TUMORIGENESIS REGULATED BY miRNAs

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TUMORIGENESIS REGULATED BY miRNAs

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EDITORIAL
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Editorial: Tumorigenesis Regulated by miRNAs

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Editorial on the Research Topic

Tumorigenesis Regulated by miRNAs

In addition to tumor-related proteins and their coding genes, non-coding genes are tightly associated with the occurrence and development of tumors. In particular, miRNAs, a class of highly conserved non-coding single-chain molecules, can inhibit gene expression at the post-transcriptional level by incomplete-complementary pairing with the untranslated regions of multiple genes, thus resulting in an imbalance of expression in a variety of tumor related genes. Therefore, miRNAs can promote or repress the expression of oncogenes or tumor-suppressor genes. These abnormal expressions detected in almost all tumor tissues can reflect their tissue origin. Previous studies demonstrated that miRNAs participate in almost every step in the occurrence and development of tumors and plays a crucial role in tumor pathogenesis.

Consequently, this topic aims to exploit novel miRNAs that could regulate the expression of tumor related genes, thus controlling the occurrence and progress of tumor. Meanwhile, the cellular, molecular and pathogenic mechanism of tumorigenesis regulation by miRNA is also to be elucidated in this topic.

The 11 articles in this Research Topic focus on the tumor genesis regulated by mRNA, which are involved in the following sections:

1) miRNAs that could promote tumorigenesis their mechanisms
2) miRNAs that could repress tumorigenesis and their mechanisms
3) The exploitation of mRNAs that could regulate tumorigenesis by bioinformatics and the demonstration of thereof function
4) Applications of miRNAs to control the occurrence and progress of tumors, and how they may affect human health.

Most articles in the Research Topic were involved in Sections 2 and 4.

For instance, the article *The Tumorigenic Properties of EZH2 are Mediated by MiR-26a in Uveal Melanoma* proposed a potential target miR-26a-EZH_2 axis for the development and treatment strategies for uveal melanoma (Li et al.). The results indicated the downregulation of miR-26a and upregulation of EZH_2 in uveal melanoma. Moreover, the overexpression of miR-26a and the knockout of EZH_2 can suppress the proliferation for uveal melanoma cells, meanwhile, the knockout of EZH_2 can counteract the tumor inhibition effect *via* the overexpression of miR-26a, suggesting that EZH_2 is the direct target for miR-26a. Furthermore, EZH_2 interacting proteins (UBC, CDK1, HDAC1, SUZ12, EED) were also found to participate in miR-26a-mediated tumor progression. However, the molecular mechanism of miR-26a-EZH_2 still needs further investigation by *in vivo* experiments.

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In 2020, there were 910,000 cases of liver cancer worldwide, which was the sixth most common malignant tumor in the world. Liver cancer kills 830,000 people, making it the third leading cause of cancer deaths. Thus it is stringent to develop novel strategies and targets for hepatocellular carcinoma. The manuscript *Delivery of miR-26a Using an Exosomes-Based Nanosystem Inhibited Proliferation of Hepatocellular Carcinoma* offers a new exosome delivery system based miRNA antitumor therapy strategy (Mahati et al.). In this article, the appearance of drug-carrying exosomes were observed by electron microscopy and dynamic light scattering. Moreover, anti-GPC3 scFv-modified exosomes can effectively and selectively delivered miR-26a to GPC3-positive hepatocellular carcinoma cells, thus inhibiting the cell migration and proliferation by the regulation of miR-26a downstream genes. The *in vitro* and *in vivo* experiments confirmed the high effeciency and selectivity of exosomes for the delivery of miR-26a and *via* the modification of exosomes with tumor specific antibodies.

In addition, there is another manuscript also described another exosomes based delivery system, which was titled as *Delivery of Anti-miRNA-221 for Colorectal Carcinoma Therapy Using Modified Cord Blood Mesenchymal Stem Cells-Derived Exosomes* (Han et al.). This article described an anti-miRNA-221 oligonucleotide (AMO) loaded exosomes, which can effectively suppress the proliferation of clonal formation of colon cancer cells *in vitro*. The results of a xenograft tumor model also showed that iRGD-modified exosomes were obviously enriched in tumor sites, exerting excellent anti-tumor efficacy. *In vivo* imaging showed that exosomes were mainly distributed in liver, spleen, and lung tissues. This article suggested that genetically modified exosomes could be served as an ideal natural nanostructure for anti-miRNA oligonucleotide delivery.

Acute myeloid leukemia causes great threaten to human health, more and more young adults suffered from acute myeloid. Therefore, it is urgent to exploit novel miRNAs to reduce the hazard of acute myeloid leukemia towards human health. The manuscript *MiRNA-142-3P and FUS can be Sponged by Long Noncoding RNA DUBR to Promote Cell Proliferation in Acute Myeloid Leukemia* reported the biological functions of lncRNA DUBR in AML pathogenic mechanism (Yin et al.). The knockdown of DUBR with small interfering RNA (siRNA) led to the suppression of survival and colony formation ability, as well as induction of apoptosis, in AML cells. And the downregulation of DUBR promoted the expression of FUS protein, targeting inhibition of FUS significantly promoted cell apoptosis in AML cell lines, indicating that DUBR is the potential target for AML therapy.

Exosome can be also employed to improve the miRNA therapy efficiency for tumor. Thus, the manuscript *Exosome-Transmitted miR-128 Targets CCL18 to Inhibit the Proliferation and Metastasis of Urothelial Carcinoma* investigated the regulatory function of exosome-transmitted miR-128 and chemokine (C-C motif) ligand 18 (CCL18) on urothelial carcinomas (UCs) (Shang et al.). The exosome-transmitted miR-128 can inhibit cell proliferation, invasion, and migration in UCs, as well as the apoptosis mediated *via* BUCT24, and these effects can be reversed by CCL18. Meanwhile, miR-128 can also inhibit the proliferation ($p < 0.05$) and metastasis ($p < 0.05$) of UCs in nude mice, thus providing a new target and therapeutic strategy for UCs treatment.

Besides the miRNA therapy for different tumor, there are also some articles involved in the diagnosis of different types of tumors, which is also important for the cancer treatment. The manuscript *miRNA Combinatorics and its Role in Cell State Control-A Probabilistic Approach* proposed an overlooked quantitative dimension for a set of genes and miRNA regulation in living cells, this study disclosed that the modest miRNA overexpression resulted in the shift of cell identity and cancer evolution (Mahlab-Aviv et al.). The findings in this study revealed that most genes are resistant to miRNA regulation, whereas the composition of the abundant miRNAs dictates the state of the cells, indicating the role of miRNAs in cell state. Moreover, FAT10 was reported as a biomarker for the tumor immune infiltration in skin cutaneous melanoma (SKCM) in the manuscript *FAT10 is a Prognostic Biomarker and Correlated With Immune Infiltrates in Skin Cutaneous Melanoma* (Wang and Zhang). *FAT10* gene expression level is positively related to immune infiltration, immune checkpoint expression, whereas negatively related to tumor cell invasion and DNA damage. These results indicated *FAT10* gene as an effective biomarker for the diagnosis and treatment of SKCM.

Furthermore, there are four review summarized the role of miRNA in the cell proliferation, apoptosis, invasion, therapeutic targets, and the pathogenicity mechanism of different types of cancers in this Research Topic, which can facilitate the diagnosis and therapy of different kinds of tumor genesis and progress employing different miRNAs. The targets of these miRNAs can thus provide more effective strategies for the treatment of different cancers.

Cervical Cancer, Papillomavirus, and miRNA Dysfunction summarized miRNAs that affected the HPV infection process and miRNAS contributing to the development and maintenance of malignant cervical tumor cells (Bañuelos-Villegas et al.). Meanwhile, the miRNAs served as biomarker for precancerous lesions or cervical tumors were also recapitulated in this review, which can facilitate the early diagnosis and treatment of cervical cancer *via* miRNAs.

Moreover, the other three reviews were prepared by Mohammad Taheri's. team. miR-1246 is the firstly recognized microRNA through a high throughput sequencing technique in human embryonic stem cells. *A Review on the Role of miR-1246 in the Pathoetiology of Different Cancers* focuses on the *in vivo* and *in vitro* oncogenic roles of miR-1246 in colorectal, breast, renal, oral, laryngeal, pancreatic and ovarian cancers as well as melanoma and glioma (Ghafouri-Fard et al.). The regulatory roles of miR-1245 in signal pathways of RAF/MEK/ERK, GSK3β, Wnt/β-catenin, JAK/STAT, PI3K/AKT, THBS2/MMP and NOTCH2 miR-1246 have been demonstrated. And this review can deepen our scientific understanding of miR-1246 systematically. miR-1290 is transcribed from *MIR1290* gene on the chromosome 1p36.13.

A Review on the Role of miR-1290 in Cell Proliferation, Apoptosis and Invasion summarized *in vitro, in vivo* and human investigations on the miR-1290 in the tumorgenesis (Ghafouri-Fard et al.). Moreover, the regulatory activity of miR-1290 in JAK/STAT3, PI3K/AKT, Wnt/β-catenin and NF-κB pathways were also summed up, which can promote the application of miR-1290 in the diagnosis and treatment in tumorgenesis. Breast cancer exhibited a higher and higher incidence in the worldwide. Angiogenesis plays crucial role in the development of breast cancer, and miRNA has been reported to serve as significant role in the angiogenesis process, thus it is important to investigate the linkage of miRNA and breast cancer. *MicroRNAs: Important Players in Breast Cancer Angiogenesis and Therapeutic Targets* described the recent progress of miRNA signature and thereof targets in the development of breast tumor (Hussen et al.). Furthermore, the miRNA-base strategies for the treatment of breast cancer targeting anti-angiogenic response were also discussed in this review, which is favorable to the future novel miRNAs-based therapeutic strategies for the breast cancer.

AUTHOR CONTRIBUTIONS

YW, TL, and GL revised the manuscript.

Conflict of Interest: Author GL was employed by company Guangzhou Bio-Gene Technology Co.,Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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The Tumorigenic Properties of EZH2 are Mediated by MiR-26a in Uveal Melanoma

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Background: The polycomb group protein enhancer of zeste homolog 2 (EZH2) has been found to be highly expressed in various tumors, and microRNA-26a (miR-26a) is often unmodulated in cancers. However, the functions of these two molecules in uveal melanoma (UM) and their relationships have not been reported.

Methods: We explored the effects of the miR-26a–EZH2 axis in UM by examining the levels of miR-26a and EZH2. The EZH2 levels in various tumor types and the correlations between EZH2 levels and overall survival and disease-free survival were reanalyzed. The binding of miR-26a to the 3′-untranslated region of EZH2 mRNA was measured using the luciferase reporter assay. The regulation of EZH2 gene expression by miR-26a was also identified, and the effect of elevated EZH2 expression on UM cell function was further examined. Results: miR-26a was downregulated and EZH2 was upregulated in UM cells. Overexpression of miR-26a inhibited cell proliferation, and knockdown of EZH2 suppressed cell growth. EZH2 was a direct target of miR-26a in UM cells. The knockout of EZH2 mimicked the tumor inhibition of miR-26a in UM cells, whereas the reintroduction of EZH2 abolished this effect. In addition, a network of EZH2 and its interacting proteins (UBC, CDK1, HDAC1, SUZ12, EED) was found to participate in miR-26a-mediated tumor progression.

Conclusion: The newly identified miR-26a–EZH2 axis may be a potential target for the development of treatment strategies for UM.

Keywords: microRNA-26a, EZH2, uveal melanoma, tumor progression, cell proliferation

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INTRODUCTION

Uveal melanoma (UM) is the most common primary intraocular malignancy in adults (Rodríguez et al., 2016). Both UM and cutaneous melanoma originate from melanocytes; however, UM is biologically and clinically different from other skin melanomas (van der Kooij et al., 2019). More than half of the main UMs are recurrent; no effective therapy exists for metastatic UM. Thus, studying the mutations associated with UM metastasis, proliferation, and survival may help researchers to understand the mechanisms of its etiology and metastasis, thereby facilitating the development of more effective therapies.

Micro-ribonucleic acids (miRNAs) are a class of small, noncoding, 21 to 25-nucleotide-long RNA molecules (Tomar et al., 2020). They can cause degradation or inhibit translation of target genes by targeting the 3′-untranslated region (3′-UTR) of mRNA. Thus, they can regulate cell proliferation, differentiation, and apoptosis (Lee et al., 2010; Matoulkova et al., 2012). A recent study found that the

abnormal expression of miRNAs is closely related to tumors, and that it exerts an effect on tumor suppressor genes and proto-oncogenes *in vivo*, thus regulating the occurrence, development, and outcome of tumors (Iorio and Croce, 2012). Low levels of the micro-RNA miR-26a were found to be expressed in liver, lung, and prostate cancers, which were closely related to tumor recurrence, metastasis, and poor prognosis (Yang et al., 2013). However, reports on the regulatory role of miR-26a on UM are rare.

Enhancer of zeste homolog 2 (EZH2) is an essential component of the epitope, genetic polycomb repressive complex 2 (PRC2) (Zhang et al., 2017; Bugide et al., 2018). EZH2 is required to maintain the characteristics of cancer stem cells (CSCs), demonstrating the ability of multiple cancers to self-regenerate, and specific transcription patterns (C. Wang et al., 2012). It is also critical for achieving cellular motility, by actively regulating genes rich in cytoskeletal components that increase invasive cell populations and promote movement and metastasis of melanoma in the skin (Moore, 2013). Elevated EZH2 levels predicted a negative prognosis, higher risk of metastasis, and shorter survival in a cohort of 89 patients with UM (Cheng et al., 2017; Jin et al., 2020). Given the effectiveness of EZH2 in enhancing CSC dryness and motility, we hypothesized that EZH2 was delivered to tumor cells with enhanced malignant characteristics during UM liver metastasis.

In this study, we explored the effects of the miR-26a–EZH2 axis in UM. The levels of miR-26a and EZH2 were examined. The regulation of EZH2 gene expression by miR-26a was also identified. Increased levels of EZH2 reversed the inhibitory effect of miR-26a in UM. This study demonstrated an important role of the miR–26a-EZH2 axis in UM and illustrated its potential application in UM therapy.

MATERIALS AND METHODS

Cell Culture

UM cell lines (92.1, Mel270, Omn2.3, and Omn1) and human choroid plexus epithelial cells (HCPEpiC) were provided by the ATCC (Manassas, VA, United States). All cells were cultured in high-glucose Dulbecco's modified medium (Invitrogen, Gaithersburg, MD, United States) with 0.1 mg/ml streptomycin, 100 U/ml penicillin, and 10% (v/v) fetal bovine serum. Cell culture was carried out in a humidified cell incubator at a temperature of 37°C, with 5% CO_2.

Transfection

The 92.1 and Mel270 cells (5×10^5 cells/well) were incubated overnight in 24-well plates. The miR-26a mimic and pcDNA3.1-EZH2 expression plasmid was synthesized at Genomics Pharmaceuticals (Shanghai, China). miR-26a mimics (50 nM), EZH2 plasmid (1 μg), and the corresponding negative control were transfected using lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. In some experiments, siRNA for EZH2 (si-EZH2) or its negative control were transfected into 92.1 and Mel270 cells. The sequences of si-EZH2 were as follows: 5′-GUC GCA ACG GAC CAG UUA A-3′. Scrambled siRNA (si-con) (5′-GGC GAU CAC GAC UAA GAC U-3′) served as a negative control.

Cell Proliferation Assays

Cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution; Dojindo, Kumamoto, Japan). 92.1 and Mel270 cells (2000 cells/well) were seeded in 96-well plates for 5 more days, then reacted in a 10% MTT solution and incubated in a cell incubator (37°C, 5% CO_2) for 2 h. Optical density values were measured at 570 nm.

Luciferase Reporter Assays

The estimated binding site of miR-26a with wild-type (wt) and mutant (mut) EZH2 3′-UTR was cloned into the pmirGLO dual luciferase reporter vector (YouBio, Changsha, China). The reporter vectors (pre-miR-control, pre-miR-26a, and wt, and mut 3′-UTR EZH2) were coinfected into 92.1 and Mel270 cells. After 48 h, luciferase activity was detected using a standardized dual luciferase assay system (Promega, Madison, WI, United States).

Quantitative RT-PCR

A TRIzol reagent (YouBio) was used to extract all RNA from the cell samples. Then, 2 μg of total RNA was reversed using the Script RT kit (YouBio). The ABI PRISM® 7900 SYBR Green PCR Master Mixture (Applied Biosystems, Waltham, MA, United States) was used in the array detection system for quantitative reverse transcription polymerase chain reaction (qRT-PCR). GAPDH and U6 snRNA were used as reference genes, respectively. The primers used were as follows: miR-26a-5p-forward: 5′-UCC AUA AAG UAG GAA ACA CUA CA-3′; backward: 5′-CAG UAC UUU UGU GUA GUA CAA-3′. EZH2-forward: 5′-CCC TGA CCT CTG TCT TAC TTG TGG A-3′; backward: 5′-ACG TCA GAT GGT GCC AGC AAT-3′. GAPDH-forward: 5′-GAA GGT GAA GGT CGG AGT C-3′; backward: 5′-GAA GAT GGT GAT GGG ATT TC-3′. U6-forward: 5′-CTC GCT TCG GCA GCA CAT ATA CT-3′; backward: 5′-ACG CTT CAC GAA TTT GCG TGT C-3′. UBC-forward: 5′-CTG GAA GAT GGT CGT ACC CTG-3′; backward: 5′-GGT CTT GCC AGT GAG TGT CT-3′. CDK1-forward: 5′-AAA CTA CAG GTC AAG TGG TAG CC-3′; backward: 5′-TCC TGC ATA AGC ACA TCC TGA-3′. HDAC1-forward: 5′-CTA CTA CGA CGG GGA TGT TGG-3′; backward: 5′-GAG TCA TGC GGA TTC GGT GAG-3′. SUZ12-forward: 5′-AGG CTG ACC ACG AGC TTT TC-3′; backward: 5′-GGT GCT ATG AGA TTC CGA GTT C-3′. EED-forward: 5′-GTG ACG AGA ACA GCA ATC CAG-3′; backward: 5′-TAT CAG GGC GTT CAG TGT TTG-3′.

Western Blotting

Cells were collected at a concentration of 30 μg/sample for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) using a pre-chilled radioimmunoprecipitation assay buffer cocktail with a phosphatase inhibitor. The polyvinylidene fluoride membrane (0.45 μm) was transferred to 1 × Tris-buffered saline solution with Tween-20 detergent (TBST) to block 2% bovine serum albumin. Then, the primary EZH2 antibody (#4905; Cell Signaling Technology) was cultured at 4°C (1:1,000) for 12 h, and the secondary antibody at 37°C for

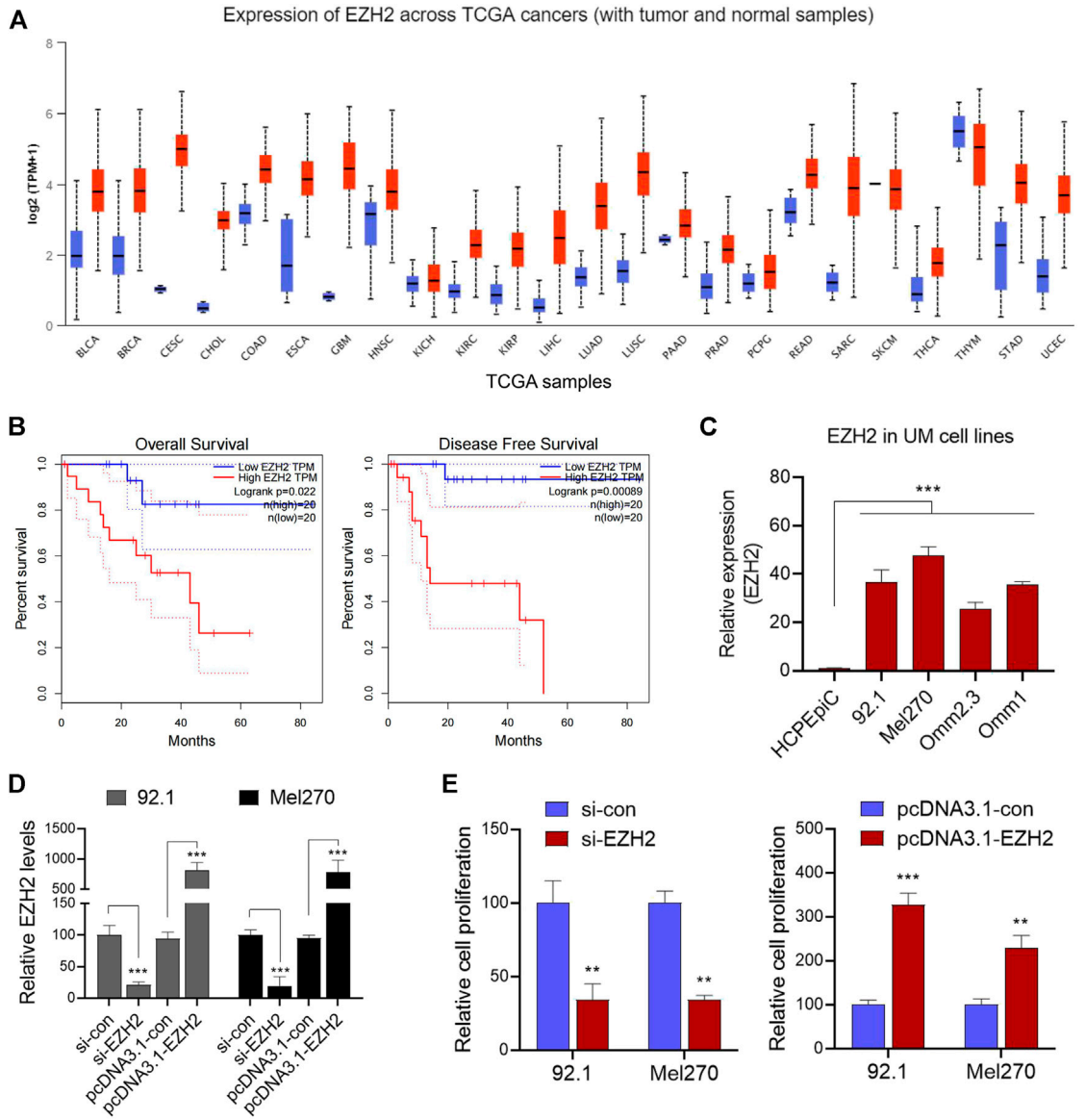

FIGURE 1 | EZH2 was upregulated and promoted tumor proliferation in UM. **(A)** In various tumor types of the TCGA dataset, UALCAN analysis was used to reanalyze the levels of EZH2. **(B)** The correlations between the expression of EZH2 and overall survival (OS) and disease-free survival (DFS) were further analyzed in patients with UM. **(C)** Relative levels of EZH2 expression in the UM cell lines and a human choroid plexus epithelial cell line (HCPEpiC) were measured. **(D)** After the knockdown of EZH2 by siRNA, or overexpression of EZH2, relative EZH2 levels were determined in 92.1 and Mel270 cells. **(E)** Cell viabilities in both cell groups were evaluated. $^{**}p < 0.01$, $^{***}p < 0.001$. Abbreviations: EZH2, enhancer of zeste homolog 2; UM, uveal melanoma; TGGA, The Cancer Genome Atlas.

1 h. After four rounds of TBST washing, the membrane was colored by chemiluminescence using the ECL Western Blotting Substrate Kit (ab65623; Abcam, Cambridge, MA, United States).

Statistical Analyses

All experimental data were analyzed and plotted using GraphPad Prism 9 (GraphPad Software, San Diego, CA, United States). All data are presented as the mean ± standard error. Statistical differences between two groups were compared using the non-response Student t-test. One-way analysis of variance was used to analyze multiple groups. A *p*-value of <0.05 was considered statistically significant.

RESULTS

Enhancer of Zeste Homolog 2 Was Upregulated and Promoted Tumor Proliferation in Uveal Melanoma

We initially analyzed the EZH2 expression levels in various tumor types and corresponding normal tissues in the Cancer Genome Atlas dataset using UALCAN analysis (http://ualcan.path.uab.edu/analysis.html). The results revealed that EZH2 was upregulated in various tumor types (**Figure 1A**). Further

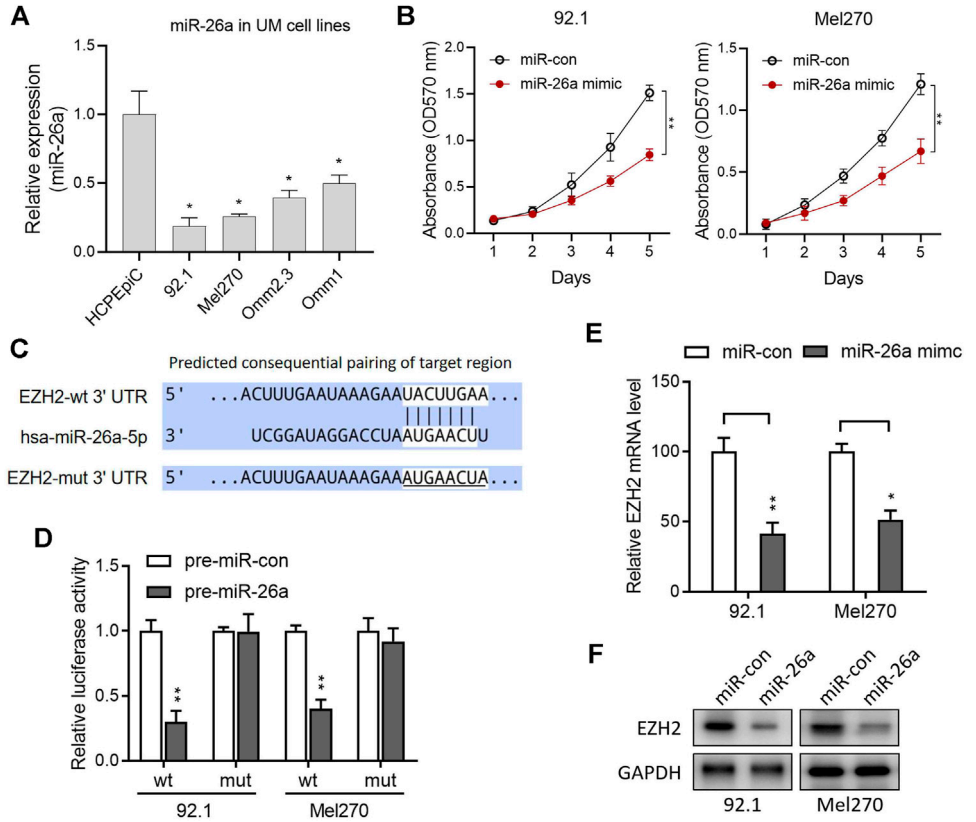

FIGURE 2 | EZH2 was identified as a target of miR-26a in UM. **(A)** Relative levels of miR-26a expression in the UM cells. *$p < 0.05$ vs. HCPEpiC. **(B)** The effect of ectopic miR-26a expression on cell proliferation in the 92.1 and Mel270 cells was evaluated by the MTT assay. **(C)** The 3′-UTR of the EZH2 mRNA included the predicted binding domain of miR-26a. **(D)** The 92.1 and Mel270 cells were transfected with pre-miR-con or pre-miR-26a, and the reporter vectors with the full-length wild-type (wt) or mutant (mut) EZH2 3′-UTR; luciferase activity was detected. **(E)** qRT-PCR was used to evaluate the EZH2 mRNA levels in 92.1 and Mel270 cells with ectopic miR-26a expression (*$p < 0.05$, **$p < 0.01$ vs. miR-con). **(F)** The effects of ectopic miR-26a expression on the EZH2 protein levels of 92.1 and Mel270 cells were detected by western blotting. Abbreviations: UM, uveal melanoma; EZH2, enhancer of zeste homolog 2; HCPEpiC, human choroid plexus epithelial cell.

analysis showed that high EZH2 expression was significantly associated with overall survival and disease-free survival in patients with UM (**Figure 1B**). Subsequently, EZH2 levels were found to be significantly higher in UM cells (92.1, Mel270, Omn2.3, and Omn1) than in normal HCPEpiC cells ($p < 0.001$, **Figure 1C**), suggesting that the upregulation of EZH2 might be associated with the development of UM. As expected, the knockdown of EZH2 by siRNA dramatically inhibited cell proliferation (**Figures 1D,E**); meanwhile, the overexpression of EZH2 significantly promoted the growth of tumor cells (**Figures 1D,E**).

Enhancer of Zeste Homolog 2 Was Identified as a Target of microRNA-26a in Uveal Melanoma

In UM cells, miR-26a expression was significantly lower than that in normal HCPEpiC cells ($p < 0.050$; **Figure 2A**). Furthermore, the miR-26a mimic significantly suppressed 92.1 and Mel270 cell proliferation, as evaluated by the MTT assay (**Figure 2B**; all $p < 0.01$). The candidate target genes of miR-26a were predicted using TargetScan (http://www.targetscan.org/) to elucidate the basic mechanism of miR-26a in UM. Potential miR-26a-binding sites in EZH2 3′-UTR were found (**Figure 2C**). A luciferase assay was used in 92.1 and Mel270 cells to confirm this prediction after transfection with wt or mut EZH2 luciferase reporter vectors in combination with pre-miR-26a or pre-miR-con. The overexpression of pre-miR-126a markedly reduced the luciferase activity of the wt reporter, rather than the mut reporter, in both cell types (**Figure 2D**). We performed qRT-PCR and western blotting to confirm this finding. The results revealed that miR-26a overexpression with the mimic significantly reduced EZH2 mRNA and protein levels in 92.1 and Mel270 cells (**Figures 2E,F**).

microRNA-26a Suppressed Tumor Progression by Targeting Enhancer of Zeste Homolog 2

A rescue experiment was conducted to further verify that EZH2, as a target gene, participated in the antitumor process induced by miR-26a. The 92.1 and Mel270 cells were transfected with a miR-

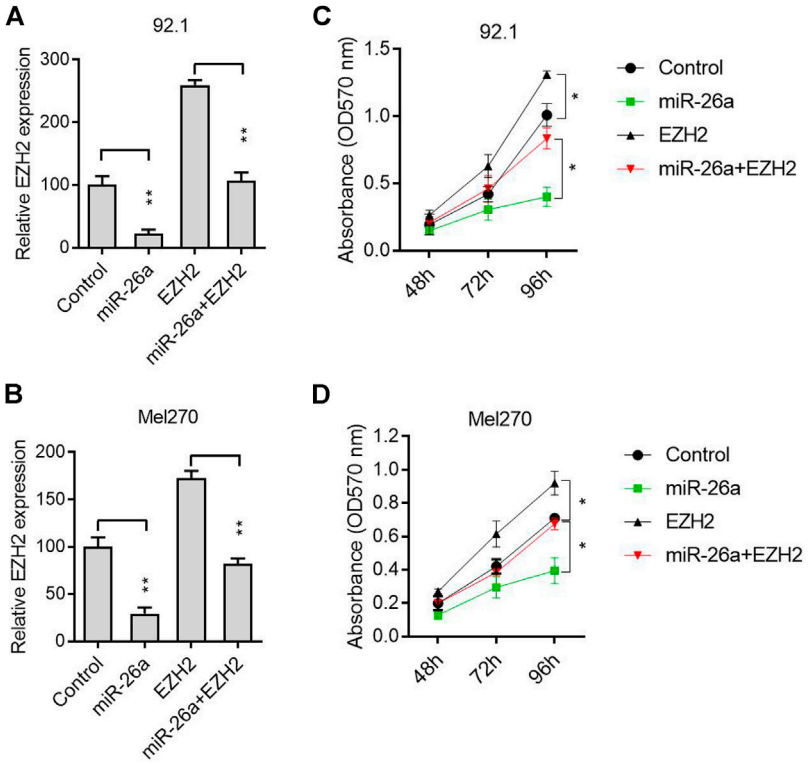

FIGURE 3 | miR-26a suppressed tumor progression by targeting EZH2. **(A)** After transfection with pcDNA3.1-EZH2 and/or miR-26a mimic in the 92.1 cells, EZH2 expression was evaluated using qRT-PCR (**$p < 0.01$). **(B)** qRT-PCR analysis was performed to evaluate EZH2 expression in Mel270 cells (**$p < 0.01$). **(C)** MTT assays were performed in 92.1 cells (*$p < 0.05$). **(D)** Cell proliferation was assessed in Mel270 cells (*$p < 0.05$).

26a mimic or EZH2 expression vector. The EZH2 mRNA level was inhibited by the miR-26a mimic, and reversed by the EZH2 expression vector, in 92.1 (**Figure 3A**) and Mel270 cells (**Figure 3B**). Furthermore, EZH2 overexpression significantly abolished the inhibition of 92.1 and Mel270 cell proliferation induced by miR-26a (**Figures 3C,D**).

A Network of Enhancer of Zeste Homolog 2 and its Interacting Proteins Participated in microRNA-26a-Mediated Tumor Progression

A Kyoto Encyclopedia of Genes and Genomes pathway analysis was performed to identify the potential interacting partners of EZH2 and describe the possible signaling pathways in UM. We found a panel of molecules involved in the EZH2-mediated signaling pathway, including UBC, CDK1, HDAC1, SUZ12, and EED (**Figure 4A**). We further analyzed the interaction between EZH2 and these molecules, using the non-log scale for calculation and the log-scale axis for visualization. Pearson correlation coefficients revealed significant positive correlations with EZH2 (**Figure 4B**). Indeed, EZH2 gene silencing significantly downregulated the mRNA expression levels of these molecules (**Figure 4C**). Notably, the results show that miRNAs may also be involved in regulating the EZH2-mediated signal network. After transfection with a miR-26a mimic, the mRNA levels of these molecules, including EZH2, UBC, CDK1, HDAC1, SUZ12, EED, were significantly downregulated in 92.1 cells (**Figure 4D**).

DISCUSSION

Most patients with UM do not seek treatment until blurred vision, metamorphopsia, or visual hallucination occurs; hence, they tend to miss the best opportunity for treatment (Yang et al., 2013). Studies have shown that miRNA expression varies at different stages of UM, suggesting that miRNAs are involved in regulating the occurrence, development, and invasion of UM (Yang et al., 2013). The involvement of miR-26a in the regulation of tumor cell proliferation, metastasis, and apoptosis has been well documented in multiple studies (Jia et al., 2014; Shao et al., 2016; Cai et al., 2017). In this study, we found that the miR-26a–EZH2 axis was involved in the occurrence and development of UM, by downregulating the expression of miR-26a and upregulating the expression of EZH2.

Initially, it was determined that miR-26a was underexpressed and EZH2 was highly expressed in UM cell lines (Bande et al., 2020). EZH2, a catalytic subunit of PRC2, is an oncogenic protein that silences the expression of various tumor-inhibitor miRNAs, such as miR-125b, miR-139-5p, miR-101, let-7c, and miR200b,

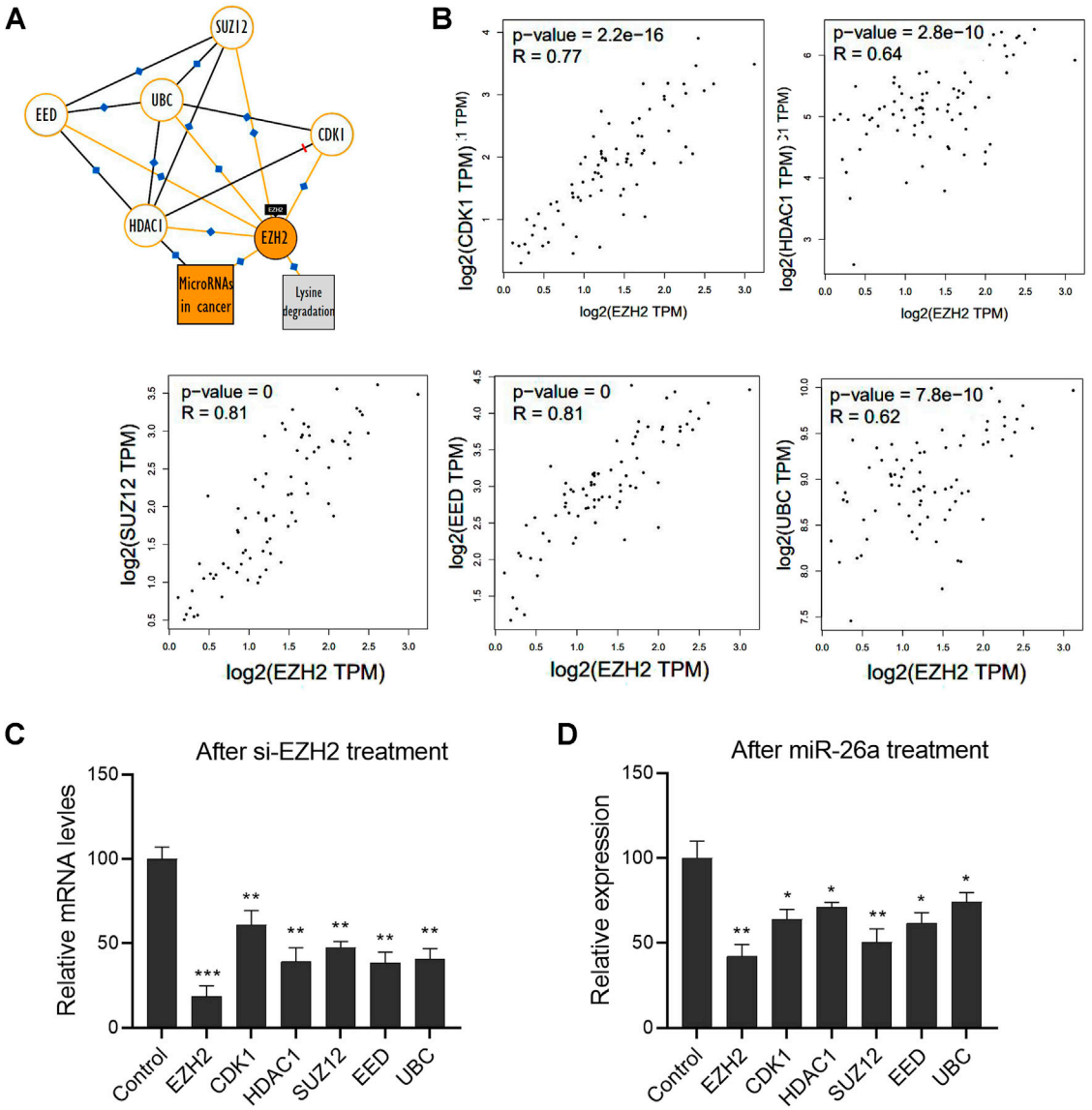

FIGURE 4 | A network of EZH2 and its interacting proteins participated in miR-26a-mediated tumor progression. **(A)** The Kyoto Encyclopedia of Genes and Genomes pathway analysis was performed to identify potential interacting partners of EZH2 in UM. **(B)** The interaction between EZH2 and these molecules was analyzed using the non-log scale for calculation and log-scale axis for visualization (Pearson's correlation coefficient). **(C)** qRT-PCR analysis was performed to evaluate mRNA expression in 92.1 cells after EZH2 gene silencing. **(D)** After transfection with a miR-26a mimic, mRNA levels of EZH2, UBC, CDK1, HDAC1, SUZ12, and EED were measured in 92.1 cells (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

and is typically increased in human cancers (Wang et al., 2016; Ma et al., 2018). Interestingly, previous studies have shown that EZH2 overexpression promotes growth within UM (Jin et al., 2020; Wu et al., 2020). The absence of EZH2 or the upregulation of miR-26a significantly impaired the proliferative capacity of UM cells in this study.

The function of miRNA depends mainly on the target gene (Mukherji et al., 2011). EZH2 plays a key role in tumor formation via epigenetic gene silencing and chromatin remodeling (Yamagishi and Uchimaru, 2017). miR-25, −26a, −30 days, -98, and let-7 interact with the defined sequences in EZH2 3′-UTR to directly diminish EZH2-regulated proteins (Zhu et al., 2016). To investigate potential mechanisms involving miR-26a and EZH2, we predicted potential miR-26a-binding sites in the 3′-UTR of EZH2 using the TargetScan database. EZH2 was further identified as a target gene of miR-26a using the luciferase assay, qRT-PCR, and western blotting.

The overexpression of miR-26a suppressed the growth of UM cells by promoting the apoptosis of UM cells, which has been reported to be related to the regulation of the p53/MDM2 pathway (Krell et al., 2013). EZH2 inhibitors suppressed the growth of UM cells by adjusting lysine methylation activity, thereby making EZH2 an effective therapeutic target (Ler et al.,

2017; Jin et al., 2020). However, the regulatory relationship between EZH2 and miR-26a in UM tumors has not been reported. We demonstrated that miR-26a inhibited cell survival by directly targeting EZH2, and that EZH2 overexpression reversed the miR-26a-dependent inhibiting effects. The potential interacting partners of EZH2 (including UBC, CDK1, HDAC1, SUZ12, EED) were predicted and identified, which constituted the PRC2. This contributed to tumorigenesis by inducing the epigenetic silencing of gene expression (Baylin and Ohm, 2006). In subsequent studies, we will further explore the effect of the miR-26a–EZH2 axis *in vivo* and evaluate the potential application value of EZH2 inhibitors and miRNA drugs in UM.

CONCLUSION

This study showed that miR-126a acts as a tumor inhibitor in UM and suppresses cell viability by targeting the EZH2 gene. Therefore, the miR-26a–EZH2 axis may be a potential target for UM therapy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

YL and MZ conceived and designed the study. HF contributed to data analysis. SM revised the manuscript. All authors read and approved the final manuscript.

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ORIGINAL RESEARCH
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Delivery of miR-26a Using an Exosomes-Based Nanosystem Inhibited Proliferation of Hepatocellular Carcinoma

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Background: MicroRNA (abbreviated miRNA)-based treatment holds great promise for application as clinical antitumor therapy, but good carriers for delivery of the miRNA drug are lacking. Exosomes secreted by mesenchymal stem cells (MSCs) have proved to be safe, and exogenously modified exosomes may potentially represent an excellent drug delivery vehicle.

Methods: In this study, we designed a delivery nano system using single-stranded variable fragment (scFv)-modified exosomes derived from human cord blood MSCs. Genetic engineering technology was used to obtain anti-Glypican 3 (GPC3) scFv-modified exosomes, which were then loaded with miR-26a mimics through electroporation.

Results: Results of electron microscopy and dynamic light scattering indicated that the diameter of the drug-carrying exosomes was about 160 nm. Furthermore, anti-GPC3 scFv-modified exosomes effectively delivered miR-26a to GPC3-positive hepatocellular carcinoma cells, thereby inhibiting cell proliferation and migration by regulating the expression of downstream target genes of miR-26a. The exosomes-based nano system displayed favorable anti-tumor effect *in vivo* with no obvious side effects.

Conclusion: Our data provided a new perspective for the use of exosome delivery systems for miRNA-based antitumor therapy.

Keywords: drug delivery, Glypican 3, exosomes, miR-26a, nanosystem

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INTRODUCTION

MicroRNAs (miRNAs) are small (20–22 nucleotides) non-coding RNA molecules that bind to partially complementary mRNA sequences and lead to degradation or translation inhibition of target genes (Rottiers and Näär, 2012). Increasing evidence has indicated that miR-26a plays an important role as an anti-oncogene in the development of a wide range of malignant tumors. miRNA-26a is found to be significantly downregulated in human hepatocellular carcinoma cells and negatively regulate both cell proliferation and of the cell cycle (Chen et al., 2011; Dang et al., 2012). Moreover, miRNA replacement therapy has been proposed as a promising treatment strategy for the treatment of malignant tumors. However, although miRNA-based approaches

may ultimately prove effective, their clinical application has been hampered by the lack of a suitable delivery system.

