tissue compared to normal cervical tissue. High NACC1 levels were associated with poor prognosis in CC patients with evidence of EMT, activation of the PI3K-AKT pathway, or hormonal abnormalities. It has been reported that high levels of NACC1 correlate with resistance to chemotherapy, tumor recurrence, poor prognosis, increased proliferation, invasion, and migration [21,28]. In cells T24 and UMUC6 of urothelial carcinoma, silencing NACC1 suppresses the expression of cyclin A1, A2, B1, and B2 involved in cell cycle control. These findings suggest that NACC1 plays a key role in cell cycle regulation and proliferation [24]. In nasopharyngeal carcinoma cells, NACC1 not only promotes proliferation but also activates the Akt/mTOR signaling pathway. This activation leads to decreased E-cadherin, increased N-cadherin, vimentin, and Snail; changes that characterize EMT, and were associated with enhanced migration and cell invasion [26]. Li et al., discovered in retinoblastoma cells that overexpression of *NACC1* triggers the phosphorylation of AKT and mTOR, increases the level of P70, T-caspase-3, and Bcl-2, while decreasing the level of p53, Bax and C-Caspasa-3. These changes result in increased cell viability, colony formation, along with inhibition of apoptosis, modulated by the activation of the Akt/mTOR pathway [23]. A recent study demonstrated that miR-1298 regulates *NACC1* mRNA in CC, with significantly reduced miR-1298 expression observed in tissues and the C-33A, SiHa, MS751, and HeLa cell lines. Overexpressing miR-1298 in SiHa and HeLa cells led to inhibition of proliferation, migration, and cell invasion, and researchers hypothesized that the results may be due to direct regulation of *NACC1* per miR-1298 [27]. This evidence indicates that NACC1 overexpression has clinical significance in patients with CC. In prostate cancer, urothelial carcinoma, and retinoblastoma, high NACC1 levels positively correlate with the upregulation of multiple genes involved in proliferation, apoptosis, invasion, and migration; processes that contribute to tumor progression [23,24,41]. While NACC1 expression in CC may be influenced by factors such as the genetic background, patient age, disease evolution, stages of progression, histological type, infection with HPV-HR, and viral genotype, the mechanisms regulating NACC1 expression in this cancer remain unknown. Bioinformatic analysis results support the hypothesis that *NACC1,* with two MREs for miR-218-5p, one each for miR-23b-3p and miR-124-3p in its 3′-UTR region (all 8-mer types), is a target of these miRNAs. Collectively, these data indicate that *NACC1* expression is modulated by miR-218-5p, -124-3p, and −23b-3p, reinforcing the hypothesis that these miRNAs synergistically regulate the proliferation and apoptosis by modulating *NACC1* mRNA translation in CC cells. Our predictions are corroborated by Li et al.'s findings, which show NACC1 regulation by miR-218-5p in WERI-Rb-1 retinoblastoma cells [23].

In C-33A and CaSki cells, levels of miR-218-5p, -124-3p, and −23b-3p are significantly lower compared to HaCaT cells, while *NACC1* mRNA levels are conversely increased. Different studies have reported that miR-218-5p, -124-3p, and −23b-3p are significantly decreased in cell lines (ME-180, C4-1, HeLa, SiHa, CaSki, C-33A), and cervical cancer tissue samples [18,42,43]. It has been reported that NACC1 is overexpressed in ovarian [44], endometrial [45], and prostate [41] cancer, in tissues from patients with CC, HeLa, and ME-180 cells [28]. The inverse relationship between the *NACC1* mRNA level with the levels of miR-23b-3p, -124-3p, and -218-5p in C-33A and CaSki cells make it possible that these miRNAs regulate *NACC1* expression.

The *NACC1* mRNA level decreased significantly in response to overexpression of each miRNA, and the reduction was greater when all three miRNAs were overexpressed. This behavior was more evident in

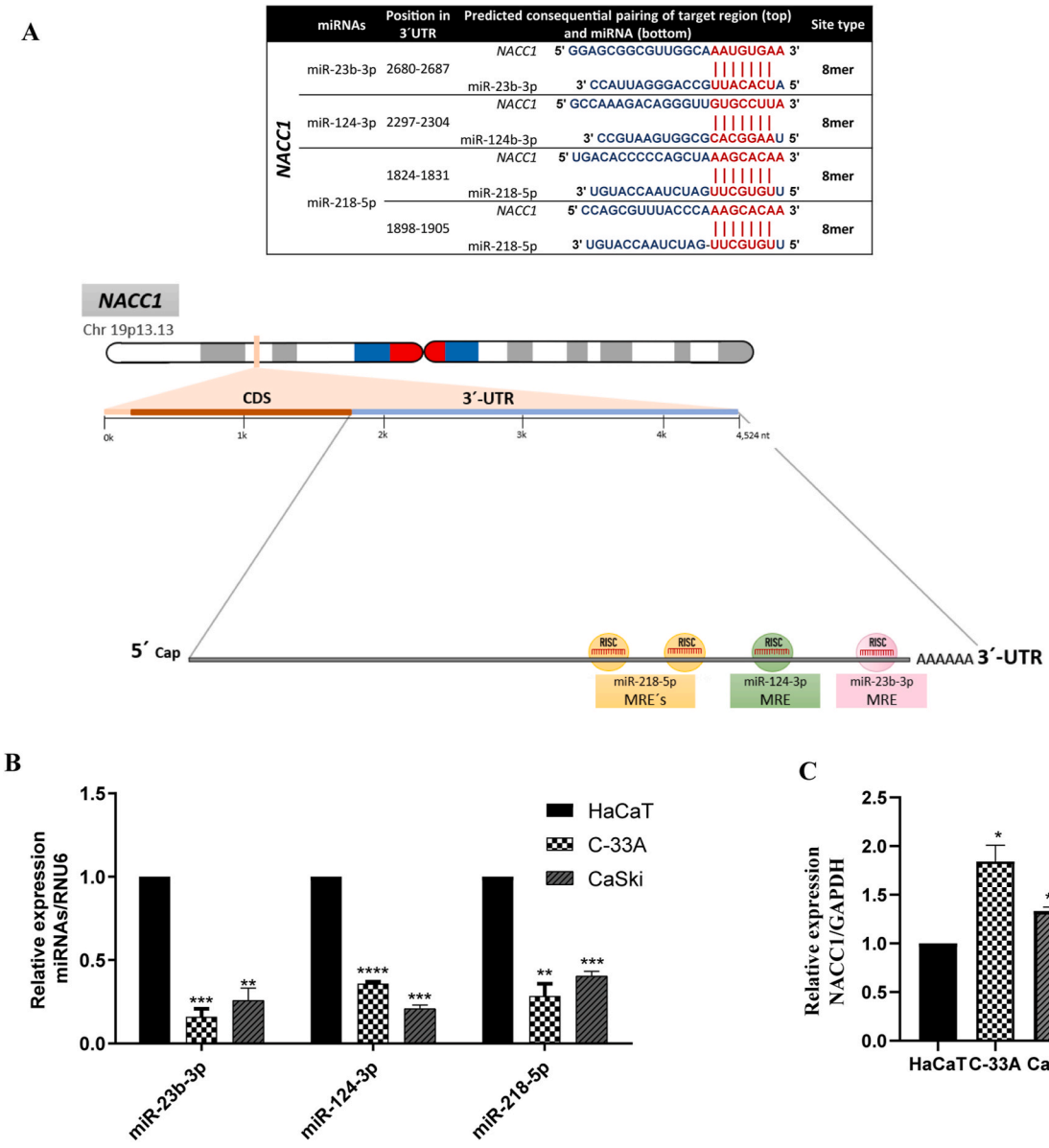

Fig. 4. *NACC1* is a putative target of miR-218-5p, -124-3p, and −23b-3p. (A) Position and recognition sequences for miR-218-5p, -124-3p, and −23b-3pin the 3′-UTR region of NACC1. (B) Basal expression of miR-23b-3p, -124-3p, -218-5p, and (C) *NACC1* in C-33A and CaSki cells compared to HaCaT cells. Statistical analysis was performed using GraphPad Prism v.8.0. A $P < 0.05$ value was considered statistically significant *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

CaSki cells compared to C-33A cells. The differences in the expression level of *NACC1* in C-33A and CaSki cells may be influenced by several factors: the concentration of mimics that entered the cells, the duration that the mimics remained viable in the cell cytoplasm, the activation of molecular mechanisms that regulate the total quantity of miRNAs in cells, the availability of MREs for each miRNA in the target mRNA, and the presence of other transcripts with MRE sites that hybridize with miR-23b-3p, -124-3p or -218-5p and compete with the MREs in the *NACC1* mRNA.