Exosomes are cell-secreted nanoscale vesicles (~100 nm) that have a key role in cell-to-cell material transport and signaling (Sluijter et al., 2014; Than et al., 2018). Exosomes are similar in size and function to synthetic nanoparticles, but as natural endogenous transporters, they have the advantages of low toxicity, lack of immunogenicity, and good permeability; thus, they may represent more promising drug delivery vehicles (Sun et al., 2013). Tumor cells release exosomes, which carry miRNAs, a phenomenon that has be used clinically in tumor diagnostics, tumor cells can also absorb miRNAs secreted by donor cells and function as exosome recipient cells (Skog et al., 2008). These data suggest that exosomes are natural carriers for miRNAs and could be used as targeted RNA drug delivery systems. However, little is known about the usefulness of exosomes as vectors in cancer treatment.

Glypican 3 (GPC3) is a heparan sulfate glycoprotein on the surface of cell membranes and is a specific antigen associated with cancer (Kolluri and Ho, 2019). GPC3 is upregulated in various types of solid tumors, but its expression is very low in normal adult tissues (Zhou et al., 2018). Thus, GPC3 is considered to be a potential target for the treatment of tumors. The development of nucleic acid-based drugs has evolved considerably over the years, and the current challenge is how to retain sufficient levels in the circulation after injection to precisely target the lesion while avoiding damage to normal cells (Hanisch and Kettenmann, 2007). The single-stranded variable fragment (scFv) retains antigen-binding activity and features low molecular weight and low immunogenicity, making it an excellent drug delivery tool with targeting capabilities (Ahmad et al., 2012).

In this study, we evaluated exosomes as drug delivery nanosystem in a cancer model. Anti-GPC3 scFv-modified exosomes effectively deliver miR-26a to GPC3-expressing hepatocellular carcinoma (HCC) cells. Our findings suggest that exosomes targeting a tumor antigen could provide a platform for miRNA replacement therapies.

MATERIALS AND METHODS

Cell Culture

HepG2 and Huh7 (HCC cells) and 293T (human embryonic kidneys cells) were provided by the American Type Culture Collection (Manassas, VA, United States) and cultured in the RPMI 1640 complete medium (Grand Island, NY, United States) containing 10% fetal bovine serum (FBS; Hyclone). Immortalized human cord blood mesenchymal stromal cells (cbMSC-hTERT) were obtained from ABM (Cat. No.: T0016; Canada) and cultured in α-MEM (Hyclone, Logan, UT, United States) with 20% FBS and a 4 ng/ml basic-fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, United States). All cell lines were cultured in an incubator (37°C, 5% CO_2).

Preparation of Exosomes

Exosomes were isolated from the cellular supernatant of mesenchymal stem cell (MSC) lines cultured in serum-free Dulbecco's Modified Eagle Medium (1% GlutaMax, Invitrogen, Carlsbad, CA, United States) for 48 h. The supernatant was centrifuged at 2,000 × g for 20 min and then at 10,000 × g for 30 min to remove cell debris. Exosomes were pelleted in small amounts by ultracentrifugation at 120,000 × g for 70 min and then washed once with phosphate buffered saline (PBS). The protein content of exosomes was measured using a rapid protein measurement kit (Wako Pure Chemicals, Osaka, Japan). The average amount of exosomes in 100 ml of the supernatant (1×10^7 cells; $n = 5$) was 75.2 μg.

Generation of Anti-Glypican 3 Single-Stranded Variable Fragment-Modified Mesenchymal Stem Cells

The coding sequence of the anti-GPC3 scFv was based on an anti-GPC3 antibody (clone GC33). A fusion gene (containing 6× His tag, anti-gpc3-scFv, full-length LAMP2B, a self-cleaved P2A linker, and puromycin) was synthesized and inserted into a pLVX-expression vector (Takara Bio, United States), which was then packaged to obtain lentivirus particles using LV-MAX Lentiviral Packaging Mix (Thermo Fisher Scientific, United States). After transduction, MSC cells expressing the scFv fusion gene (scFv-MSCs) were selected with 3 μg/ml puromycin (Invitrogen, Carlsbad, CA, United States).

Flow Cytometry Analysis

The expression of anti-GPC3 scFV on the surface of MSC cells was confirmed by flow cytometry. 1×10^6 unmodified negative control MSCs (nc-MSCs) and anti-GPC3 scFv-modified MSCs (scFv-MSCs) were collected. After being washed once with PSB, cells were incubated with 0.5 μg FITC-labeled human GPC3 protein (Fc Tag,Cat. No. GP3-HF258, Acrobiosystems) at 4°C for 30 min. To detect the expression of the GPC3 antigen in HCC cells, an APC-labeled anti-glypican 3 antibodies (clone 024, ab275695, abcam) was used. Fluorescence intensity was measured using the Cytoflex flow cytometer (Beckman Coulter, Brea, CA, United States).

Engineering Exosomes With miR-26a Mimics

The miR-26a mimics (5'-UUC AAG UAA UCC AGG AUA GGC U-3'; Sigma-Aldrich Co. LLC) and negative control (5′-GGU UCG UAC GUA CAC UGU UCA-3′; Sigma-Aldrich Co. LLC) were loaded into MSC exosomes using the Neon electroporation system (Life Technologies, Carlsbad, CA, United States) as reported previously (Zhang et al., 2017). Briefly, after ultracentrifugation, exosomes were dissolved in the R Buffer to ensure a concentration of at least 1 mg/ml, and 10 μL miR-26a mimic (0–50 nM) was then added to 90 μL of the electro transfer solution at a mass ratio of 1:1. This mixture was electrically transformed (1,000 V, 10 ms, 2 pulses), and 1 ml of PBS was added before incubation at 37°C for 1 h to promote exosome repair. Engineered exosomes with miR-26a mimics ($Exo^{miR\text{-}26a}$) were obtained by collecting exosomes after ultrasonication.

The Particle Size of Stranded Variable Fragment-Exo$^{miR-26a}$ Was Evaluated by Dynamic Light Scattering

The sample was prepared in the sample particle reserve solution as described in previous studies (Liang et al., 2018). A homogeneous solution was obtained by vertexing, and dynamic light scattering (DLS) was used to evaluate the homogeneous solution (1.5 ml) by transferring the sample to a square colorimetric cup, which was then subjected to the DynaPro NanoStar for DLS measurement (Wyatt, Santa Barbara, United States).

Quantitative Real-Time PCR

RNA was extracted from exosomes or cells using a Quick-RNA MagBead kit (Zymo Research, Orange, CA) according to the manufacturer's protocol, and the concentration of total RNA was measured using the Nanodrop micro-spectrophotometer. TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific Carlsbad, CA, United States) was used to obtain single-stranded cDNA. qRT-PCR was then performed using TaqMan MGB probes (Applied Bioscience) for miR-26a-5p. Amplification was carried out using TaqMan Universal PCR Master Mix (Applied Biosystems) for 40 cycles. The levels of miR-26a-5p were measured using stem-loop qPCR. The data obtained after three independent experiments were analyzed using the relative quantitative (RQ) formula RQ = $2^{-\Delta\Delta Cq}$. U6 was used as the reference gene for normalization. Each sample was repeated three times. The primers used were as follows: miR-26a-5p-forward: 5′-UCC AUA AAG UAG GAA ACA CUA CA-3′, reverse: 5′-CAG UAC UUU UGU GUA GUA CAA-3′, and U6-forward: 5′-CTC GCT TCG GCA GCA CAT ATA CT-3′, reverse: 5′-ACG CTT CAC GAA TTT GCG TGT C-3′.

Cellular Uptake of Exosomes

Carboxyfluorescein succinimidyl ester (CFSE) was used to stain the exosomes. Briefly, 2.5 μM CFSE was added into extracted 100 μg exosomes, supplemented with PBS to 50 μL, and incubated in a dark room at room temperature without stirring for 60 min. During incubation, the Exosome Spin Columns (Invitrogen) kit was prepared according to the instructions and the powder resin was hydrated at room temperature for 15–30 min. The incubated CFSE-labled exosomes were then added to the resin. Centrifuge the column in a 1.5 ml microcentrifuge tube at 750 × g for 3 min, and excess free dye could be absorbed by the resin. Fluorescence labeled exosomes were slowly added with 100 μL DMEM without phenol red, filtered in a 0.22 μm filter membrane, and then separated and stored at −80°C for later use. HepG2, Huh7 and 293T cells were seeded into 12-well plates at a concentration of 2×10^5 cells/well. After being cultured overnight, the cells were treated with CFSE-labeled scFv-Exo$^{miR-26a}$ (10 and 20 μg) for 6 h. The green fluorescence signals were observed and photographed under an inverted fluorescence microscope (Olympus Corp.). Then cells were digested and analyzed using the Cytoflex flow cytometer (Beckman Coulte) to measure the fluorescence intensity.

MTT Assay

HepG2, Huh7, and 293T cells were seeded in 96-well plates with 3,000 cells per well and then treated with nc-Exo$^{miR-26a}$ (unmodified MSC-derived exosomes carrying miR-26a), nc-Exo$^{miR-con}$ (unmodified MSC-derived exosomes carrying control miRNA), scFv-Exo$^{miR-26a}$ (anti-GPC3 scFv-modified exosomes carrying miR-26a) and scFv-Exo$^{miR-con}$ (anti-GPC3 scFv-modified exosomes carrying control miRNA) for 0, 24, 48, 72, and 96 h. The MTT solution (5 mg/ml, 20 μL) was added, and the cells were incubated for an additional 4 h. Then, the medium was replaced with dimethyl sulfoxide (200 μL, DMSO, Sigma, St. Louis, Missouri, United States). The absorbance of the resulting solution was measured at a wavelength of 450 nm using a microplate reader.

Colony-Forming Assay

A total of 10^3 HepG2 and Huh7 cells were inoculated in six-well plates. After an overnight culture, the cells were treated with scFv-Exo$^{miR-26a}$ and scFv-Exo$^{miR-con}$. After 2 weeks of continuous culture, the cell supernatant in each well was discarded, and cells were rinsed twice with PBS. Four percent paraformaldehyde was added to fix cells for 15 min at 25°C, and the cells were then stained with 2 ml 0.5% crystal violet for 30 min at 25°C. After being dried, the plates were photographed, and the clones were counted.

Scratch Wound Assay

Cell mobility was measured *via* a scratch wound-healing assay. HepG2 and Huh7 cells were inoculated in 12-well plates overnight. A 200 μl pipette tip was used to make scratches on the single cell layer. After gentle washes with PBS, the cells were then cultured in a cell incubator for another 48 h. Cells were photographed (0 and 48 h cell scratches) and the scratch area was measured using Image J software. Migration area ratio = (0 h scratch area − 48 h non migration area)/0 h scratch area.

In Vivo Study

In order to determine the performance of scFv-Exo$^{miR-26a}$ *in vivo*, 4–5 weeks old female B-NDG mice (NOD-*Prkdcscid Il2rgtm1*/Bcgen, Biocytogen Jiangsu Co., Ltd, Jiangsu, China) bearing HCC were established by subcutaneously injection of HepG2/luciferase cells (1×10^6) in the right flank. Mice were divided into two groups randomly, and administered intratumorally for three times (on day 0, 3, 5) with scFv-Exo$^{miR-26a}$ and scFv-Exo$^{miR-con}$ at 100 μg/mouse, respectively. Afterwards, mice were imaged every 7 days by IVIS live imaging, and raised tumors were measured using a caliper according to the following formula: V = length × width^2/2. The body weight was measured simultaneously as an indicator of systemic toxicity. At the end of the experiments, the mice were sacrificed, and tumors were resected, photographed and weighed, their organs (liver and kidney) were stained with hematoxylin-eosin (HE) to check for toxicity. The study was approved by the ethics committee of Xinjiang Medical University, and laboratory animal care as well as the user guide were followed. All animals were kept in accordance with Chinese animal health and welfare norms.

Statistical Analysis

Data were analyzed and graphed using GraphPad Prism 9 (GraphPad Software, Inc., CA, United States). All data were presented as means ± SEMs. The two groups were statistically compared using the Welch's

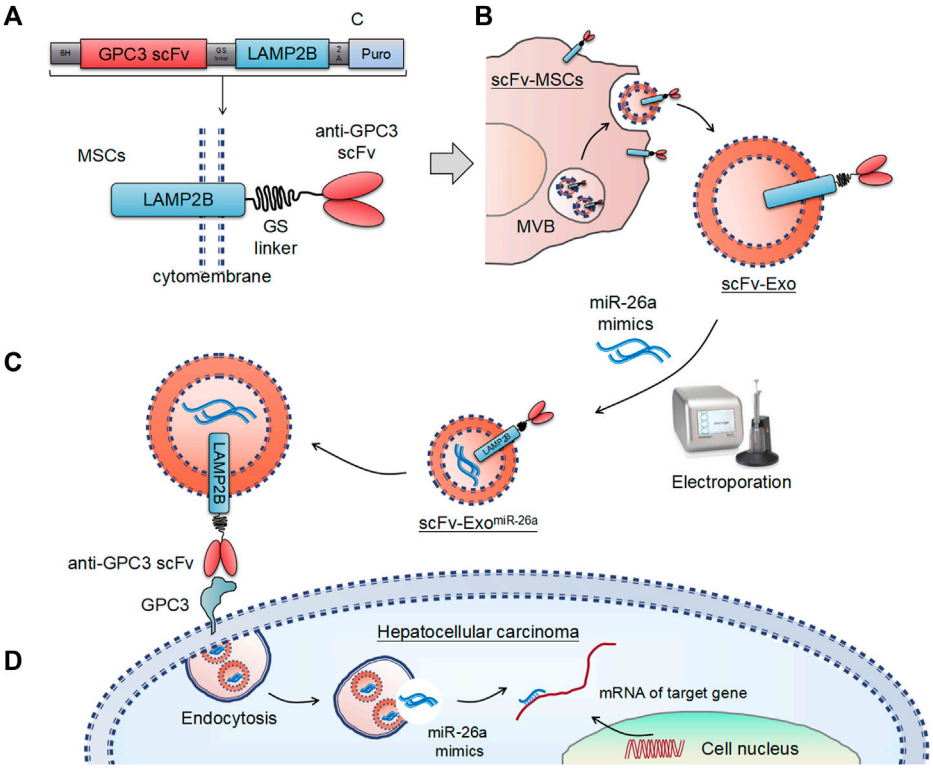

FIGURE 1 | Schematic illustration of the exosome-based drug delivery systems. **(A)** Design of the anti-GPC3 scFv-Lamp2b fusion constructs. **(B)** Schematic representation of the exosomes-based nanosystem preparation. **(C)** Uptake of anti-GPC3 scFv-modified exosomes by tumor cells. **(D)** Schematic illustration of the antitumor mechanisms of miR-26a mimics.

unpaired *t*-test. Multiple groups were analyzed using one-way ANOVA. A p-value <0.05 was considered statistically significant.

RESULTS

Schematic Illustration of the Exosome-Based Drug Delivery Systems

Schematic illustration of the preparation of anti-GPC3 scFv-modified exosomes and anticancer drug delivery for the suppression of hepatocellular carcinoma were showed in **Figure 1**. The structural diagram of the anti-GPC3 single-chain antibody (scFv) and the lysosome-associated membrane protein 2b (LAMP2B) fusion gene was presented in **Figure 1A**. Fusion constructs were configured 5′→3′ as per the following: a signal peptide (SP), an anti-GPC3 single-chain antibody (scFv), a (Gly4-Ser)3 linker, the full-length Lamp2b lacking the endogenous SP sequences, followed by an in-frame self-cleaving peptide from porcine teschovirus-1 (P2A), a puromycin-resistant gene, and a stop codon. Fusion of a protein of interest to Lamp2b is very common for displaying the protein on the surface of exosomes. A Lamp2b-based fusion protein can be used to display anti-GPC3 scFv on the surface. **Figure 1B** showed a proposed model illustrating how the anti-GPC3 scFv-Lamp2b fusion protein participates in exosomes in mesenchymal stem cells (MSCs). Ectopic expression of the fusion protein occurs at the rough endoplasmic reticulum (ER) and becomes concentrated in lamp2b-enriched exosomes, which are that are stored in a multivesicular bodies (MVB) prior to release from the cell. The scFv-modified exosomes (scFv-Exos) were isolated from the supernatant *via* ultracentrifugation, and then transfected with miR-26a mimics using electroporation. The miR-26a-loaded exosomes (scFv-Exo$^{miR-26a}$) were then uptake by hepatocellular carcinoma by binding to GPC3 on the tumor cell surface (**Figure 1C**). The exosomes were then incorporated into HCC cells by endocytosis, and the miR-26a mimics were then released and bind to the 3' untranslated regions (UTRs) of its target mRNAs to suppress expression (**Figure 1D**).

Identification of Exosomes Loaded With miR-26a Mimics

The fusion gene of anti-GPC3 scFv-Lamp2b was packaged with lentivirus and used to infect the mesenchymal stem cells (MSCs). After 14 days of selection using puromycin, the anti-GPC3 scFv-modified MSCs (scFv-MSCs) were obtained. Compared with the unmodified negative control MSCs (nc-MSCs), scFv-MSCs did not exhibit any changes in morphology and had the typical MSC fusiform shape (**Figure 2A**). In addition, flow cytometry analysis indicated that anti-GPC3 scFv was highly expressed in scFv-MSCs by detecting their binding with GPC3 protein (**Figure 2A**). Then,

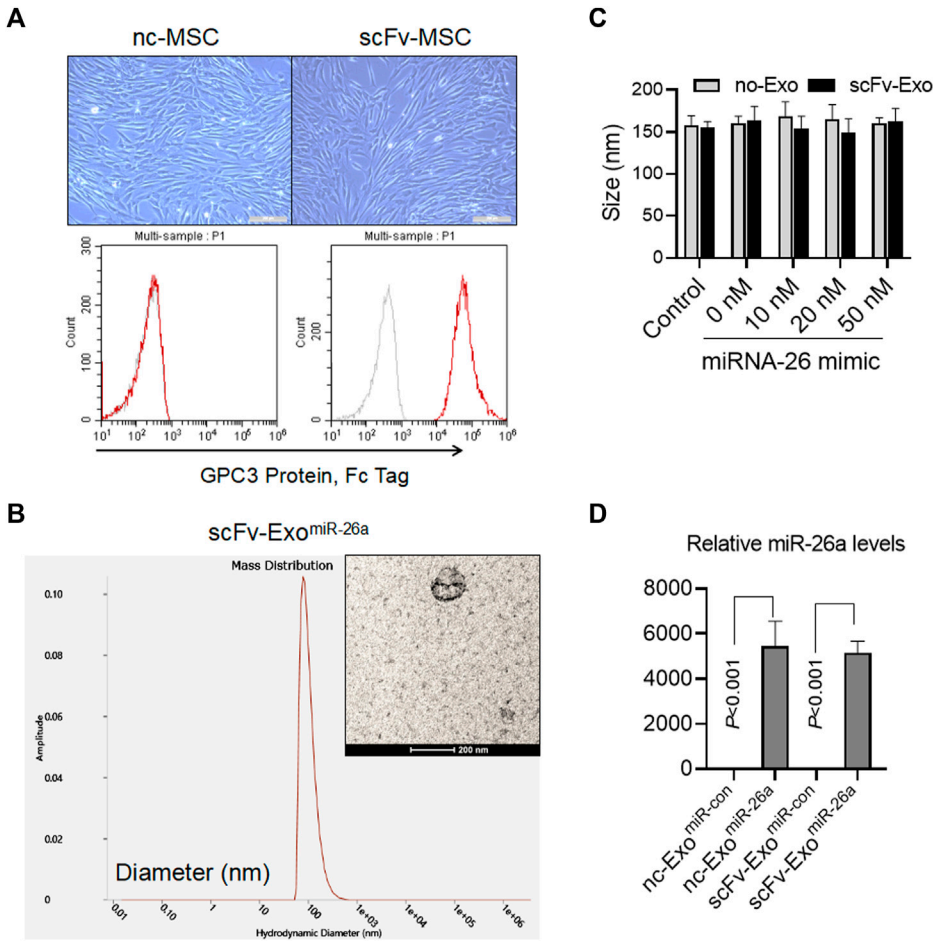

FIGURE 2 | Identification of exosomes loaded with miR-26a mimics. **(A)** Morphological characteristics of the fusion gene-modified MSCs were observed, and the expression of anti-GPC3 scFv on the surface of MSCs was detected by flow cytometry. Scale bars = 50 μm. **(B)** The morphology of exosomes loaded with 20 nM miR-26a mimics (scFv-Exo$^{miR-26a}$) as determined by electron microscopy. The particle size of exosomes was evaluated using dynamic light scattering. Scale bars = 200 nm. **(C)** Different amounts of miR-26a mimics (0–50 nM) were electrotransferred into exosomes, and the particle size of exosomes was evaluated using dynamic light scattering. **(D)** Detection of miR-26 levels in engineered exosomes using quantitative real-time PCR (qRT-PCR).

miR-26a mimics were transfected into the exosomes derived from scFv-MSCs using electroporation, and the resulting morphology is presented in the **Figure 2B**. The particle size of the scFv-Exo$^{miR-26a}$ exosome was evaluated by DLS, with a size of approximately 160 nm. Moreover, electroporation of different amounts of miR-26a mimics (0–50 nM) did not affect the particle size of exosomes (**Figure 2C**). The levels of the miR-26a loaded in the exosomes were further confirmed by qRT-PCR, and the results indicated that miR-26a levels were markedly higher in miR-26a-transfected nc-Exo and scFv-Exo than those in miR-con-transfected nc-Exo and scFv-Exo, respectively (all $p < 0.001$, **Figure 2D**).

Exosomes-Based Nanosystem Effectively Delivered miR-26a Into Hepatocellular Carcinoma Cells

We next examined whether the drug-loaded exosomes could improve the miR-26a expression in HCC cells. Firstly, we evaluated the expression of GPC3 on the surface of human HCC lines. Indeed, HepG2 and Huh7 cells, rather than 293T, exhibited higher levels of GPC3 expression (**Figures 3A,B**). It was revealed that CFSE-labeled scFv-Exo$^{miR-26a}$ was only able to be uptake by both HepG2 and Huh7 tumor cells, and effectively displayed green fluorescence (CFSE) (**Figures 3C,D**). After co-culture with exosomes for 24 h, the levels of miR-26a expression were higher in the scFv-Exo$^{miR-26a}$-treated HepG2 and Huh7 cells than in the GPC3 negative 293T cells (**Figure 3E**). Notably, the exosomes derived from the unmodified negative control MSC (nc-Exo) was not effective in delivering miRNAs to tumor cells.

Exposure to miRNA Drug-Loaded Exosomes Inhibited Hepatocellular Carcinoma Cells Proliferation

To determine the effects of modified exosomes on the proliferation of HepG2 and Huh7 cells *in vitro,* the cell lines

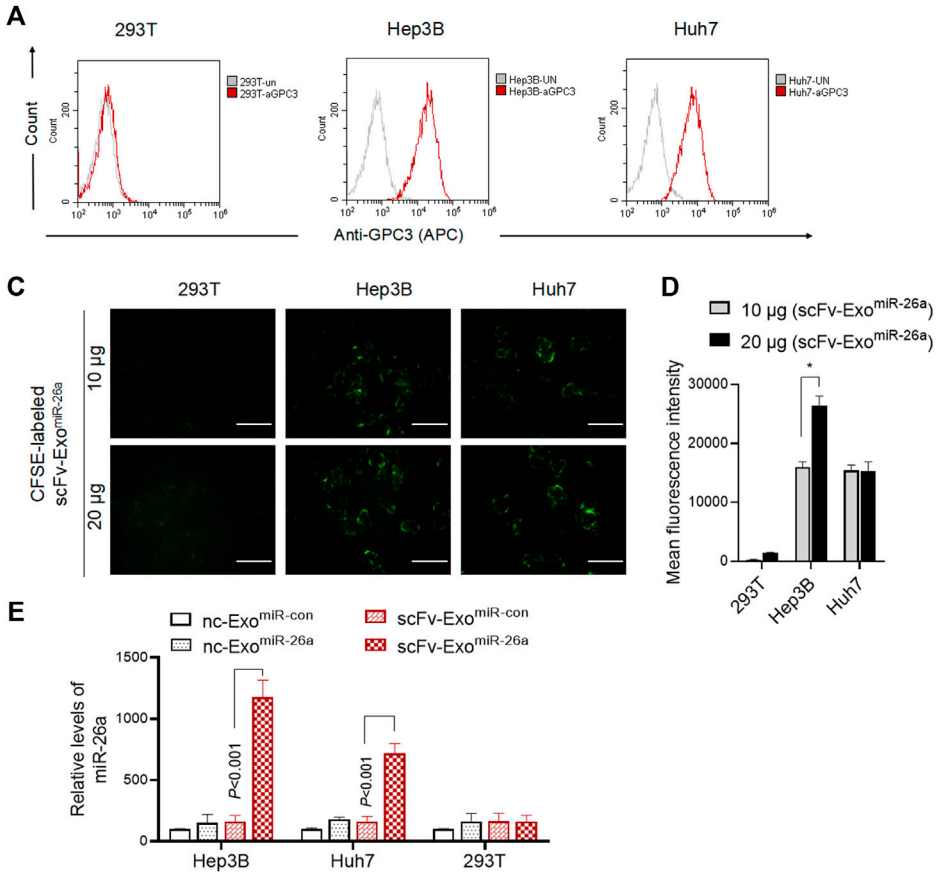

FIGURE 3 | Exosomes-based nanosystem effectively delivered miR-26a into HCC cells. **(A)** Flow cytometric detection of the expression of GPC3 antigen protein on the surface of 293T, HepG2 and Huh7 cells. **(B)** And the percentage of HCC cells expressing GPC3 was showed. ***$p < 0.001$ vs 293T. **(C)** CFSE-labeled scFv-Exo$^{miR\text{-}26a}$ (10 and 20 μg) exosomes were added into 293T, HepG2 and Huh7 cells, and the green fluorescence signals were observed and photographed after 6 h. Scale bars = 50 μm. **(D)** The fluorescence intensity of CFSE was measured by flow cytometry. ***$p < 0.001$ vs 293T. **(E)** Detection of the abundance of miR-26a mimics in cells using quantitative real-time PCR (qRT-PCR).

were treated with scFv-Exo$^{miR\text{-}26a}$ and scFv-Exo$^{miR\text{-}con}$. The cell viability of HepG2 and Huh7 cells was not altered after scFv-Exo$^{miR\text{-}con}$ treatment, whereas scFv-Exo$^{miR\text{-}26a}$ obviously inhibited cell proliferation of both HepG2 and Huh7 cells (**Figure 4A**). Clone formation also revealed a decrease in the number of cell clones among scFv-Exo$^{miR\text{-}26a}$-treated HepG2 and Huh7 cells, as compared to scFv-Exo$^{miR\text{-}con}$-treated cells (**Figure 4B**), thus suggesting that scFv-Exo$^{miR\text{-}26a}$ could effectively inhibit tumor cell proliferation in HCC. Cell mobility was measured *via* a scratch wound-healing assay, and the results demonstrates that exposure to scFv-Exo$^{miR\text{-}26a}$ inhibited the cell migration in both HepG2 and Huh7 cells (**Figure 4C**).

Exosomes-Based Nanosystem Displayed Favorable Anti-Tumor Effect *in Vivo* With No Obvious Side Effects

To evaluate the antitumor effects of exosomes-based nanosystem *in vivo*, B-NDG mice bearing HepG2/luciferase cells were subjected to three treatments with scFv-Exo$^{miR\text{-}26a}$ and scFv-Exo$^{miR\text{-}con}$ at 100 μg/mouse within 7 days, respectively (**Figure 5A**). The results of live-imaging showed lower fluorescent signals in mice receiving scFv-Exo$^{miR\text{-}26a}$ than that in scFv-Exo$^{miR\text{-}con}$ treated mice (**Figure 5B**). The tumor volume was smaller in the mice treated with miR-26a-loaded exosomes compared to control group (**Figure 5C**). Meanwhile, all mice had a normal diet and their body weights increased in the whole process of experiment, and the body weights of the two groups showed no significant change (**Figure 5D**). At the end of the experiments, the tumor size (**Figure 5E**) and tumor weight (**Figure 5F**) were significantly decreased, but the liver and kidney (**Figure 5G**) had no morphological differences, when compared to the control.

DISCUSSION

Although miRNAs have been proved to be potential antitumor drug candidates, the lack of a specific delivery system has impeded their clinical application. In this study, we found that antibody-modified exosomes, as a drug delivery nanosystem, could represent a potential carrier to efficiently deliver antitumor miRNAs to HCC.

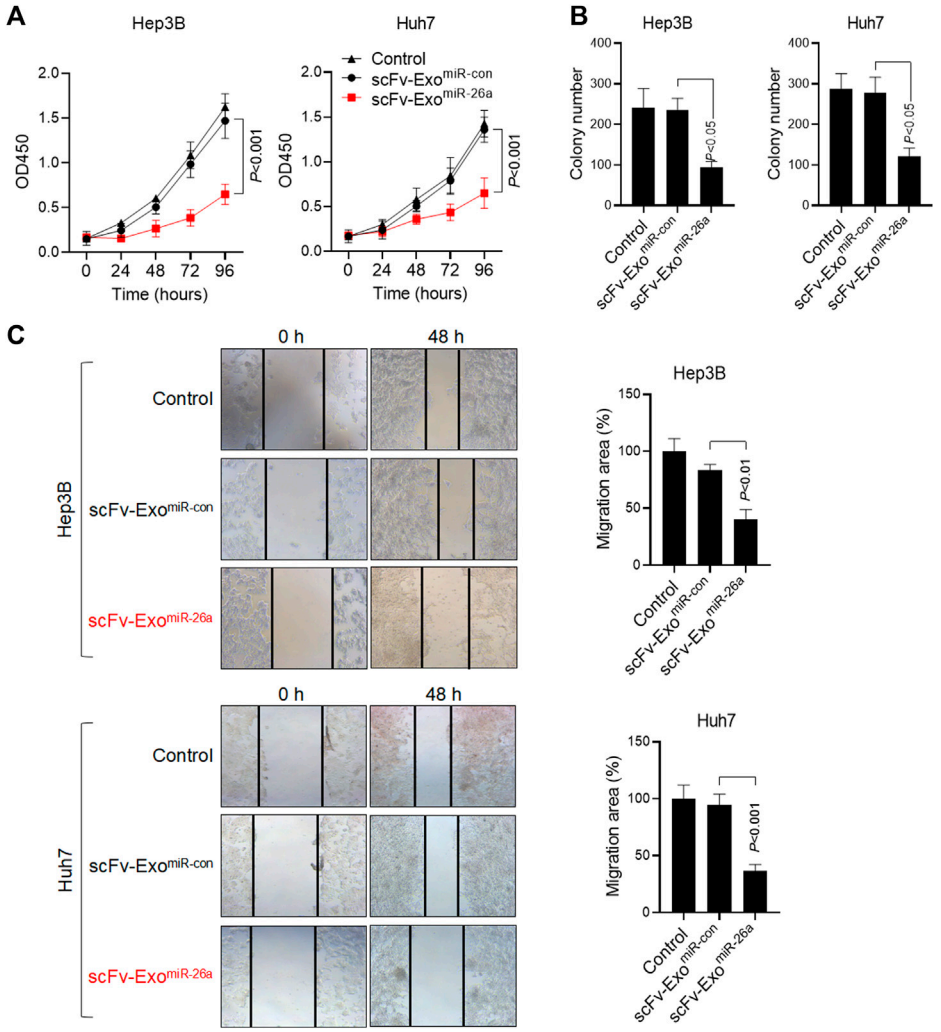

FIGURE 4 | Exposure to miRNA drug-loaded exosomes inhibited HCC proliferation. **(A)** MTT detection of cell proliferation in 293T, HepG2, and Huh7 cells after the addition of 20 μg miRNA drug-loaded exosomes. **(B)** The number of cell clones was counted after the addition of drug-loaded exosomes. **(C)** The migration of HepG2 and Huh7 cells in different treatment groups was detected by scratch assay (100×). Relative migration area was quantified (bar charts).

Exosomes are a nano-sized follicles originating from cell endocytosis and are released by different cell types (Koppers-Lalic et al., 2013). Exosomes essentially act as a communication medium between cells by delivering protein and RNA (Vlassov et al., 2012). Therefore, the application of exosomes as a biological delivery medium is very promising. Accumulating research has indicate that MSCs are highly suitable for the mass production of exosomes, and artificially modified exosomes can be obtained by harvesting supernatants from genetically modified MSC cells (Phan et al., 2018). When antibodies are fused with LAMP2B, an exosome-associated transmembrane protein, they are also highly efficiently expressed on the surface of the exosomes (Limoni et al., 2019). In the present study, genetic engineering and protein technology were used to obtain anti-GPC3 scFv-modified exosomes for GPC3-specific targeting and drug delivery.

miRNAs exert a strong influence on cell behavior through the regulation of extensive gene expression networks (Rottiers and Näär, 2012). Therefore, the therapeutic regulation of one miRNA can affect multiple pathways and, at the same time, can achieve clinical benefits. To date, most *in vivo* translation studies investigating miRNAs have been aimed at preparations using antisense reagents (such as locked nucleic acid oligomers and other qualified oligonucleotides), which inhibit the function of endogenous miRNAs (Van Rooij, 2011). However, most tumors are characterized by an overall decrease in miRNA expression compared to their normal counterpart tissues, and experimental interference with miRNA expression has been demonstrated to promote cell transformation and tumorigenesis (Xu et al., 2006; Paquet et al., 2016). Furthermore, carcinogenic lesions can result in widespread miRNA inhibition (Berti et al., 2019). Therefore, targeted miRNA delivery will enable therapeutic recovery of physiological regulatory programs lost in cancer and other disease conditions.

MiR-26a is a potentially excellent miRNA proposed as a treatment for hepatoma cancer (Yang et al., 2013). MiR-26 family members have multiple antitumor properties in various

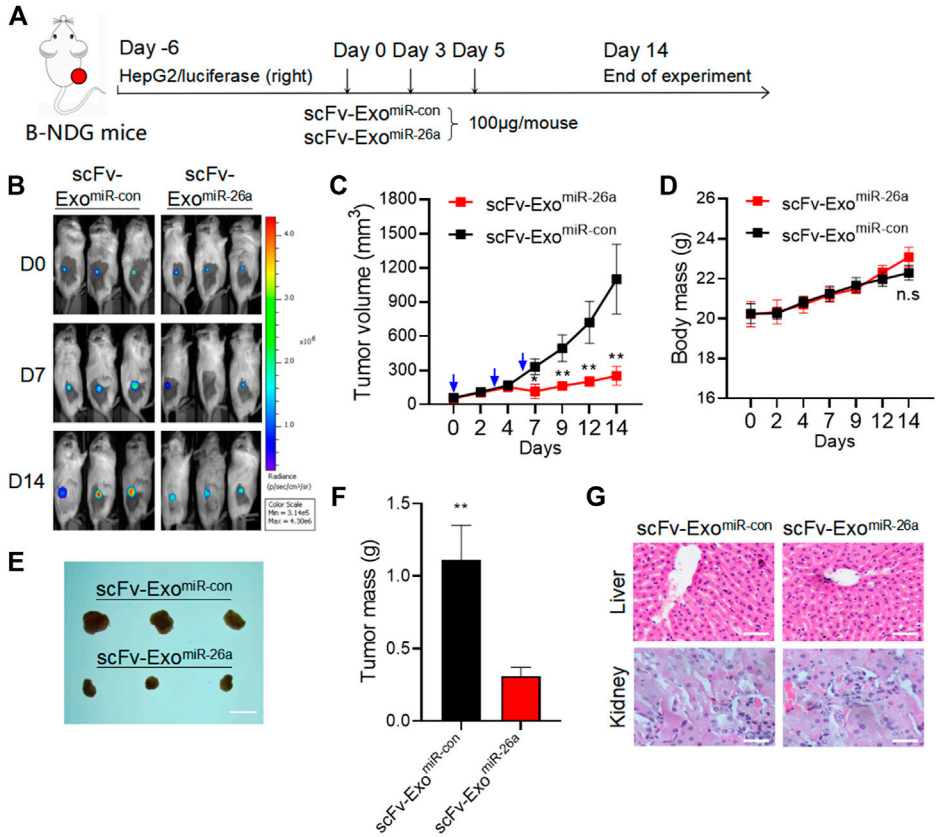

FIGURE 5 | Exosomes-based nanosystem displayed favorable anti-tumor effect *in vivo* with no obvious side effects. **(A)** Schematic representation of the experimental arrangement for animal experiments. **(B)** *In vivo* live imaging of mouse with HCC tumor formed by HepG2/luciferase cells. **(C)** Tumor volume was measured over time. $^{*}p < 0.05$, $^{**}p < 0.01$. **(D)** These mice were fed for 14 days for weight analysis. **(E)** Tumors collected from mice were exhibited. Scale bars = 1 cm. **(F)** Average tumour weight in each group was calculated. $^{**}p < 0.01$. **(G)** Mice were sacrificed after *in vivo* imaging, and HE staining of liver and kidney tissues was then performed. Scale bars = 50 μm.

tumor contexts (Yang et al., 2014). In liver cancer, miR-26a was reported to induce cell-cycle arrest associated with direct targeting of cyclins D2 and E2. However, if future studies determine that the role of miR-26 delivery is selective for the treatment of Myc disorders, then the efficient delivery of this miRNA remains an important objective to achieve, as the therapeutic delivery of this miRNA will be effective for a large number of human cancer subtypes (Paquet et al., 2016). Since physiological gene expression networks have evolved to adapt to endogenous miRNA regulation, the risk of off-target gene silencing may be less than the risks associated with artificial RNAi triggers (Xu et al., 2006). In this study, we designed a drug delivery system that used anti-GPC3 scFV-modified exosomes to load miR-26a and inhibited the proliferation and migration of GPC3-positive HCC cells *in vitro*. To further evaluate the efficacy of exosomes-based nanosystem in HCC, we conducted an antitumor efficacy study in a HepG2 cell mouse xenograft model. We found that nanosystem mediated miR-26a delivery effectively enhanced the curative effects in HCC tumor, which coincided with the results from those results *in vitro*. Also, a future comparison between intravenous and intratumoral viral administration may be warranted.

CONCLUSION

Our study indicated that miR-26a delivery *via* scFv-modified exosome carriers could inhibit the growth of HCC cells and thus represents a new treatment strategy for hepatoma. Technological improvements aimed at enhancing the preparation of exosomes and reducing potential immunogenicity should be explored to further develop this effective method of drug delivery.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the ethics committee of Xinjiang Medical University.

AUTHOR CONTRIBUTIONS

SM and XF conceived and designed the study. XM contributed to data analysis. HZ and LX revised the manuscript. All authors read and approved the final manuscript.

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Delivery of Anti-miRNA-221 for Colorectal Carcinoma Therapy Using Modified Cord Blood Mesenchymal Stem Cells-Derived Exosomes

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Background: Exosomes, as natural intercellular information carriers, have great potential in the field of drug delivery. Many studies have focused on modifying exosome surface proteins to allow drugs to specifically target cancer cells.

Methods: In this study, human cord blood mesenchymal stromal cell-derived exosomes were used in the delivery of anti-miRNA oligonucleotides so as to be specifically ingested by tumor cells to perform anti-tumor functions. Mesenchymal stem cells modified by the fusion gene iRGD-Lamp2b were constructed to separate and purify exosomes, and the anti-miRNA-221 oligonucleotide (AMO) was loaded into the exosomes by electroporation.

Results: The AMO-loaded exosomes (AMO-Exos) effectively inhibited the proliferation and clonal formation of colon cancer cells *in vitro*, and it was further found that AMO-Exos was taken up by tumor cells through interaction with the NRP-1 protein. The results of a xenograft tumor model also showed that iRGD-modified exosomes were obviously enriched in tumor sites, exerting excellent anti-tumor efficacy. *In vivo* imaging showed that exosomes were mainly distributed in liver, spleen, and lung tissues.

Conclusion: Our results suggest that genetically modified exosomes could be an ideal natural nanostructure for anti-miRNA oligonucleotide delivery.

Keywords: anti-miRNA oligonucleotides, anti-miRNA-221, iRGD nanoparticles, delivery platform, exosomes, human cord blood mesenchymal stromal cells

INTRODUCTION

Anti-microRNA oligonucleotides (AMOs), or anti-miRNAs, bind miRNAs through complementary sequences and inhibit miRNA functions in cancer cells (Rupaimoole and Slack, 2017). The development of these drugs is based on in-depth descriptions of the biological pathogenesis between target miRNA and diseases (Li and Rana, 2014). Many preclinical studies have been performed on miRNA or anti-miRNA therapy since the first AMO drug, miravirsen (a specific inhibitor of miR-122), and entered clinical trials (Elmen et al., 2008). However, the biggest challenge

to developing miRNA therapeutics is designing a miRNA delivery vector to prevent the degradation of nuclease and the escape of drug molecules from endocytosis (Xie et al., 2018). Furthermore, the dilemma of a miRNA drug delivery system lies in its potential immune stimulation and the lack of target specificity for the lesion (Taniguchi et al., 2019).

The homing ability of mesenchymal stem cells (MSCs) endows them with global positioning system navigation. It has been reported that MSCs using oncolytic herpes simplex virus can effectively kill malignant glioblastoma cells and prolong the median survival time (Cheng and Slack, 2012). MSC-derived exosomes have broad application possibilities because of their small size, low complexity, simple production, and easy storage (Zhao et al., 2019). It is an ideal delivery vector that can protect enzymes or RNA from degradation by wrapping molecules in a membrane and promoting intracellular uptake by endocytosis. In addition, exosomes are simple to transport in blood and easily pass through the blood-brain barrier. It was found that anti-miRNA-9 MSC exosomes can reverse the expression of multidrug transporters in resistant glioblastoma and reverse chemoresistance (Munoz et al., 2013).

The iRGD peptide is a specific peptide composed of nine amino acid residues (Yan et al., 2016). As a ligand, the iRGD peptide can interact with tumor cells with high expression of the neuropilin-1 (NRP-1) receptor, mediate the cell membrane penetration effect, and effectively kill the tumor (Nie et al., 2014). MicroRNA is a potential therapeutic target for many solid tumors. A significant number of studies have shown that miR-221 cancer-related miRNA, which is upregulated in colorectal cancer, liver cancer, lung cancer, and other malignant tumors (Park et al., 2011; Sun et al., 2011). MiR-221 downregulates key tumor suppressors, such as p27kip1, PTEN, and TIMP3, and has a significant effect on the cell cycle, apoptosis, and the Wnt signaling pathway (Fornari et al., 2008; Garofalo et al., 2009; Howe et al., 2012). The development of anti-miR221 has great significance in the treatment of solid tumors. In preclinical studies, however, it is difficult to deliver miRNA-related drugs efficiently to tumor cells, which hinders their broader application.

In this study, we loaded anti-miR221 into the exosomes of human cord blood MSCs (cbMSCs), which expressed the iRGD peptide. The anti-miR221 encapsulated in exosomes targeted NRP-1 receptor-positive tumor cells and might therefore play important roles in clinical application.

MATERIALS AND METHODS

Cell Cultures

Human embryonic kidney cells (HEK293T) were obtained from Clontech (Mountain View, CA, United States). The human colon cancer cell lines Caco2 and HCT116 were obtained from the American Type Culture Collection. All cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 U/mL, Gibco), and streptomycin (0.1 mg/ml, Gibco). The cells were cultured under standard conditions of 5% CO_2 at 37°C. Immortalized cbMSCs (T0016; cbMSC-hTERT) were obtained from Applied Biological Materials Inc. (abm, Richmond, BC, Canada), and were maintained in Prigrow III medium (Cat. No. TM003; abm) containing 20% FBS and 4 ng/ml recombinant basic fibroblast growth factor at 37°C in a 5% CO_2 incubator. The liquid was changed every 2–3 days after the suspension cells were discarded.