Variations in miRNA levels after mimic transfection may be related to the specific characteristics of each cell line. C-33A cells originate from HPV-negative cancer *in situ*, whereas CaSki cells come from metastatic HPV-16-positive CC. These cell lines exhibit different expression profiles and, therefore, different miRNA regulatory mechanisms, including deregulation of miRNA processors and altered expression of p53, c-myc, and p63. HPV-16 modifies miRNA expressions [20,43,46,47]. Long noncoding RNAs HOTAIR, MALAT1, and HULC are overexpressed in CC biopsies and cell lines. HOTAIR has a binding site for miR-23b-3p, MALAT1 for miR-124-3p, and HULC for miR-218-5p. Therefore, these lncRNAs could negatively regulate the level of those miRNAs in C4-1, Hela, CaSki, C-33A, and SiHa cells [19,48,49]. The secondary or tertiary structure of an mRNA influences the availability of the MRE for a specific miRNA, and other miRNAs can compete for MREs in the 3′-UTR region of the messenger. When the miR-218-5p mimic was transfected, and also when miR-218-5p + miR-124-3p + miR-23b-3p were overexpressed in CaSki cells, the level of *NACC1* mRNA showed a greater decrease in this cell line compared to C-33A. This can be partially explained by the existence of two binding sites for miR-218-5p in the 3′UTR region of the *NACC1* transcript. The level of *NACC1* mRNA in C-33A and CaSki cells transfected with all three miRNAs suggests a synergistic effect in the regulation of *NACC1*.

Since NACC1 modulates apoptosis and proliferation, changes in those processes were determined in C-33A and CaSki cells that overexpress miR-23b-3p, -124-3p, and -218-5p. In both cell lines, the overexpression of the three microRNAs was related to the decrease in proliferation and increase in apoptosis. These results confirm that all