Flow Cytometry

MSCsurface antigen markers were detected by flow cytometry. Briefly, 1 × 105 cells were washed and resuspended in 200 μL phosphate buffered saline (PBS). Then, the cell suspension was added with fluorescein isothiocyanate (FITC)-conjugated anti-CD73, allophycocyanin (APC)-conjugated anti-CD105, phycoerythrin-conjugated anti-CD34, and APC-conjugated anti-CD45 and incubated in the dark for 15 min. Subsequently, the cell suspension was washed with 2 ml PBS and centrifuged at 1000rpm for 5 min to remove the supernatant. Lastly, the cell suspension was added with 200 μL buffer solution and detected by a CytoFLEX flow cytometer (Beckman Coulter, United States). The expression of NRP-1 on 293T, Caco2, and HCT116 cells were detected using FITC-conjugated anti-human CD304 (neuropilin-1) antibody (Clone 12C2, Biolegend).

Transfection and Lentiviral Transduction

The iRGD-Lamp2b fusion gene encodes (N to C) the signal peptide of Lamp2b (1-28aa), GNSTM glycosylation motif (GNSTM), 3 residue spacer (GSG), iRGD peptide (CRGDKGPDC), 10 residue spacer (GSGSGSGGSS), and Lamp2b (exosomal transmembrane protein, 29-410aa). The iRGD-Lamp2b fusion gene was constructed into lentivirus vector pLVX-IRES-Puro and transfected into 293T cells together with packaging plasmids psPAX2 and pMD2G. The cell supernatant was collected and the lentivirus particles (LV-iRGD-Lamp2b) were purified. cbMSCs were then infected by LV-iRGD-Lamp2b: 1×10^5 cbMSCs cells were spread into 24-well plates 18 h before transfection. The next day, the original culture medium was replaced with fresh medium containing 6 μg/ml polybrene and LV-iRGD-Lamp2b with a multiplicity of infection (MOI) of 5. cbMSCs expressing the iRGD-Lamp2b fusion gene (MSC-iRGD) were obtained after continuous culture with 5 μg/ml puromycin (Sigma-Aldrich, United States).

Isolation and Characterization of Exosomes

cbMSCs and cbMSC-iRGD cells at were cultured in DMEM with 10% FBS without exosomes. The supernatant of the cell culture (500 ml) was collected and centrifuged at low speed at 300×g for 10 min, then the supernatant was centrifuged at 2000×g for 10 min to remove the dead cells. The remaining supernatant was obtained and centrifuged at 10,000×g for 30 min to remove the cell debris. After that, the supernatant was centrifuged at high speed, 100,000×g for 70 min, and the crude exosome precipitation (containing a small amount of heteroproteins) was obtained. Lastly, the exosomes were resuspended with PBS and centrifuged at 100,000×g for 70 min to obtain pure exosomes. The exosomes' morphology was identified by transmission electron microscopy (FEI Tecnai G2 Spirit Bio TWIN).

Loading miRNAs Into Exosomes Using Electroporation

We employed AMOs with the following sequences: 5′GAAACC CAGACAGACAAUGUAGCU′3, with 2′ O-methyl modification. AMOs or FAM-labeled-miR-221 inhibitor (FAM-AMO) was loaded onto exosomes using electroporation methods. Briefly, 200 μg exosomes and 10 μL AMO were pre-mixed and added into the perforation dish. After electroporation (100 V, 125 μF, 1 ms), the exosome samples were incubated in a cell incubator for 1 h to promote exosome membrane repair. Subsequently, the mixed exosome samples were centrifuged twice at 100,000×g for 70 min each, and the supernatant was discarded. The sediment contained the compound exosomes loaded with miR-221 inhibitor (AMO-Exos), and stored at −80°C for standby use.

Exosome Uptake

Various concentrations (0, 0.625, 1.25, 2.5, 5, 10, 20, 40 μg) of exosomes containing FAM-labeled-anti-miRNA-221 (iRGD-$Exo^{FAM-221}$) were added into 293T, Caco2, and HCT116 cells. After culture for 24 h, FAM fluorescence was detected by flow cytometry after washing with PBS twice to assess the uptake efficiency of exosomes by tumor cells. The green FAM fluorescence was observed and photographed under an inverted fluorescence microscope (MF52-N; MSHOT, Guangzhou, China).

Cell Proliferation Assay

We evaluated cell proliferation with a CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) kit (Promega; G1112). We inoculated 3,000 Caco2 and HCT116 cells into flat 96-well plates. After overnight culture, various concentrations of AMO-Exos and their controls (AMO-NCs) were added. We continued to culture them for 24 h, with a small amount of CellTiter 96® AQueous single solution reagent added to the culture pores. After incubation for 4 h, the absorbance at 490 nm was recorded on a microplate reader. The absorbance value measured at 490 nm is directly proportional to the number of living cells in the culture.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted using the RNeasy Plus Universal Mini Kit (73,404, QIAGEN, Duesseldorf, Germany) or the miRNeasy Mini Kit (217,004, QIAGEN). Reverse transcription was performed to obtain complementary DNA with the QuantiTect Reverse Transcription Kit (205,311, QIAGEN). Real-time quantitative polymerase chain reaction (PCR) was performed using ABI7500 (7500, ABI, United States). The mRNA and miRNA expression data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6, respectively. The primers used were as follows: miR-221-forward: 5′-CCT GAA ACC CAG CAG ACA A-3′, backward: 5′-CAG GTC TGG GGC ATG AAC-3′. U6-forward: 5′-CTC GCT TCG GCA GCA CA-3′, backward: 5′-AAC GCT TCA CGA ATT TGC GT-3′. NRP-1-forward: 5′-GGC GCT TTT CGC AAC GAT AAA-3′, backward: 5′-TCG CAT TTT TCA CTT GGG TGA T-3′. GAPDH-forward: 5′-GGA GCG AGA TCC CTC CAA AAT-3′, backward: 5′-GGC TGT TGT CAT ACT TCT CAT GG-3′.

Colony Formation Assay

A total of 700 cells were pre-inoculated in a 6-well plate. After overnight culture, 20 μg exosomes containing AMO-Exos or AMO-NCs were added to Caco2 and HCT116 cells for another 14 days. When obvious cell cloning was observed in the culture dish, the culture medium was abandoned, and the clones were fixed with formaldehyde for 15 min, stained with gentian violet for 30 min, and the number of clones was determined under the microscope. Each experiment was repeated 3 times.

Xenograft Tumor Model

We purchased 4-week-old B-NDG (NOD-$Prkdc^{scid}$ $Il2rg^{tm1}$/Bcgen) female mice from Biocytogen (Biocytogen Co., Ltd., Beijing, China) and raised them in an SPF grade environment. After 7 days of adaptive feeding, the mice were subcutaneously inoculated with HCT116-luc cells (stably expressing firefly luciferase). Ten days after inoculation, obvious xenograft formation could be seen. Ten minutes after intraperitoneal injection of D-Luciferin (3 mg/mouse), the mice were imaged, and the fluorescence values were analyzed. The mice were randomly divided into 3 groups (n = 3) according to the fluorescence values, and received intratumoral injection with AMO-Exos (200 μg, 50 μL), NC-Exos (200 μg, 50 μL), or PBS (50 μL) on day 0, day 3, and day 7, respectively. *In vivo* imaging was performed on the mice every 7 days. The size of the transplanted tumor was measured with vernier caliper twice a week, and the mental state and diet of the mice were observed daily.

Statistical Analysis

Data drawing and analysis were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, United States). The data were described as mean ± standard error of the mean. The analysis of data between groups was performed using an analysis of variance or a t-test. Tumor growth in the mice was compared using Mauchly's test of sphericity.

RESULTS

Production and Identification of MSC-Derived Exosomes

We first identified the morphology and molecular markers of the cbMSCs. As shown in **Figure 1A**, under the light microscope, the cbMSCs were spindle-shaped and wall-attached. The flow cytometry results showed that the specific cbMSC surface markers, CD73 and CD105, were highly expressed in the cbMSCs, whereas the levels of CD34 and CD45, the specific surface markers of hematopoietic stem cells, were low (**Figure 1B**). In order to obtain iRGD peptide modified exosomes (iRGD-Exos), plasmids containing the iRGD-Lamp2b fusion gene were further transferred into cbMSCs using lentivirus (**Figure 1C**). Exosomes from iRGD-modified cbMSCs were collected and purified, and the AMOs or corresponding NCs

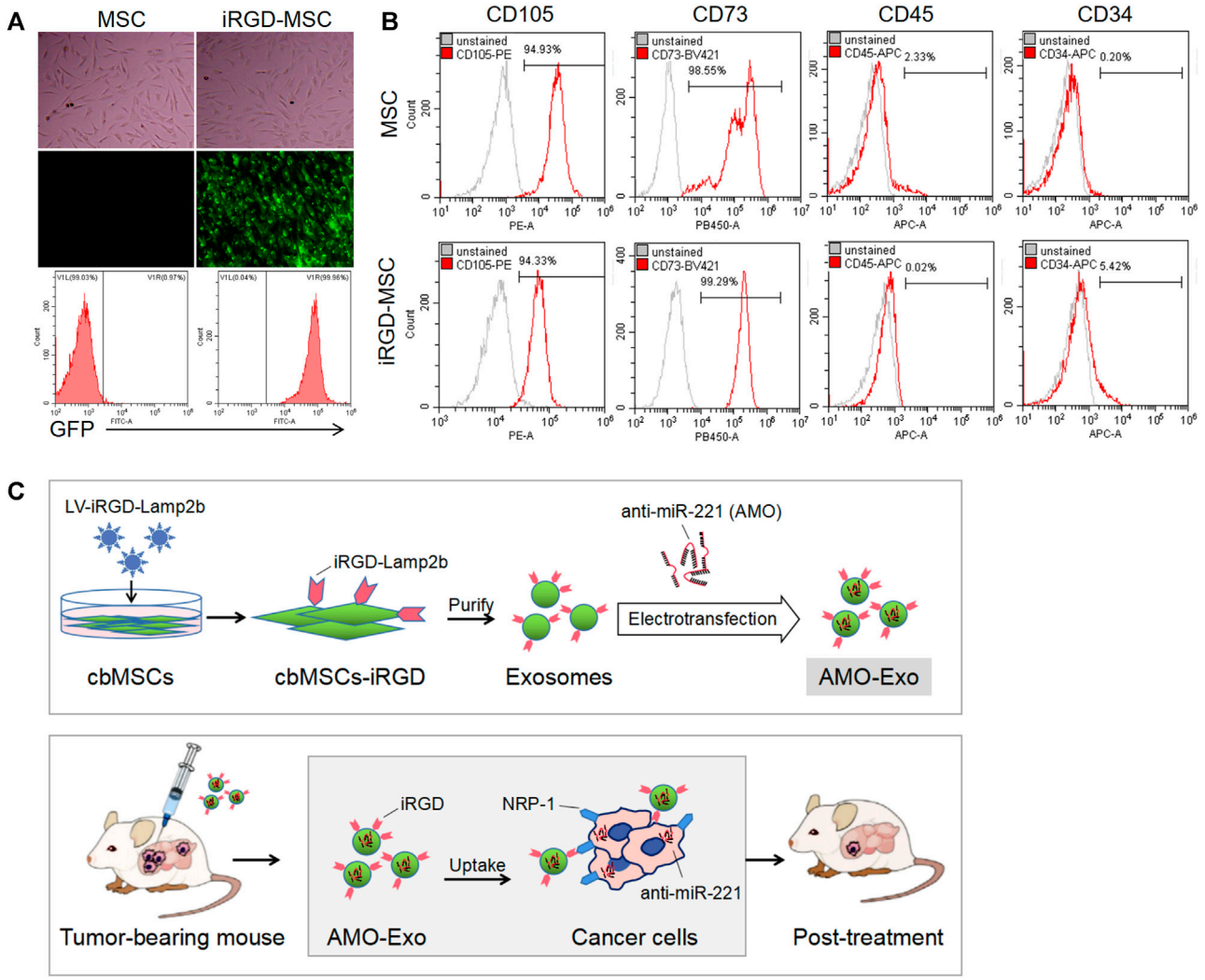

FIGURE 1 | Production and identification of MSC-derived exosomes **(A)** Cell morphology and GFP expression of mesenchymal stem cells.**(B)** The expression of molecular markers on the surface of mesenchymal stem cells after iRGD peptide modification was detected by flow cytometry. **(C)** Schematic diagram of the anti-tumor mechanism of exosomal drug delivery.

were loaded into the exosomes by electric transduction. Subsequently, the iRGD peptide was bound to NRP-1, a receptor protein of tumor cells, to mediate the anti-tumor effect.

AMO-loaded exosomes were taken up by colon cancer cells through NRP-1.

The morphological identification of AMO-Exos was performed by transmission electron microscopy (**Figure 2A**). Considerable research has shown that the residual peptide, CRGDK/R, the product of iRGD hydrolyzation, is the specific ligand of NRP-1, which is highly expressed in tumor tissues. As shown in **Figure 2B**, the expression levels of NRP-1 in various tumors were analyzed. The results suggested that NRP-1 was highly expressed in malignant melanoma, breast cancer, liver cancer, and colon cancer. Quantitative PCR and flow analysis were performed, and the results showed that NRP-1 was highly expressed in the colon cancer cells Caco2 and HCT116, rather than the 293T cells (**Figures 2C,D**). To investigate whether iRGD-Exos could combine with NRP-1-positive colon cancer cells and deliver AMO into tumor cells, AMO was pre-labeled with fluorescein amidite (FAM) before being loaded into exosomes, and was co-cultured with human colon cancer cell lines and 293T cells. The results showed that FAM green fluorescence was increased in Caco2 and HCT116 cells in a dose-dependent manner (**Figure 2E**), indicating that exosomes containing FAM-AMO were taken up by colon cancer cells. Furthermore, 4 h after incubation, we observed strong green fluorescence in the HCT116 cells, but almost no fluorescence in the 293T cells (**Figure 2F**).

AMO-Loaded Exosomes Inhibited Colon Cancer Cell Proliferation

We further investigated the anti-tumor function of exosomes loaded with AMO-Exos. Compared with the exosomes loaded

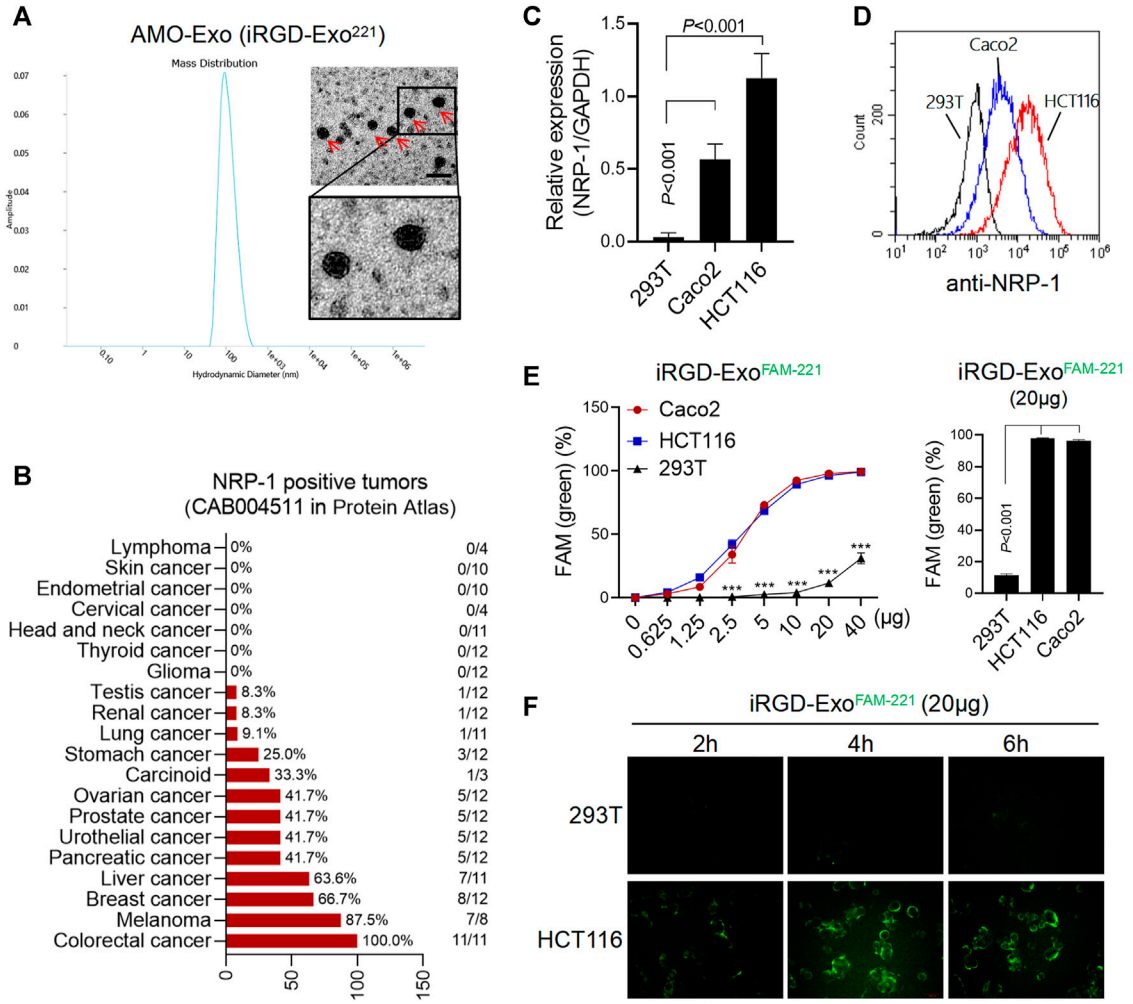

FIGURE 2 | AMO-loaded exosomes were taken up by colon cancer cells through NRP-1. **(A)** Electron microscope analysis and particle size analysis of exosomes, scale bars = 200 μm. **(B)** Tissue expression of NRP-1 in a online database (The Human Protein Atlas). A majority of cancer tissues showed weak to moderate cytoplasmic immunoreactivity with a granular pattern, and the expression of NRP-1 was highest in colon cancer. **(C)** Q-PCR was used to detect the relative expression of NRP-1 in colon cancer cells (HCT116 and Caco2) and 293T cells. **(D)**The surface expression of NRP-1 in colon cancer cells was detected by flow cytometry. **(E)** After co-incubation with the FAM-labeled exosomes, the positive rate of cells expressing FAM green fluorescence was detected by flow cytometry. ***$p < 0.001$. **(F)** The green fluorescence signal of cells was observed by fluorescence microscopy.

with the NC-Exos, AMO-Exos could significantly downregulate the level of miRNA-221 in colon cancer cells Caco2 and HCT116 (**Figure 3A**). The results showed that AMO-Exos significantly inhibited cell proliferation, whereas no significant inhibition was observed under NC-Exo treatment (**Figure 3B**). Furthermore, AMO-Exos markedly suppressed the clonogenic abilities of Caco2 and HCT116 (**Figures 3C,D**).

AMO-Loaded Exosomes Suppressed Tumor Growth *In Vivo*

Subcutaneous HCT116 tumor-bearing mice were established to investigate the potential anti-tumor effects of AMO-Exos *in vivo*. When the tumors formed, the mice were divided into 3 groups and received intratumoral treatment with NC-Exos or AMO-Exos 3 times (at days 0, 3, and 7). The mice were imaged *in vivo* using bioluminescent imaging every 7 days and tumor volume was measured. The results indicated that the fluorescence intensity of the mice was significantly suppressed after AMO-Exo treatment (**Figure 4A**), and the tumor volume also grew significantly more slowly than the control group (**Figure 4B**). Quantitative PCR detection of the xenograft revealed that AMO-Exo treatment significantly reduced the expression of miR-221 (**Figure 4C**), suggesting that anti-miR-221 transferred by exosomes acted as a tumor suppressor *in vivo*. Further analysis of the expression of downstream genes of miR-221 showed that the expression of several genes (BMF, CDKN1B, CDKN1C, PTEN, TIMP3, and MET) was significantly upregulated after AMO-Exo treatment (**Figure 4D**).

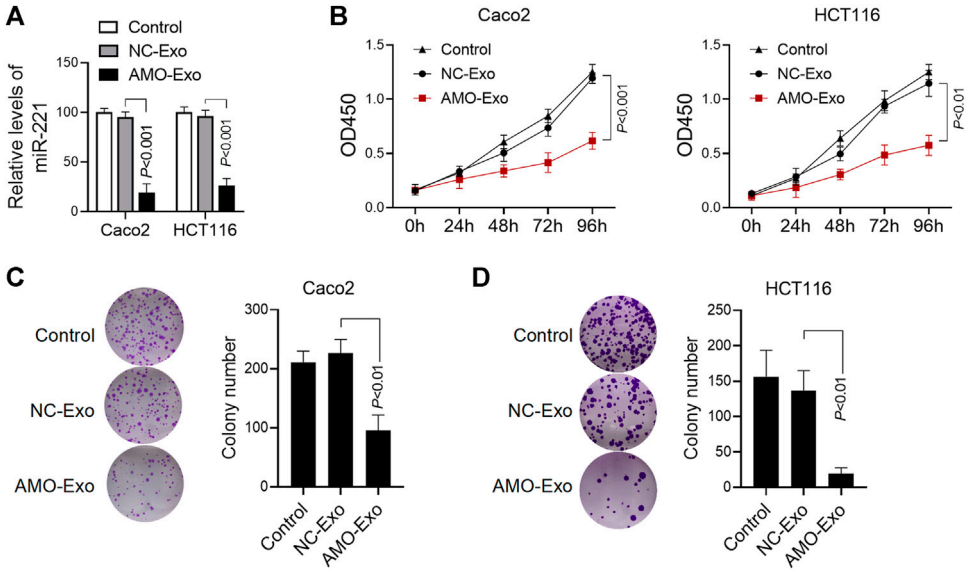

FIGURE 3 | AMO-loaded exosomes inhibited colon cancer cell proliferation. **(A)** The relative expression level of miR-221 in colon cancer cells was detected by Q-PCR. Caco2 and HCT116 cells were treated with 20 μg exosomes containing anti-miRNA-221 (AMO-Exos) or their controls (AMO-NCs) for 24 h, and then lysed to detect the expression level of miR-221. **(B)** Caco2 and HCT116 cells were treated with 20 μg exosome drugs. After continuous culture for different times, the cell proliferation was evaluated. **(C)** Effect of exosome drugs on clone formation ability of Caco2 cells. Caco2 cells were treated with 20 μg exosome drugs and cultured for 14 days. The number of clones was calculated and histograms were shown. **(D)**Effect of exosome drugs on HCT116 cell clone formation ability.

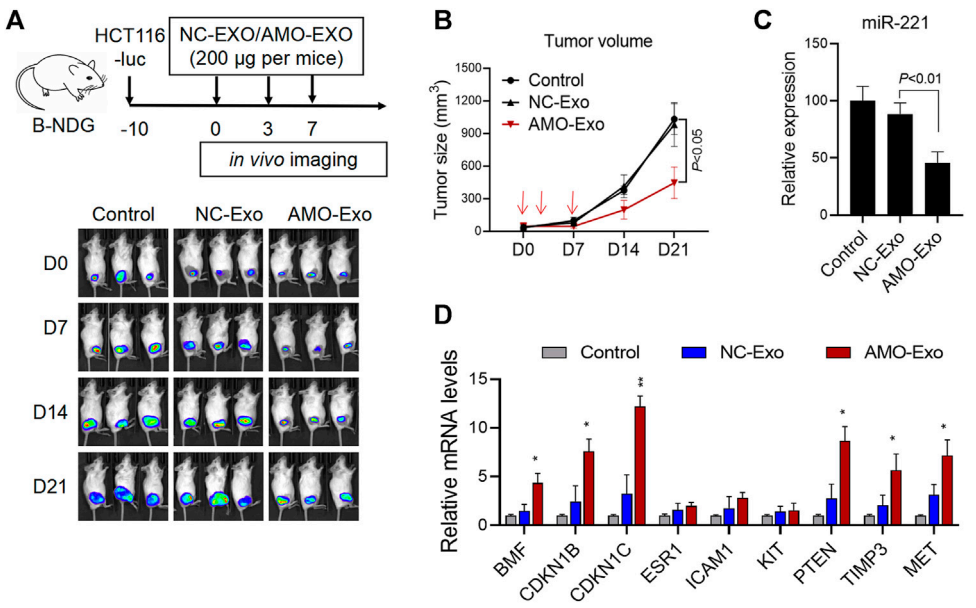

FIGURE 4 | AMO-loaded exosomes suppressed tumor growth *in vivo*. **(A)** HCT116 tumor-bearing mice received 3 intratumoral injections of exosome drugs on days 0, 3, and 7. *In vivo* imaging of the mice was performed once a week to observe the change of fluorescence value. **(B)** Tumor changes in mice were measured with a vernier caliper. **(C)** The level of miR-221 in the dissected mouse tumor tissues was detected by quantitative PCR. **(D)** The mRNA levels of downstream target genes of miR-221 in the dissected mouse tumor tissues were detected by quantitative PCR. $^{*}p < 0.05$, $^{**}p < 0.01$.

DISCUSSION

For nucleotide-based drugs, the largest obstacles to their functioning *in vivo* are the degradation of nucleases and the escape of drug molecules from the endocytes during endocytosis. In this study, cbMSC-Exos were used as micoRNA drug transport carriers. Natural exosomes lack the ability to specifically bind tumor cells because they lack a targeting property, which results in poor efficacy in tumor-targeted therapy. In our study, the iRGD-Lamp2b fusion protein was stably expressed on the surface of cbMSCs, enabling MSC-Exos to display a large number of iRGD peptides. Thus, iRGD-Exos were used as the carrier of miRNA drugs, endowing them with highly efficient tissue penetration abilities and a specific binding ability to tumor cells.

Many studies have reported that iRGD peptide accelerates drug delivery into tumor cells (Tian et al., 2014). After its combination with integrins, the enzyme hydrolysate promotes the tissue penetration of the drug (Zuo, 2019; Zhou et al., 2021). We found that NRP-1 was highly expressed in colon cancer tissues and cell lines, enabling the iRGD-Exos to be effectively ingested by colon cancer cells.

The exosomes' lipid bilayer membrane can protect them from degradation in the blood circulation; however, this membrane structure makes it difficult for exosomes to carry "cargo." In this study, the modified miRNA-221 was effectively loaded into exosomes by electroporation, due to easy control of the electrorevolution parameters. Wang et al. (2017) studied extracellular vesicles as a targeted delivery system for small RNA. They used electroporation to load siRNA/miRNA into the vesicles modified by nucleic acid aptamer AS1411, and then delivered siRNA/miRNA to breast cancer tissues through exosomes. Schindler et al. (Schindler et al., 2019) loaded doxorubicin into exosomes by electroporation to achieve a new drug delivery system.

The administration of exosomes is an important determinant of the efficacy and metabolic distribution of exosomes. In the HCT116 transplanted tumor mouse model, we explored local administration (intratumoral injection) and intravenous administration of exosomal drugs. The former was mainly used to observe the efficacy of the drug and the latter to assess its metabolism. Our results showed that 3 intratumoral injections inhibited the growth of the transplanted tumor to a certain extent; however, the tumor eventually recurred. Increasing the dose and frequency of administration will be further studied. Preliminary experiments have also been done on the distribution of exosomes, and exosomes were observed in the liver, spleen, and lung through *in vivo* imaging (data not shown), which was consistent with previous studies (Yi et al., 2020). The toxicity and duration of exosome drugs *in vivo* should be explored in subsequent studies.

Previous studies have confirmed that miR-221 also inhibits the expressions of the PTEN and cyclin-dependent kinase inhibitor family members (Le Sage et al., 2007; Park et al., 2011; Callegari et al., 2012), whereas anti-miR-221 upregulates the expression levels of corresponding tumor suppressor factors, which could be the molecular mechanism underlying the anti-tumor effect induced by anti-miR-221 in this study. This finding is consistent with the latest understanding of miRNA, i.e., that miRNA and its target mRNA are not in a simple "one-to-one" direct linear relationship, but constitute a complex network regulation pattern (Liu et al., 2018).

CONCLUSION

In this study, we described a novel drug delivery system that infiltrated anti-miRNA-221 into solid tumors using cbMSC-derived exosomes. The modified exosomes showed high binding ability to NRP-1-positive colon cancer cells and significantly inhibited tumor growth *in vitro* and *in vivo*. Our data suggest that iRGD-modified cbMSCs-derived exosomes appear to be one of the best candidates for the specific transport of miRNA drugs to tumors.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of the Guangzhou Institutes of Biomedicine and Health (GIBH).

AUTHOR CONTRIBUTIONS

SH, GL, MJ, CC, and XC conceived and planned the experiments. SH, GL, MJ, YZ, CH, MH, and LJ carried out the experiments. MH, LJ, MW, JY, XJ, and XL contributed to sample preparation. SH, GL, LJ, MW, JY, XJ, and XL contributed to data analysis and the interpretation of the results. SH, GL, MJ, and CC took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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MiRNA-142-3P and FUS can be Sponged by Long Noncoding RNA *DUBR* to Promote Cell Proliferation in Acute Myeloid Leukemia

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Acute myeloid leukemia (AML) represents a frequently occurring adulthood acute leukemia (AL). Great progresses have been achieved in the treatment of AML, but its pathogenic mechanism remains unclear. This study reported the biological functions of lncRNA *DUBR* in AML pathogenic mechanism. As a result, lncRNA *DUBR* showed high expression level within AML, resulting in poor prognosis, especially in M4 AML. *In vitro* studies elucidated that knockdown of *DUBR* with small interfering RNA (siRNA) resulted in the suppression of survival and colony formation ability, as well as induction of apoptosis, in AML cells. RNA pull-down assay and computational revealed that *DUBR* could sponge with miRNA-142-3P and interact with FUS protein. MiRNA-142-3P have a negative correlation with *DUBR* and overexpression of miRNA-142-3P inhibited cell growth in AML. Meanwhile, *DUBR* promoted the expression of FUS protein, targeting inhibition of FUS significantly promoted cell apoptosis in AML cell lines. In conclusion, these results revealed new mechanism of lncRNA *DUBR* in AML malignant behavior, and suggested that the manipulation of *DUBR* expression could serve as a potential strategy in AML therapy.

Keywords: DUBR, miRNA-142-3p, FUS, AML, sponge

INTRODUCTION

Acute myeloid leukemia (AML) represents the heterogeneous myeloid cancers with high aggressiveness. It has the features of fast cell proliferation, aggressiveness, and great mortality (Löwenberg et al., 1999). Its incidence rate is approximately 1.62/1 million worldwide and increases with age (Döhner et al., 2015). The French-American and British (FAB) classification of acute myeloid leukemia (AML) is based on the recognition of granulocytic (M1, M2, and M3), granulocytic-monocytic (M4), monocytic (M5a, M5b), erythroid (M6), and megakaryocytic (M7) types of cells (Chen et al., 2021). This classification has been widely accepted due to its reproducibility and true morphological correlation. At present, AML can be treated by allogeneic stem cell transplantation (ASCT) and intensive chemotherapy, but these treatments can only be applied in some young and fit patients (Shallis et al., 2019). Recently, some AML molecular biology-based novel treatments are proposed, yet the prognosis of disease is still dismal (Yen et al., 2017). Therefore, it is necessary to identify drug targets, novel biomarkers, along with underlying molecular mechanisms to prevent, diagnose and treat AML.

Non-coding RNAs (ncRNAs), the short RNAs, can be classified into circular RNAs (circRNAs), long ncRNAs (lncRNAs), and microRNAs (miRNAs). These ncRNAs can not encode proteins and may be used for diagnosis, prognosis, and therapy (Esteller, 2011). LncRNAs generally contains over 200 nucleotides (nt). And, they have important functions in physiology, cell growth, and human disorders, especially those that are malignant (Wei and Wang, 2015; Izadirad et al., 2021). The discovery of lncRNAs has also provided new insights into the management of AML. Emerging evidence suggests that lncRNAs, such as *HOTAIRM1*, *UCA1* (Li et al., 2020), and *MEG3* (Sellers et al., 2019), function as key regulators of the differentiation and maturation of myeloid cells, participating in regulating AML cell viability and apoptosis.

MiRNAs are the small, single-stranded, endogenous ncRNAs that are 19–25 nt in length (Dolff et al., 2021). They show negative effect on regulating the levels of target genes at post-transcriptional level, especially through combining with 3′-untranslated region (3′UTR) in mRNAs, resulting in gene silencing (Cheng et al., 2020). Abnormal expression of certain miRNAs is suggested to facilitate the occurrence of leukemia. For instance, miRNA-181a act as the biomarker in CML (Gu et al., 2019). The miRNA expression profiling data are suggested as the efficient part for the prediction of AML prognosis. Over-expressed miR-98 level predicts the superior prognostic outcome for AML cases who received chemotherapy (Hu et al., 2019). The miR-99a up-regulation while miR-29/miR-20b down-regulation have been identified as the factors to predict poor prognosis of AML (Cheng et al., 2018; Cheng et al., 2020). The miR-142-3p (miR-142) gene is located at chromosome 17q22. MiR-142 is initially found to participate in the invasive B-cell leukemia that harbors t (8; 17) translocation (Gauwerky et al., 1989). MiR-142 shows low expression within hematopoietic stem/progenitor cells (HSPCs) (Kramer et al., 2015). AML-related miR-142 loss-of-function mutations will destroy the negative signal transduction pathway, which gives rise to the persistent HOXA9/A10 expression within myeloblasts/myeloid progenitors, finally facilitating the transformation of leukemia (Trissal et al., 2018). MiR-142 expression was a prognostic marker within the AML intermediate cytogenetic risk group as AML patients with a high miR-142 expression in their blasts showed a survival benefit compared to patients with low miR-142 expression (Dahlhaus et al., 2013).

The interaction of lncRNA-proteins has attracted wide attention in diverse fields in AML (Rinn et al., 2007; McHugh et al., 2015). For example, lncRNA-*PCAT-1*/FZD6 interaction facilitates AML cell proliferation through activating the Wnt/β-catenin pathway (Yuan et al., 2019). And lncRNA *HOXB-AS3* interacts with EBP1, thereby increasing AML cell proliferation (Papaioannou et al., 2019).

The functions of numerous lncRNAs are identified, but many of them are not completely understood so far. In addition, *DUM*, the lncRNA *DUBR*, shows down-regulation within many tumor cell types, which predicts poor prognosis (Utnes et al., 2019; Nie et al., 2021). However, the role of *DUBR* in AML are unclear, thus, further exploration is required to clarify the molecular mechanisms underlying its role.

The present work examined *DUBR* level within AML for ascertaining its relations with clinic pathological characteristics and prognostic outcome of AML. As a result, lncRNA *DUBR* showed up-regulation within AML cells, which knocking down *DUBR* with siRNA inhibited cell viability and colony formation of AML. Regarding the mechanism, we identified that *DUBR* sponge miR-142 and promotes upregulation of the fused in sarcoma (FUS) protein, which could potentially be an essential biomarker and therapeutic target for AML.

MATERIALS AND METHODS

Public Data Analysis

Public RNA-seq data and clinical manifestations of LAML (which corresponds to AML) were downloaded from The Cancer Genome Atlas (TCGA) project (https://portal.gdc. cancer.gov/). This study utilized the online bioinformatics analysis tool Gene Expression Profiling Interactive Analysis 2 (GEPIA2) to analyze differentially expressed genes (DEGs) as well as patient overall survival (OS) and to identify correlations. To analyze DEGs, we utilized normal TCGA and Genotype-Tissue Expression (GTEx; https://gtexportal.org/home/) data for reference. To analyze OS, we compared distributions by log-rank test, where median level was adopted to be the threshold. Finally, we use the Xiantao academic website to make figures.

Cell Culture

This study acquired human AML cell lines KG-1 and Molm-13 at the Cell Bank of Type Culture Collection of Chinese Academy of Sciences. Then, cells were cultivated within the Iscove's modified Dulbecco's medium (IMDM; Gibco, Grand Island, NY, United States) containing 1% penicillin–streptomycin and 10% fetal bovine serum (FBS; Gibco) under 37°C and 5% CO_2 conditions.

Transfection of siRNAs and miRNA Mimic

AML cells were transfected with scramble, *DUBR*, *FUS* siRNA, and miRNA-142-3P mimic, purchased from RiboBio (Guangzhou, China) by the use of Lipofectamine 3000 (Invitrogen, Waltham, MA, United States) in line with specific protocols. At 48 h later, we harvested cells in later analysis.

Colony Formation Assay

This study conducted colony formation analysis on dispersed single cells. First of all, we inoculated single cells (KG-1 or Molm-13) in the 24-well plates, followed by complete mixing with 0.6% Agar solution within IMDM that contained 20% FBS. Later, we randomized single cells, followed by even distribution in all wells. When cells were incubated for 1–2 weeks under 37°C and 5% CO_2 conditions, colony formation was observed. Then, colonies (>50 cells) were observed and counted under the light microscope (Yin et al., 2020a).

Flow Cytometric Assay

Cells were transfected with scramble, *DUBR* siRNA, *FUS* siRNA, for 48 h, over 10^5 of cells were harvested by EDTA-free trypsin, washed with PBS and re-suspended in 55 μl of binding buffer containing 5 μl of Annexin V-FITC. 450 μlof binding buffer and 10 μl of PI were then mixed with cells and stained in the dark for 5 min. The apoptotic cells were immediately analyzed and quantified with flow cytometry within 1 h (NovoCyte, ACEA Biosciences, Hangzhou, China) (Yin et al., 2020b).

RNA Extraction and Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from cells and tissues using the RNeasy kit (Qiagen, Grand Island, NY) in line with specific protocols. In qRT-PCR assay, MCE qRT-PCR Kits were used to prepare cDNA using 1 μg of the extracted total RNA from reverse transcription as per manufacturer recommendations. For miR-142-3P detection, Qiagen miRNA extraction kits (CeKunBio, Changsha, Hunan, China) were employed. The levels of miRNAs were determined by applying Qiagen miRNA detection kits (CeKunBio, Changsha, Hunan, China) according to the kits' protocols. The expressing levels of lncRNA, gene and miRNA were defined based on the threshold cycle (Ct), and determiend by $2^{-\Delta\Delta CT}$ approach, with U6 and GAPDH being references for lncRNA, gene, or miRNA. Besides, data were normalized relative to GAPDH. Data were expressed in a form of normalized mean ± SD.

Western Blotting

The radioimmunoprecipitation assay (RIPA) buffer that contained the protease inhibitor cocktail (Roche, Basel, Switzerland) was utilized for cell lysis. Total protein was quantified using the bicinchoninic acid (BCA) protein detection kit (Biosharp, Shanghai, China). Later, SDS-PAGE was conducted to separate proteins, then proteins were transferred on PVDF membranes, and membranes were probed with the corresponding primary antibodies at 4°C overnight. After washing, secondary antibodies were utilized to incubate membranes for another 1 h under ambient temperature. Then, enhanced chemiluminescence reagent (Millipore, MA, United States) was utilized to visualize protein bands.

RNA Pull-Down Assay

The Biotin RNA Labeling Mix (Roche Molecular Systems, Inc., Hague Road, IN, United States) was used for biotin labeling of RNA, and later T7 RNA polymerase (Roche) was utilized for transcription *in vitro*. After purification, the protein lysates were adopted for cultivating biotinylated cells. Thereafter, streptavidin agarose beads (Life Technologies, Carlsbad, CA, United States) were utilized for treating cells for another 1 h under ambient temperature, followed by bead elution within Biotin Elution Buffer three times and boiling within SDS buffer. Later, we conducted qRT-PCR and WB assays for RNA and protein analyses, with IgG being the reference.

Statistical Analysis

Data were displayed in a form of mean ± SD from at least three biological duplicates. This study adopted GraphPad Prism software (Systat Software, San Jose, CA, United States) for statistical analysis. Significant differences were determined by unpaired student's t-test (two-tailed). $p < 0.05$ stood for statistical significance.

RESULTS

DUBR is Abnormally Expressed and Closely Associated With Patient Survival in Human Pan-Cancer.

To determine the correlation between *DUBR* expression and cancer, we analyzed GEPIA data and found that *DUBR* was significantly upregulated in LAML, cholangiocarcinoma (CHOL), brain lower grade glioma (LGG), sarcoma (SARC), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), pancreatic cancer (PAAD), gastric cancer (STAD), and thymoma (THYM). In contrast, *DUBR* was markedly down-regulated in bladder urothelial carcinoma (BLCA), kidney chromophobe (KICH), endocervical adenocarcinoma and cervical squamous cell carcinoma (CESC), lung squamous cell carcinoma (LUSC), lung adenocarcinoma (LUAD), prostate adenocarcinoma (PRAD), ovarian serous cystadenocarcinoma (OV), skin cutaneous melanoma (SKCM), rectum adenocarcinoma (READ), thyroid carcinoma (THCA), testicular germ cell tumors (TGCT), uterine carcinosarcoma (UCS) and uterine corpus endometrial carcinoma (UCEC) (**Figure 1**). These results indicate that *DUBR* is abnormally expressed in the tumor tissue. Then we measured the association of *DUBR* expression with cancer prognosis in these cancers. For overall survival analysis, only high expression of *DUBR* in LAML had an unfavorable prognosis (**Figure 2A**). Therefore, *DUBR* can serve as the factor to predict the poor prognosis of LAML.

DUBR Expression is Significantly Higher and Associated With Poor Prognosis in AML

For investigating *DUBR*'s effect on the genesis and progression of AML, this study examined the *DUBR* levels within 173 AML and 70 normal tissue samples derived from GEPIA, the extensively utilized online bioinformatic approach. As a result, *DUBR* level significantly increased within AML tissues (**Figure 2C**). To further confirm these results, we first determined *DUBR* expression in normal peripheral blood mononuclear cells (PBMCs) and different AML cell lines (MV-4-11, Molm-13, U937, and KG-1 cells) via qRT-PCR. The results demonstrated that *DUBR* level significantly higher within AML cells compared with healthy PBMC cell lines, especially in Molm-13 and KG-1 cells, so we choose these two cell lines for the further experiments (**Figure 2E**). Thereafter, this study examined OS between cases with high vs. low *DUBR* expression; as a result, those showing *DUBR* up-regulation had poor prognostic outcome (**Figure 2B**).

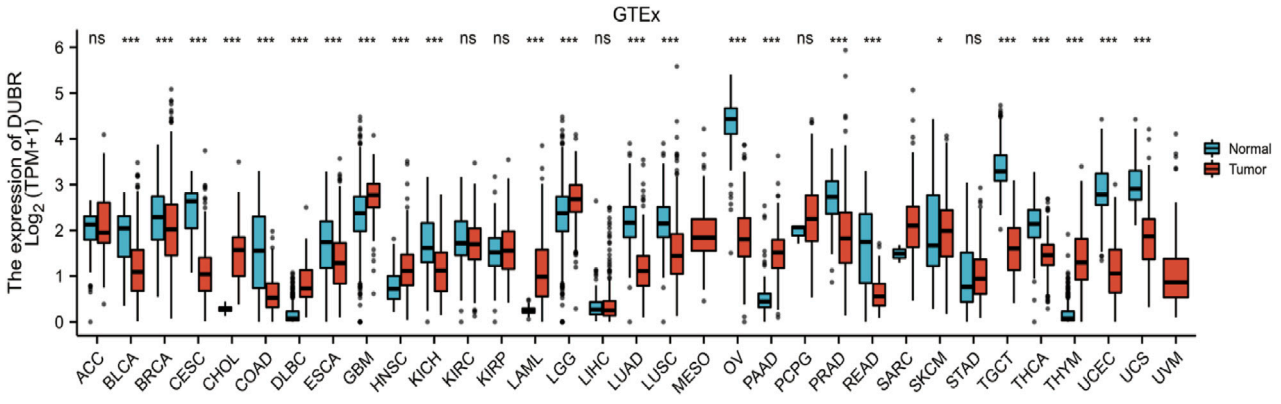

FIGURE 1 | DUBR levels within diverse cancers. DUBR levels within 33 different human cancers were measured according to The Cancer Genome Atlas (TCGA) cancer and normal data. Tumor tissues and matched normal TCGA and GTEx tissues were compared. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

We analyzed the predictive ability of *DUBR* for the prognosis of patients in the M0 to M5 FAB subtypes of 151 patients with AML. We found the *p* value of prediction rates are M1 (0.589), M1 (0.331), M2 (0.626), M3 (0.943), M4 (0.003), and M5 (0.07), it means *DUBR* can accurately predict the malignancy of M4 patients (**Figure 3**). This study applied ROC curve in evaluating the role of *DUBR* in prognosis prediction. As presented in **Figure 2D**, the AUC of *DUBR* according to the ROC curve was 0.878, which indicates that the expression level of *DUBR* can be used to predict the AML process. Our data shows that *DUBR* showed high expression level within AML, which predicted poor prognostic outcome, indicating the role of *DUBR* as the oncogene of AML.

DUBR Knockdown Inhibits Proliferation and Induces Apoptosis

For assessing *DUBR*'s biological effect on AML, we explored the effect of *DUBR* knockdown by siRNA (target sequence: AGC AGAGAAAAGGAAAGAAAACT) on the colony-forming, proliferation and apoptosis assays of KG-1 and Molm-13 cells (The efficiency of *DUBR* siRNA in KG-1 and Molm-13 cells are shown in **Supplementary Figure S1**). As revealed by cell counting kit-8 (CCK-8) assay, *DUBR*-siRNA-transfected cell proliferation was markedly suppressed (**Figures 4A,B**). According to colony formation analysis, *DUBR*-siRNA suppressed colonies formed in AML cells (**Figures 4C,D**). Moreover, Annexin-V-FITC/PI double staining analysis indicated that the downregulation of *DUBR* greatly facilitated apoptosis in these cells (**Figures 4E,F**), suggesting that lncRNA *DUBR* can affect the behavior of AML cells.

DUBR Acted as a ceRNA via Sponging miR-142-3p in AML Cells

Next, we attempted to elucidate the mechanisms by which *DUBR* facilitated the AML tumorigeneses. Many studies revealed that lncRNAs might be involved in the progression of diverse cancer types via competitively binding to miRNAs. Then, the "Starbase (Li et al., 2014)" "DIANA (Paraskevopoulou et al., 2013)" program showed that *DUBR* possibility bind with miR-142-3P, miR-107, and miR-104-3P (**Figure 5A**). And the overall survival analysis of miR-142-3P (**Figure 5B**), miR-107 (**Figure 5C**) and miR-104-3P (**Figure 5D**) in AML were applied. These results showed that low miR-142-3P expression related with worse prognosis, it may be the *DUBR* binding miRNA in AML. Furthermore, RNA-pull down results confirmed that *DUBR* bind with miR-142-3P in AML cells (**Figure 5F**). The last, the correlation between *DUBR* and miR-142-3P expression were applied, the results indicated that *DUBR* negative regulated miR-142-3P in AML (**Figure 5E**). To further reveal that *DUBR* bind with miR-142-3P in AML, we measured the miR-142-3P in *DUBR* overexpression KG-1 cells, we found compared with the empty vector group, the miR-142-3P were significantly downregulated by *DUBR*. Meanwhile, we transfected *DUBR* siRNA in KG-1 cells, and we found the expression of miR-142-3P could be upregulated by *DUBR* siRNA (**$p < 0.01$) (**Supplementary Figure S5**). Taken together, these results demonstrate that *DUBR* sponges miR-142-3P in AML.

miR-142-3P Inhibited Cell Proliferation in AML Cells

To explore the roles of miR-142-3P in AML cell lines, miR-142-3P mimic was transfected into Molm-13 and KG-1 cells, then CCK8 assay was performed to measure the cell viability. As a result, miR-142-3P treatment markedly suppressed cell proliferation in both two cells, compared to the control (**Figures 5G,H**).

DUBR Interacts With FUS Protein in AML

According to the above description, *DUBR* significantly affected AML growth, yet the underlying mechanism of *DUBR* in affecting cancer growth remains unknown. While exploring this, we first predicted the RNA-binding protein (RBP) FUS as the candidate target for lncRNA *DUBR*, based on StarBase (http://starbase.sysu.edu.cn/index.php), RBPsuit (http://www.csbio.sjtu.edu.cn/bioinf/RBPsuite/), and RBPDB (http://rbpdb.ccbr.utoronto.ca/)

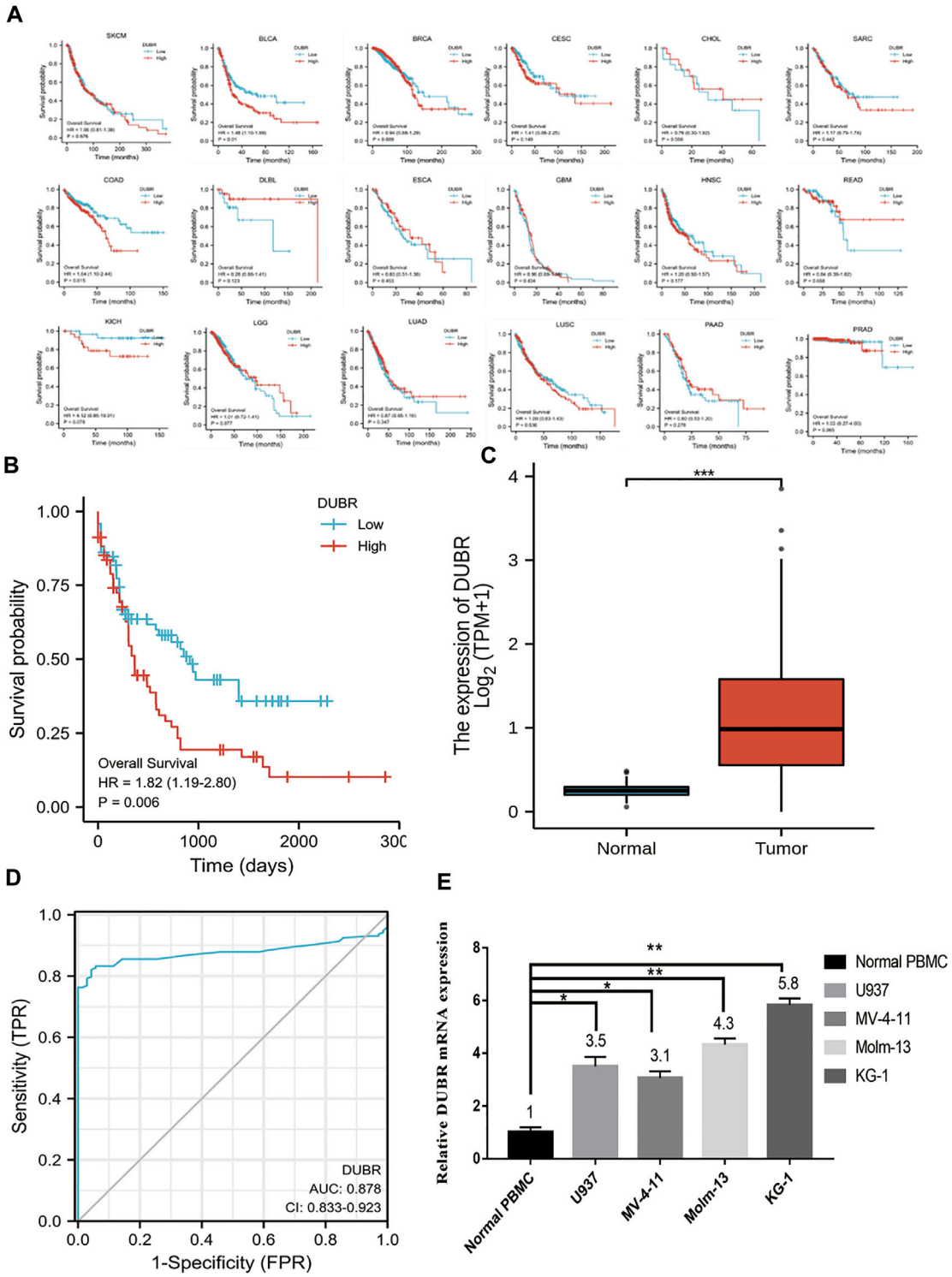

FIGURE 2 | Role of DUBR in predicting OS of different human cancers measured using the Gene Expression Profiling Interactive Analysis (GEPIA) database. **(A)** Kaplan-Meier analysis of overall survival based on *DUBR* expression levels in SKCM, BLCA, BRCA, CESC, CHOL, SARC, COAD, DLBL, ESCA, HNSC, READ, GBM, KICH, LGG, LUSC, PAAD, PRAD, and LUAD. **(B)** Kaplan-Meier plot of OS of patients with AML having high *DUBR* expression, which was defined as *DUBR* RNA expression higher than the median level, and lncRNA *DUBR* down-regulation ($p < 0.001$). **(C)** lncRNA *DUBR* levels within LAML cells in comparison with normal subjects ($p < 0.001$). TCGA-derived data were analyzed by GEPIA (http://gepia2.cancer-pku.cn). with the use of RNA-seq data from GTEx-derived healthy tissues. TPM: Transcripts Per Kilobase Million. **(D)** Predictive ability of DUBR expression for AML sensitivity. **(E)** RT-PCR results of *DUBR* in normal tissue and LAML cell line. $^{*}p < 0.05$; $^{**}p < 0.01$.

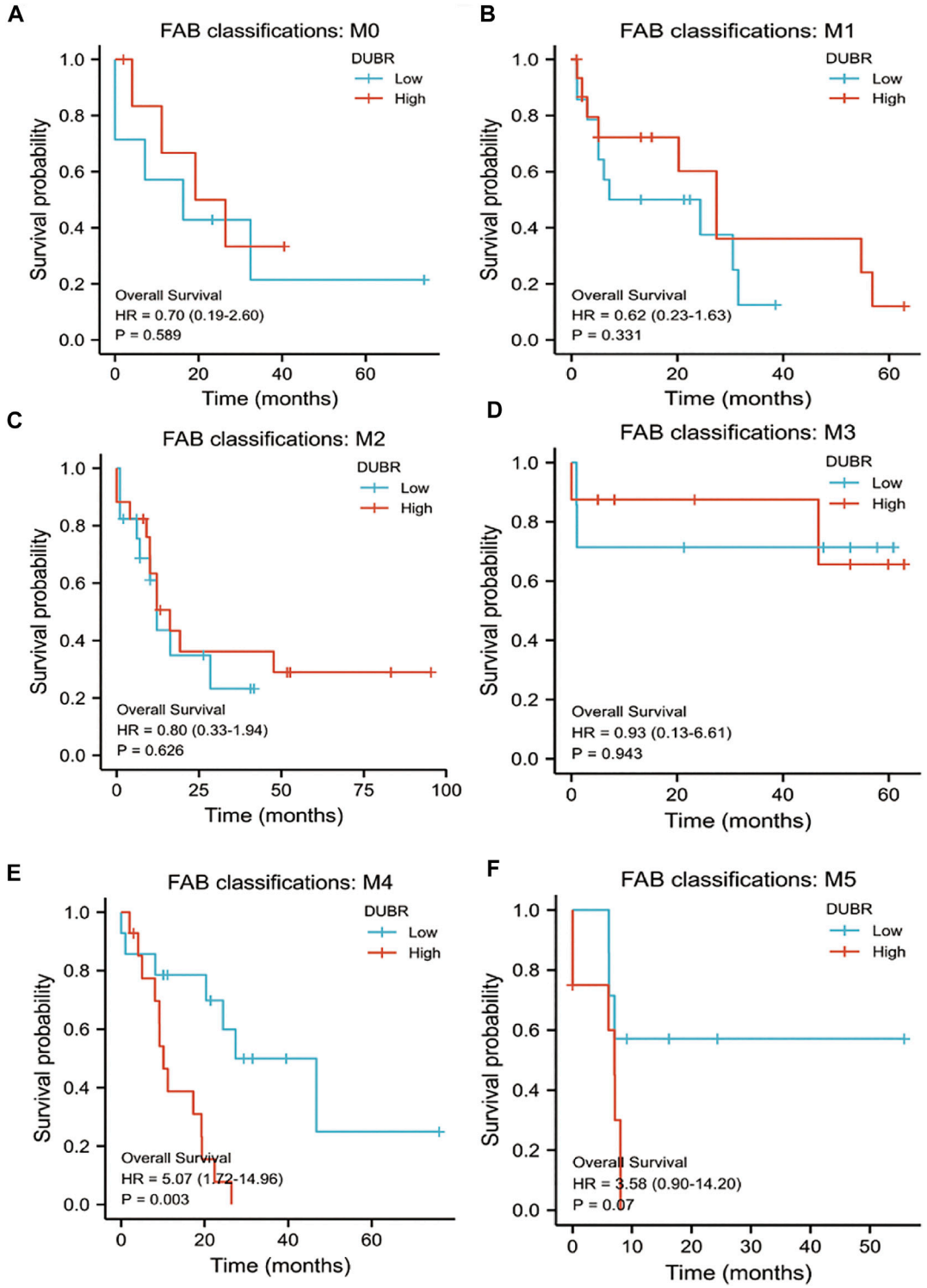

FIGURE 3 | K-M survival analysis to compare *DUBR* up-regulation and down-regulation within diverse FAB classification types of AML in Kaplan-Meier Plotter by GEPIA database. **(A)** M0; **(B)** M1; **(C)** M2; **(D)** M3; **(E)** M4; **(F)** M5.

databases. Two possible binding proteins of *DUBR* were found, including quaking (QKI) and FUS (**Figure 6A**, Left). Additionally, the level of *DUBR* in AML was positively correlated with *FUS* mRNA levels (R = 0.22, p = 0.007) (**Figure 6A**, middle). These results suggest the presence of one *FUS*-binding motif within the lncRNA *DUBR* sequence.

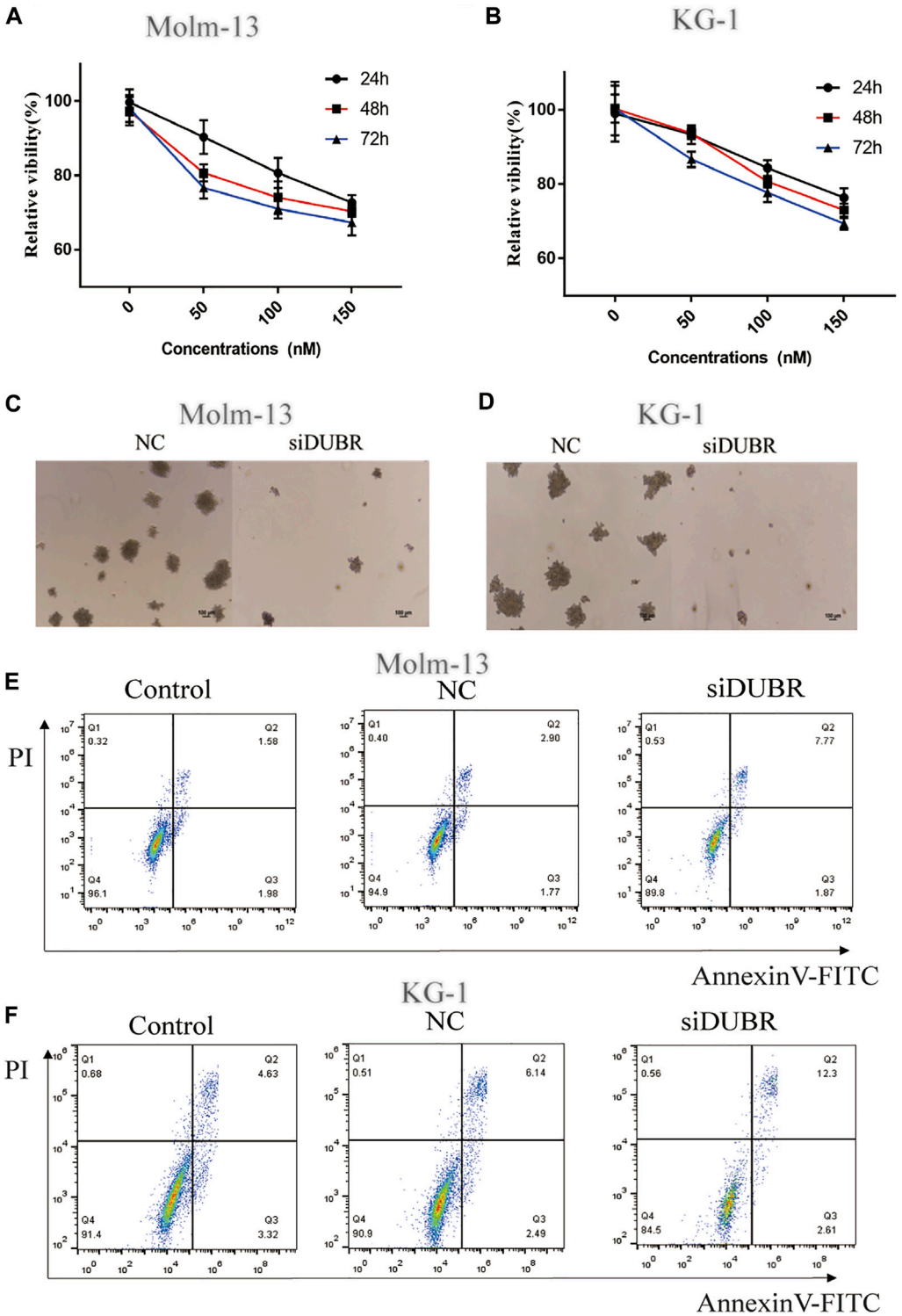

FIGURE 4 | DUBR induces the promotion of cell viability and inhibition of apoptosis in AML cells. Cell growth in control and *DUBR*-knockdown (using *siDUBR*) Molm-13 **(A)** and KG-1 cells **(B)** was measured using cell counting kit-8 assay. Downregulation of *DUBR* by *DUBR*-siRNA inhibited the colony-forming ability of Molm-13 **(C)** and KG-1 cells **(D)**. Apoptosis rate in Molm-13 **(E)** and KG-1 cells. **(F)** assessed using flow cytometry.

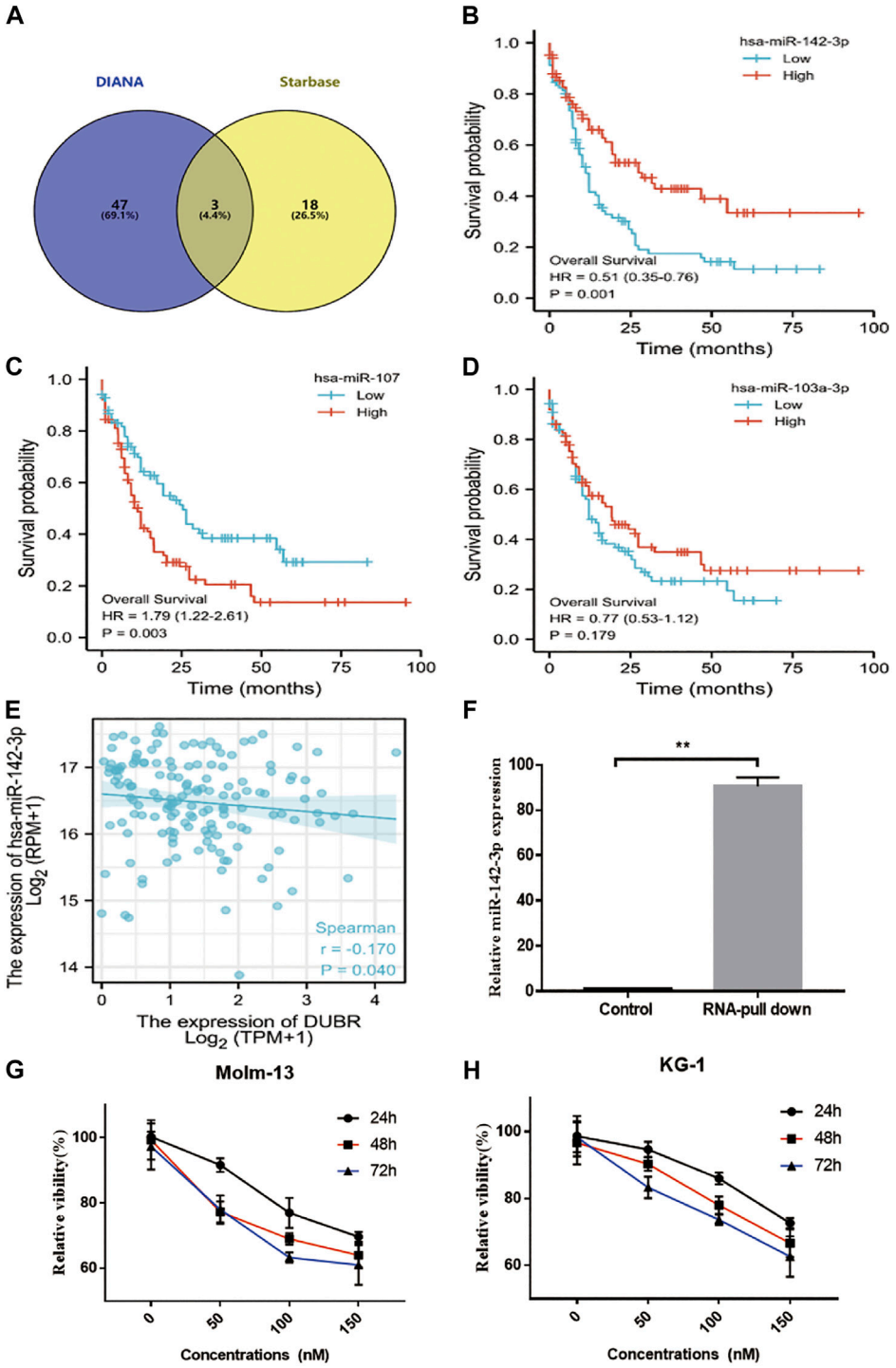

FIGURE 5 | DUBR sponged miR-142–3P and negatively modulated miR-142–3P expression. **(A)** Starbase2.0 and DIANA were used to predict the *DUBR* candidate sponge miRNA (miR-142-3P, miR-107, and miR-103-3P were involved in them). The OS plot of miR-142-3P. **(B)**, miR-107 **(C)** and miR-103-3P **(D)** in AML. **(E)** Spearman correlation analysis of relative expression between miR-142-3P and *DUBR* within AML bone marrow, $p < 0.05$. **(F)** RNA pull-down assay and qRT-PCR analysis for the detecting of endogenous miR-142-3P related to the *DUBR* transcript. SCR or miR-142-3P was transfected into Molm-13 **(G)** and KG-1 **(H)** cells. Then, cell viability was measured through CCK8 assay.

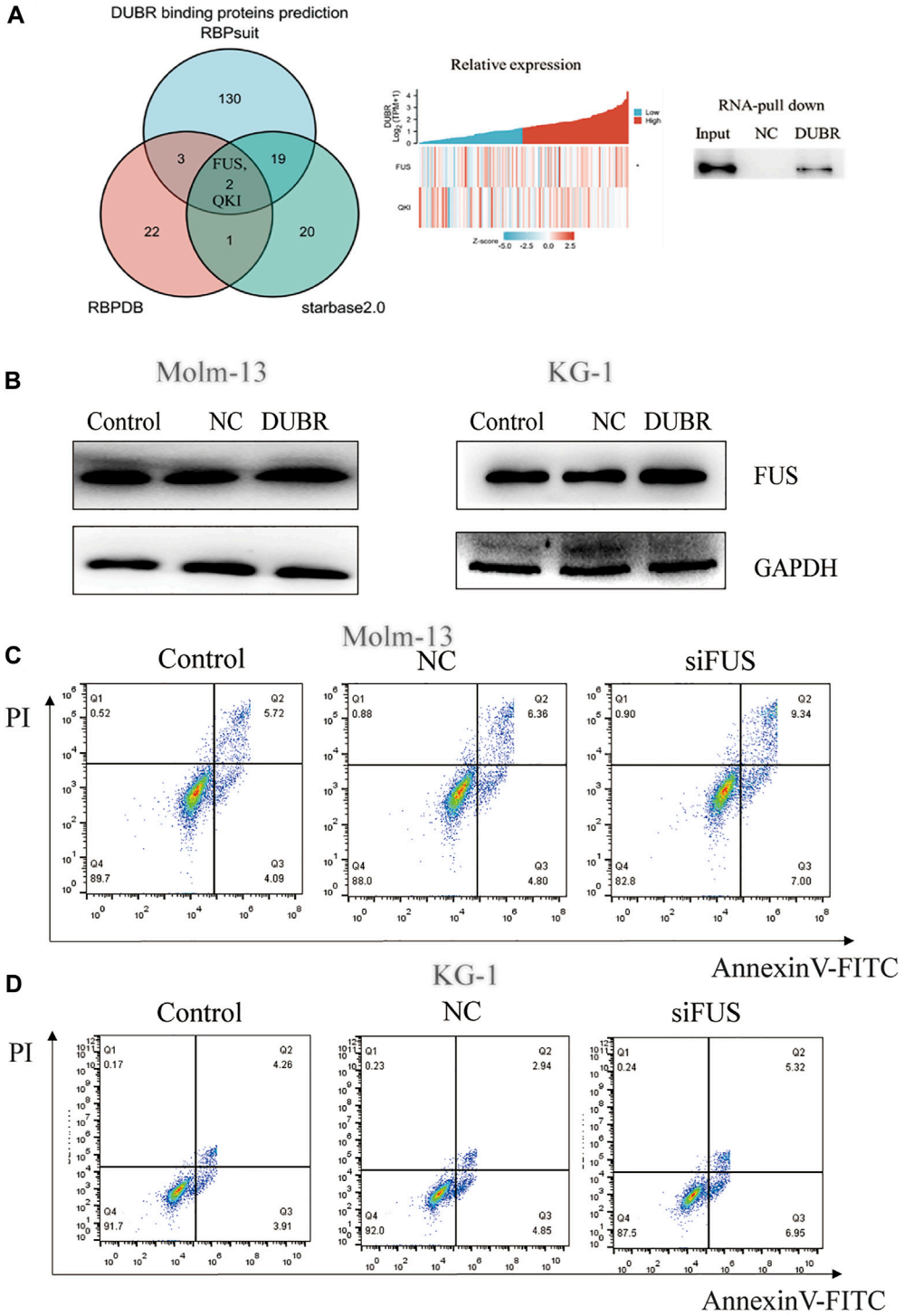

FIGURE 6 | *DUBR* binds to FUS protein in AML. **(A)** Starbase2.0, RBPDB, and RBP suit online were adopted for predicting the relations of lncRNA *DUBR* with FUS protein **(Left).** Pearson correlation analysis on the relative expression between FUS and *DUBR* within AML bone marrow, $p < 0.05$ **(Middle)**. Binding of *DUBR* and FUS was validated via RNA pull-down assay **(Right)**. **(B)** *DUBR* promotes FUS protein expression in AML. *DUBR* was knocked in via transfection with *DUBR* plasmid, and in Molm-13 and KG-1 cells, *DUBR* promoted FUS expression in AML. FUS knockdown induced AML cell apoptosis. FUS effect on the apoptosis of Molm-13 **(C)** and KG-1 cells **(D)** was evaluated by flow cytometric analysis.

Furthermore, RNA pull-down assay demonstrated an interaction between *DUBR* and FUS (**Figure 6A**, right). Taken together, these findings suggest that *DUBR* binds to FUS, which may be important for the regulation of proliferation in AML.

DUBR Promotes Oncoprotein FUS Expression in AML

To explore the association of *DUBR* with FUS protein, this study examined FUS protein level following *DUBR* overexpression in AML cells. We found that the FUS protein was upregulated by *DUBR* overexpression in KG-1 and Molm-13 cells, which implies that *DUBR* promoted the FUS expression (**Figure 6B**; **Supplementary Figure S2**).

Targeted Inhibition of FUS Attenuates the Malignant Properties of AML Cells

To reveal the underlying mechanism of the interaction of FUS with *DUBR*, this study analyzed the effect of FUS on the proliferation of AML cells. Specific siRNAs targeting FUS (*siFUS,* target sequence: AGCAGAGTTACAGTGGTTATAGC) were transfected into KG-1 and Molm-13 cell lines to reduce FUS expression (The efficiency of *FUS* siRNA in KG-1 and Molm-13 cells are shown in **Supplementary Figure S1**). The apoptotic rate of AML cells increased upon FUS inhibition (**Figures 6C,D**). Thus, the targeted inhibition of FUS attenuated malignant properties of AML.

MiR-142-3P Mimic in Combination With siFUS Could Have a Synergistic Effect on the Inhibition of AML Proliferation

To measure the inhibition effect of miR-142-3P mimic in combination with siFUS, we cotranfected 100 nM miR-142-3p mimic and 100 nM siFUS in KG-1 cells for 48h, then, we found the inhibition rate of the combination is better (**$p < 0.01$) than miR-142-3p mimic or siFUS alone (*$p < 0.05$) (**Supplementary Figure S4**).

DISCUSSION

LncRNAs are becoming the research hotspot in the field of cancer recently. It has been increasingly suggested that lncRNAs have important functions in cancers through regulating gene levels epigenetically, transcriptionally or post-transcriptionally (Bhan et al., 2017). Additionally, lncRNAs have emerged as the candidate cancer biomarkers, because of the disease and tissue specificity (Liu et al., 2016). *DUBR* is first suggested to participate in regenerating and differentiating muscle cells (Wang et al., 2015). Nonetheless, no previous studies have suggested *DUBR*'s biological roles in AML and the underlying mechanism. Here, we showed that *DUBR* was upregulated in AML cell lines and that its expression was related to AML prognosis. Our study indicated that *DUBR* played a role of an oncogene in AML via *DUBR*-miRNA-142-3P and *DUBR*-FUS interaction.

DUBR is identified as the potent *cis*-regulatory element within muscle cells (Wang et al., 2015). Negligible research has been conducted to test the function of *DUBR* in cancer. Nie et al. (2021) found that *DUBR* suppressed lung adenocarcinoma (LUAD) cell growth and metastasis by inhibiting oxidative phosphorylation regulated by ZBTB11 (Nie et al., 2021). The authors suggested the down-regulation of *DUBR* might serve as the tumor suppressor in lung adenocarcinoma cells. Utnes et al. found that the expression of *DUBR* had a strong relationship with high-risk recurrent neuroblastoma (Utnes et al., 2019). We also noted downregulation of *DUBR* in many cancers; however, in AML, *DUBR* expression was significantly higher than that in normal samples, which was related to poor prognosis in AML patients.

MiRNAs have been identified as the key post-transcriptional regulating factors, which are associated with tumor occurrence (Lee and Dutta, 2009). miR-142-3p, the miRNA specific to hematopoietic tissues, has key function in regulating T cell differentiation induced by antigen (Gebeshuber et al., 2009). Bellon and others revealed the abnormal miR-142-3p levels within uncultured ATL cells (adult T cell leukemia) *in vitro* and cells infected with HTLV-I (human T cell leukemia virus type-I) *in vitro* (Wu et al., 2007). miR-142-3p can serve as the key regulatory factor in the maintenance of healthy hematopoiesis, which can serve as the candidate factor for the diagnosis of leukemia (Bellon et al., 2009). Here, we reported that DUBR function by sponging with miR-142-3P through computer prediction and RNA pull down results. miR-142-3P over-expression suppressed AML cell proliferation.

Furthermore, we found that *DUBR* directly bound to FUS and promoted the level after regulating *DUBR* level within AML cells. RBPs binding represents a major mechanism where lncRNAs exert their roles through several cancer-related pathways, while RBPs show diverse effects on gene expression-related processes; for instance, lncRNA *SOX2OT* combines with FUS by promoting pancreatic cancer proliferation (Chen et al., 2020). Another study shows that lncRNA *HOTAIR* induces Runx3 ubiquitination through the interaction with Mex3b while enhancing gastric cancer (GC) cell proliferation (Xue et al., 2018). As discovered by Zhang, lncRNA *GAS5* promoted the arrest of cell cycle at G1 stage through combining with YBX1 for regulating p21 level within GC (Liu et al., 2015). Herein, bioinformatics and RBP pull-down assays were conducted, which suggested that *DUBR* directly interacted with FUS. In addition, RBP FUS (referred to as TLS) is suggested to relate to several RNA metabolic processes, such as splicing, transcription, local translation, miRNA processing, and mRNA transport (Lagier-Tourenne et al., 2010). According to previous report, FUS enhances cancer cell growth and invasion (Ward et al., 2014; Deng et al., 2018). Brooke and others discovered that FUS served as the important process connecting prostate cancer (PCa) cell cycle progression with androgen receptor signal transduction (Brooke et al., 2011). FUS has also been implicated in leukemia (Panagopoulos et al., 1997). FUS serves as the BCR-ABL oncoprotein downstream target. BCR-ABL is suggested to avoid proteasome-induced FUS decomposition; besides, the FUS mutant that shows resistance to proteasome-induced

decomposition suppresses 32D myeloid cell differentiation, thus promoting cell growth (Perrotti et al., 1998). The rearranged 16; 21 chromosomes within AML cells contribute to forming the *TLS/FUS-ERG* fusion gene, owing to which patients are resistant to conventional chemotherapy (Kong et al., 1997), and FUS has a certain effect on the development of APL cell resistance to retinoic acid as a consequence of mutations in the binding domain (Walsby et al., 2007). A number of lncRNA-related research on AML exists; for example, *LINC00319* regulates post-transcriptional SIRT6 expression by the FUS-mediated pathway (Zhang et al., 2019). Our results carried out CCK-8 assays by using the AML cells transfected with *siFUS*; as a result, FUS exerted an important effect on enhancing AML growth.

In order to observe the candidate target genes of miR142-3P, we applied target prediction based on miWalk, starbase, mitarbase, and miRDB database (**Supplementary Figure S3**), FUS is not involved in it. We think there are independent pathway between *DUBR*, miR-142-3P and FUS, it is not a *DUBR*/miR-142-3P/FUS axis, it is meaningful because we will get a better inhibition rate by regulation of FUS and miR142-3P genes simultaneously.

Based on the work presented here, we confirmed that *DUBR* expression increased within AML cells, which was related to AML prognosis via DUBR-miRNA-142-3P and DUBR-FUS interaction. We suggest that the DUBR-miRNA-142-3P and DUBR-FUS interaction promotes AML cell proliferation, which can serve as the candidate anti-AML therapeutic target.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

SL, RO, CG, YH, ZY, QZ, and ZL conceived and designed the project; YH, FW, HS, and JH collected the data, performed the interpretation of data and statistical analysis; ZY, QZ, MZ, and ZL wrote the article; ZY, FW, JH, RO, and SL revised the paper. All authors read and approved the final article.

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SUPPLEMENTARY MATERIAL

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REVIEW
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MicroRNAs: Important Players in Breast Cancer Angiogenesis and Therapeutic Targets

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The high incidence of breast cancer (BC) is linked to metastasis, facilitated by tumor angiogenesis. MicroRNAs (miRNAs or miRs) are small non-coding RNA molecules that have an essential role in gene expression and are significantly linked to the tumor development and angiogenesis process in different types of cancer, including BC. There's increasing evidence showed that various miRNAs play a significant role in disease processes; specifically, they are observed and over-expressed in a wide range of diseases linked to the angiogenesis process. However, more studies are required to reach the best findings and identify the link among miRNA expression, angiogenic pathways, and immune response-related genes to find new therapeutic targets. Here, we summarized the recent updates on miRNA signatures and their cellular targets in the development of breast tumor angiogenetic and discussed the strategies associated with miRNA-based therapeutic targets as anti-angiogenic response.

Keywords: breast cancer, microRNA, vascular endothelial growth factor, angiogenesis, therapeutic target 3

INTRODUCTION

Breast cancer (BC) is one of the more prevalent occurring forms of cancer in females and the second most frequently occurring type of cancer worldwide (Siegel et al., 2021), and 90 percent of breast cancer deaths are due to the formation of distant organ metastases (Chaffer and Weinberg, 2011). In breast cancer, angiogenesis, or the development of new blood vessels, is essential for both local tumor growth and distant metastasis (Folkman, 1971). Angiogenesis is a multi-step complicated process characterized by 1) MMP damages the basement membranes of tissues on a local basis, causing degradation and hypoxia immediately, 2) endothelial cells migrate in response to angiogenic factors, 3) proliferation and stabilization of endothelial cells, and 4) angiogenic factors still influence the angiogenic mechanism. The development of new vessels is regulated by a concerted action of multiple cytokines and growth factors such as anti-angiogenic and proangiogenic factors (**Figure 1**). miRNAs regulate tumor angiogenesis in two ways: They can inhibit or promote it (Leone et al., 2019) (**Figure 2**). Victor Ambros' lab was the first to publish an article regarding miRNAs (Lee et al., 1993), which have a length of 21–25 nt, are known as short non-coding RNAs (short-RNAs) and regulate the expression of a variety of cellular proteins by modulating their messenger RNA levels (Lewis et al.,

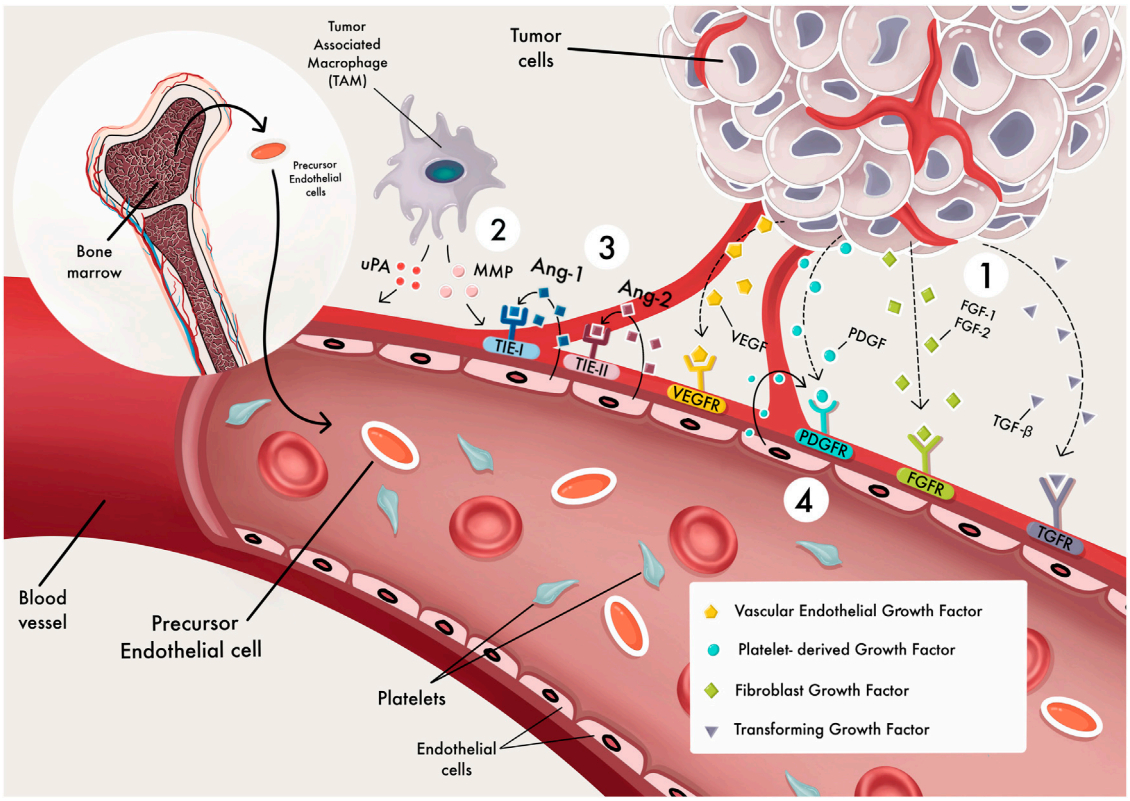

FIGURE 1 | angiogenesis processes. The different stages of blood vessel development (Siegel et al., 2021). angiogenesis factors activate signal transduction pathways by binding to endothelial cells (EC) receptors (Chaffer and Weinberg, 2011). MMPs degrade the extracellular matrix, allowing ECs to migrate and proliferate outside the pre-existing capillary wall (Folkman, 1971). Endothelial cells express Tie-2 receptors for binding with Angiopoietin-1 (Ange-l); this might promote vessel sprouting, pericyte acquisition, vessel survival, and stabilization (Leone et al., 2019). ECs secrete PDGF, which attracts pericyte precursors as a chemoattractant. These cells attach to endothelial cells and grow into pericytes.

2005). They regulate gene expression in health and disease cells by binding to the 3′-untranslated region (UTR) or other regions such as the 5′ UTR, gene promoters, and the coding sequence (Broughton et al., 2016). Also, it has been established that crosstalk exists between long non-coding RNAs and miRNAs, where these two networks interact and form complex networks in gene regulation pathways (Ghafouri-Fard et al., 2021a; Ghafouri-Fard et al., 2021b; Dastmalchi et al., 2021).

According to TargetScan studies, miRNAs regulate one-third of all human genes (Lewis et al., 2005) and are essential regulators of a variety of processes, including cancer-related developments like angiogenesis (Goradel et al., 2019), metastasis (Martello et al., 2010), and drug resistance (Taheri et al., 2021). For example, global miRNA depletion inhibits the angiogenic process because miRs control the angiogenesis process (Chen et al., 2014). In addition, several investigations have described miRNA signatures in clinical breast specimens and cell lines. In particular, miRNAs can influence angiogenesis directly by affecting endothelial cell function or indirectly by changing the production of proteins that prevent or promote angiogenesis (Wang et al., 2018a). Consequently, miRNAs have attracted attention as potential targets for new anti-angiogenic therapies.

In this review, we summarized the recent updates on miRNA signatures and their cellular targets in the development of breast tumor angiogenetic and discussed the strategies associated with miRNA-based therapeutic potential as anti-angiogenic response.

In the current review, the key questions attempted to be answered were “What are the miRNA’s contribution to breast cancer angiogenesis?”, “How can miRNAs contribute to BC angiogenesis? and “What are miRNA-based therapies for angiogenesis in BC?.”

“The terms “microRNA” or “miRNA,” “breast cancer,” “therapeutic target,” and “angiogenesis” were searched in PubMed and Google Scholar. To determine whether the retrieved papers were relevant to the subject, we evaluated the abstracts of all articles. Then, all related papers (*in vitro*, *in vivo*, and human-based research) to the subject were selected to be included in the study.

BIOGENESIS OF MICRORNA

MiRNAs are small, single-stranded non-coding RNAs that come from pri-miRNA, which is an early-stage transcript produced by

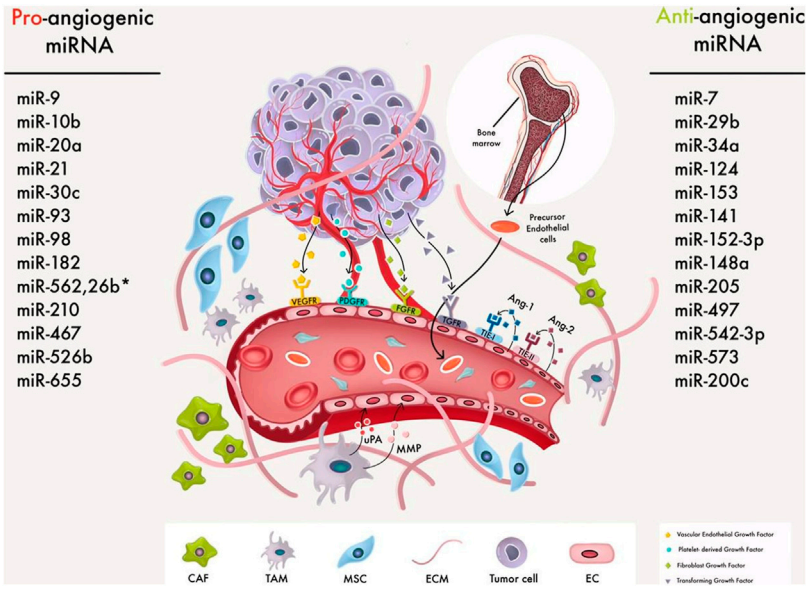

FIGURE 2 | MiRNAs that regulate angiogenesis in BC cells. An anti-angiogenic miRNA can either slow or promote angiogenesis by affecting the many growth pathways involved in cancer management. For example, angiogenesis is a critical stage in tumor metastasis that might aid tumor spread, and angiogenesis in breast cancer involves TME and cancer cells communication. So miRs regulate BC angiogenesis via macrophages, MSCs, ECM, and CAFs.