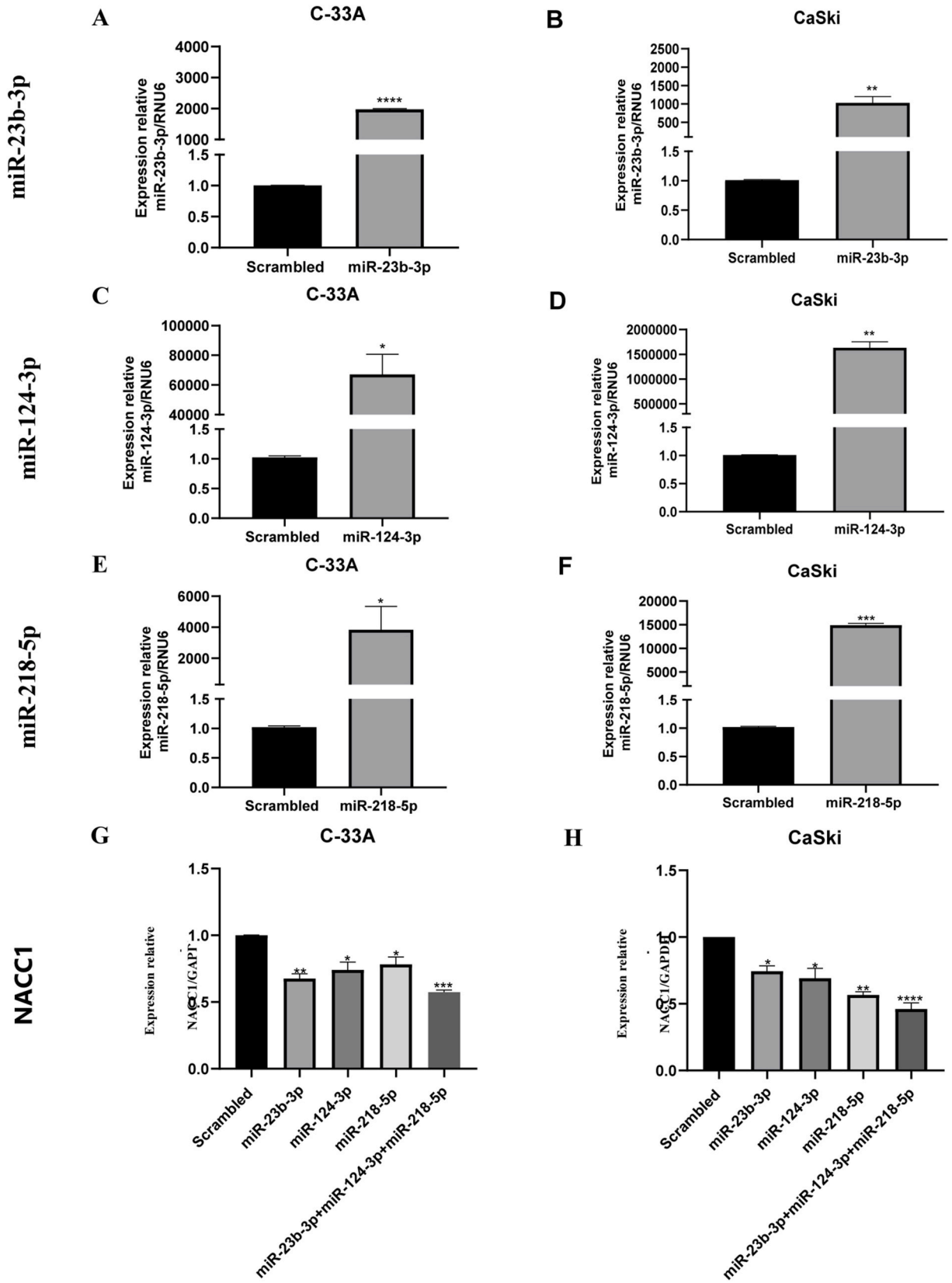

Fig. 5. *NACC1* mRNA level in response to overexpression of miR-218-5p, -124-3p, and −23b-3p. (A–F) Levels of miR-23b-3p, -124-3p and -218-5p in C-33A and CaSki cells transfected with each mimic. (G) *NACC1* mRNA level in response to overexpression of miR-23b-3p, -124-3p, -218-5p or to the co-expression of the three microRNAs, compared to scrambled, in C-33A cells and (H) CaSki cells. The levels of miRNAs and *NACC1* were normalized with RNU6 and GAPDH, respectively. Statistical analysis was performed using GraphPad Prism v.8.0. When the value was $p < 0.05$, differences were considered statistically significant. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