RNA polymerase II (Zeng and Cullen, 2006). The pri-miRNAs are defined by the presence of one or many incomplete hairpin structures with a stem of about 33 base pairs (Bartel, 2004). Drosha and Dicer, two RNase III family ribonucleases, process the pri-miRNA precursor in two steps (Kuehbacher et al., 2008). First, Drosha cleaves the pri-miRNA in the nucleus to create a pre-miRNA of roughly 70 nucleotides in length transported to the cytoplasm through an exportin-5 (XPO5) process (Bohnsack et al., 2004; Wu et al., 2018). Then, Dicer converts the pre-miRNA into a mature, functional, double-stranded (ds) miRNA (Carthew and Sontheimer, 2009). The mature miRNA is then covalently coupled to RISC, a multiprotein complex that includes the AGO protein, necessary for RNA silencing. Through Watson-Crick base pairing, RISC utilizes the leading strand to target the mRNA complementary, and the other strand is eliminated (Gregory et al., 2005). The miRNA binding to a 3′-UTR causes mRNA destruction or translational inhibition. The level of mRNA degradation or translational repression is determined by the degree of miRNA complementarity to the 3′-UTR. In addition, RISC can target and trigger 5′-UTR mRNA translation (Vasudevan et al., 2007). **Figure 3** shows the biogenesis of miRNA.

TUMOR ANGIOGENESIS

Angiogenesis is a multi-step and complex process to form new blood vessels from pre-existing ones. It begins with the stimulating, migrating, proliferating, and differentiating endothelial cells in response to signals from the surrounding tissue, such as hypoxia (low oxygen levels) (Bentley and Chakravartula, 2017). Each step is progressed and controlled by several pro-and anti-angiogenic factors.

VEGF, PDGF, and FGF promote new blood vessel development. VEGF-A, VEGF-B, VEGF-C, and VEGF-D are the primary forms of VEGF, and they bind to VEGF tyrosine kinase receptors (VEGFR-1-2-3) to regulate angiogenesis (Holmes and Zachary, 2005). Additionally, angiopoietins have a role in controlling endothelial cell signaling pathways. They associate with other angiogenic factors to bind with Tie-2 tyrosine kinase receptors and help the formation of endothelial tubes.

Despite the angiogenic factors, the body also produces endogenous anti-angiogenic substances such as TSP1 (Good et al., 1990), proteolytic fragments of basement membrane or extracellular matrix that comprises an inhibitor of blood vessel formation, an anti-angiogenic factor. Another anti-angiogenic factor is Endostatin, a proteolytic fragment of collagen XVIII (O'Reilly et al., 1997). The last two anti-angiogenic factors are canstatin (Magnon et al., 2005) and tumstatin (Maeshima et al., 2002), cleavage fragments of collagen IV. Furthermore, the body also produces endogenous anti-angiogenic soluble substances like IFN- α and IFN-β and angiostatin, a proteolytic fragment of plasmin (Baeriswyl and Christofori, 2009).

The balance between pro- and anti-angiogenic factors together with differential expression, release, or activation of the numerous factors control new blood vessel formation under pathological or physiological situations (**Figure 4**). Under physiological conditions, stromal and endothelial cells and released chemicals constitute a dynamic system that constantly alters and produces anti-angiogenic substances that keep the vasculature quiet. First, the "angiogenic switch" is turned on because proangiogenic factors are overabundant within tumor

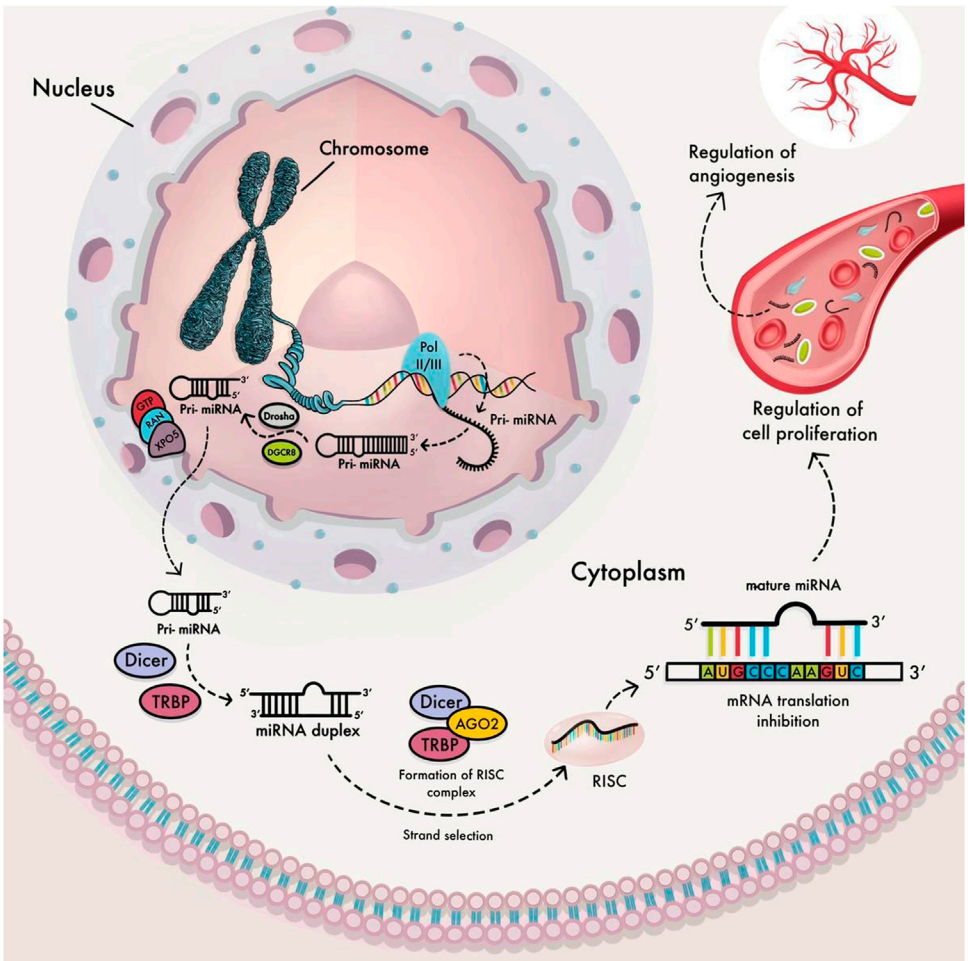

FIGURE 3 | miRNA biogenesis pathway. In the nucleus, RNA polymerase II transcripts miRNA genes to produce primary miRNA transcript (pri-miRNA), then by the microprocessor complex Drosha-DGCR8 cleaved into precursor miRNA (pre-miRNA). After that, Exportin-5 transports the pre-miRNA into the cytoplasm, where it is processed into a miRNA duplex by Dicer and its interaction protein, TRBP. Once the miRNA duplex has been unwinding, it is divided into two single-stranded miRNAs for further processing. Mature miRNA is attached to the RNA-induced silencing complex (RISC) and mediates mRNA degradation or repression of protein translation within the cytoplasm. Mature active miRNAs inter to cell proliferation regulation by controlling the angiogenesis process in the tumor cells.

cells, and angiogenesis occurs as a response. Then, tumor cells and invading inflammatory/immune cells can release proangiogenic factors (Baeriswyl and Christofori, 2009) (**Figure 1**).

A key feature of tumor development is the ability of the tumor to generate its blood supply. It is an essential step in tumor initiation, which can vary based on the characteristics of the tumor and its microenvironment (Carmeliet and Jain, 2000). In addition, this tumor vasculature facilitates the tumor's ability to obtain appropriate oxygen, nutrition, and waste removal. Moreover, neovascularization has the potential to create an "escape route" for tumor cells to enter the circulation, allowing them to disseminate and metastasis (Aguilar-Cazares et al., 2019).

Physiological angiogenesis and tumor angiogenesis are significantly different. First, tumor angiogenesis (the formation of new blood vessels to nourish the tumor) is a long-term process. Secondly, tumor blood vessels are disorganized, chaotic vascular networks, irregular in shape, dilated, hyper permeable, and not made up of distinctive arterioles, venules, and capillaries like normal vascular systems (Siemann, 2011). Additional aspects of tumor angiogenesis, tumor cells can undergo a process known as vasculogenic mimicry, during which they alter their gene expression to look like endothelial cells (Luo et al., 2020). Vasculogenic mimicry may be induced by hypoxia due to mass tumor growth (Wei et al., 2021), which occurs when tumor cells are far away from blood vessels and consequently do not receive enough oxygen and nutrients. Individuals with malignancies that display the characteristics of vasculogenic mimicry have a poor prognosis (Yang et al., 2016). The microenvironment in anoxic conditions collaborates with oncogenic pathways to trigger new blood vessel formation and leads to cancer development (Dayan et al., 2008). Hypoxia-inducible factor-1 (HIF-1) is a major transcriptional factor influencing the development of hypoxia-induced conditions. HIF-1 is composed of α and β polypeptides. HIF-1α induces

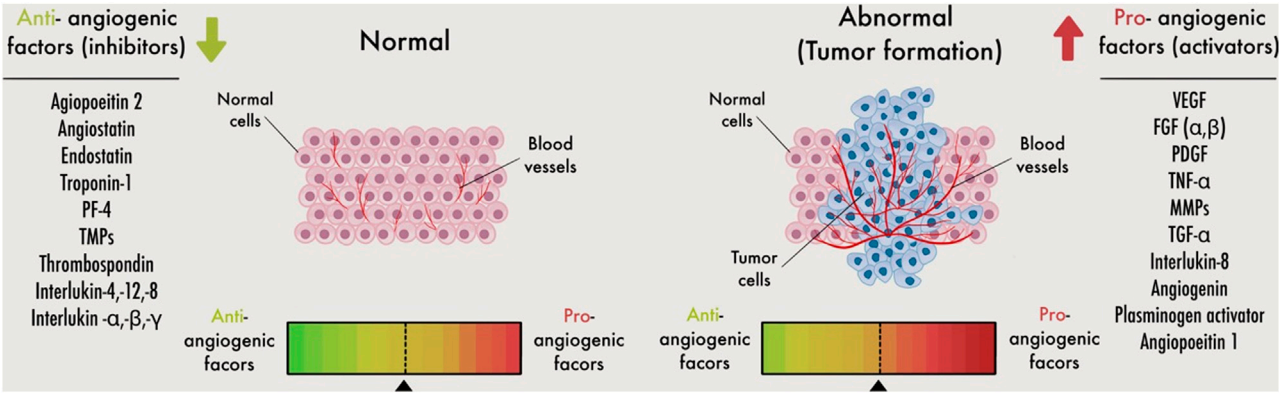

FIGURE 4 | Schematic image for the angiogenic switch. Angiogenesis is controlled by a careful balance between pro-angiogenic and anti-angiogenic molecules in the bloodstream. Inadequate angiogenesis leads to a defect in the angiogenic balance, with a subsequent angiogenesis change toward excessive or reduced angiogenesis. Pro-angiogenic supports to stimulate the production of endothelial cells (ECs)during angiogenesis. Whereas anti-angiogenic factors, on the other hand, inhibit EC activation. More angiogenic than anti-angiogenic factors are involved when the angiogenic switch is activated.

angiogenesis by upregulating the production of proangiogenic factors, including VEGF-A (Hashimoto and Shibasaki, 2015). von Hippel-Lindau (VHL) regulates the proteasome via ubiquitin degradation in normoxic conditions to keep HIF-1 levels low (Tanimoto et al., 2000). While hypoxia causes HIF-1α breakdown, the effect is that HIF-1α levels increase, and angiogenesis is promoted (Jin et al., 2020).

KEY MICRORNAS IN BREAST CANCER ANGIOGENESIS

In the human genome, about 2,300 mature miRNAs have been identified (Alles et al., 2019), 10% of these have been shown to target EC activity and angiogenesis (Sun et al., 2018), like let7a, let-7b, let-7days, miR-16, miR-9, miR-21, miR-20, miR-23a, miR-34a, miR-29, miR-99a, miR-93, miR-100, miR-124, miR-125-5p, miR-126, miR-146, miR-135a, miR-195, miR-276, miR-181a, miR-181b, miR-199b, miR-204, miR-200b, miR-221 and miR-222, miR-296, miR-320, miR-361-5p, miR-874. Moreover, they are upregulated in ECs and linked to angiogenesis control (Poliseno et al., 2006; Kuehbacher et al., 2007; Suárez et al., 2007; Caporali and Emanueli, 2011). Thus, miRNAs are divided into two categories based on miRNA expression and angiogenesis function: 1) miRNAs that control the angiogenesis process primarily through well-characterized target genes, and 2) miRNAs controlled by pro- or anti-angiogenic factors or hypoxia (Caporali and Emanueli, 2011).

Anti-angiogenic microRNAs in Breast Cancer

The expression of miRNAs, which regulate oncogenes and tumor suppressor genes, is changed in different types of human cancers (Hussen et al., 2021a). Therefore, these molecules have been termed "oncomiRs." MiRNAs that are directing the process of angiogenesis are called angiomiRs, as they govern angiogenic processes in pathological and physiological circumstances (**Table 1**) (Suárez and Sessa, 2009). Overexpression of miRNAs is usually associated with a poor prognosis, chemotherapy resistance, and low survival (Babashah and Soleimani, 2011; Bitaraf et al., 2020). Various research groups have discovered many different miRNA expression patterns and individual miRNAs in breast cancer patients, and they have been linked to angiogenesis, invasion, and prognosis.

microRNA-126

miRNA-126 is expressed mainly in ECs, and it is significantly linked with angiogenesis throughout normal development and wound healing (Harris et al., 2008). The two mature strands of the pre-miR-126 are miR-126-3p and miR-126-5p, which have distinct cell-type and strand-specific functions in angiogenesis (Zhou et al., 2016).

MiRNA-26, which targets both VEGFA and PIK3R2, plays a significant role in angiogenesis in human breast cancer, and its expression was decreased (Zhu et al., 2011). In addition, Overexpression of miR-26a in MCF7 cells has been found to reduce cancer development, tumor angiogenesis and induce apoptosis by inhibiting VEGF/PI3K/AKT signaling pathway (Fish et al., 2008; Zhu et al., 2011). Furthermore, ectopic expression of miR-126 in BC cells reduced CD97, a G-coupled receptor that promotes cell invasion and angiogenesis via integrin signaling. (Lu et al., 2014). Another study found that miR-126 controls metastasis and angiogenesis through targeting the pro-angiogenic genes (IGFBP2, PITPNC1, and c-Mer kinase) (Png et al., 2011). These findings suggest that a single miRNA (miR-126) regulates cell survival and angiogenesis, with the possibility to help control vascular function and development.

microR-497

According to the miRNA growing evidence, miR-497 has been found that regulate the proliferation, migration, and survival of BC cells (Liu et al., 2016). In a mouse xenograft model, miR-497 inhibits tumor development and endothelial cell tube formation

TABLE 1 | miRNAs that inhibit angiogenesis in Breast cancer (ANTs: adjacent non-cancerous tissues).

miRNA	Numbers of clinical samples	Assessed cell line	Animal model	Targets/ Regulators	Signaling Pathways	Association with patients' outcome	Function	References
miR-126-3p	—	MDA-MB-231, HCC193, MCF-10A	—	RGS3	G-protein signaling 3	—	miR126-3p serves as a tumor suppressor via controlling RGS3, an essential regulator of TNBC.	Hong et al. (2019b)
miR-126	—	MCF7	—	VEGF-A	—	—	VEGF-A is downregulated due to the expression of miR-126 in breast cancer	Alhasan, (2019)
miR-126-3p	—	MCF7	Rat	VEGFA and PIK3R2	VEGF/ PI3K/AKT	—	The expression of miR-126 has been shown to regulate the VEGF/PI3K/AKT signaling pathway	Zhu et al. (2011)
miR-206	—	MDA-MB-231 MCF-7	—	*VEGF*, *MAPK3*, and *SOX9*	—	—	miR-206 downregulated in TNBC through targeting *VEGF*, *MAPK3*, and *SOX9*	Liang et al. (2016)
miR-206	50 formalin-fixed and paraffin-embedded BC tissue	MDA-MB-231, SKBR3, MDA-MB-1739, HCC70, MDA-MB-361 MCF-7	—	VEGF MAPK3	—	—	In TNBC cells, miR-206 controls VEGF and MAPK3 expression	Salgado et al. (2018)
miR-4500	—	MCF10A, MCF7, BT474, MDA-MB-231, MDA-MB-468	Nude mice	RRM2	MAPK	tumor suppressor in BC	miRNA-4500 suppresses MAPK signaling via regulating RRM2	Li et al. (2020a)
miR-497	45 pairs of clinical samples and ANTs	MCF-7, T-74D, MDA-MB-468, MDA-MB-453, MDA-MB-435	—	HIF-1α	—	suppresses the proliferation and tube formation	miRNA-497 suppresses BC angiogenesis by direct targeting HIF-1α	Wu et al. (2016)
miR-100	Six healthy persons	MCF-7, MDA-MB-231, and T47D	—	mTOR	mTOR/HIF1α/ VEGF	—	miR-100 regulates neovascularization via mTOR.	Pakravan et al. (2017)
miR-153	—	HEK293T MCF10A MDA-MB-231 HCC1937	13 Nude mice	HIF1α	IRE1α-XBP1	—	Hypoxia promotes miRNA-153 to fine-tune HIF-1α/ VEGFA-stimulated angiogenesis in BC.	Liang et al. (2018a)
miR-190	12 pairs of tumors and ANTs	MCF-7, MCF-10A, T47D, MDA-MB-231, Bcap37	36 Nude mice	STC2	AKT-ERK	Overexpression of miRNA-190 leads to inhibition of tumor growth in BC.	Angiogenesis in BC is inhibited by miR190, which targets STC2	Sun et al. (2019)
miR-200 family	—	MDA-MB-231, T47D, H578T, BT-549, MCF-7	Nude mice	IL-8 and CXCL1	—	—	Through targeting interleukin-8 and CXCL1, miRNA-200 inhibits angiogenesis	Pecot et al. (2013)
miR-145	—	MDA-MB-231	Nude mice	IRS1	PI3K/Akt and Ras/Raf/MAPK	-	miR-145 inhibits angiogenesis by inhibiting vascular endothelial cell tube formation	Pan et al. (2021)
miR-148a-3p		MDA-MB-231	Nude mice	IGF-1R and VEGF	—	—	Angiogenic factors, such as VEGF and IGF-1R, are targeted by miR-148a-3p, which decreases the expression of these factors	Lacerda et al. (2019)
miR-148a	—	Hs-578T, MDA-MB-231	—	Wnt-3′- UTRs	Wnt/β-catenin signaling pathway	—	miR-148a inhibits angiogenesis by suppressing GLA via the Wnt/-catenin pathway	Mu et al. (2017)
miR-148a	68 breast tissues and 22 pairs of tumors and ANTs	MCF-7, T47D, MDA-MB-231	—	IGF-IR and IRS1	AKT and MAPK/ERK signaling pathways	—	miR-148a inhibits tumor growth by binding to IGF-IR and IRS1	Xu et al. (2012)

(Continued on following page)

TABLE 1 | (*Continued*) miRNAs that inhibit angiogenesis in Breast cancer (ANTs: adjacent non-cancerous tissues).

miRNA	Numbers of clinical samples	Assessed cell line	Animal model	Targets/ Regulators	Signaling Pathways	Association with patients' outcome	Function	References
miR-152	—	MDA-MB-468	Nude mice	IGF-IR IRS1	—	—	miRNA-152 suppresses HIF-1 and VEGF via modulating IGF-IR and IRS1	Marques et al. (2018)
miR-153	Seven pairs of tumors and ANTs	MCF7, MDA-MB-231, and HCC1937	—	ANG1	—	—	miR-153 reduced endothelial cell migration and tube formation by targeting ANG1in breast cancer	Liang et al. (2018b)
miR-139	—	HCC180, MCF-10A Six and BT549	48 Nude mice	SOX8	—	—	miR-139 reduced TNBC cell growth by targeting SOX8	Dong et al. (2020)
miR-10a	—	MCF-7, MCF-10A and MDA-MB-231	—	p-Akt p-mTOR, p-p70S6K, PIK3CA Cyt C	PI3K/Akt/ mTOR	—	miRNA-10a inhibited phosphoinositide/Akt/ mTOR signaling in breast cancer cells	Ke and Lou, (2017)
miR-195	—	MDA-MB-231, MCF7, MDA-MB-435, MDA-MB-453, SK-BR3 T47D, ZR-75-30	—	IRS1	—	—	miR-195 reduces tumor angiogenesis by inhibiting the IRS1-VEGF axis	Wang et al. (2016)
miR-19b-1	—	MDA-MB-231 MCF-7	Nude mice	VEZF1 VEGF	—	—	miR-19b-1 can activate VEGFR to decrease angiogenesis in BC.	Yin et al. (2018)
miR-199b-5p	-	T47D, MCF-7, and BT474	Nude mic	ALK1	ALK1/ Smad/Id1	—	miR-199b-5p targets ALK1 to suppress angiogenesis in BC.	Lin et al. (2019)
miR-542-3p	72 breast cancer patients	HMECs, SVEC4-10, 4T1 and HEK293T	—	Ang2	—	With the late stages of BC, miRNA-542-3p could be useful for tracking disease progression	Breast cancer miR-542-3p targets Angiopoietin-2 to decrease tumor angiogenesis	He et al. (2014)
miR-542-3p	52 breast cancer patients	MDA-MB-231 and HEK293T	Nude mice	Angpt2	—	Lower miR-542-expression was linked to increased ANG expression.3p expression	Breast cancer miR-542-3p targets angiopoietin-2 to decrease tumor angiogenesis	He et al. (2015)
miR-497	45 pairs of tumors and ANTs	MCF-7 T-74D, MDA-MB-453, MDA-MB-435 MDA-MB-468	BALB/c nu/nu mice	HIF-1α and VEGF	miR-497/HIF-1α pathway	—	miR-497 overexpression reduced the expression of HIF-1 and VEGF.	Wu et al. (2016)
miR-519c	34 breast cancer tissues	MCF-7, SKBR3, MDA-MD431, MDA-MB231, T47D	BALB/c Nude mice	HIF-1α	HGF/c-Met signaling pathway	—	miR-519c controls angiogenesis by inhibiting HIF-1 *in vitro* and *in vivo*	Cha et al. (2010)
miR-573 and miR-578	43 FFPE	HEK293, MCF-7 and SUM149PT	—	VEGFA and ANGPT2	VEGF, FAK, and HIF-1 signaling pathways	—	miR-573 and miRNA-578 can control angiogenic markers like HIF-1, VEGF, and focal adhesion kinase	Danza et al. (2015)
miR-29b	—	MDA-MB-231	Nude	Akt3	—	—	miR-29b slows tumor development by targeting Akt3 and decreasing angiogenesis	Li et al. (2017)
miR-199b-5p	—	MDA-MB-231		ALK1	ALK1/ Smad/Id1	—	miR-199b-5p overexpression decreased tumor development and angiogenesis	Lin et al. (2019)

(Continued on following page)

TABLE 1 | (*Continued*) miRNAs that inhibit angiogenesis in Breast cancer (ANTs: adjacent non-cancerous tissues).

miRNA	Numbers of clinical samples	Assessed cell line	Animal model	Targets/ Regulators	Signaling Pathways	Association with patients' outcome	Function	References
miR-204	58 breast cancer samples	MCF-7, MDA-MB-231, ZR-75, MDA-MB-453, T457-D	Nude mice	FOXC1, RAB22A, SMAD4	—	Deregulated expression of microRNAs has been associated with angiogenesis	miR-204 reduces angiogenesis in BC cells by targeting FOXC1, RAB22A, and SMAD4	Flores-Pérez et al. (2016)
miR-204	—	MCF-7, MDA-MB-231, T457-D, ZR-75, MDA-MB-453	Athymic nu/nu mice	ANGPT1 and TGFβR2 genes	angiopoietin signaling	—	miR-204 targets proangiogenic genes (ANGPT1 and TGF-R2) to decrease cell proliferation, invasion, and angiogenesis	Flores-Pérez et al. (2016)

(Wu et al., 2016). Furthermore, it was shown that the levels of VEGF and HIF-1 protein were decreased due to the overexpression of miR-497 (Wu et al., 2016). Tu and his group (Tu et al., 2015) described that increasing levels of miR-497 in 4T1 cells suppressed the growth of BC cells, angiogenesis, and VEGFR2 expression when injected in transgenic mice with VEGFR2-luc. Further, it has been recommended (Wu et al., 2016) that miR-497 may serve as a novel treatment approach for BC via inhibiting proangiogenic molecules (HIF-1α and VEGF).

microR-155

MiR-155 expression is considerably increased in BC and is highly associated with high tumor grade, progressing stage, and lymph node, but is negatively associated with cancer survival (Chen et al., 2012). Tumors respond to hypoxia by activating a genomic pathway, including miRNAs dependent on HIF1α and the hypoxia-induced pathway. Recent data showed that miR-155 has a crucial role in HIF1α-induced angiogenesis, and its expression is differentially regulated in BC (Chang et al., 2011). In BC tissues, the comparative expression of miR-155 was substantially greater than in normal tissues. Increased levels of miR-155 were all associated with tumor grade, tumor stage, and lymph node metastasis (Chen et al., 2012). Furthermore, the levels of miR-155 are negatively related to VHL protein; an E3 ubiquitin ligase inhibits members of the HIF1 family (Kong et al., 2014). Additionally, in patients with breast cancer, an increase in the expression of miR-155 may offer a prognostic marker and therapeutic target.

microR-542-3p

The control of Angpt2 by miRNAs has been established, indicating that miRNAs are key modulators of angiogenesis (He et al., 2014). MiR-542-3p has been shown to serve as a tumor suppressor gene and has been linked to the control of various cancers (Long et al., 2016; Wang et al., 2018b), including breast cancer (Lyu et al., 2018). The level of miR-542-3p is inversely correlated to the clinical progression of patients with advanced-stage of BC. The use of an *in-vitro* BC mice model revealed that the overexpression of miR-542-3p could suppress tumor growth, formation of tubules, and metastasis (He et al., 2015). He et al. miR-542-3p downregulates the expression of angiogenin, allowing it to be overexpressed in tumor cells and promote angiogenic activation in both *in vitro* and *in vivo* models (He et al., 2015). CEBPB and POU2F1, which are transcription factors for miR-542-3p, were shown to be suppressed by angiogenin, which might function as a new tumor-endothelial cell signal pathway (He et al., 2015). As a result, miR-542-3p presents novel targets for BC prevention and therapy.

microR-568

The miR-568 has been found to be present in the circulation of women with breast cancer (Leidner et al., 2013). lncRNA Despite being well-known for promoting metastasis in several malignancies, such as BC, Hotair has the potential to alter the expression of gene patterns and inhibit the production of miR-568, a critical tumor suppressor gene (Li et al., 2014). Furthermore, it appears that miR-568 is causing low levels of NFAT5 expression, which in turn sustains VEGFC and S100A4 expression, both of which are angiogenic and metastatic transcriptional activators of NFAT5 (Li et al., 2014). Despite these findings, additional study is required to identify or better understand the connection between lncRNAs and miRNAs for more efficient treatment approaches.

microR-204

miR-204 is a new multi-target anti-angiogenic miRNA that fights BC. in MCF7 BC cells, miR-204 mediates tumor-suppressing effect, and expression of miR-204 induces the inhibition of proliferation, invasion, and metastasis through targeting p-AKT and p-PI3K significantly (Fan et al., 2019). *In vivo* vascularization and angiogenesis were similarly reduced in nu/nu mice by miR-204, which is consistent with previous findings (Salinas-Vera et al., 2019). The levels of proangiogenic ANGPT1 and TGFβR2 proteins were decreased in MDA-MB-231 BC cells after treating with miR-204 (Salinas-Vera et al., 2019). Furthermore, ectopic expression of miR-204 exhibits decreasing vascular endothelial growth factor (VEGF) levels and a reduced number of brunches of capillary tubes (Salinas-Vera et al., 2018). Conclusively, multiple proteins associated with the PI3K/AKT, RAF1/MAPK, FAK/SRC, and VEGF pathways were downregulated and phosphorylated due to increased levels of miR-204 (Salinas-Vera et al., 2018; Hong et al., 2019a). This

new finding reveals miR-204 has a new, yet-unproven role in regulating the crucial synergy of the PI3K/AKT/FAK mediators that are important in VM development.

microR-29

The family of miR-29a, miR-29b, and miR-29c genes have similar structures, have a high degree of sequence similarity, and serve as a target-identifying foundation for molecules (Sun et al., 2018). miR-29b acts as a tumor suppressor, which inhibits angiogenesis and tumor development. However, in several malignancies, including endometrial carcinoma (Chen et al., 2017), HCC (Fang et al., 2011), ovarian cancer (Sugio et al., 2014), and BC (Li et al., 2017), miR-29b levels are downregulated. miRNA-29b Expression in breast cancer impairs the development of capillary-like tubular structures in HUVECs, as well as their ability to proliferate, migrate, and stop tumor progression (Li et al., 2017). Moreover, miR-29b acts to target Akt3 and inhibit angiogenesis and tumor growth by acting as an anti-angiogenesis and anti-tumorigenesis agent through the VEGF and C-myc arrest in BC cells (Li et al., 2017). Importantly, miR-29b might be an efficient anti-cancer treatment by way of therapeutic administration.

microR-4500

MiR-4500 was expressed poorly in BC cell lines, and RRM2 was a target gene (Li et al., 2020a). Additionally, a high expression of miR-4500 is seen in BC cells. Its expression in BC cells impairs the MAPK signaling through control of RRM2, which decreases proliferation, invasion, and angiogenesis while causing apoptosis. (Li et al., 2020a). Based on the findings that therapeutic methods should target the elevation of miR-4500, which might be a therapeutically feasible target in breast cancer treatment, this implies that therapies should focus on raising miR-4500 levels. However, to completely understand the precise processes and mechanisms of miR-4500 in BC, further studies are required on tissues from patients and additional BC cell lines.

microR-200

It has been determined that miR-200 can inhibit angiogenesis in breast cancer's environment and make it a therapeutic potential substance. Pecot et al. showed that miR-200 reduces angiogenesis via targeting interleukin-8 and CXCL1, which are produced by the tumor endothelium and cancer cells, and indirectly by targeting interleukin-8 (Pecot et al., 2013).

Furthermore, miR-200 family members have been shown to regulate the formation of blood vessels and angiogenesis by suppressing VEGF signaling ((Choi et al., 2011)). *In vitro*, angiogenesis was inhibited by miR-200b, which caused activation of the Notch system, which then triggered wound healing (Qiu et al., 2021). Thus, by modulating the expression of VEGF, the miR-200 family may offer a possible anti-angiogenesis treatment for treating cancer and other illnesses dependent on angiogenesis.

microR-190

In BC cells, miRNA-190 has the potential to target STC2 negatively, and through suppressing the AKT-ERK pathway, it has the potential to impede migration, invasion, EMT, and angiogenesis ((Sun et al., 2019)). Angiogenesis is influenced by the tumor microenvironment, which alters the cellular mechanisms required for vascular growth. MiRNA-190 suppresses the tumor microenvironment by targeting a set of angiogenic genes including RAS2, TCF4, HGF, Smad2, Smad4, IGF1, Jak2, and VEGF *in vivo* and *in vitro* (Hao et al., 2014). Furthermore, these genes, targeted by miRNA-190, have been shown to control VEGF expression (Hao et al., 2014). Moreover, it has been demonstrated that miR-190 substantially inhibits tumor metastasis, particularly angiogenesis (Hao et al., 2014). These findings collectively suggest that miR-190 is a promising anticancer target in therapeutic applications.

microR-148a

In various kinds of cancer, including BC (Yu et al., 2011), HCC (Pan et al., 2014), and ovarian cancer (Wang et al., 2018c), miRNA-148a-3p works as a tumor suppressor that is substantially downregulated. miRNA-148b-3p has also been implicated in the control of carcinogenesis, according to recent research. Interestingly, both miRNA-148a and miRNA-148b are essential regulators of EC migration in responses to VEGF, and they are also important regulators of EC proliferation. Because miRNA-148a/b-3p targets NRP1, upregulating its expression in ECs from its normally low endogenous levels has a significant inhibitory effect on VEGF-induced activation of VEGFR2 and downstream signaling (Kim et al., 2019). HIF-1a expression, which is required to form a functional HIF-1 transcription factor, was likewise reduced by MiR-148a. HIF-1 is a transcription factor that regulates the production of VEGF and other angiogenesis regulators (Semenza, 2003). As a result, we believe that miR-148a suppresses angiogenesis in breast tumors.

Furthermore, it has been established that miR-148a overexpression inhibited the angiogenesis produced by MCF7 cells in BC by directly targets ERBB3 (Yu et al., 2011). Additionally, overexpression of miR-148a targets IGF-IR and IRS1 suppresses BC cells proliferation and tumor angiogenesis by suppressing their downstream AKT and MAPK/ERK pathways (Xu et al., 2013). Following these findings, it appears that miRNA-148a might potentially be a promising therapeutic target in cancer treatment in the upcoming years.

microR-199b-5p

According to previous studies, miRNA-199b-5p is a tumor suppressor. It was revealed to be downregulated in various BC cell lines and reduced in VEGF-induced human umbilical vein epithelial cells (HUVECs) (Lai et al., 2018; Lin et al., 2019). Interestingly, the migration and angiogenesis of HUVECs were decreased by ectopic expression of miRNA-199b-5p, whereas inhibition of miRNA-199b-5p induced the reverse effect. Similarly, HUVECs treated with high levels of miR-199b-5p exhibited suppressed ALK1 mRNA and protein production due to direct binding to the 3′UTR of ALK1 (Lin et al., 2020). Aside from that, high levels of miR-199b-5p in HUVECs reduced the activation of the ALK1/Smad/Id1 signaling by BMP9 in BC. As a result of these findings, miR-199b-5p, which primarily affects ALK1, has been identified as an anti-angiogenic factor, suggesting that miR-199b-5p might be used as an anti-angiogenic strategy in treating cancer.

TABLE 2 | miRNAs that promote angiogenesis in Breast cancer (ANTs: adjacent non-cancerous tissues).

miRNA	Numbers of clinical samples	Assessed cell line	Animal model	Targets/ Regulators	Signaling Pathways	Association with patients' outcome	Function	References
miR-20a	breast cancer patients (n = 108)	MCF7 MDA-MB-231	—	VEGFA	—	Promoting metastasis	miR-20a promotes aberrant vascular mesh size and excessive VEGFA expression	Luengo-Gil et al. (2018)
miR-20a/b	32 breast cancer patients 16 controls	—	—	VEGF-A and HIF-1alpha	—	metastatic heterogeneity	In BC patients, VEGF-A and HIF-1alpha target proteins correlated negatively with miR-20a/b	Li et al. (2012)
miR-20b	23 paired clinical breast cancer tissues and ANTs	MCF7, SK-BR-3 T-47D ZR-75-30	Nude mice	PTEN	PTEN-PI3K-Akt pathway	—	miR-20b acted as a tumor promoter by targeting PTEN expression	Zhou et al. (2014)
miR-21	—	MVT1 and E0771	FVB/N Nude mice	Col4a2, Spry1, and Timp3	CSF1-ETS2 pathway	—	miR-21 promotes tumor metastasis and angiogenesis by suppressing the CSF1-ETS2 pathway	Isanejad et al. (2016)
miR-29	79 breast cancer samples and 60 pairs of tumors	MCF-10A, MCF-7 MDA-453, MDA-231	BALB/c Nude mice	—	TET1	poorer prognosis	miR-29a stimulates BC cell proliferation and EMT through TET1	Pei et al. (2016)
miR-93	—	MT-1	Nude mice	LATS2	—	—	miR-93 targets LATS2 to promote angiogenesis and metastasis	Fang et al. (2012)
miR-10b	-	HMEC-1, MDA-MB-231	Nude mice	HoxD10	—	—	miR-10b targets HoxD10 mRNA to induce angiogenesis	Shen et al. (2011)
miR-655/ 526b	105 tumor tissue samples 20 non-cancerous tissues	MCF7 and MDAMB231	—	VEGFA, VEGFD, VEGFC, COX-2 *PTEN* LYVE-1	PI3K/Akt and ERK pathways	—	miR-526b-655 induce both angiogenesis and lymph angiogenesis in BC.	Hunter et al. (2019)
miR-155	231 breast cancer patients	BT474 HS578T MDA-MB-157	nude mice	VHL	—	poor prognosis	miR-155 stimulates tumor angiogenesis and proliferation by targeting VHL.	Kong et al. (2014)
miR-9	—	BT-474, MDA-MB-231	—	LAMC2, ITGA6, and EIF4E	—	—	miR-9 targets mRNA from genes involved in VEGF expression (LAMC2, ITGA6, EIF4E)	Kim et al. (2017)
miR-10b	130 patients	—	—	HOXD10	—	breast cancer aggressive behavior, distant metastasis, and poor prognosis	miR-10b expression associated with metastases and angiogenesis in node-negative breast cancer	Liu et al. (2017)
miR-182	45 pairs of tumors and ANTs	MCF-7 EA. hy926 H184B5F5/M10	—	FBXW7	HIF-1α-VEGF-A pathway	promote breast cancer progression	miR-182 promotes breast cancer angiogenesis by increasing HIF-1 expression	Chiang et al. (2016)
miR-183-5p	50 pairs of BC tissues and neighboring non-tumor breast tissues	BT549, MCF-10A SK-BR-3, MDA-MB-231, MCF7, MDA-MB-453, BT20	—	FHL1	—	poor prognosis	The miR-183-5p inhibits FHL1 and hence increases tumor proliferation and angiogenesis	Li et al. (2020b)
miR-373	196 breast cancer patients 76 Benign patients 49 Healthy controls	—	—	VEGF and cyclin D1	—	miRNA-373 expression level has unfavorable prognostic factors for breast cancer	By targeting VEGF and cyclin D1, miR-373 increases angiogenesis and metastasis	Bakr et al. (2021)
miR-210	299 paraffin-embedded breast cancer tissue	—	—	HIF-1α	—	breast cancer progression	miR-210 induced angiogenesis by targeting HIF-1α -VEGF signaling	Foekens et al. (2008)

microRNA-195

miRNA-195 is one of the genetic markers found on chromosome 17p13.1, known as the origin of intron 7. miRNA-195 has been characterized as a tumor suppressor molecule that is often dysregulated in many malignancies, such as BC (Yu et al., 2018). miR-195's anti-cancer activity is attributed to its target molecules, FASN, ACACA, HMGCR, and IRS1, which help slow down BC cell growth, migration, angiogenesis, and metastasis. (Singh et al., 2015; Wang et al., 2016). In both BC cell lines and BC tissues, miR-195 is negatively linked to Insulin receptor substrate 1 (IRS1) (de Sales et al., 2021). IRS1 expression is downregulated by induction miR-195 using a miR-195 oligo transfection method or infection with a lentivirus containing the miR-195 gene (Wang et al., 2016; Lai et al., 2018). These findings imply that miR-195 replicas are promising BC treatment agents.

Pro-angiogenic microRNAs in Breast Cancer

miRNAs are involved in the initiation and progression of several tumor characteristics, such as tumor invasion, angiogenesis, and metastasis. Recent investigations have suggested that several miRNAs suppress angiogenesis in the BC. These studies present novel therapy options for treating angiogenesis in BC (**Table 2**).

microR-20b

miRNA-20 is a member of the miRNA-17–92 cluster, and it has been characterized as an oncogenic miRNA molecule that is often dysregulated in many malignancies, such as BC (Li et al., 2012). miRNA-20b is a potential oncogene that affects the control of VEGF expression in MCF-7 breast cancer cells by targeting HIF-1α and STAT3 (Cascio et al., 2010). The other study found that miR-20b was elevated in human BC tissues and predicted that the anti-oncogenic PTEN gene might target miRNA-20b (Zhou et al., 2014). These data suggest that miR-20b may be used as a potential biomarker and a viable target for diagnosing and treating breast cancer.

microR-29a

In several types of malignancies, including esophageal (Liu et al., 2015a), colon cancer (Leng et al., 2021) and BC (Rostas et al., 2014) miR-29a was shown to be upregulated. The essential process that leads to metastasis is the EMT. miR-29a, which is upregulated in many types of cancer, has been shown to promote EMT in BC (Rostas et al., 2014). Furthermore, *in vivo* and *in vitro*, miRNA-29a upregulation led to TET1 reduction, which increased cell proliferation and EMT in BC (Pei et al., 2016). Thus, it appears that miR-29a is a novel biomarker for BC detection and a possible treatment target.

microR-9

The miRNA-9 expression is abnormal in many cancers; however, the Role of miR-9 in cancers is still debated, in some studies indicating that it is a proangiogenic oncomiR as in BC (Bertoli et al., 2015) or as a tumor suppressor such as in melanoma (Bu et al., 2017). Kim et al. recently discovered that miRNA-9, an angiogenic mediator, selectively targets mRNA from genes involved in stimulating VEGF expression (Kim et al., 2017). Their study observed that miR-9 could inhibit the production of VEGFA by binding to the products of the ITGA6 gene, which encodes α6β4 integrin complex subunit in BC cell lines (Kim et al., 2017). *In vitro*, it has already been established that the integrin (α6β4) subunit enhances the VEGF expression by activating the mTOR pathway (Soung et al., 2013). Similarly, miR-9-mediated E-cadherin increases VEGFA expression in breast cancer via activating beta-catenin signaling in animal models and cell lines (Ma et al., 2010).

Furthermore, the same study found that miRNA-9 targets the CDH1 expression, which is translated into E-cadherin protein and increases the nuclear localization and activity of β-catenin (Ma et al., 2010), which are both essential in tumorigenesis (Yu et al., 2019). Lastly, they observed that upregulation or elevated levels of miRNA-9 in breast cancers promotes angiogenesis (Ma et al., 2010). These findings provide evidence that miRNA-9 has a proangiogenic function for the development of cancer.

microR-10b

The miR-10b genomic location is in front of the HOXD4 gene and has more attention because of its highly conservative (Tehler et al., 2011). *In vivo* and *in vitro* studies have revealed the importance of miR-10b in angiogenesis (Lin et al., 2012). It was shown that axillary lymph node-negative breast cancer patients had an increased microvessel density (MVD), which was correlated with raised miRNA10b (Liu et al., 2017). Plummer and his colleagues found that miR-10b shows increased expression and promotes stimulation of VEGF in high-grade human breast cancer (Plummer et al., 2013). The increased expression of miR-10b is considered to have a role in increasing the capacity of endothelial cells to create blood vessels by reducing the anti-angiogenic pathway gene expression (Shen et al., 2011). By binding to the 3′ UTR of HoxD10 mRNA, miRNA-10b is capable of targeting HoxD10 mRNA and inhibits the production of HoxD10 protein (Shen et al., 2011). Interestingly, FLT1 is believed to inhibit VEGF and VEGFR2 interaction from stopping the development of new blood vessels (Fong et al., 1999). It has been observed that inhibiting the expression of miR-10 in HUVECs exposed to low concentrations of VEGF decreases the VEGFR2 phosphorylation, which inhibits the VEGF-dependent angiogenesis (Hassel et al., 2012).

microR-21

miR-21, a hypoxia-inducing miRNA, has participated in developing cancer, angiogenesis, and stimulation of VEGF signaling in patients with BC (Foekens et al., 2008). Oncogenic mi R-21, which is related to the advanced tumor stage, lymph node, and poor patient mortality, was found as a potential molecular prognosis mark for BC development (Yan et al., 2008). In a VEGFR2-Lucent mice model of BC, a miR-21 antagomir substantially decreased cancer growth and tube formation by directly inhibiting the VEGF/VEGFR2/HIF1 pathway (Zhao et al., 2013). Interestingly, this work demonstrated that miR-21 inhibition causes apoptosis in BC

TABLE 3 | Phytochemicals and their target miRNAs regulating tumor angiogenesis.