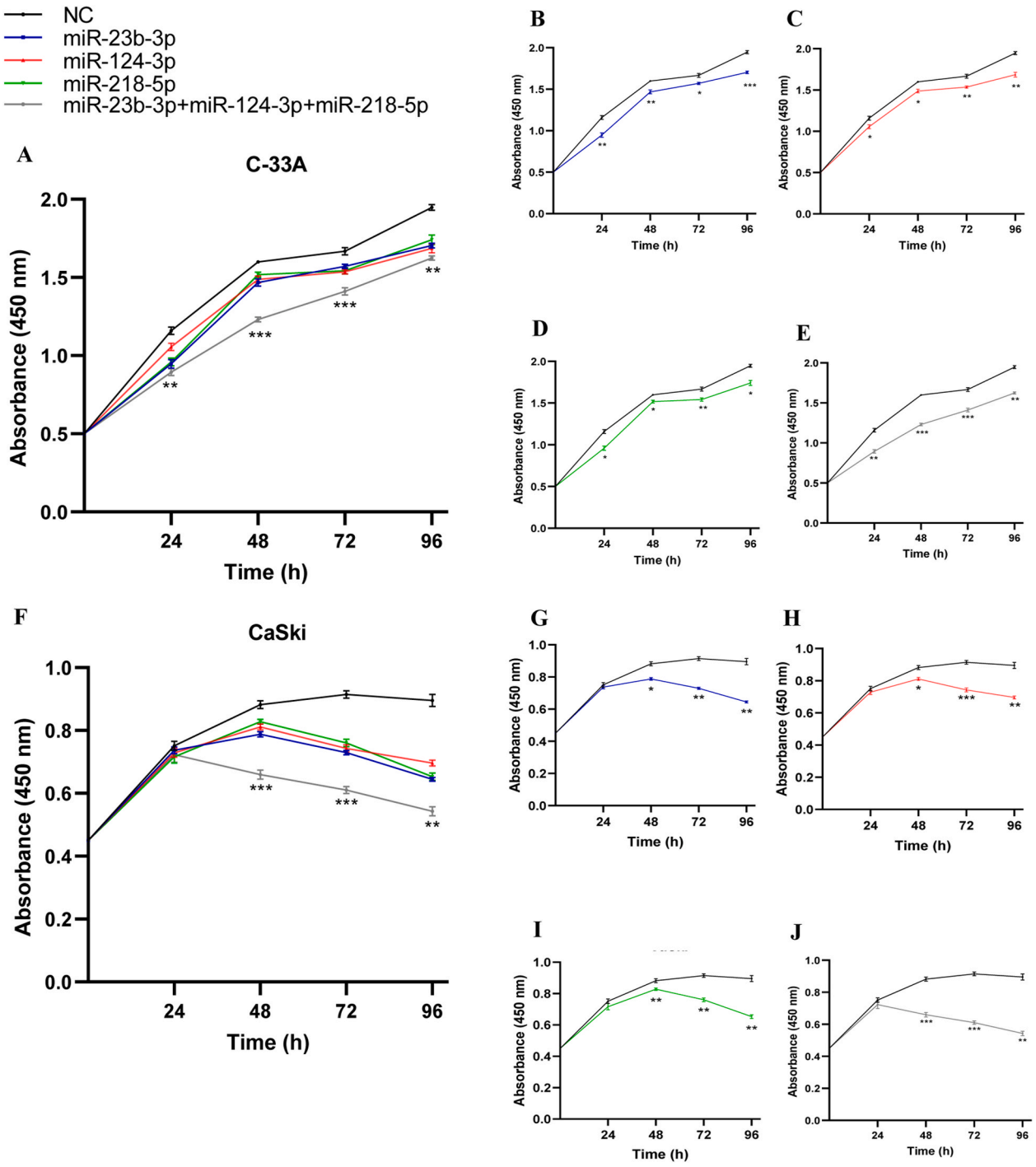

Fig. 6. Effect of miR-218-5p, -124-3p, and −23b-3p overexpression on proliferation in CaSki and C-33A cells.
Cell proliferation decreased at 48 h, 72 h, and 96 h after transfection with miR-23b-3p (B and G), miR-124-3p (C and H), or miR-218-5p (D and I) mimics in both cell lines. The percentage of inhibition of proliferation was higher when the three miRNAs were overexpressed in C-33A (A and E) and CaSki cells (F and J). When the value was $p < 0.05$, the differences were considered statistically significant. *P < 0.05, **P < 0.01, ***P < 0.001.

three miRNAs are negative regulators of proliferation and promoters of apoptosis in CC, as suggested by the Gene Ontology analysis in the previous bioinformatics analysis [31]. C-33A and CaSki cells transfected with all three miRNAs exhibited lower proliferation and a higher rate of apoptosis than when transfected with any of the individual miRNAs. The results indicate that miR-218-5p, -124-3p, and −23b-3p act synergistically to modulate proliferation and apoptosis [50]. The differences in the decrease in proliferation and the increase in apoptosis can be attributed to the number and function of the target genes that each miRNA regulates [31]. The repression of genes by a miRNA can have limited effects on cellular processes, but the impact is significantly greater when a set of miRNAs regulate common processes through their specific target genes and through shared targets, such as *NACC1* for miR-23b-3p, -124-3p, and -218-5p [31,50].

Overexpression of miR-23b-3p decreases the proliferation and invasion of C-33A and CaSki cells through the regulation of c-Met, a tyrosine kinase receptor that activates the RAS and PI3K signaling pathways. In SiHa and CaSki cells, the increase in miR-23b-3p induces a

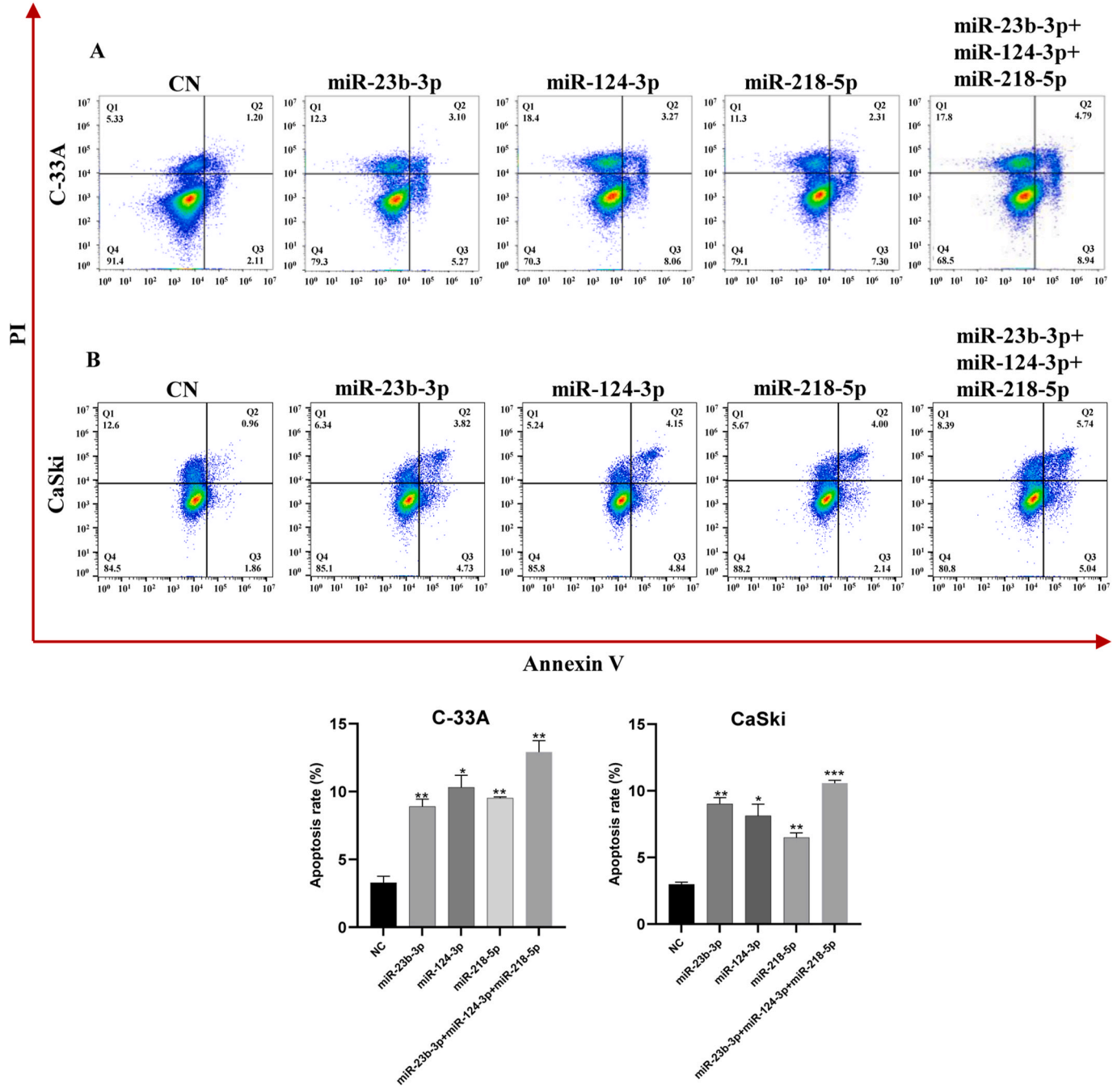

Fig. 7. miR-218-5p, -124-3p, and −23b-3p modulate apoptosis in C-33A and CaSki cells. Changes in apoptosis were determined by flow cytometry. C-33A (A) and CaSki (B) cells were transfected with miR-218-5p + miR-124-3p + miR-23b-3p mimics or with each individual miRNA. When the value was $p < 0.05$, the differences were considered statistically significant. *P < 0.05, **P < 0.01, ***P < 0.001.