Compound	miRNA	Assessed cell line	Animal model	Target genes	Signaling Pathways	References
Melatonin	↑miR-152-3p	MDA-MB-468	Female BALC/c Nude mice	↓ angiogenesis by ↓IGF-1R ↓HIF-1α, ↓VEGF	miR-152/IGF-IR, HIF-1α, VEGF pathway	Marques et al. (2018)
	↑miR-148a-3	MDA-MB-231	Nude mice	↓ angiogenesis by ↓IGF-1R, ↓VEGF ↓ migration, ↓ invasion of BC cells	IGF-1R/VEGF pathway	Lacerda et al. (2019)
Metformin	↓miR-21	HUVEC	—	↓ migration, ↓ proliferation ↓ angiogenesis, ↓ TGF-β ↑PTEN, ↑SMAD7	TGF-β/PTEN/PI3k/AKT pathway	Luo et al. (2017)
	↑miR-26a	MDA-MB-468 MDA-MB-231 MCF-7	—	↓ cell viability, ↓ Bcl-2 ↓PTEN, ↓EHZ2	PTEN/AKT/PKB pathway	Cabello et al. (2016)
	↓miR-181a ↑miR-let-7a ↑miR-96	MCF-7	—	↓TGFβ and ↓ mamosphere-forming efficiency, ↓EMT	TGFβ-signaling pathway	Oliveras-Ferraros et al. (2011)
	↓miR-21-5p	MCF-7 BT-549 BT-474, SUM159PT	—	↑ AMPK, ↑CAB39L, ↑Sestrin-1 → ↓ mTOR synergistically with everolimus → ↓ cell invasion and growth	AMPK/mTOR pathway	Pulito et al. (2017)
	↓miR-21 ↓miR-155	MDA-MB-23 MCF-7	—	↓ ROS → ↑ SOD ↓ MMP-2, ↓MMP-9, ↓ Bcl-2 ↑ apoptosis and ↓ proliferation	ROS-independent pathway	Sharma and Kumar (2018)
	↑miR-200c	MDA-MB-231 MCF-7, BT549, T-47-D	SCID mice	↓ growth, migration and invasion, ↑ apoptosis ↓AKT2, ↓ c-Myc, ↓Bcl-2	metformin/c-Myc/miR-200c/AKT2/Bcl-2 pathway	Zhang et al. (2017)
Cardamonin	↓miR-21	HUVEC	—	↓VEGF mediated angiogenesis ↓ proliferation and migration of endothelial cells	miR-21/VEGF signaling	Jiang et al. (2015)
(Silibilin + EGCG)	↓ miR-17 ↓ miR-18a ↓ miR-20a ↑miR-19b ↑miR-92a	HUVEC A549	—	Synergistically ↓VEGF and ↓VEGFR2	VEGF/VEGFR2 pathway	Mirzaaghaei et al. (2019)
Curcumin	↑miR-15a ↑miR-16	MCF-7	—	↓Bcl-2	miR-15a/16-Bcl-2 apoptotic pathway	Yang et al. (2010)
	↑miR-34a	MDA-MB-231 MDA-MB-435	—	↓ proliferation, survival, invasion and ↑apoptosis ↓ Bcl-2, ↓Bmi-1	Bcl-2 mediated apoptotic pathway	Guo et al. (2013)
	↑miR-181b	MDA-MB-231	—	↓proliferation, ↑apoptosis by ↓ Bcl-2, ↓survivin, ↓MMP-1, ↓MMP-3 Anti-metastatic effect by ↓CXCL1, ↓CXCL 2	Bcl-2 mediated apoptotic pathway	Kronski et al. (2014)
	↑miR-34a	MCF-10F MDA-MB-231	—	↓ cell viability, ↓ cell migration and invasive ness ↓EMT (Axl, Slug, CD24) ↓Rho-A	Rho -signaling pathway	Gallardo et al. (2020)
	↓miR-21	MCF-7	—	↑ caspase 3/9→ ↑ apoptosis ↑ PTEN, ↓ p-AKT	miR-21/PTEN/Akt pathway	Wang et al. (2017)
Hesperidin and luteolin	↓miR-21 ↑ miR-16 ↑ miR-34a	MCF-7	—	↓ cell viability, ↑ apoptosis, ↓Bcl-2 ↑ Bax	Bcl2/Bax apoptotic pathway	Magura et al. (2020)
Quercetin	↓miR-21	MCF-7	—	↑Maspin, ↑PTEN, ↓ cell viability	PTEN/maspin pathway	Panahi, (2018)
betulinic acid	↓miR-27a	MDA-MB-231, BT-549	—	↑ ZBTB10, ↓Sp1, Sp3, Sp4 ↑ Myt-1	miR-27a/ZBTB10/Sp-axis	Talcott et al. (2008)
	↓miR-27a	MDA-MB-231	Female athymic Nude mice	↓ angiogenesis by ↓Sp1, Sp3 and Sp4, ↑ ZBTB10 ↑ cell cycle arrest in G2/M phase ↑ Myt-1, ↓VEGFR, and ↓survivin in mice ↓ hβ2G in lung of mice	miR-27a/ZBTB10/Sp-axis	Mertens-Talcott et al. (2013)

(Continued on following page)

TABLE 3 | (*Continued*) Phytochemicals and their target miRNAs regulating tumor angiogenesis.

Compound	miRNA	Assessed cell line	Animal model	Target genes	Signaling Pathways	References
Glabridin	↑miR-148a	MDA-MB-231 Hs-578T	—	↓ angiogenesis by ↓Wnt/β-catenin pathway and ↓ VEGF secretion	miR-148a/Wnt/β-catenin signaling	Alhasan, (2019)
Pomegranate	↓ miR-27a	BT474	Female athymic BALB/c Nude mice	↓ Sp1, Sp3 and Sp4 → ↑ ZBTB10	miR-27a/ZBTB10/Sp-axis	Banerjee et al. (2012)
	↓miR-155	MDA-MB-231		↓ cyclin D1, ↓Bcl-2, ↓surviving ↓ VEGF and VEGF1-R, ↓ NF-$_k$B ↑ SHIP-1→↓ pPI3K and ↓ pAKT ↓ NF-$_k$B	miR-155/SHIP1/PIP3/AKT/NF-kB- axis	
Mango	↑miR-126	BT474	Female athymic BALB/c Nude mice	↓PI3k/AKT pathway, ↓HIF-1α, ↓VEGF, ↓ NF-$_k$B, ↓ mTOR	miRNA-126/PI3K/AKT -axis	Banerjee et al. (2015)

cells and HUVECs via upregulating the PTEN gene. This microRNA has also been shown to be effective in inhibiting angiogenesis.

Evolving angiogenesis in mice carrying BCs with a luciferase gene that activates the VEGF/VEGFR2 pathway may be stopped if a molecule called miR-21 is inhibited by blocking HIF-1α/VEGF/VEGFR2 signaling (Zhao et al., 2013). Furthermore, this work demonstrated that blocking miRNA-21 leads to PTEN overexpression, contributing to BC cell and HUVEC death. The presence of miRNA-21 has also been shown to inhibit the growth of new blood vessels. Liu et al. exposed that a BC oncogene, metadherin (MTDH), can promote angiogenesis by stimulating the miR-21/ERK-VEGF-MMP2 pathway (Liu et al., 2015b). According to recent research, exercise and hormone treatment decreased tumor growth and angiogenesis in a mouse model of invasive breast cancer by reducing levels of miR-21, ER, HIF-1, VEGF, and raising levels of miR-20 IL-10, let-7a, and PDCD4 in tumor tissue (Isanejad et al., 2016). Thus, reductions in miR-21 levels are associated with an anti-angiogenic response in breast cancer. Additionally, miR-21 can enhance tumor metastasis and angiogenesis by inhibiting anti-angiogenic genes such as TIMP3, COl4a2, and Spry1 in tumor-infiltrating myeloid cells (Isanejad et al., 2016).

microR-93

It is located on the 7th chromosome and is part of the miRNA-106b-25 cluster. miR-93 is one of the miRNAs frequently found to be overexpressed in tumors (Sun et al., 2018). Previous research has discovered that miRNA-93 is increased in BC and that it functions as an oncomiR, promoting angiogenesis. (Fang et al., 2012; Liang et al., 2017; Sun et al., 2018). Fang and his team found that miRNA-93 is required to promote angiogenesis, enhanced EC proliferation and migration, and tube formation (Fang et al., 2012). Also, miRNA-93 is upregulated in breast cancer, stimulating new blood vessel growth by blocking the homology 2 gene (LATS2) (Fang et al., 2012). MiR-93, on the other hand, appears to have a function in the inhibition of angiogenesis in some pathological conditions (Fabbri et al., 2016). According to the study by Liang et al., TNBC specimens with greater levels of miR-93-5p had increased blood vessel density. They also revealed that overexpressing miRNA-93-5p in HUVECs enhanced proliferation, migration, and cell sprouting *in vitro*, but inhibiting miRNA-93-5p reduced migration and angiogenic ability (Liang et al., 2017). miR-93 is involved in tumor angiogenesis by inhibiting several targets, particularly VEGF, EPLIN, integrin-β8, IL-8, and LATS2 in TNBC tissues (Liang et al., 2017). Fang et al. have demonstrated that tumor xenografts formed from breast cancer cell lines transfected with miR-93 showed increased vascular density and metastatic ability and a greater capacity for lung metastasis than tumors transfected with a vector without miR-93 (Fang et al., 2012).

Additionally, they found that miR-93 might promote invasion and tumor angiogenesis by silencing LATS2 expression. Furthermore, miR-93, an oxygen-responsive microRNA, might disrupt NCOA3, an epigenetic factor that mediated tumor suppression and inhibits cGAS-mediated antitumor immunity in breast cancer (Wu et al., 2017). Due to this, tumor angiogenesis may be promoted by the hypoxia-regulated miRNAs like miR-93, which also participates in immunosuppression. Altogether, miRNA-93 has a dual impact on angiogenesis in various human tissues and cells, and these effects are mediated through a variety of molecular pathways.

microR-182

The miRNA-182-183-96 cluster contains miR-182, which is located on chromosome 7q32. Overexpression of miR-182 has been found in BC cells, and this miRNA inhibits FOXO1, MTSS1, MIM, and BRCA1 and, therefore, negatively impacts cell proliferation angiogenesis and DNA damage response (Guttilla and White, 2009; Lei et al., 2014). Furthermore, increasing miR-182 expression leads to an increase in HIF-1α and VEGFA activation by direct targeting FBXW7 induces angiogenesis in BC tissues (Chiang et al., 2016). In addition to its involvement in regulating ubiquitin ligase (SCF) activity, tumor suppressor FBXW7 is essential for SCF complex activity, which controls the degradation of a variety of oncogenic proteins, such as HIF-1, Notch, cyclin E, and c-myc (Flügel et al., 2012). Thus, we conclude that miR-182 contributes to breast cancer invasion, angiogenesis, and metastasis based on the above studies.

microR-210

miR-210, a hypoxia-inducing miRNA, has been primarily described as an oncomiR. Overexpression of miRNA-210 is a critical component of EC survival, angiogenesis, and differentiation in response to hypoxia (Fasanaro et al., 2008). In research conducted by Jung et al. (Jung et al., 2017), HIF-1 and miR-210 were overexpressed in exosomes produced from mouse BC cells during a hypoxic microenvironment. Exosomes carrying miR-210 were transfected into HUVEC cells, efficiently decreasing PTP1B and Ephrin-A3 expression and promoting angiogenesis by targeting VEGF signaling (Jung et al., 2017). MiR-210 expression was highly correlated with VEGF expression, hypoxia, and angiogenesis in breast cancer patients, suggesting that miR-210 may play a role in tumor angiogenesis. Although this association is significant, it is not adequate to evaluate whether miR-210 is a functional regulator of BC angiogenesis due to its substantial increase under hypoxic circumstances. An additional miR-210 target, the protein tyrosine phosphatase Ptp1b, has been discovered as a factor that promotes angiogenesis and suppresses cellular death in the setting of a mouse myocardial infarction (Hu et al., 2010). Earlier studies demonstrated that PTP1B might bind to and inhibit the activation of a VEGF receptor, VEGFR2, and inhibit the tyrosine phosphorylation of VEGFR2 in endothelial cells stabilizing cell-cell adhesions (Nakamura et al., 2008). Taken together, because of its ability to suppress Ptp1b and Efna3, miR-210 might facilitate angiogenesis.

microR-467

The miR-467 was found to be a specific inhibitor of TSP-1, which was reported to be elevated in the BC cells after glucose stimulation (Soheilifar et al., 2021). It was demonstrated that the miR-467 mimic increased the number of BC cells in the matrigel plugs in mice, indicating the proangiogenic activity of miR-467 *in vivo*. MiR-467, on the other hand, was unable to stimulate angiogenesis in the absence of TSP1Additionally, it was demonstrated that the amount of miR-467 in BC tumors increased, and a link between the expression miR-467 and tumor mass was shown in STZ-treated hyperglycemic mice. Similar results were found in the hyperglycemic Leprdb/db mice, with miRNA-467 hyperactivity leading to increased tumor growth and angiogenesis (Krukovets et al., 2015). Also, in the animal models, it was discovered that miRNA-467 blockers reduced tumor development and angiogenesis indicators (Krukovets et al., 2015). These findings show that hyperglycemia causes angiogenesis by increasing the expression of miR-467.

microR26b and microR562

Both miR26b and miR562, tumor suppressor microRNAs, are found on human chromosomes 2q37.1 and 2q35, respectively. The expression of both NF-κB1 (p105) and NF-κB subunit RELA (p65) are directly repressed by miR26b and miR562, and this is linked with angiogenesis in breast cancer patients (Anbalagan et al., 2014). Many pathways, including the PI3K/AKT pathway, are associated with NF-κB signaling (Ghafouri-Fard et al., 2021a; Hussen et al., 2021b). In addition, the phosphatidyl inositol 3-kinase/Akt signaling is involved in the production of HIF-1 and VEGF, and it is essential for the development of blood vessels in the BC (Karar and Maity, 2011; Li et al., 2015). Because of this, miR-26b and miR-562 lead to BC angiogenesis through the activation of NF-κB, PI3K/AKT, HIF-1α, and VEGF pathways.

microR-655/526b

The expression of miRNA-655/526b is considerably greater in human BC, and the higher expression of miR-655/526b is linked with a poorer prognosis (Gervin et al., 2020). *In vitro* research showed that COX2, an inflammatory enzyme elevated in BC, increases the expression of miR-655/526b (Majumder et al., 2015; Majumder et al., 2018). Furthermore, the researchers discovered that miR-655/526b transfection led to increases in the levels of angiogenic molecules like VEGFC, VEGF-D, COX2, and LYVE1 (Hunter et al., 2019). It was also shown that the expression of VEGFR1, which controls the growth of blood vessels, was increased in cell lines treated with both miRNAs. Additionally, HUVEC cells treated with medium containing miR-655/526b generated tubular structures (Hunter et al., 2019).

ANTI-ANGIOGENIC MICRORNA-BASED THERAPY

One of the most essential proposed strategies to combat and prevent cancer metastasis is to target angiogenesis pathways (Lee et al., 2015). Tumors start to produce a myriad of proangiogenic factors early during tumorigenesis to form their vasculature (Carmeliet and Jain, 2011), the anti-angiogenesis strategy, which was first suggested by Judah Folkman in 1971 now a day is considered an effective and promising antitumor strategy (Folkman, 1971), (Ebos and Kerbel, 2011). Therefore, targeting angiogenic miRNAs can be gained by either 1) miRNA-based therapeutics or 2) drugs and phytochemicals that already affect angiogenic miRNA. This category has additional advantages of being available and almost safe, and their toxicity and side effects are well studied (Varghese et al., 2020).

microRNA-Based Therapeutics in Breast Cancer

miRNA-based therapeutics with antitumor and/or anti-angiogenic effects are achieved by either substituting or restoring tumor suppressor miRNAs activity or silencing overexpressed endogenous oncogenic miRNAs (Boca et al., 2020). In which mimic sequencing is used for restoring tumor suppressor miRNA (miRNA mimics) and exogenous delivery of antagonists (oligonucleotides that are chemically modified) for silencing endogenous oncogenic miRNAs (antagomir) (Caporali and Emanueli, 2011). Achieving efficient and targeted delivery of miRNAs mimics or antagomirs to targeted cancer tissues is of paramount importance. Some successful modalities have been researched extensively, yet a major obstacle still in progress is to be translated more successfully into clinical practice (Ganju et al., 2017). miRNA therapeutics can be delivered by either viral or non-viral vectors. Nano-technology delivery of miRNA

therapeutics, a non-viral vector, sounds promising with less systemic toxicity and several types of nanocarriers being available in practice, each with a unique formulation, advantages, and disadvantages (Boca et al., 2020).

Drugs and Phytochemicals Harboring Anti-angiogenic Activity in Breast Cancer

Melatonin

Melatonin is a flexible anti-cancer agent that has been studied extensively in various malignancies, including breast cancer. According to a recent study, melatonin has anticancer effects ranging from antiproliferative to increased apoptosis of breast cancer cells at physiological and pharmacological doses. Melatonin also has anti-metastatic properties and can reduce antitumor resistance and toxicity (Kong et al., 2020). Cheng et al. showed that melatonin reduces cellular viability and has an anti-angiogenic effect on HUVECs via the downregulation of the HIF1/ROS/VEGF axis, in which melatonin exerts these effects by directly inhibiting hypoxia-induced HIF1 and indirectly acting as a free radical scavenger, resulting in a reduction of ROS and VEGF crosstalk (Cheng et al., 2019).

Additionally, melatonin can also disrupt the development of vasculogenic mimicry (VM) via breast cancer cell lines in both normoxic and hypoxic conditions. VM mediates resistance toward anti-angiogenic drugs and has a significant role in breast cancer metastasis (Maroufi et al., 2020). Another study found that melatonin not only downregulates VEGF mRNA but also simultaneously downregulates ANG1 and ANG2 in breast cancer cells (González-González et al., 2018). miR-148a/152 overexpression is associated with marked inhibition of breast cancer cell proliferation and angiogenesis by targeting IGF-1R and IRS1 and consequently their downstream signaling pathway (Xu et al., 2013). Melatonin can also regulate the aforementioned angiogenic miRNAs, and it can upregulate expression of miR-152-3p, "a tumor suppressor found to be downregulated in breast cancer," which in turn reduces the protein expression of IGF-insulin-like-like growth factor-1 receptor), HI, F-1,α, and VEGF (Marques et al., 2018). In another study, melatonin upregulated miR-148a-3 and reduced gene expression of IGF-1R and VEGF (Lacerda et al., 2019).

Metformin

Metformin, a widely used anti-diabetic, has been very extensively investigated in recent years for having several anti-cancer effects in breast cancer (DeCensi et al., 2014), (Goodwin et al., 2008), (Alimoradi et al., 2021). The most extensive ongoing clinical trial (NCT01101438) for using metformin in breast cancer will reveal metformin's Role in breast cancer in detail. Metformin has several mechanisms for its beneficiary effect in breast cancer; among them, it affects miRNAs. Metformin exerted an anti-angiogenic effect in breast cancer models by inhibitiHER2-mediated VEGF upregulation and HIF-1α-mediated VEGF up-regulation, suggesting a novel mechanism of metformin targeting the HER2/HIF-1α/VEGF signaling axis (Wang et al., 2015).

Additionally, it was found out that metformin inhibited proliferation, tube formation, and migration of HUVECs by downregulation of miR-21 and TGF-β protein expression, consequently increasing PTEN and SMAD7 protein expression (Luo et al., 2017). Furthermore, Metformin was found to reduce breast cancer cell viability, upregulated miR-26a, and reduced expression of miR-26a targets PTEN and EHZ2 in several breast cancer cell lines (Cabello et al., 2016). Although, metformin might have an impact on metastasis, potentially via altering the levels of miR-21 in various cancer cell lines and breast cancer patients (Pulito et al., 2017). Metformin was also found to suppress miR-21 and miR-155 and up-regulate miR-200c in breast cancer cells, accordingly suppressing proliferation and metastasis of breast cancer (Alimoradi et al., 2021), (Sharma and Kumar, 2018), (Zhang et al., 2017).

These metformin effects showed synergism with everolimus, providing a potential role for metformin to be used in conjunction with breast cancer treatments.

Phytochemicals

Cardamonin suppressed miR-21 in HUVECs and accordingly suppressed VEGF-induced angiogenesis and cell migration (Jiang et al., 2015). Epigallocatechin-3-gallate (EGCG) from green tea and silibinin from milk thistle is widely consumed shown to have a powerful anti-angiogenic effect. Both synergistically, they were found to downregulate VEGF and miR-17–92 cluster while upregulated anti-angiogenic miR-19b in HUVECs (Mirzaaghaei et al., 2019). Interestingly EGCG was also found to inhibit tumor cell growth and angiogenesis via suppressing HIF-1α, NFκB a, and VEGF (Gu et al., 2013). Meanwhile, silibinin downregulated miR-21 and miR-155 in T47D breast cancer cells (Zadeh et al., 2015).

Furthermore, curcumin was found to suppress the proliferation and induce apoptosis of cancer cells (Ghaderi et al., 2021), (Sobhkhizi et al., 2020) as well as impinge MCF-7 cells by upregulating miR-15a and miR-16, which caused downregulation of Bcl-2 (Fix et al., 2010). Curcumin also upregulated miR-34a and miR-181b in breast cancer cell lines and inhibited invasion and metastasis of them (Guo et al., 2013), (Kronski et al., 2014), (Norouzi et al., 2018). In addition, curcumin efficiently targeted and elevated protein expression of miR-34a in MCF-10F and MDA-MB-231 cell lines and consequently affected regulatory genes of EMT and Rho-A and attenuated tumor cell migration and invasiveness (Gallardo et al., 2020). Beside of the above, several additional phytochemicals, such as resveratrol, luteolin, and betulinic acid, have been shown to influence angiogenic miRNAs (Varghese et al., 2020) (**Table 3**).

CONCLUSIONS AND FUTURE PERSPECTIVES

MicroRNAs regulate tumor angiogenesis, a key component in cancer growth and metastasis. Identification of novel molecular features of angiogenesis regulation, and a greater understanding of cancer progression strategies, will allow the development of new therapeutic options. Numerous genes which involved in angiogenesis are regulated by miRNAs, therefore identifying

miRNA-target interaction networks might be useful in describing anti-angiogenic therapy and novel diagnostic biomarkers in BC. Angio-regulatory miRNAs may be used to produce a new generation of medicines such as nano-based therapeutics. Additionally, phytochemical medicines might modulate the expression of angio-regulatory miRs, which in turn could enhance survival in BC patients. Furthermore, we revealed the inhibiting and stimulating pathways of angio-regulatory miRNAs in cancer-related angiogenesis process, which may be useful in the developing anti-angiogenic methods in cancer therapy.

AUTHOR CONTRIBUTIONS

MT, SF and HJ wrote the draft and revised it. BH, SA, MR and AS collected the data, designed the figures and tables. All the authors read and approved the submitted version.

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Cervical Cancer, Papillomavirus, and miRNA Dysfunction

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Cervical cancer is the leading cause of death by cancer in women from developing countries. Persistent infection with high-risk human papillomavirus (HPV) types 16 and 18 is a major risk factor for cervical carcinogenesis. Nevertheless, only a few women with morphologic expression of HPV infection progress into invasive disease suggesting the involvement of other factors in cervical carcinogenesis. MicroRNAs (miRNAs) are conserved small non-coding RNAs that negatively regulate gene expression including genes involved in fundamental biological processes and human cancer. Dysregulation of miRNAs has been widely reported in cervical cancer. This work focuses on reviewing the miRNAs affected during the HPV infection process, as well relevant miRNAs that contribute to the development and maintenance of malignant cervical tumor cells. Finally, we recapitulate on miRNAs that may be used to distinguish between healthy individuals from patients with precancerous lesions or cervical tumors.

Keywords: cervical cancer, human papillomavirus, HPV, miRNA, microRNA

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INTRODUCTION

In 2020 there were 604,127 new cases and 341,831 deaths of women with cervical cancer worldwide (WHO, 2020). Clinically, cervical tumors can be classified as squamous cell carcinoma (SCC), corresponding to 70–80% of the cases, and adenocarcinoma (AC) with a 10–25% occurrence (Babion et al., 2020a). Other rare cervical tumors exhibit a variety of histological types including malignant adenoma, endometrioid carcinoma, clear cell carcinoma, papillary adenocarcinoma, adenoid cystic carcinoma, adenosquamous carcinoma, and undifferentiated carcinoma, which collectively represent less than 1% of the newly diagnosed cases (Berman and Schiller, 2017; Hull et al., 2020).

Infection with high-risk human papillomaviruses (HR-HPVs) is the primary risk factor for the development of cervical cancer (Castellsagué, 2008; Li and Xu, 2017). Through the expression of two oncogenic viral proteins E6 and E7, HR-HPVs are capable of orchestrating diverse molecular mechanisms that may result in progression to malignant disease. Although persistent infection by HR-HPVs is a necessary cause of cervical cancer, only a few women with morphologic expression of HPV infection progress to invasive disease (Castellsagué, 2008). Hence, other tumor promoter factors must be involved. The identification of such factors is crucial for early diagnosis and could be useful for more efficient treatment and prognosis of cervical cancer.

Most studies generally focus on the genetic dysregulation of host-cell proteins associated with promoting cervical carcinogenesis. In recent years, research turned towards non-coding RNAs (ncRNAs), especially microRNAs (miRNAs). The miRNAs are highly-conserved small double-stranded ncRNAs 19 to 24 nucleotides long that negatively regulate the expression of coding genes through hybridization with complementary or near-complementary sequences within the 3′-UTR of

target mRNAs (Felekkis et al., 2010; Bhaskaran and Mohan, 2014). Such binding specifically blocks translation or enables mRNA degradation (He and Hannon, 2004). Because of their functions, miRNAs are essential regulators of many biological pathways associated with cancer development.

HPV Infection and Cervical Cancer

HPVs are non-enveloped double-stranded DNA (dsDNA) viruses belonging to the *Papillomaviridae* family (Yuan et al., 2021). Most HPVs infect cells from the basal epithelium and have been classified into cutaneous or mucosal types (Burd, 2003). HPV viral particles are relatively small (approximately 60 nm), consisting of an icosahedral capsid containing a single molecule of circular dsDNA of 8,000 base pairs (Harden and Munger, 2017). In general, the HPV genomes encode for six early (E1, E2, E4, E5, E6, and E7) and two late proteins (L1 and L2) (Bansal et al., 2016). There is a third non-coding region in the HPV genome known as the Long Control Region (LCR) or Upstream Regulatory Region (URR) containing the origin of DNA replication as well as transcription control sequences (Harden and Munger, 2017).

The early genes encode for proteins needed for viral replication and transcription. E1 is a DNA helicase essential for the viral replication and E2 works as a regulatory protein modulating the expression of E6 and E7 and regulating viral transcription, replication and genome partitioning (Ryndock and Meyers, 2014). E4 is embedded within the E2 gene and promotes the viral escape from the cornified epithelial layers by attaching to cytokeratin filaments, disrupting their structure (Harden and Munger, 2017). The E5 protein enhances proliferation and may contribute to cancer progression through controlling trafficking of proteins and the vacuolar ATPase in endosomes to modulate epidermal growth factor receptor turnover, thus maintaining a constitutive proliferative signaling (Moody and Laimins, 2010). The E6 and E7 proteins are the principal viral transforming factors. E6 binds and degrades p53 and cellular PDZ proteins and activates telomerase activity thus inhibiting the host cell p53-mediated apoptosis and senescence while E7 binds and degrades the retinoblastoma protein (pRB) leading to continuous cell-cycle rounds contributing to malignant progression by inducing genomic instability and abnormal and sustained host cell proliferation (Harden and Munger, 2017).

The viral late region encodes for the structural proteins L1 and L2. L1 is the major component of the virion capsid produced at the upper layer of the differentiated cervical epithelium during infection and is necessary for binding of the viral primary receptor heparan sulfate (HS). L2 is the minor capsid protein and is involved in the entrance of the viral genome into the host cell (Szymonowicz and Chen, 2020).

There are approximately 30–40 HPV types that infect the anogenital tract (Yuan et al., 2021). Depending on the association with cancer and precursor lesions, the HPV types were grouped in two main classifications: high risk (HR-HPVs) and low risk types (LR-HPVs). HR-HPVs types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 are commonly found integrated to the host cell genome in cervical tumor cells. Notably, most cervical tumors contain HPV16 or HPV18 which contribute to ~70% of invasive cervical carcinoma cases globally (Bhatla and Singhal, 2020). HPV strains 6, 11, 40, 42, 43, 44, 54, 61, and 72 are considered as low-risk HPVs (LR-HPVs), often associated with benign anogenital warts and laryngeal papilloma (Okunade, 2020). It is presently unclear whether HPV types 53, 66, 70, 73 MM9, and 82 MM4 belong to high or low-risk classification.

Remarkably, about 80% of people will get an HPV infection at some point in their lives and usually become infected with HPV shortly after the onset of sexual activity. However, over 90% of those infected will spontaneously clear the infection within 9–12 months suggesting an active participation of the immune system in HPV infection (Szymonowicz and Chen, 2020).

Persistent infection by HR-HPVs can lead to benign Squamous Intraepithelial Lesions (SIL) (**Figure 1**). These lesions are classified as Low-grade Squamous Intraepithelial Lesions (LSIL) encompassing HPV infection or mild dysplasia also known as CIN1 (cervical intraepithelial neoplasia grade 1) and High-grade Squamous Intraepithelial Lesions (HSIL) encompassing moderate (CIN grade 2) and severe dysplasia (CIN grade 3). In CIN1 the abnormal proliferative cells are present only in the lower one-third of the epithelium. CIN2 and CIN3 are characterized by the expansion of the neoplasia to the lower two-thirds (CIN2) or more (CIN3) of the epithelium. Sometimes, CIN3 involves the full thickness of the epithelium, and it is also known as *in situ* cervical carcinoma (ISCC). HSILs are developed in 10–20% of women with LSILs, and less than 30% of the ISCC patients may progress to cervical tumors when the neoplasia invades into the stroma underneath (Wang et al., 2014; Shen et al., 2020).

Starting with an HPV infection, the transformation of normal cervical tissue to invasive cervical cancer (ICC) is a multistep process that implicates the sustained action of the oncogenic viral proteins E6 and E7 (Wang et al., 2018). Thus, E6 and E7 promote the transformation of normal cervical keratinocytes into pre-malignant and LSIL states and then to an HSIL with a subsequent transformation to ICC.

miRNAs and Cancer

Most miRNAs are involved in the regulation of fundamental biological processes, such as proliferation (Lenkala et al., 2014), differentiation (Yao, 2016), inflammation (Zhou W. et al., 2018), apoptosis (Taghavipour et al., 2020), cell cycle (Mens and Ghanbari, 2018), and immune response (Chandan et al., 2020). Due to their wide range of functions, miRNA dysregulation has significant consequences in terms of cellular outcomes enhancing the development of a wide range of diseases, including cardiovascular diseases (Zhou S.-S. et al., 2018), neurological disorders (Hussein and Magdy, 2021), autoimmune disorders (Salvi et al., 2019) and cancer (Peng and Croce, 2016).

In the specific case of cancer, it is well established that miRNA dysregulation plays a pivotal role in the initiation, progression, and dissemination of tumorigenesis. The miRNAs are capable of influencing the main features of the carcinogenic process such as continued proliferative capacity, apoptosis resistance, invasion and metastasis induction, increased angiogenesis and the evasion of growth inhibitor signals (Peng and Croce, 2016).

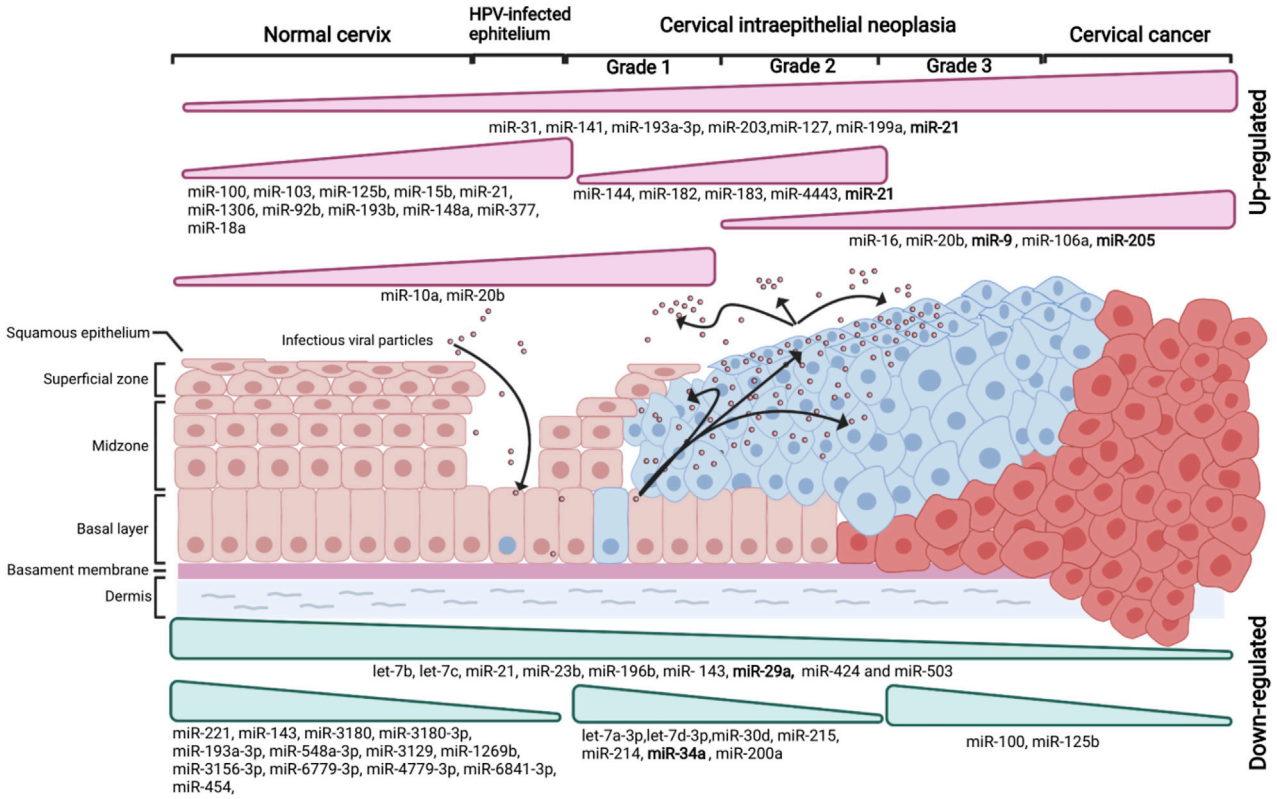

FIGURE 1 | Survey of dysregulated miRNAs in the different stages of cervical carcinogenesis (Created with BioRender.com). Variations in miRNA expression provide a signature of miRNAs in almost every stage of cervical cancer (CC) development. These miRNA signatures might be advantageous in the diagnosis and the monitoring of CC. The normal cervical epithelium morphology is shown in the left followed by intraepithelial lesions (Grade 1, Grade 2, and Grade 3) and CC. The miRNAs essential for the development and maintenance of CC with a diagnostic potential are shown in boldface.

In several tumor types, miRNAs function by targeting transcripts from tumor suppressors or proto-oncogenes (Wang et al., 2008). Thus, miRNAs are subdivided into oncogenic miRNAs (oncomiRs) and tumor suppressor miRNAs (tsmiRs), respectively (Svoronos et al., 2016). OncomiRs are usually highly expressed promoting tumor progression and maintaining the tumor phenotype. On the other hand, the tsmiRs inhibit tumorigenesis by regulating cell proliferation, invasion, promoting apoptosis, and other processes related to cancer development. These tsmiRs are frequently downregulated in most human cancers (Ali Syeda et al., 2020).

The established connection between miRNAs and cancer is deeply associated with aberrant miRNA expression in different cancer types. The miRNA profile in any tumor type can provide various applications such as therapeutic targets or as diagnostic and prognostic markers. However, for the miRNAs to be used for diagnosis or therapy, there is a need for the understanding of their oncogenic or tumor-suppressive roles and how their dysregulation affects tumor progression. OncomiRs may play a meaningful role in cancer treatment through intervening with their expression by silencing or modulation, while the directed overexpression of tsmiRs may exhibit therapeutic effects.

In the specific case of ICC, several OncomiRs have been described, such as miR-499, which promotes cervical tumor progression by enhancing proliferation, migration, invasion, and apoptosis resistance via tumor suppressor SOX6 targeting (Chen Y. et al., 2020). Another example is miR-18a which is involved in the oncogenic transformation of HPV^{+} cervical cells. A study found that HPV E6 and E7 increased the expression of miR-18a (Morgan et al., 2020). This overexpression leads in turn to higher proliferation, migration, and invasion via silencing of tumor-suppressive Hippo pathway STK4 mRNA.

MiR-125 is a well-known tsmiR in cervical cancer. Several authors established that miR-125 inhibited cervical cancer progression by targeting VEGF, thus inhibiting migration and invasion of cervical cancer cells (Fu et al., 2020). Also, miR-375 exerts tumor suppressor effects in cervical tumors via MELK downregulation, thus promoting cell apoptosis while impairing the proliferation, migration, and invasion (Ding et al., 2020).

Some miRNAs can play dual roles as OncomiRs and tsmiRs in cervical carcinogenesis. For example, miR-9-5p is upregulated in the $HPV16^{+}$ CaSki and SiHa tumor cell lines originating from a SCC. In SCC, miR-9-5p plays an oncogenic role by generating downregulation of TWIST1 and CDH1, thus enhancing EMT induction. However, miR-9-5p expression is low in the $HPV18^{+}$ cervical AC cell line HeLa. In this scenario, TWIST is not repressed, so CDH2 activation might be necessary to maintain a malignant phenotype in cervical AC (Babion et al., 2020a).

HPV and miRNAs

During infection, viruses play a significant role in regulating host gene expression. Part of the gene regulation mediated by viruses is due to changes in miRNA expression triggered by infection (Girardi et al., 2018). Persistent HPV infection induces abnormal miRNA expression (Zheng and Wang, 2011), which could trigger the transformation processes observed from HPV infection to cervical cancer.

Many researchers have studied the miRNA expression profile related to HPV infection and the development of cervical cancer. In 2020 Babion et al. using microarrays determined the miRNA expression profile associated with HPV infection in eight different passages of HPV-transformed keratinocytes representing different stages of cell transformation induced by HPV infection. In total, 106 mature miRNAs were found differentially expressed through the different cell passages. The most representative miRNAs validated by RT-qPCR were miR-15b-5p, miR-100-5p, miR-103a-3p, miR-125b-5p, found upregulated and only miR-221-5p presented downregulation (Babion et al., 2020b).

Another study correlated the severity of the intraepithelial lesions from women infected with HPV-16 to the expression of a set of four miRNAs. The comparison between $HPV16^+$ LSIL and cervical cancer patients showed an increased expression of miR-16, miR-21, miR-34a, and miR-143 relative to the HPV-negative group. In the case of HSIL patients, miR-16 and miR-34a expression changes resulted irrelevant compared to the HPV-negative control group. However, miR-21 expression significantly increased while miR-143 decreased compared to the HPV-negative group (Norouzi et al., 2021).

Nunvar et al. identified dysregulated miRNAs exclusive for HPV-dependent SCC. The authors evaluated miRNA expression profiles in a group of HPV^+ anogenital tumors by next-generation sequencing (NGS). The set included cervical, vulvar, anal, and tonsillar tumors. A variation among tumor types on the total number of dysregulated miRNAs was observed. However, the highest number of miRNAs differentially expressed was detected in cervical HPV^+ tumors compared to the other SCCs. They also found more downregulated than upregulated miRNAs in HPV^+ cervical tumors (Nunvar et al., 2021).

In a separate report, Tong et al. identified a subset of miRNAs associated with HPV status. They performed small RNA-seq in cervical tumor lines (2 HPV-negative and 5 HPV^+) and the exosomes (EXOs) secreted by these cell lines. Specifically, EXOs presented a total of 32 miRNAs differentially expressed in HPV-positive relative to HPV-negative cell lines. The five most commonly upregulated were miR-92b-5p, miR-92b-3p, miR-193a-5p, miR-193b-5p, and miR-1306-5p, while downregulation was reported for miR-548a-3p, miR-1269b, miR-3129-5p, miR-3180-5p, and miR-3180-3p. In tumor cell lines, 70 miRNAs were found differentially expressed in HPV-positive cells including miR-34a-5p, miR-199b-5p, miR-193a-3p, miR-193a-5p, and miR-365b-5p. Meanwhile, miR-431-5p, miR-432-5p, miR-816a-5p, miR-3180-5p, and miR-3180-3p were observed downregulated in HPV^+ relative to HPV-negative cells. Six miRNAs (miR-146-5p, miR-193a-5p, miR-4661-5p, miR-410-3p, miR-3180-5p and miR-3180-3p) were associated with HPV status in both EXOs and cells (Tong et al., 2020). All these findings suggest a potential miRNA expression deregulation associated with HPV-mediated cervical carcinogenesis.

Dysregulated miRNA expression in HPV infection is widely associated with the viral oncoproteins E5, E6, and E7. Downregulation of miR-454-5p, miR-656-5p miR-3156-3p, miR-4779-3p, miR-6779-3p and miR-6841-3p, was observed by microarrays from HPV-negative HT-3 cells ectopically expressing HPV16 E6 and E7. Further validation with RT-qPCR in HT-3 and C33-A (HPV-negative) cells transfected with HPV16-E6/E7 showed a decrease in miR-3156-3p expression in both cell types. A reduction of miR-3156-3p in HPV16/18-positive cervical tumor samples were also reported. These findings indicate that miR-3156-3p expression is associated with HR-HPV infection and the HR-HPV oncoproteins (Xia et al., 2017).

Silencing of E5, E6, and E7 oncoproteins in the $HPV16^+$ cervical tumor lines CaSki and SiHa showed that the loss of the viral oncoproteins upregulates miR-148a-3p. Silencing of only E6 and E7 in SiHa cells showed an increased expression of miR-199b-5p and miR-190a-5p. Overexpression of miR-190a-5p was observed by silencing E5 alone in CaSki. Thus, these three miRNAs might be used as biomarkers for the diagnosis of cervical cancer in HR-HPV-infected patients (Han et al., 2018). Additionally, in a cervical tumor microvesicles (CC-MVs) study, HeLa cells transfected with siRNAs against HPV18 E6/E7 showing increased expression of miR-377. These findings suggested that miR-377 may play a role in E6/E7-mediated oncogenesis (Zhang et al., 2020).

Morgan et al. evaluated the role of HPV16 and 18 E6/E7 oncoproteins in the transformation of HeLa (HPV-18) and CaSki (HPV-16) cells. Upregulation of miR-18a was observed after silencing E6/E7. MiR-18a directly targets the STK4 3′-UTR, a tumor suppressor gene that correlates with the Hippo pathway involved in the hyperproliferative state of the tumor cells (Morgan et al., 2020).

HPV-Coded miRNAs in Cervical Cancer

There is little evidence that HPV is capable of coding its own viral miRNAs. A bioinformatic study predicted novel putative HPV pre-miRNAs among different HPV types. Surprisingly, HPV16 showed the coding potential for three unique pre-miRNAs HPV16-miR-1, HPV16-miR-2, and HPV16-miR-3 located at E6, E1, and L2 ORFs, respectively. Using the miRTar tool, the putative target genes for HPV16-miR-1 were predicted as ARID5B, ZEB2, THBS1, genes involved in cell motility and migration, and STAT5B related to cell adhesion. For HPV16-miR-3, the predicted targets were SYNE1, PDE1B, GATA6, and GULP1 associated with cell death. However, the predicted targets for HPV16-miR-2 (AFF3, FRMD7, IGDCC4, MYRIP, NRN1, PMP22, RBPMS) were not associated with cervical cancer progression. With this approach, the authors suggested that viral miRNAs might facilitate the host immune response evasion through advocating the latent phase of the HPV life cycle, thus increasing the risk for cancer development although no experimental evidence of these viral miRNAs has been provided so far (Weng et al., 2017).

Although it appears that viral miRNAs could play a significant role in HPV infection and cervical cancer development, there is a controversy about the existence and possible function of these putative viral miRNAs. Accordingly, further research is required to establish if HPV is indeed capable to express its miRNAs and define their functions for the diagnosis, prognosis, or therapy against cervical cancer.

Dysregulation of miRNAs in cervical cancer

Most miRNAs are encoded as individual genes or gathered in clusters along with other miRNAs and either expressed from their own promoters or as passenger transcripts within introns (Cai et al., 2009). Canonical miRNA biogenesis is a stringently regulated process starting with a primary transcript (pri-miRNA) produced by RNApolII transcription containing 5′ cap and 3′ polyA tail (Ha and Kim, 2014).The pri-miRNA structures as a defined stem-loop determined by internal base-pairing and is processed in a structure-driven manner within the nucleus by the *Microprocessor* complex, containing the RNAse III-like enzyme *Drosha* and the RNA binding protein DGCR8, that cleaves the base of the stem generating a 2 nt overhang at the 3' end. The resulting precursor miRNA (pre-miRNA) retains the stem-loop structure and is exported to the cytoplasm by the Exportin 5/Ran-GTP complex (Gregory et al., 2004). There, a cytoplasmic RNAse III-like enzyme, *Dicer*, cuts out the loop leaving a 21 nt double-stranded miRNA molecule. The miRNA is bound by an RNA-induced silencing complex (RISC) composed of TRBP, *Dicer* and AGO2 in humans (Chendrimada et al., 2005) and one of the strands is used as guide sequence to mediate sequence-dependent silencing of complementary target mRNAs (Lai, 2002). Therefore, mutations on the miRNA promoters, sequence, biogenesis regulatory regions or target sites and alterations on the miRNA biogenesis proteins may cause dysregulation, loss of specificity or even silencing of the miRNA function with deleterious consequences for the cell.

Numerous researchers have characterized some of the mechanisms causing miRNA dysregulation and their expression pattern in cervical cancer. Compelling evidence suggests that alterations in miRNA expression might result from genomic variations of miRNA genomic loci, such as genetic deletions, amplifications, or mutations. For example, 45 deregulated miRNAs were identified in advanced SCC cells associated with high expression of Drosha and a gain of chromosome 5p. These 45 miRNAs include miR-31, miR-141, miR-203 upregulated, and miR-193a-3p downregulated in clinical samples. Additionally, a majority of miRNAs upregulated were identified in Drosha-overexpressing cells, with only five being down-regulated (Muralidhar et al., 2011).

It is well-known that alteration of transcriptional activators and repressors and abnormal DNA methylation modification of miRNA genomic loci often causes an aberrant miRNA expression profile in cancer (Hussen et al., 2021). In addition to the genomic alterations and patterns of methylation of the miRNA gene sequence, dysregulation of the proteins implicated the miRNA biogenesis contributes to the alterations of miRNA expression in cervical cancer (Ali Syeda et al., 2020).

Another factor that modifies miRNA expression in cervical tumors is single nucleotide polymorphisms (SNPs). SNPs within miRNA genes alter their maturation process by destabilizing the miRNA precursor secondary structure, interfering with Drosha and Dicer processing and strand choice (Wang Y. et al., 2021). Therefore, SNPs in miRNAs deregulate their expression, thus affecting the binding to their target genes. These alterations are well associated with cancer development. Zhi, et al. explored the association between the SNPs in miR-21, miR-26b, miR-221/222, and miR-126 in healthy, CIN, and CC patients. The authors showed that rs1292037 in miR-21 might be involved in the development of CIN or CC. In the case of miR-126, they found that rs2297538 and rs2297537 SNPs might be implicated in the progression of CIN to cervical carcinoma. Finally, the SNP rs2745709 in miR-221/222 might be associated with the development of a CIN lesion (Yang et al., 2021).

Furthermore, a case-control study in the Chinese population evaluated the influence of SNPs in the promoter of the miR-17-92 cluster (miR-17, miR-18a, miR19a, miR-19b, miR-20a, and miR-92-1) in the development of SCC. The authors found a lower level of oncogene miR-20a in patients with the rs9588884 GG genotype. Since miR-20a promotes the proliferation, migration, invasion, and suppression of apoptosis of cervical carcinoma cells, the rs9588884 GG genotype seemingly reduces the risk of SCC (Huang et al., 2020). Another case-control study carried out by the same authors found that miR-34b/c rs4938723 C/T polymorphism is also associated with cervical cancer risk in the Chinese population (Yuan et al., 2016). Liu et al. associated the downregulation of let-7i with a higher risk of cancer development by the presence of SNPs in the let-7i promoter (Liu and Ni, 2018).

Infection with HR-HPV causes alterations in miRNA expression which are essential to maintain a transformed phenotype and subsequent progress to invasive carcinoma. Different studies evaluated miRNAs expression in a range of cell lines, biopsies, cervical mucus, exfoliated cervical cells, and serum from women diagnosed with SCC. These studies showed highly variable miRNA expression during the different stages of cervical cancer.

Lui et al. by direct sequencing found downregulation of miR-196b, miR-23b, miR-143, let-7b, let-7c, and an upregulation of miR-21 in cervical carcinoma cells compared with non-tumorigenic cervical samples (Lui et al., 2007). In a different report, miR-127 and miR-199a were found upregulated in invasive SCC compared with non-tumorigenic cervical squamous epithelial samples. The upregulation of miR-127 was associated with lymph node metastasis. Likewise, miR-199 promoted tumorigenesis by enhancing cell growth (Lee et al., 2008).

Variations in miRNA expression also have been reported during the transition from LSIL to HSIL and invasive CC. Zheng et al. described eight differentially secreted exosomal miRNAs; miR-30d-5p, let-7a-3p, let-7d-3p, miR-215-5p downregulated, and miR-144-5p, miR-182-5p, miR-183-5p, miR-4443 upregulated between patients presenting HSILs from those with LSILs. The same study identified miR-30d-5p and let-

TABLE 1 | Differential expression of miRNAs in cervical carcinogenesis. CIN, cervical intraepithelial neoplasia; SCC, squamous cervical carcinoma.

	Differential expression	miRNAs	References
		HPV infection	
VPH infected cells vs normal cervical cells	Upregulated	miR-100-5p, miR-103a-3p, miR-125b-5p, miR-15b-5p, miR-143-5p, miR-1306-5p, miR-92b-5p, miR-92b-3p, miR-193a-5p, and miR-193b-5p	Babion et al. (2020b); Norouzi et al. (2021); Tong et al. (2020)
	Downregulated	miR-221-5p, miR-3180-5p, miR-3180-3p, miR-548a-3p, miR-3129-5p and miR-1269b, miR-432-5p, miR-816a-5p, miR-431-5p, miR-3156-3p, miR-148a-3p	Babion et al. (2020b); Tong et al. (2020); Xia et al. (2017); Zhang et al. (2020)
		Cervical intraepithelial neoplasia	
CIN2/3 vs CIN1	Upregulated	miR-21, miR-144-5p, miR-182-5p, miR-183-5p, miR-4443, miR-10a	Wang et al. (2019); Pardini et al. (2018); Norouzi et al. (2021)
	Downregulated	let-7a-3p, let-7d-3p, miR-30d-5p, miR-215-5p, miR-214, miR-34a, miR-200a, miR-143	Zheng et al. (2019); (Wang et al. (2019); (Norouzi et al. (2021)
CIN2/3 vs normal samples	Upregulated	miR-125a, miR-21	Wang et al. (2019); Bayramoglu Tepe et al. (2021)
	Downregulated	miR-30e, miR-96, miR-30a, miR-130a, miR-302a, miR-143, miR-372, miR-17, miR-375, miR-30c, miR-520e, miR-548c, miR-373, miR-214, miR-34a and miR-200a	Wang et al. (2019); Bayramoglu Tepe et al. (2021)
		Cervical cancer	
SCC vs normal cervical samples	Upregulated	miR-21, miR-127 and miR-199a	Lui et al. (2007); Lee et al., (2008)
	Downregulated	let-7b, let-7c, miR-23b, miR-196b, miR-143	Lui et al. (2007)
SCC vs CIN1	Upregulated	miR-96-5p, miR-17-5p, miR-130a-5p and miR-520b-5p miR-16-5p, miR-20b-5p, miR-9-5p, miR-106a-5p, miR-205-5p	Bayramoglu Tepe et al. (2021); Pardini et al. (2018); Norouzi et al. (2021)
	Dowregulated	miR-30e-5p, miR-30a-5p and miR-30c-5p, miR-143-5p, miR-372-5p, miR-375-5p, miR-520e-5p	Bayramoglu Tepe et al. (2021); Norouzi et al. (2021)

7d-3p as strong predictors for clinical diagnosis due to the highly differential expression between patients with CIN1 and CIN2 (Zheng et al., 2019). Moreover, Wang et al. found that overexpression of miR-21 and downregulation of miR-214, miR-34a, and miR-200a expression in plasma is associated with the severity of the cervical lesion. Expression of these miRNAs in HSILs was significantly different from those patients with lower-grade lesions (Wang et al., 2019).

Using a bioinformatics approach, Dai et al. identified the downregulation of KFL4 and ESR1 closely related to poor prognosis triggered by the high expression of miR-21 and miR-16 in CC (Dai et al., 2019). Likewise, a systematic study by Pardini et al. identified miR-29a downregulation and miR-21 upregulation. The same study found miR-10a, miR-9, miR-20b, miR-106, and miR-16 as frequently CC dysregulated miRNAs (Pardini et al., 2018).

Downregulation of miR-188 and upregulation of miR-223 is linked with short survival of cervical cancer patients, while downregulation of miR-125b and miR-99a was associated with the 5-years survival rate (Gao et al., 2018). Qi et al. by bioinformatics assays validated the signature of a set of miRNAs correlated to cancer prognosis (miR-585-5p, miR-216b-5p, and miR-7641) and validate these miRNAs through *in vitro* assays (Qi et al., 2020). Moreover, it was established that miR-503 and miR-424 are expressed as a cluster and it was downregulated in CC cells. Upregulation of miR-424/miR-503 impaired cell proliferation, epithelial-mesenchymal transition (EMT), migration, and invasiveness, but improve autophagy (Chen X. et al., 2020).

Tepe et al., explore the potential diagnosis and prognosis of miRNAs involved in autophagy. To achieve this goal, they evaluate the expression of such miRNAs in SCC, founding miR-30e-5p, miR-30a-5p and miR-30c-5p, miR-143-5p, miR-372-5p, miR-375-5p, miR-520e-5p downregulated in the SCC samples compared with HSILs, whereas miR-96-5p, miR-17-5p, miR-130a-5p and miR-520b-5p were upregulated. Finally, they identified miR-30a-5p, miR-520e-5p, miR-548c-5p and miR-372-5p as significant diagnostics markers for SCC, due to the significant association of this miRNAs with the overall survival of SCC patients (Bayramoglu Tepe et al., 2021).

MiRNAs, in development, progression, and maintenance of cervical cancer

As shown above, miRNA dysregulation may play a significant role in the onset and progression of cervical cancer. Nevertheless, every study appears to suggest different miRNAs associated with the initiation and progression of cervical cancer (**Table 1**). For clarity, we enlisted the most frequently dysregulated miRNAs in the different stages of cervical cancer progression and their effect on cellular pathways (**Table 2**).

MiR-9-5p

In humans, miR-9 is coded by three different genes (miR-9-1, miR-9-2, and miR-9-3) located at different chromosomes: 1q22, 5q14.3, and 15q26.1, respectively. The presence of miR-9 sequences in three different loci coupled with the high level of sequence conservation, suggests an important role of this miRNA on the cell cycle and the development of cancer (Nowek et al., 2018).

Expression of miR-9 is upregulated in HPV16-infected LSIL compared with the HPV- negative healthy population, while is significantly reduced in HPV52 and HPV58-infected dysplasia

TABLE 2 | Relevant target genes for dysregulated miRNAs in cervical cancer.

miRNA	Clasification	Target	Pathway	Associated events	Reference
miR-9-5p	OncomiR	CDH1	Angiogenesis	Down-regulation of CDH1 leads to activation of β-catenin, resulting in the up-regulation of VEGFA, a proangiogenic factor	Farzanehpour et al. (2019)
miR-9-5p	OncomiR	TWIST1	Metastasis	Suppression of epithelial protein CDH1 and transcriptional activation of mesenchymal marker CDH2	Babion et al. (2020a)
miR-9-5p	OncomiR	SOC5/	Angiogenesis/ Radiosensitivity	Increasing EMT transition and tube formation	Wei et al. (2019)
miR-21-5p	OncomiR	PDCD4	Immune evasion	Down-regulation of PDC4 resulting in suppression of the inflammation process via NF-kB activating the anti-inflammatory cytokine interleukin 10	Asangani et al. (2008)
miR-21-5p	OncomiR	PTEN	Tumorigenesis	PTEN down-regulation amplifies PI3K signaling resulting in sustaining proliferative signaling	Bumrungthai et al. (2015)
miR-34a-5p	TsmiR	WNT1	Proliferation/Invasion	Induction of an E-P cadherin switch via the WNT1/β-catenin pathway	(Li et al. (2020))
miR-34c-3p	TsmiR	MAP2	Proliferation	Microtubule stabilization of MAP2 leads to proliferation inhibition and cell death in tumor cells	Córdova-Rivas et al. (2019)
miR-29a	TsmiR	STIR1	Migration and invasion	SIRT1 suppressed E-cadherin expression and promoted N-cadherin expression	Nan et al. (2019)
miR-29a	TsmiR	HSP47	Metastasis	Overexpression of molecular chaperone HSP47 leads to cell migration and invasion	(Yamamoto et al. (2013))
miR-16-1	OncomiR	CCNE1	Cell cycle	Cyclin E1 (CCNE1) promotes transition of cells from G1 to S phase	Zubillaga-Guerrero et al. (2015)
miR-148b	TsmiR	CASP3	Apoptosis	Caspase-3 activated death protease, catalyzing the specific cleavage of many key cellular proteins	Mou et al. (2016)
miR-182	TsmiR	DNMT3a	Apoptosis	Hypermethylation by DNMT3a resulted in the silencing of tumor suppressor genes	Sun et al. (2015)
miR-155-5p	OncomiR	TP53INP1	Invasion	TP53INP1 blocks tumor progression via p53-dependant and -independent pathways	Li et al. (2019a)
miR-452b-5p	OncomiR	WTX, β-Catenin	Invasion/Migration	WTX, β-catenin, β-TrCP2, APC and AXIN1 form a complex that could inhibit cancer progression by ubiquitination of β-catenin protein	Li et al. (2019c)
miR-204	TsmiR	ATF2	Autophagy	Phosphorylated ATF2 bind to promoter region of genes involved in cell cycle and autophagy	Li et al. (2019b)
Let-7a	TsmiR	PKM2	Invasion/Migration	PKM2 in the role of protein kinase contribute to development of tumorigenesis	Guo et al. (2017)

(Liu et al., 2020). These differences suggest that different HPVs may have different infection patterns, and thus the levels of key miRNAs may be differentially affected. This situation reflects the challenges faced by investigations aimed to find differences in the expression levels of miRNAs in cervical cancer, as the natural history of HPV infection is not yet fully understood and it is suspected that this may vary depending on the viral subtype, and that it is practically impossible to determine when the infection has started on a patient tested positive for HPV and how long the infection has been ongoing at the time of sampling (Woodman et al., 2007). Once normal tissue has progressed to HPV-associated dysplasia, it has been observed downregulation of miR-9, resulting in high levels of E-cadherin what leads to activation of β-catenin. The final result is the up-regulation of VEGFA, a proangiogenic factor (Pardini et al., 2018; Farzanehpour et al., 2019). Nevertheless, Babion et al. explored in more detail the role of miR-9 in the most common CC types (SCC and AC), finding that miR-9 expression is influenced by tumor histotype and by HR-HPV type. They found that miR-9-5p could be acting like an oncomiR, by targeting TWIST1 and CDH1. In contrast, miR-9-5p acts as tsmiR in AC, since the EMT phenotype is achieved by low levels of miR-9-5p, which facilitated the upregulation of CDH2 via TWIST1 (Babion et al., 2020a).

MiR-21-5p

The locus for pri-miR-21 is found within the intronic region of the VMP1 gene located in chromosome 17 (Kumarswamy et al., 2011). MiR-21 is abundantly expressed in mammalian cells and its up-regulation is associated with many cancers.

Lui et al. reported the up-regulation of miR-21 expression in HPV-positive CC compared with that in LSILs, especially in HPV16-infected tumors. Conversely, in cervical tumors infected with HPV52 and HPV58, the miR-21 levels are not statistically different compared with LSILs infected with the same type. These results indicate that miR-21 dysregulation could be useful as a biomarker particularly in HPV16-infected tumors (Liu et al., 2020). Mir-21 targets several genes associated with cancer such as PTEN, PDCD4, RECK, and STAT3 (Asangani et al., 2008; Bumrungthai et al., 2015; Bautista-Sánchez et al., 2020). The role of miR-21 in CC was deepened by Bumrungthai et al. where miR-21 expression in HPV-negative normal cytology was significantly lower than in HPV-positive samples in abnormal tissue and SCC. This result demonstrates that induction of miR-21 might be involved in HPV infection, and cervicitis (Bumrungthai et al., 2015). It has been proposed that the miR-21 overexpression could lead to the down-regulation of PDC4 resulting in suppression of the inflammation process via NF-kB

activating the anti-inflammatory cytokine interleukin 10, leading to immune evasion and cervical cancer progression (Asangani et al., 2008). MiR-21 can also control the PI3K/AKT/mTOR pathway by inhibiting AKT activation increasing NF-kB activity leading to inflammation.

MiR-34a and miR-34c

The miR-34 family consists of miR-34a, located on chromosome 1p36, miR-34b, and miR-34c co-transcribed from chromosome 11q23 (Navarro and Lieberman, 2015). MiR-34a is a classical tumor suppressor gene and is frequently down-regulated in gastric cancer, liver cancer, prostate cancer, and cervical cancer (Zhang et al., 2016). The study of miR-34a in cervical cancer began in 2009 when Wang et al. showed that cervical tumors and cervical cancer-derived cell lines containing oncogenic HPVs displayed reduced expression of tumor-suppressive miR-34a, attributed to the expression of the viral protein E6, which destabilizes the tumor suppressor p53, a known miR-34a transactivator (Wang et al., 2009). Later, Li et al. observed a reduced pri-miR-34a expression in cervical tumors and precancerous lesions. Such diminution could be detected in a very early stage, suggesting that miR-34a inhibition is induced by HR-HPV E6 oncoprotein through the p53 pathway, constitute an early-onset event in the development of CC (Li et al., 2010). The mechanism that has been proposed for miR-34 to promote the proliferation and invasion of cervical cells with SCC is through its target WNT1 by induction of an E-P cadherin switch via the WNT1/β-catenin pathway (Li et al., 2020).

miR-34c is another miR-34 family member it's been associated with CC. Lopez et al. found an inhibitory proliferation effect of miR-34c-3p and miR-34c-5p in CC SiHa cells. Further, the authors establish that only miR-34c-3p is involved in, apoptosis, cell migration, and invasion. Thus, suggesting that miR-34c-3p and miR-34c-5p may target different mRNAs and thus harbor dissimilar functional roles (López and Alvarez-Salas, 2011). These results were complemented by Rivas et al., who demonstrated that miR-34c-3p mimic transfection led to the clear downregulation of MAP2 protein, as well as of MMP9 activity (Córdova-Rivas et al., 2019). Also found that 5p and 3p strands of miR-34 family members have differential effects in cell proliferation, migration, and invasion in cervical cancer cells, however, the mRNA targets regulated by 5p and 3p arms of miR-34 family members are needed to clarify, and this could elucidate the differential effects of this miRNAs on cell processes.

MiR-29a

The miR-29 family consists of four members (miR-29a, miR-129b-1, miR-29b-2, and miR-29c) encoded in two gene clusters. In humans, miR-29b-1 and miR-29a are located at chromosome 7, and miR-29b-2 and miR-29c are situated on chromosome 1 (Wang et al., 2015). It has been shown that miR-29a is down-regulated in tumors and HPV-positive LSILs (Yamamoto et al., 2013; Jia et al., 2015; Servín-González et al., 2015). In 2019, Zamani et al. reported a significant decrease in miR-29 in tumor samples relative to the control groups, suggesting that miR-29a could operate as a tumor-suppressor in CC progression (Sara et al., 2019). MiR-29a participated in the migration, invasion, and EMT directly targeting the 3′-UTR of SIRT1 mRNA (Nan et al., 2019). Recently it has been demonstrated that miR-29a regulates the p16 methylation pattern in CC by downregulating DNMT3A and DNMT3B (Wang A. et al., 2021). Also, it has been found that miR-29a regulates HSP47 having a key role in the maturation of collagen molecules. The diminution of HSP47 inhibited cancer cell migration and invasion, suggesting that the miR-29a-HSP47 pathway contributes to the metastasis of cervical SCC (Yamamoto et al., 2013).

Role of miRNAs as biomarkers in early stages of cervical cancer

Currently, histopathology and cytology (Pap-smear) are the gold standard for detection of HPV-associated dysplasia and cervical cancer. Although efficient and low-cost, these methods have a relatively low sensitivity (about 50%) and heavily rely on interpretation, sample recollection and technician training (WHO, 2014). Recent updates in cervical cancer screening guidelines include the addition of HPV testing to cervical cytology, which provides 60–70% greater protection against ICC compared to Pap-smear alone (Bedell et al., 2020). Nevertheless, more sensitive approaches are needed for early and opportune diagnosis. In this regard, liquid biopsy-based approach may theoretically represent a valid additional (or alternative) model for CC screening, diagnosis, and follow-up (Palmirotta et al., 2018). In liquid biopsy, smalls non-coding RNAs gain a central role in cervical cancer diagnosis and prognosis, being major components in circulating RNA detection and exosome identification (**Table 3**). The need for markers that indicate the presence of cervical dysplasia in early stages is urgently required, as early stages of cervical carcinogenesis are usually asymptomatic, and in advanced cervical carcinoma the symptoms are general and shared with several conditions (Jia et al., 2015).

Because miRNAs are highly stable ncRNA species compared with mRNAs and some IncRNAs and have a relatively high average half-life (5 days on average), miRNAs can be considered as prognostic biomarkers of cervical cancer (Jiang et al., 2020). The potential clinical relevance of certain miRNAs in cervical tissue samples was explored by Park et al., demonstrating the potential to miR-21 and miR-155 combined with an HPV E6/E7 mRNA assay as biomarkers for diagnosis and management of both HPV positive and HPV negative LSILs and cervical tumors (Park et al., 2017). In a study conducted in Chinese population, miR-21 was notably upregulated and suggested as a biomarker to distinguish cervical tumors from LSILs. Another miRNA involved in the differentiation between LSILs and CC is miR-34a, especially in HPV16 infected patients. (Liu et al., 2020).

Recently, Zamanni et al. explored the differences between the levels of miR-21 and miR-29a in HPV^{+} and cervical tumor groups using liquid-based cytology samples (LBCs) in Iranian women. Both miR-21 and miR-29a can serve for cervical cancer diagnosis because of the existing correlation between miR-21 upregulation and the downregulation of miR-29a in tumor samples (Sara et al., 2020). Another study using LBCs showed the up-regulation of miR-205 in HSILs, suggesting the utility of miR-205 expression as

TABLE 3 | Molecular models for early cervical cancer diagnosis and screening.

Method	Description	Role in cervical cancer diagnosis and prognosis	Reference
Circulating tumor cells (CTCs)	Isolation of tumor cells in bloodstream utilizing their physical differences compared with leukocytes	Identification and quantification of HPV oncogenes and epithelial markers, by using molecular and/or immunofluorescence procedures	Palmirotta et al. (2018)
Circulating Cell-Free DNA (ctDNA)	Detection of tumor DNA free in the circulatory system by extremely sensitive detection methods	Finding of distinctive mutated genes in cervical cancer or viral DNA by NGS panels or dPCR.	Palmirotta et al. (2018)
Cell-Free Circulating non-coding RNA	Detection of tumoral ncRNAs in the bloodstream, active release by cancer cells	Searching for lncRNA or miRNA characteristic of CC taking advantage of stability of ncRNAs in plasma compared with other nucleic acid	Cafforio et al. (2021)
Exosomal miRNAs	Analysis of miRNAs shuttled in extracellular vesicles that can be easily detected in body fluids thanks to their abundance and stability	Identify signature DEmiRs in patients with CINs and CC.	Cafforio et al. (2021)
Detection of aberrant methylation pattern	Analysis of methylation pattern in cervical scraps or biopsy in genes	Detection of aberrant DNA methylation of oncogenes and ts-genes using affinity capture of methylated DNA.	Yang et al. (2020)

a novel triage marker to supplement HR-HPV testing in patients with LSILs (Xie et al., 2017). Analyzing the levels of miR-205 can serve as a supplement to the screening of HR-HPVs, which can help determine which patients with LSILs will progress to HSILs.

Circulating miRNAs are attractive as biomarkers in cervical cancer because this eliminates the need to take solid tissue samples, simplifying sample collection. In this regard, You et al. analyzed three plasma miRNAs (miR-127, miR-205, and miR-218) for cervical cancer detection. The results showed that miR-205 had a higher predictive value with an AUC of 0.843, a sensitivity of 72.00%, and a specificity of 82.35% (You et al., 2015). Comparing these results with those obtained by Xie et al., it is likely that miR-205 could serve as a biomarker both in plasma and in samples from cervical tissue, although further research would be necessary to examine the levels of miR-205 in both sample types.

In another study, Farzanehpour et al. monitored the levels of miR-9, miR-192, and miR-205 in serum and tissue of cervical cancer and LSIL patients infected by HPV in comparison with normal tissue showing an increased expression level of miR-192 in tumor and L-SIL tissues, concluding that miR-192 can be used as a potential biomarker for the early detection of CC (Farzanehpour et al., 2019).

Finally, several research groups focused in establishing a miRNA profile for the detection of cervical cancer. For example, Jia et al. described a panel of 5 serum miRNAs (miR-21, miR-29a, miR-25, miR-200a, and miR-486-5p) as a cervical cancer biomarker. The ROC curves indicated that this panel may constitute a useful fingerprint test for early diagnosis. Interestingly, miR-29a and miR-200a may indicate tumor histological grade and progression stage (Jia et al., 2015).

DISCUSSION

The impact of miRNAs in cancer has been widely studied because of their capacity to influence many tumorigenic processes, such as sustained proliferative capacity, apoptosis resistance, invasion and metastasis induction and increased angiogenesis. Thus, several studies have focused on the expression profile of miRNAs in different types of cancer and their functions in the tumorigenic process.

In this review, we highlighted the expression and function of four extensively studied miRNAs. Mainly, miR-21 was found up-regulated in HPV infection and through regulation of several target genes is capable of enhancing progression to cervical cancer. Because of the well-established overexpression in cervical tumor tissue, miR-21 is described as a good diagnostic and prognostic marker. Also, miR-9a has been found upregulated in SCC associated with EMT through regulation of TWIST1 and CDH1 contributing to invasive CC development. In the case of miR-34a and miR-29, they are both downregulated in cervical carcinogenesis and are well known for their tumor suppressor functions. These miRNAs are associated with an ICC state through the regulation of genes involved in migration, invasion, and EMT (WNT1, SIRT1, and HSP47).

Due to the discovery of miRNA aberrant expression associated with cervical cancer, the new molecular mechanism of cervical tumorigenesis has emerged providing opportunities for miRNAs become useful for clinical applications. Accordingly, it is important to categorize the expression and the function of miRNAs in the different stages that lead to cervical cancer (**Figure 1**); from the infection with HR-HPV, followed by subsequent LSIL to malignant transformation. Aberrant miRNA expression begins with HPV infection and despite most infections are spontaneously cleared in many patients, the HPV infection persists. Consequently, the expression profile of miRNAs continues changing throughout the cervical carcinogenesis development process. As mentioned above, the upregulation of miR-205 could be a marker for the diagnosis of HPV^{+} intraepithelial lesions. The use of this miRNA could be more accurate than the determination of the HR-HPV oncoproteins in cervical tissue. In invasive SCC the overexpression of miR-21 and downregulation of miR-29 stands out in tumors compared to normal cervical tissue providing a molecular signature useful for cervical cancer diagnosis.

Most miRNAs possess some advantages that could turn them into ideal candidates as biomarkers for the diagnosis and prognosis of cervical cancer. For example, their stability, accessibility for measurement, specificity for the tissue or cell

type and a putative capability to be more informative than other biomolecules. Although these advantages suggest a promising clinical implication of miRNA-based diagnosis the validation of miRNA as biomarkers has not been successful. This can be explained by many methodological challenges including sample collection, storage, extraction methods, quality controls, differences in methodology of the studies, the lack of standard methods for normalization, and the inability to discriminate among closely related miRNAs (Saliminejad et al., 2019). Besides the potential technical biases mentioned above, other critical variables that could have deep implications in the accurate interpretation of miRNA biomarker studies are related to the intrinsic variability, such as the heterogenicity of the tumor itself since miRNA expression pattern may fluctuate among different patients with the same type of cancer due to the individual variability such as race and gender, life-style and external factors, drugs, smoking habits, and other conditions (Tiberio et al., 2015).

Thus, considering all these limitations, every miRNA-based diagnosis study requires further steps of validation and a proper standardization of all analytical procedures, to control for all potential technical biases. Furthermore, deeper studies with a larger number of patients enrolled are required to discover a signature of specific and sensitivity miRNAs capable of discriminate between HPV-infection, dysplasia and cancer patients from healthy subjects.

In addition, the individual variability between different cervical cancer patients is not negligible and can influence miRNA application in clinical practice. Accordingly, miRNAs could be used as a complementary but not definitive diagnostic tool requiring the input from standard diagnosis practices such as Pap-smear cytology, histopathology, and HPV testing.

Besides diagnosis, some clinical trials have focused on the miRNA therapeutic efficacy in cervical cancer. Potential miRNA-based cervical cancer therapies focus on the delivery of miRNA mimics to restore the functions of tsmiRs or delivering antagomiRs to repress oncomiRs (Hanna et al., 2019). However, there are some challenges to overcome to develop miRNA-based therapies, such as degradation by nucleases upon addition into biological systems, poor delivery to target cells, off-target effects, and activation of immune responses (Segal and Slack, 2020). Despite the off-target effects that miRNA-based therapies may have, they are promising tools due to their many advantages compared with the highly unspecific and toxic therapies currently applied such as chemotherapy and radiotherapy. For example, because the anatomic location of cervical tumors, miRNAs can be easily delivered in the specific tumor site reducing the damage to normal cells.

The clinical implications of miRNA-based diagnostic and therapeutic strategies are necessary to reduce the mortality and successful management of cervical cancer. Thus, further studies are required to elucidate the role of miRNAs in cervical carcinogenesis and the mechanisms through which miRNAs regulate cellular process enhancing tumorigenesis. Those studies might provide suitable evidence of the role of miRNAs as diagnostic and prognosis biomarkers, as well as treatment molecules for cervical cancer.

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EGB-V and MFP-yP contributed equally to literature research and figure artwork. LMA-S provided funding and critical review. Manuscript correction and original idea.

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miRNA Combinatorics and its Role in Cell State Control—A Probabilistic Approach

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A hallmark of cancer evolution is that the tumor may change its cell identity and improve its survival and fitness. Drastic change in microRNA (miRNA) composition and quantities accompany such dynamic processes. Cancer samples are composed of cells' mixtures of varying stages of cancerous progress. Therefore, cell-specific molecular profiling represents cellular averaging. In this study, we consider the degree to which altering miRNAs composition shifts cell behavior. We used COMICS, an iterative framework that simulates the stochastic events of miRNA-mRNA pairing, using a probabilistic approach. COMICS simulates the likelihood that cells change their transcriptome following many iterations (100 k). Results of COMICS from the human cell line (HeLa) confirmed that most genes are resistant to miRNA regulation. However, COMICS results suggest that the composition of the abundant miRNAs dictates the nature of the cells (across three cell lines) regardless of its actual mRNA steady-state. *In silico* perturbations of cell lines (i.e., by overexpressing miRNAs) allowed to classify genes according to their sensitivity and resilience to any combination of miRNA perturbations. Our results expose an overlooked quantitative dimension for a set of genes and miRNA regulation in living cells. The immediate implication is that even relatively modest overexpression of specific miRNAs may shift cell identity and impact cancer evolution.

Keywords: CLIP-Seq, miRNA-target prediction, TargetScan, ceRNA, Markov chain, cell line, cell simulation, miRNA binding site

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1 INTRODUCTION

Mature microRNAs (miRNAs) are non-coding RNA molecules that regulate genes through base complementarity with their cognate mRNAs, at the 3′-untranslated regions (3′-UTR) (Moore et al., 2015). Within cells, miRNAs act by destabilization of mRNAs and interfering with the translation machinery (Chekulaeva and Filipowicz, 2009; Eichhorn et al., 2014). It was established that alteration in the relative abundance of miRNAs may lead to transition between cell states and the establishment of cell identity (Peláez and Carthew, 2012).

The human catalog of miRNA includes about 2500 mature miRNAs derived from ~1900 genes (Kozomara and Griffiths-Jones, 2013). However, in each human cell, only a few dozens of miRNAs are expressed in substantial amounts. The miRNA distribution has a long tail of lowly expressed miRNAs. A reduced set of miRNA families (~250 representatives) combines miRNAs with a substantial overlap in binding properties. In reality, ~60% of the human coding genes are postulated as targets for miRNA regulation (Ha and Kim, 2014; Jonas and Izaurralde, 2015). Many miRNAs

carry the potential for targeting hundreds of transcripts (Rajewsky, 2006; Balaga et al., 2012). Looking from the transcripts' angle, at the 3′-UTR of an mRNA, there are tens of predicted miRNA binding domains (MBS) (Landgraf et al., 2007). Experimental results using CLIP-based deep sequencing protocols provide quantitative amounts of miRNAs and mRNAs in living cells (Li et al., 2014). Unfortunately, these protocols suffer from poor consistency (Lu and Leslie, 2016).

The quantitative aspect of miRNAs within living cells is understudied. It includes the stoichiometry on miRNA and mRNAs and the combinatorics of MBS. For a given mRNA, the composition and relative positioning of MBS along the transcript dictate the potential of a fruitful interaction (Jens and Rajewsky, 2015), but not necessarily the contribution of any specific miRNA to the overall suppression of gene expression (Agarwal et al., 2015). From the perspective of the miRNA, a fundamental player in the regulation is AGO, the catalytic component of the RNA silencing complex (RISC) within cells, and its availability (Wen et al., 2011; Janas et al., 2012). This many-to-many relation of the miRNA-mRNA network calls for developing a probabilistic model that will capture the design principle of miRNA regulation within the context of any cell type.

In this study, we present a stochastic, probabilistic model that operates at the cellular level. Furthermore, we substantiate a quantitative view on miRNA regulation that assesses the impact of changes in the quantities and diversity of miRNAs along with changes in cell behaviors. Technically, we applied the iterative simulator (called COMICS) on a selected human cell lines while exhaustively testing the outcome of *in silico* miRNA overexpression manipulations. We confirm the robustness of cells to the combinatorial effects of miRNA manipulations while calculating the retention level of each mRNA at the end of a simulation run (100 k iterations). We identify genes that are sensitive to the rate of mRNA degradation (i.e., cell dynamics) and others that respond to the actual elevation in the amounts of expressed miRNAs. In this study, we expose overlooked properties of miRNA regulation that are highly relevant to the maintenance of cell identity and the progression of cancer.

2 METHODS

2.1 Probabilistic Map for miRNA-mRNA Pairing

The probabilistic framework for the interaction between miRNAs and their matched mRNAs was defined according to TargetScan (Agarwal et al., 2015). Accordingly, a high probability of miRNA-mRNA interaction (values ranging from 0 to 1) complies with numerous features from the sequence, secondary structure, and evolutionary conservation. Altogether, a complete miRNA-mRNA interaction table includes 8.22 M pairs covering as well poorly conserved interactions. We used a compressed version of the interaction table that reports on evolutionarily conserved miRNA pairs. This table includes 1,183,166 pairs which cover 18,953 genes and 289 miRNA families. Interaction scores were mapped to binding probabilities according to TargetScan score: $p = 1–2^{score}$.

2.2 Normalizations of mRNA Expression and miRNA Families

For the mRNA expression profile, we extracted data from RNA-seq experiments of HeLa cells that reliably report on 16,355 mRNAs and 539 miRNAs (Mahlab-Aviv et al., 2019). All genes pass the minimum threshold of >=1 reads (for experimental details see (Mahlab-Aviv et al., 2019)). Based on accepted quantification, we define a cell to display a 2:1 ratio of miRNAs to miRNAs, with a predetermined amount of 50 k and 25 k miRNAs and mRNAs per cell, respectively. Applying a strict threshold of ≥1 molecule per cell resulted in 110 miRNAs and 3666 mRNAs. We limited the expression level to 5 mRNA molecules per cell from a total amount of 25 k molecule (e.g., 0.02%), to improve the robustness of the analysis. Following this threshold, 753 genes remained for further analyses.

2.3 Probabilistic miRNA-mRNA Simulator

The input to COMICS (Competition of miRNA Interactions in Cell Systems) includes a normalized number of molecules from the RNA-seq results, and the values reported for the miRNA-mRNA interaction probabilities (see above). In each run, a random miRNA is chosen from the predetermined available miRNAs distribution. Next, a target is randomly chosen according to available targets' distribution. mRNA that is already bound by miRNA molecules can still be a putative target for another miRNA if the two MBS do not overlap on the same molecule. This is defined as a minimal legitimate distance (≥50 nucleotides apart) between two neighboring RISC. Upon a binding event, the free miRNA and mRNA distributions are updated, with bound mRNA molecules marked as occupied. An occupied molecule (i.e., at least one bound miRNA) is removed after 1 k iterations following a successful binding event (to mimic the destabilization, leading to transcript degradation). Following mRNA removal, the bound miRNAs return to the general pool of free miRNAs.

2.3.1 Configuration of COMICS

COMICS simulator supports a broad set of configurable parameters (see Mahlab-Aviv et al., 2019) that provide a high level of flexibility: 1) the number of total miRNAs; 2) the number of mRNA molecules in the cell; 3) the number of iterations to complete the run; 4) the number of iteration interval between the miRNA-mRNA binding event and the mRNA removal; 5) random removal of unbound mRNAs according to a predetermined decay rate of the mRNAs; 6) addition of newly transcribed mRNAs along with the iterations interval; 6) incorporation of alternative miRNA-target mapping. It is also possible to activate COMICS with a random set of genes, or a pre-existing iteration as a starting point, before the simulation run. In this study, we used default parameters. For mimicking cell manipulation: 7) miRNAs or genes overexpressed according to a selected multiplication factor. Specifically, we tested 7 multiplication steps (from x1 to x1000). If miRNA was undetected in the naïve cell, an arbitrary starting amount of 0.02% (the equivalent of 10 miRNA molecules per cell) is artificially added to the naïve cell (marked as x1).

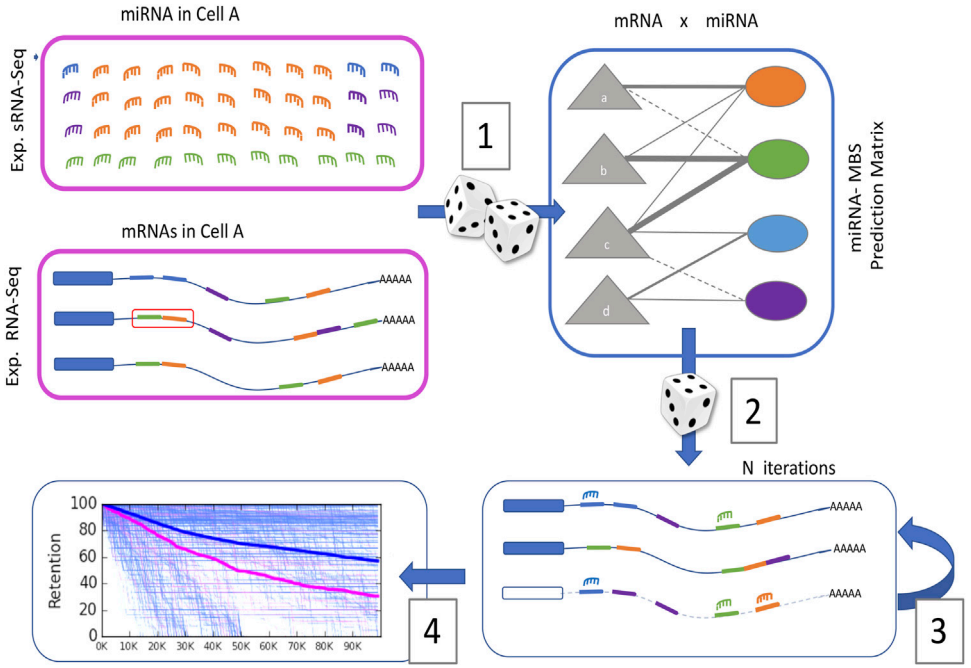

FIGURE 1 | Schematic procedure of the probabilistic nature of COMICS (see text).

2.3.2 Analytical Methods

Statistical values for correlations were determined using standard Python statistical package. For annotation enrichment statistics and visualization, we used Gene Ontology platform (Huang et al., 2007). Clustering was performed by k-means classification method. We used the unsupervised Elbow method to test consistency within clusters by the percentage of variance explained. (i.e., the ratio of the between-group variance to the total variance). A change in the slope indicates the preferred number of clusters in that dataset. Standard statistical tests were applied to provide *p*-value for protein set comparisons.

3 RESULTS

3.1 Assessment of a Probabilistic Approach for Cell States

Our previous study modeled the outcome of the miRNA-mRNA network in living cells by simulating the stochastic nature of miRNA regulation (Mahlab-Aviv et al., 2019). We observed that the relative ratio of miRNA to mRNA dictates the kinetics and the steady-state of expressed genes as measured by tracing the mRNA decay where no new expressed mRNA is considered.

In this study, we extend the analysis by clustering genes by their distinct kinetics and by testing the sensitivity of gene classes to changes in miRNAs' relative quantities. The nature of miRNA regulation in living cells is depicted by the absolute quantities, composition, and stoichiometry of miRNAs and mRNAs (Balaga et al., 2012). Systematic analysis of the miRNA-mRNA interaction network shows that miRNA regulation operates under tight stoichiometric constraints in living cells. Classifying genes into sets that are unified by a common property, reduces complexity, and provides new insights on genes that are unified by their sensitivity to miRNA regulation.

Experimental data from HeLa cells for miRNAs and mRNAs are extracted from repeated NGS experiments (Mahlab-Aviv et al., 2019). A total of 539 miRNA types and 16,236 expressed mRNAs were mapped according to a predetermined expression threshold. As the molecular interaction of miRNA and mRNA within a cell is a stochastic process, we developed COMICS as an iterative simulator that attempts to capture such interactions (Mahlab-Aviv et al., 2019).

3.2 COMICS Performance

Figure 1 illustrates a scheme from a cellular perspective while focusing on the probabilistic framework. COMICS iterations capture the stochastic process in cells according to predetermined quantities and partition of miRNAs and mRNAs. The sampling process (**Figure 1**; Moore et al., 2015) is driven by the distribution of miRNAs and mRNAs that can be monitored experimentally (pink frames). Each mRNA is characterized by the types and positioning of its miRNA binding sites (MBS) on the transcript. The interaction table contains estimates of the probability-based scores for any pair of miRNA and MBS in the context of a specific mRNA. These calculated probabilities do not account for the fact that the expression of miRNAs and mRNAs are cell-type specific.