reduction in EMT, migration, invasion, and proliferation, through the regulation of Six1, a transcription factor that modulates the expression of MYC and CCND1 oncogenes [17,42]. The restoration of miR-124-3p in HeLa and SiHa cells induces downregulation of the AEG-1 protein that contributes to the progression and development of different cancers. AEG-1 activates oncogenic signaling pathways, PI3K/Akt, MAPK, Wnt, and NF-κB between them; thus, the subexpression of AEG-1 decreases proliferation, invasion, and migration, and increases the rate of apoptosis [18,51]. Additionally, overexpression of miR-218-5p decreases proliferation and increases apoptosis through the regulation of transcription factor Gli3, related to the increase in the expression of cyclins B1 and D1 in SiHa cells [52]. Thus, we propose that the changes in proliferation and apoptosis in C-33A and CaSki cells, which overexpress miR-23b-3p, -124-3p, and -218-5p, are determined by the regulation of specific and common target genes of these miRNAs, including *NACC1*. There is little information on the role of NACC1 in CC. In this regard, Yeasmin et al. reported that NACC1 is overexpressed in cancerous cervical epithelium and cell lines, and its inhibition induces decreased cell proliferation, invasion, and migration, and increased apoptosis [28], However, the specific molecular mechanisms that regulate these processes in CC are unknown.

Little is known about the effect of overexpression of more than one miRNA in cancer cells. Existing information suggests that miRNAs may have a synergistic effect in the control of biological processes through common target mRNAs [53–56]. In renal carcinoma cells, the overexpression of miR-124-3p or miR-203 decreases the translation of ZEB2,

and this decrease was greater when the two miRNAs were co-expressed. The authors proposed that the decrease in ZEB2 mRNA in cells transfected with both miRNAs denotes a synergistic effect between miR-124-3p and miR-203. In cells with increased levels of both miRNAs, the decrease in proliferation and migration was more pronounced, responding to the downregulation of ZEB2, a transcription factor that plays a fundamental role in the development of EMT and its associated processes. Thus, ZEB2 participates in the regulation of apoptosis, survival, cell cycle arrest, migration, invasion, metastasis, and drug resistance [50,57]. In this investigation, the decrease in *NACC1* transcript levels, alongside reduced proliferation, and increased apoptosis due to the co-expression of miR-23b-3p, -124-3p, and -218-5p, supports the hypothesis of a synergistic effect of all three miRNAs and the possibility that NACC1 mRNA is a common target.

5. Conclusion

In conclusion, the results of this investigation indicate that miR-218-5p, -124-3p, and −23b-3p act synergistically to regulate NACC1 expression, proliferation, and apoptosis in CaSki and C-33A cells. This effect on cellular functions can likely be explained by modulation of the expression of specific and common target genes, among which is probably NACC1. The expression levels of NACC1 in the tissue of CC patients and the experimental results indicate that NACC1 is an important player in the progression of cervical cancer. The expression levels of NACC1, miR-218-5p, -124-3p, and −23b-3p could serve as prognostic indicators for CC patients. However, further studies involving patients with various histological types of CC and stages of disease progression are needed, along with confirmatory experiments in vitro and animal model experiments.

Funding

This study was supported by the Fondo Sectorial de Investigación para la Educación/FON.SEC.349/2021 (F003), CONAHCYT, annountcement 2017–2018 de Ciencia Básica, Project: A1-S-43684. Manuel Joaquín Romero López, Merlin Itsel Cruz de la Rosa, Gladys Wendy Valente Niño, and José Manuel Tinajero Rodríguez were supported by CONAHCYT (scholarship for doctoral studies).

CRediT authorship contribution statement

Manuel Joaquín Romero-López: Writing – review & editing, Formal analysis, Conceptualization. **Hilda Jiménez-Wences:** Project administration, Funding acquisition, Conceptualization. **Merlin Itsel Cruz-De La Rosa:** Methodology. **Judit Alarcón-Millán:** Methodology, Investigation. **Miguel Ángel Mendoza-Catalán:** Methodology. **Elizabeth Ortiz-Sánchez:** Supervision. **José Manuel Tinajero-Rodríguez:** Methodology. **Daniel Hernández-Sotelo:** Methodology. **Gladys Wendy Valente-Niño:** Methodology. **Dinorah Nashely Martínez-Carrillo:** Writing – original draft, Visualization, Formal analysis. **Gloria Fernández-Tilapa:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ncrna.2024.02.016.

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