In each iteration, a miRNA is sampled randomly, according to the cell's miRNA abundance and composition. Next, one of its target genes is chosen randomly according to the measured expressed mRNAs distribution. In the following stochastic step

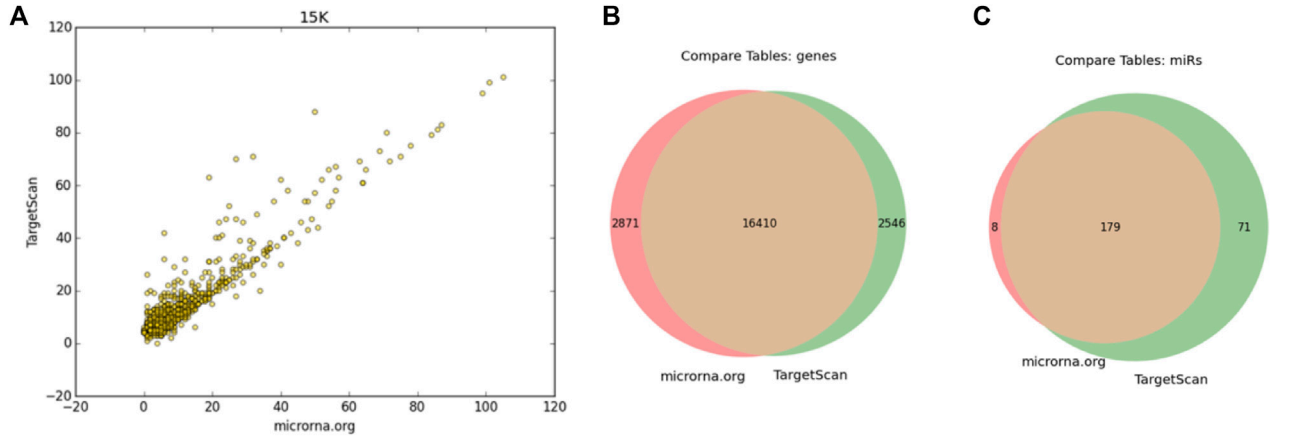

FIGURE 2 | Comparing COMICS results by applying MBS-miRNA interaction table from TargetScan and microRNA.org **(A)** Correlation plot following 15 k iterations with r = 0.93. The interaction table from microRNA.org includes 1,097,065 pairs that covers 19,281 genes and 249 miRNAs, and TargetScan includes 1,183,166 pairs, 19,327 genes and 1741 miRNAs. Venn diagrams of the mRNAs **(B)** and miRNAs **(C)**.

(**Figure 1**; Eichhorn et al., 2014), a randomly chosen miRNA and its target get paired by the sparse table of miRNA-MBS interactions (~1.2 M pairs, see **Section 2**). Each miRNA-MBS interaction is associated with a probabilistic score that is a proxy for the level of confidence for that interaction and can be considered the probability of effective binding for any specific pair. Following a successful binding event, the distribution of the miRNAs and the mRNAs get updated (**Figure 1**; Chekulaeva and Filipowicz, 2009). Following a successful pairing, the status of the mRNA becomes "ready for degradation". Upon binding, it may be engaged in the additional binding of miRNAs, but MBS interactions at close physical proximity are excluded. Our protocol supports cooperative binding on a target by imposing a degradation delay (e.g., 1 k iterations) that allows multiple miRNAs on the same mRNA. When an occupied mRNA is removed from the system, all its bounded miRNAs return to the miRNA pool. Consequently, the stoichiometry of miRNA to mRNA changes gradually with an increase in miRNAs to free mRNAs ratio. Note that additional variables that potentially impact miRNA regulation were omitted from the model for simplicity. These include cases in which binding of the miRNA to its target does not convert into mRNA degradation, instances of competitive endogenous RNA (ceRNA) (Thomson and Dinger, 2016), mRNA with an alternative or edited 3′-UTRs (Zhang et al., 2016), and subcellular partition of miRNAs (e.g., exosomes, nuclei, or cytoplasm) (Mahlab-Aviv et al., 2020).

The results of such a simulation are illustrated in **Figure 1** (Peláez and Carthew, 2012). A decay rate for all genes is shown and the non-target (blue) and the genuine targets (pink) are signified with different dynamics and endpoints. Cell state is defined as the retention levels (%) of the unbounded genes at the end of the simulation run. The overall agreement of COMICS simulator protocol with experimental results was confirmed (Mahlab-Aviv et al., 2019).

The sensitivity of the values chosen to run COMICS was assessed by changing the total molecules in a cell, the initial ratio of miRNAs to mRNAs, the number of iterations required for reaching a steady-state, and more (see detailed in Mahlab-Aviv et al., 2019). Testing COMICS results by changing the input of miRNA-mRNA interaction matrix (see **Figure 1**) is fundamental for assessing the robustness of COMICS. To this end, we activated COMICS under two different mRNA-miRNA pair probabilities extracted from TargetScan and microRNA.org (Betel et al., 2010). The latter provides a MirSVR score for mRNA-miRNA pairs. The score is a result of a machine learning method that was trained on numerous contextual features allowing to rank MBS by their score for downregulation extracted from confirmed target sites.

Figure 2 shows the results of the comparison between the use of conservative TargetScan (see **Section 2**) and MirSVR scores following 15,000 COMICS iterations. A strong correlation (0.93) in using these alternative matrices confirmed the robustness of the analysis (**Figure 2A**). This is substantiated by the large overlap in COMICS results (at 100 k iterations) using these two alternative interaction matrices as input (**Figures 2B,C**).

TarBase v8 (Karagkouni et al., 2018) compiles the current knowledge on miRNA-target pairs from a broad range of experimental methodologies and conditions. This resource also includes cases on cell-type specific miRNA regulation. We performed a COMICS run (100 k iterations) and found a moderate correlation of the retention at the end of the run (382 genes shared genes; Pearson correlation = 0.38, p-value = 5.3e-14). Such correlation was completely lost following miRNA-mRNA pair randomization (Pearson correlation = 0.02).

We conclude that COMICS is quite robust to the use of a particular interaction miRNA-MBS scores (**Figure 2**) and corroborates with validated experimental knowledge on cell regulation by miRNAs.

3.3 miRNA Expression Dominates Cell Identity

Hundreds of cell lines were established for advancing cancer research (Ghandi et al., 2019). Each of these cell types is

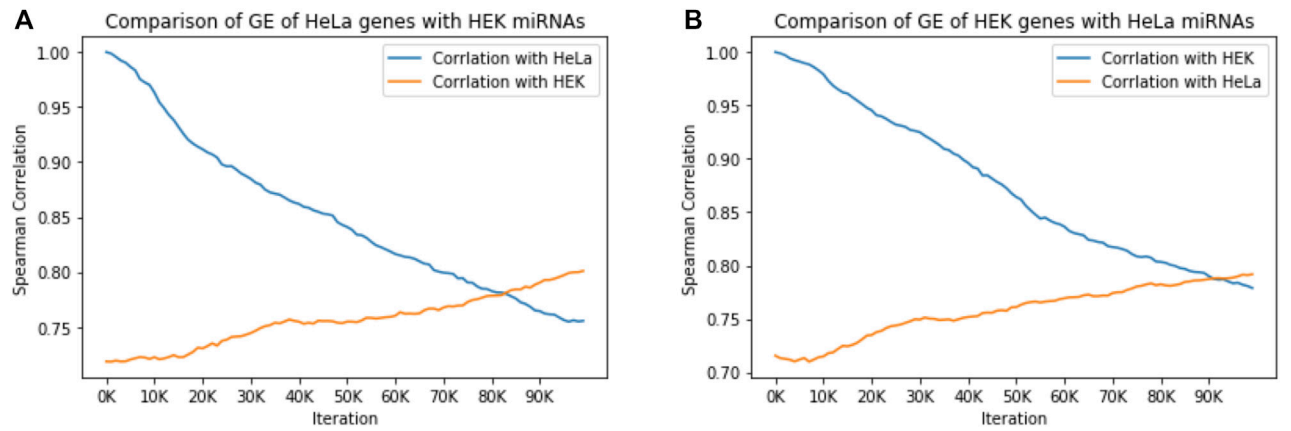

FIGURE 3 | miRNAs govern cell identity. The effect of miRNA expression of one cell type on gene expression (GE) of a different cell type is measured by Spearman correlation of gene expression across the COMICS 100,000 iterations. **(A)** The correlation of HeLa regulated by its own miRNA expression profile (746 genes), with HeLa gene expression regulated by the miRNA expression profile from HEK-293 (514 genes). **(B)** The correlation of HEK-293 regulated by its own miRNA expression profile (516 genes), with HEK-293 gene expression regulated by the miRNA expression profile from Hela (514 genes). All correlation *p*-values are < 1e-15.

considered as a feature in the space of a multidimensional cell. In recent years, mRNAs and miRNAs expression profiles became available, thus providing a solid base to assess the contribution of cell-specific molecular landscape to tumorigenesis. The high correlation between biological samples of miRNA profiles across different cell types and platforms (e.g. (Lu et al., 2005)) was established. We utilize the molecular landscape of established cell lines (HeLa and HEK-293) that represent carcinoma lineages (of cervical and renal cell, respectively) to evaluate the dependency of miRNAs profile and COMICS outcomes.

To this end, we normalized the absolute RNA-seq data to 50 k of miRNA and 25 k of mRNA per cell and monitored COMICS results along the run (100 k iterations). Assessment of the miRNA regulation in the cellular context relies on: 1) Expression of abundant miRNAs differs between cell lines (Mahlab-Aviv et al., 2019). 2) Only a handful of miRNAs account for 90% of the total cellular miRNA molecules in any specific cell. **Figure 3** shows the results from comparing the trend of the gene expression by Pearson correlation across the 100 k iterations of a naïve cell and the cell in which the miRNA profile had been switched to that of the other cell type. **Figure 3A** shows the correlation between HeLa cells and an in-silico hybrid in which the miRNA profile was replaced with that of HEK-293 cells. Under such artificial setting, the correlation is markedly reduced (**Figure 3A**, blue). However, the reverse scenario in which the correlation between HeLa and HEK-293 was measures (Pearson correlation of 0.72, iteration = 0), we observed a monotonic increase in the correlation from 0.72 to 0.81 along the simulation run. **Figure 3B** shows a similar trend when the correlation of naïve HEK-293 is tested with respect to a hybrid setting (HEK-293 with miRNA profile of HeLa). The quantities of miRNAs in each of the two cell lines (normalized values) are listed in **Supplementary Table S1**. These results show that miRNAs largely dictate cell identity. We conclude that the molecular composition of miRNAs governs the dynamics and the steady state. Thus, tracing cell behavior across the progression of the simulation is informative.

3.4 Dynamic Gene Classes

Figure 4 shows the results from HeLa cells and following k-means classification of genes according to their retention profiles throughout the simulation run. **Figure 4A** presents the clustering result for k = 5. We refer to each cluster as a Dyn-class. The average behavior of the dynamic classes of all genes is shown (total ~750 genes). Genes are non-uniformly associated with the different dynamic classes, with cluster #1 covering 69% of the genes and only <4% being associated with the fast decaying cluster (cluster #5). Inspecting the decay rate of the lower retention clusters (cluster #4 and #5, 7% of total genes) show that despite differences in degradation rates, the endpoint almost coincides. While cluster #1, the most stable one, contains genes of the translation machinery, ribosomal subunits, chaperones, and cytoskeletal components, Cluster #5, with the lowest retention rate, contains genes that are enriched in the annotation of transcription regulators and splicing factors. We conclude that genes' dynamics carries valuable functional information while reducing the complexity of the cell regulation model.

3.5 Exhaustive Perturbations of miRNAs

Accumulating evidence argues that an abrupt change in the expression of specific miRNA (or a set of miRNAs) may lead to a switch in cell identity tumorigenesis. Thus, we performed a set of manipulations in HeLa cells using a systematic approach covering all expressed miRNAs. We applied COMICS simulations by *in silico* overexpression of each of the 248 miRNA-MBS predictions (from TargetScan table, see

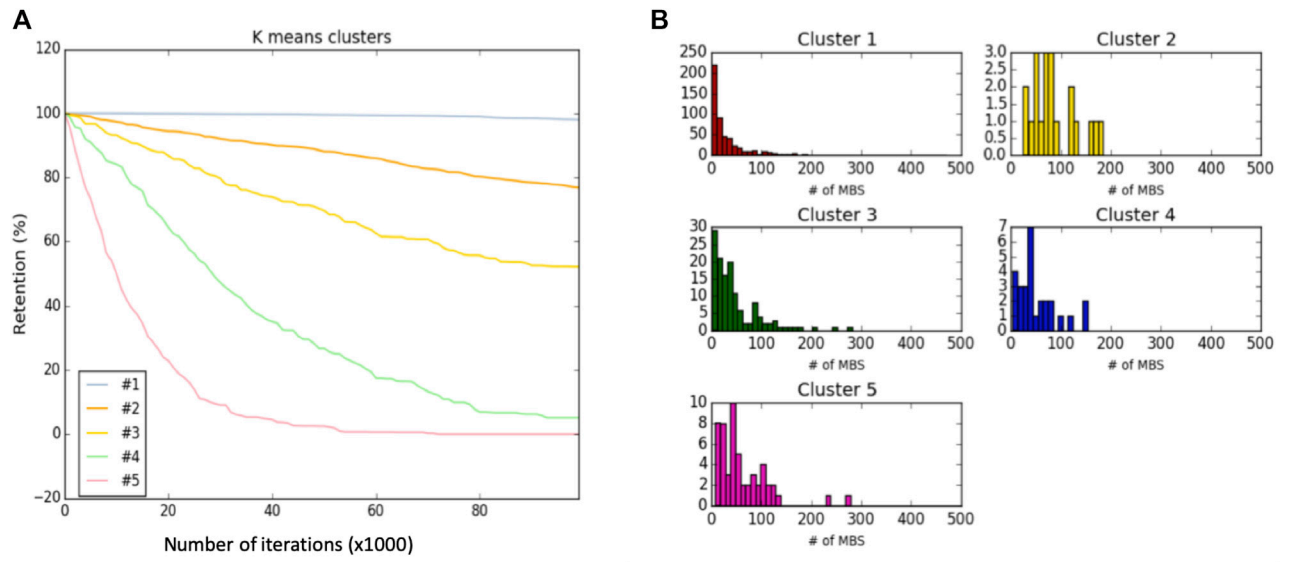

FIGURE 4 | Dynamic classification and analysis. **(A)** Dynamic classes of Hela cells along 100 k iterations of a single simulation run. **(B)** The distribution of number of MBS per gene in the 5 dynamic classes (Dyn-class).

Section 2). We multiply the basal abundance (x1) of each miRNA family by the following factors: x3, x9, x18, x90, x300, and x1000. For each such multiplication factor (f), final retention was computed, and cell state at the end of the COMICS run was monitored.

Figure 5 shows the pattern of the mRNA retention (%) for a matrix of miRNAs (columns) and genes. The panels show Mfij with factors x3, x9, x90, and x1000, where each of the listed miRNAs was overexpressed by the indicated multiplication factor. Therefore, each cell in the matrix Mfij is the final % of retention of gene i after 100 k iterations of COMICS for the overexpressed experiment of miRNA j (**Figure 4**). As the Mfij matrix reveals, genes are naturally clustered by their final retention level. For example, top "red" rows represent genes that were very sensitive to manipulation by any miRNA type (red = 0% retention; purple = 100% retention).

Several observations can be drawn from inspecting these matrices (**Figure 5**): 1) Large number of coordinated behaviors is evident. This is reflected by observing monochromatic rows across most miRNA columns. 2) As the overexpression factor increases (towards x1000), the pattern of the columns (i.e., specific miRNAs) becomes more informative and distinct. This is qualitatively seen as the increasing number of monochromatic columns at x90 and x1000 relative to their number in the moderate overexpression setting (x3 and x9). We present the result of the miRNA profile for overexpression of x300 (normalized) for 248 different miRNAs (**Supplementary Table S2**).

3.6 Perturbation by miRNA Overexpression of Pair Ratio Classes

Given the observation that genes behave similarly for their retention level at a broad range of Mfij, we tested the possibility of classifying genes by their sensitivity to miRNA abundance.

Following the relative changes of each gene retention by each miRNA, and an overexpression factor, we computed the retention ratio between any overexpression multiplications. Formally, we computed the value of Mfij/Mkij. This is the ratio of the retention of a specific gene (simulation at 100 k iterations) in a specific miRNA overexpressed by factor f, and its retention in the same miRNA overexpressed by factor k (**Figure 6A**). For visualization purposes, a discretization was applied for which ratio is > 2 folds. It implies that the retention of genes i in the overexpression of miRNA j by factor f is higher than its retention where miRNA j was overexpressed by factor k (**Figure 6B**, blue). However, a ratio that is < 0.5 implies that in factor f the gene is more prone to degradation for factor k (**Figure 6B**, red). Clusters with coherent behavior with respect to the ratio of two consecutive overexpression ratios were defined as OXR-classes.

Figure 6C illustrates the retention ratios of three selected genes (for illustrative purposes). It is shown that following overexpression of hsa-mir-155 the gene TPI1 (Triosephosphate Isomerase 1) remains stable throughout all tested retention ratios. As expected, TPI1 belongs to the dynamic class of genes that are extremely stable in the system (according to the Dyn-class). Different behavior is observed for ITGB1 (Integrin subunit beta 1) whose expression is very unstable and sensitive to a relatively minor change in amounts (the ratio of x18/x9). The non-monotonic behavior of ITGB1 and DSTN (Destrin, actin-depolymerizing factor) are evident.

Figure 6D illustrates the gene sensitivity as measured by the retention rate of different genes in the case of hsa-mir-155 for 6 pairs of factors: (x1, x3), (x3, x9), (x9, x18), (x18, x90), (x90, x300), and (x300, x1000). The results of COMICS retention for a

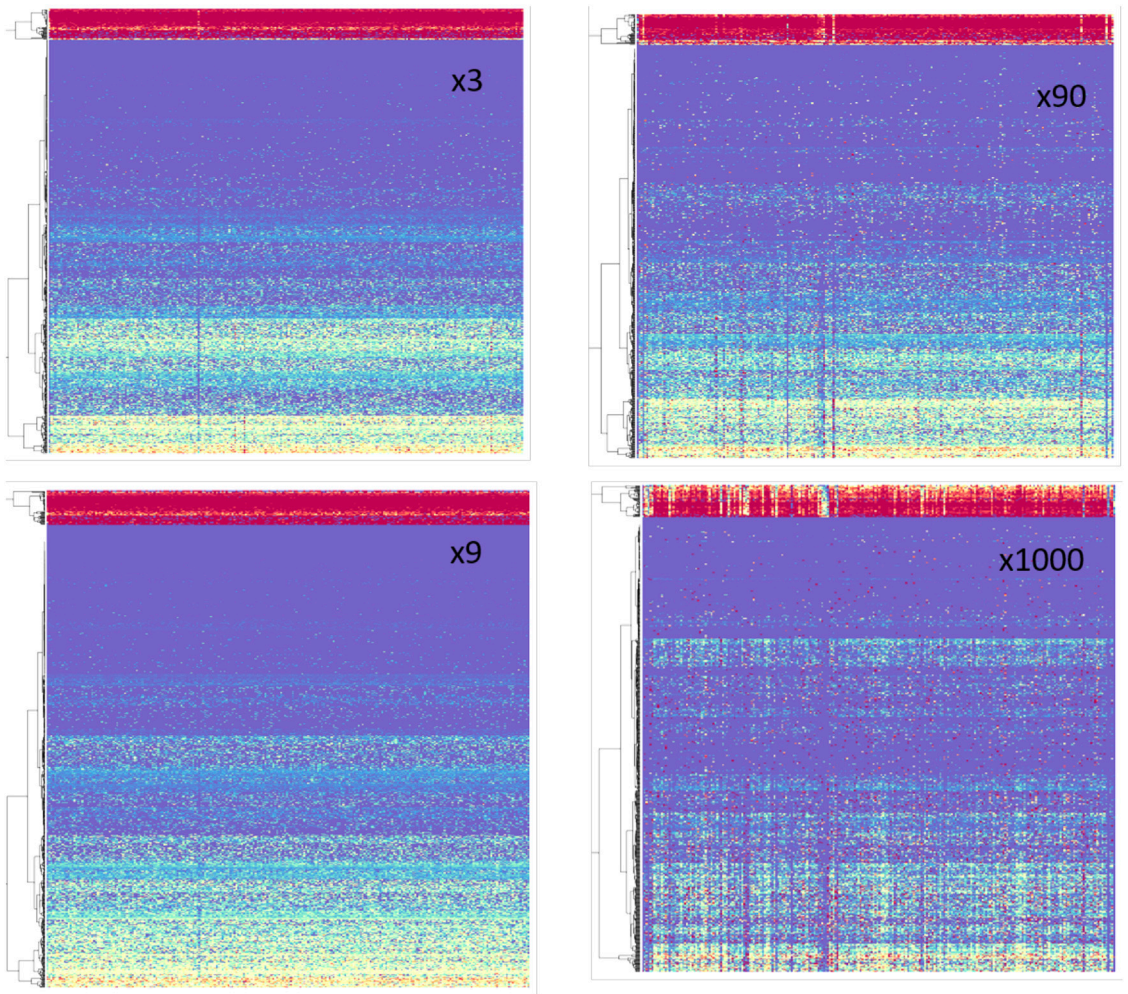

FIGURE 5 | Retention values following *in silico* overexpression from HeLa cells. Rainbow color code: red = 0 to purple = 100% retention level at the end of the simulation run (100 k iterations). Each panel consists of 248 miRNAs (columns) and 750 genes (rows). The overexpression factors are indicated (x3 x9, x90, x1000). The miRNAs (columns) are sorted alphabetically, matrices are clustered by genes.

specific miRNA overexpression were clustered by the K-means clustering algorithm (a cluster must contain >5 genes). The analysis reveals that OXR-classes display different sensitivity patterns for the pair-overexpression retention ratio. **Figure 6D** shows the partition of all genes into 5 clusters (marked #1 to #5). The majority of the genes (~71%, cluster #1, colored grey) are indifferent to the levels of overexpression factors. However, the rest (~29%) of the genes are sensitive to some extent to the overexpression factors that were used. For example, cluster #3 (**Figure 6D**, yellow) contains genes whose retention rate is drastically decreased as the overexpression factor increases (f). It is satisfying to note that most hsa-mir-155 expressed target genes (52%) belong to cluster #3.

The illustration of **Figure 6D** was extended to show miRNAs that are candidates for strong cell-behavior dependency. **Figure 7** shows 4 selected miRNAs according to their OXR-class across a 6-consecutive matrix ratio. The selected represented miRNAs are expressed at a different order of magnitudes. miRNAs that are highly expressed (e.g., hsa-mir-7, 4.2% of total miRNA in the cell), and others that are were analyzed and compared to low level expressing miRNA (hsa-mir-123, 0.006%).

Figure 7 exhibits several behaviors associated with the OXR-classes: 1) For all miRNAs, the largest OXR-class includes 82–91% of the analyzed genes. This cluster is quite stable, implying that most genes are insensitive to perturbation according to OXR. 2) The OXR-class that includes most targets of the subjected miRNA, a monotonic decrease is observed with a maximal effect seen for a ratio of the highest overexpression pair. This is shown for hsa-mir-7 in cluster #2. Note that for some miRNAs, no target is detected in the list of the analyzed genes (e.g., hsa-mir-92). 3) Some OXR-classes show a non-monotonic behavior that cannot be trivially anticipated, as

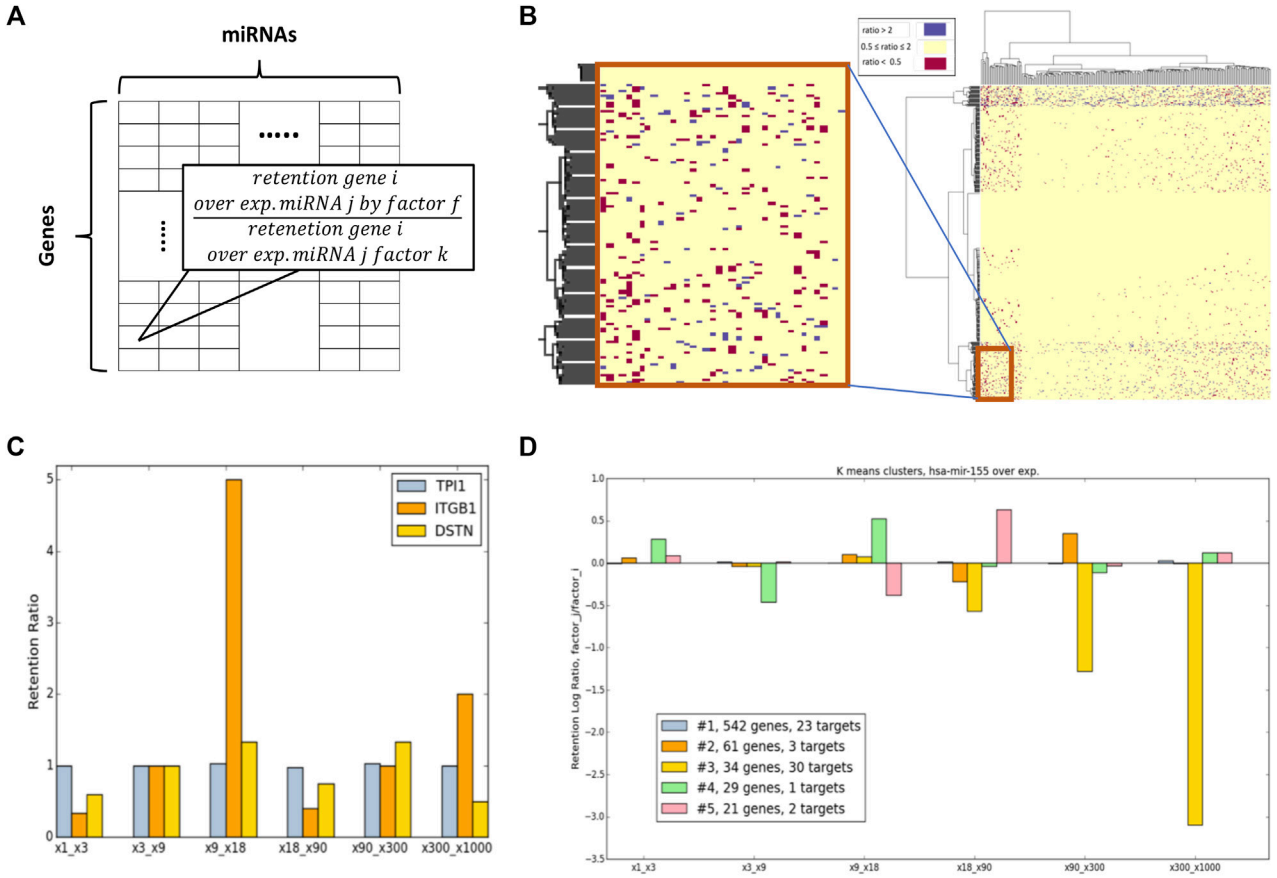

FIGURE 6 | Overexpression ratio (OXR) classes. **(A)** Scheme for the presented Mfij/Mkij ratio retention matrix. **(B)** Ratio matrix for x90/x9 and a zoon-in for a small section. Colors only marked genes that show a significant ratio difference (by the predetermined differential folds of >2, and <0.5). **(C)** Examples of 3 selected genes according to the retention ratio from the 6-consecutive ratio-matrices. **(D)** A case study of hsa-mir-155 manipulation *via* OXR-clusters. A measure of the genes associated with five OXR-classes represented for 6-consecutive ratio matrices (x-axis). The numbers of expressed targets of hsa-mir-155 that are included in each OXR-class (including the numbers of direct targets) are indicated.

shown for hsa-mir-99 cluster #4. 5) Some clusters show extreme increases or decreases in retention rates. The gene sets in such clusters exhibit high sensitivity to a specific miRNA abundance, as demonstrated for hsa-mir-132, cluster #3. **Figure 7** illustrates OXR-classes for 6-consecutive ratio-matrices. A complete comparison and analyses are beyond this illustration. It includes each miRNA (total 248), for 21 pairs the 7 overexpression factors tested.

4 DISCUSSION

The detailed quantitative considerations of miRNA and mRNA govern the dynamics and the steady-state of a gene expressed in cells (Bosson et al., 2014; Hausser and Zavolan, 2014). Cells' behavior cannot be extracted from the direct measurements of miRNAs or mRNAs (Landgraf et al., 2007; Arvey et al., 2010). Still, insights on the regulation of gene expression by miRNAs in the complexity of the cells are improving as more experimental results become available (e.g., CLIP-Seq, CLASH (Li et al., 2014)) and the maturation of single-cell technologies. Most of the knowledge about specific miRNA in cancer samples relies on *in vitro* studies on the effect on an oncogene, tumor suppressor, or transcription factors. Despite progress in data collection that proposed a specific role of miRNAs in tumorigenesis, the underlying rules for regulation by miRNAs in the context of cell identity are still fragmented (Erhard et al., 2014).

The OXR-classes aim to capture the miRNA-dependent system dynamics (rather than the gene expression dynamics). We were able to cluster genes to their OXR-classes by performing hundreds of simulations that yield a robust assessment of cell states. For most instances, under all conditions, the majority of the expressed genes are not sensitive to the matrix-ratio measures. Namely, the final retention that is achieved in all conditions of overexpressed miRNAs is unchanged (**Figure 7**, y-axis = 0). In a smaller set of genes, a switch in the abundance of a specific miRNA may dramatically change target regulation (see examples in **Figures 7B,D**).

In this study, we consider two sets of gene classes: dynamic-classes (Dyn-class, **Figure 4**) and overexpression-ratio ratio

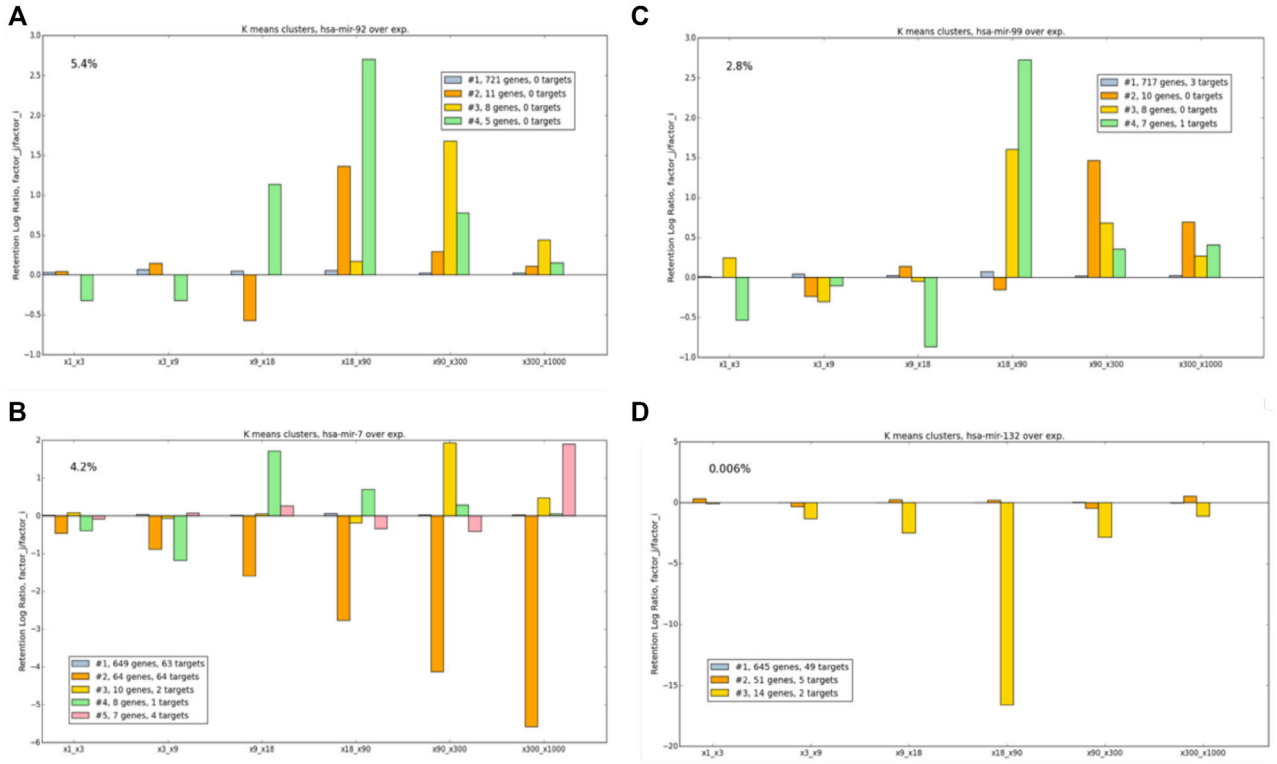

FIGURE 7 | Example of OXR classes for different miRNAs, following manipulations of **(A)** has-mir-92, **(B)** hsa-mir-7, **(C)** hsa-mir-99, and **(D)** hsa-mir-132. The numbers of the targets of each tested miRNA are indicated (see color legend). miRNA expression (% of all miRNA in the cell) is marked. The values at y-axis are expressed by log2.

(OXR-classes, **Figures 6**, **7**). These two complementary types of classes capture different aspects of miRNA regulation. Results from the dynamic class show that genes which are likely to be successfully targeted are those with a relatively large number of MBS at their 3′-UTR (**Figure 4B**). However, Dyn-classes #2 to #5 are not distinguished by such features. Specifically, cluster #2, #3, #4, #5 are associated with 50.3, 71.6, 63.3, and 47.6 average MBS per gene.

There are numerous limitations in using COMICS to determine the sensitivity of cells to combinatorial regulation. For example, we assume all the measured miRNAs are accessible for regulation by miRNAs. However, a substantial fraction is not available in the cell cytosol. Partition of miRNAs in subcellular location is not addressed by our model (Mahlab-Aviv et al., 2020). Moreover, the TargetScan interaction table is restricted to major transcripts, and alternatively, spliced variants that potentially affect MBS are ignored. The addition of cell-specific genes list and gene versions will benefit the refinement of classification.

It was shown that miRNA profile is carefully regulated to promote and stabilize cell fate choices (Shenoy and Blelloch, 2014). Unfortunately, many experiments that use overexpression (and other perturbations like RNAi) do not measure or report the extent of miRNA overexpression. We have shown (**Figure 5**) that different genes exhibit different level of response to the absolute amount of the studied miRNA. We anticipate that inconsistencies among experimental results may be attributed to the missing overexpression factors.

Notwithstanding 2 decades of research in the miRNA field, basic principles remain unknown. Most current knowledge on the specificity of the miRNA-mRNA regulatory network is based on computational prediction tools (Peterson et al., 2014) that suffer from a flood of false positives (Pinzón et al., 2017). Experimental methodologies (e.g. CLASH and CLIP-Seq) that are based on capturing the interactions followed by sequencing show poor reproducibility (Lu and Leslie, 2016). miRNA regulation is often cell specific and changes in response to change in cellular conditions (as in differentiation, infection etc). Despite such variability, COMICS results agree with TarBase v8 (Spearman correlation = 0.38, p-value = 5.3e-14), arguing that the simplistic model implemented in COMICS provide valuable information that was further used to classify genes to various resultion classes. We suggest that a representation of genes by their Dyn- and OXR-classes can yield an accurate yet simple model for miRNA regulation. This study illustrates the use of COMICS results for gene classification. It further stresses the importance of a quantitative view for miRNA regulation modeling.

In pathological cells, such as in cancer, a quantitative change in the amounts of miRNAs is often the most significant molecular change observed in the early phase of cancer development (Peng and Croce, 2016). Assessing changes in the behavior of representative genes from OXR-classes could benefit cancer

diagnosis. Some OXR-classes may serve as an indicator for a shift in cell states and identity. In all our classification approaches, only a very small number of coherent gene classes are reported. Cells likely display unexpected robustness concerning miRNA regulation (Ebert and Sharp, 2012). Revisiting our model on cell line encyclopedia will allow generalizing our observations to the collection of cancerous cells (primary and established) (Ghandi et al., 2019). The ability to classify genes according to dynamic overlooked features carries its potential to improve cell modeling and the understanding of cellular miRNA regulation in health and disease.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

The research is part of the PhD research of SM-A, NL, and ML served as mentors. SM-A, NL, and ML contributed to conceptualization, analysis, curation, visualization and manuscript draft writing. All authors are accountable for the content of this work.

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REVIEW
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A Review on the Role of miR-1290 in Cell Proliferation, Apoptosis and Invasion

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MicroRNAs (miRNAs) have been shown to affect expression of several genes contributing in important biological processes. miR-1290 a member of this family with crucial roles in the carcinogenesis. This miRNA is transcribed from *MIR1290* gene on chromosome 1p36.13. This miRNA has interactions with a number of mRNA coding genes as well as non-coding RNAs SOCS4, GSK3, BCL2, CCNG2, KIF13B, INPP4B, hMSH2, KIF13B, NKD1, FOXA1, IGFBP3, CCAT1, FOXA1, NAT1, SMEK1, SCAI, ZNF667-AS1, ABLIM1, Circ_0000629 and CDC73. miR-1290 can also regulate activity of JAK/STAT3, PI3K/AKT, Wnt/β-catenin and NF-κB molecular pathways. Most evidence indicates the oncogenic roles of miR-1290, yet controversial evidence also exists. In the present review, we describe the results of *in vitro*, animal and human investigations about the impact of miR-1290 in the development of malignancies.

Keywords: miR-1290, cancer, biomarker, miRNA, expression

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INTRODUCTION

MicroRNAs (miRNAs) are a group of small-sized transcripts with a wide range of regulatory roles. They are mostly produced through a multistep mechanism. These steps include transcription from DNA sequences into primary miRNAs and processing into precursor miRNAs and subsequently into mature miRNAs. The majority of bind with the 3′ untranslated region (3′ UTR) of target transcripts to either degrade mRNA or repress its translation. In some circumstances, miRNAs can induce translation or control transcription (O'Brien et al., 2018). Approximately 50% of all miRNAs are transcribed from intragenic regions. These miRNAs are mainly produced from introns and a number of exons of protein coding genes. Other miRNAs are intergenic and are produced in an independent manner from a host gene. Thus, these miRNAs have their own promoters (Kim and Kim, 2007; De Rie et al., 2017). miRNAs partake in the regulation of important biological functions, such as cell proliferation, differentiation and apoptosis, thus being involved in the pathoetiology of several disorders, particularly neoplastic disorders (Peng and Croce, 2016). These transcripts participate in the pathoetiology of diverse cancers (Abolghasemi et al., 2020).

miR-1290 is transcribed from *MIR1290* gene on chromosome 1p36.13. The primary transcript (NR_031622.1) has 78 nucleotides (GAGCGUCACGUUGACACUCAAAAAGUUUCAGAUUUUGGAACAUUUCGGAUUUUGGAUUUUUGGAUCAGGGAUGCUCAA). The mature transcript

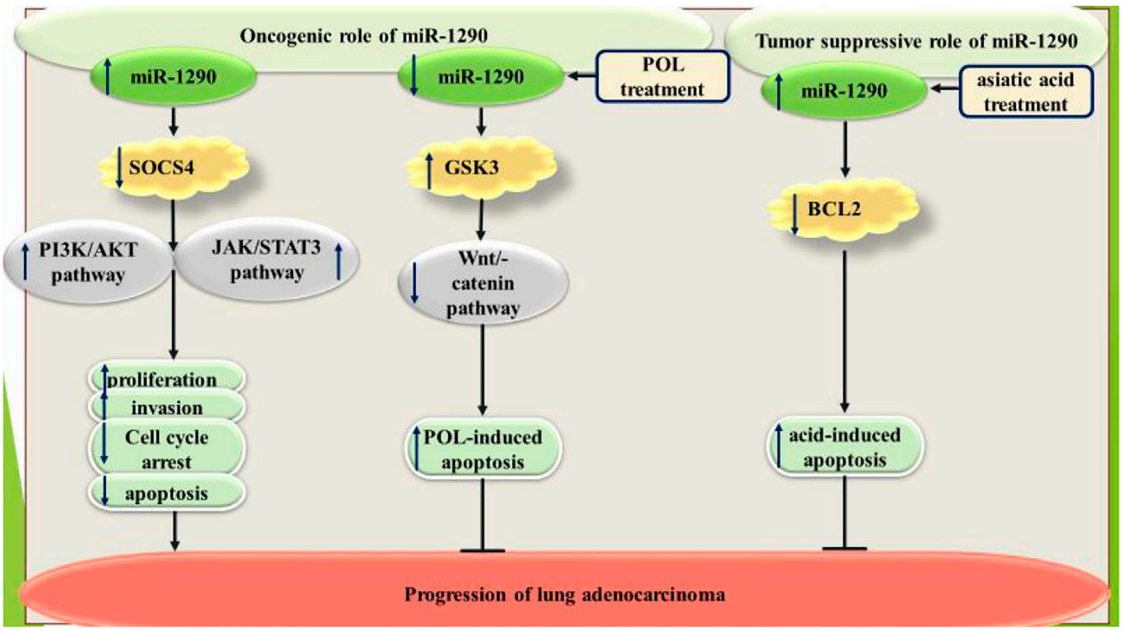

FIGURE 1 | Dual roles of miR-1290 in the pathoetiology of lung cancer.

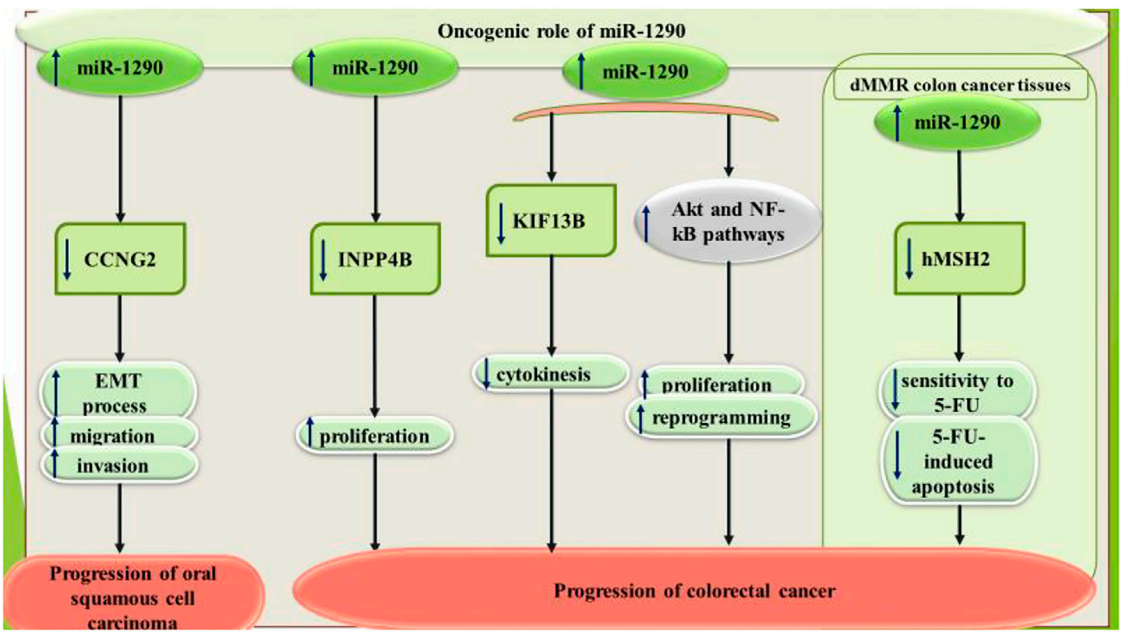

FIGURE 2 | Oncogenic influence of miR-1290 in squamous cell carcinoma and colorectal cancer.

of hsa-miR-1290 (MIMAT0005880) has 19 nucleotides (UGG AUUUUUGGAUCAGGGA). This miRNA has important functions in the carcinogenesis. Several *in vitro* studies have assessed function of miR-1290. Moreover, animal studies in lung, colon and liver cancer models have assessed functional consequences of up-regulation or silencing of this miRNA. However, some inconsistencies exist regarding the role of miR-1290. In the present manuscript, we describe the results of *in vitro*, animal and human assays about the influence of miR-1290 in the development of cancers.

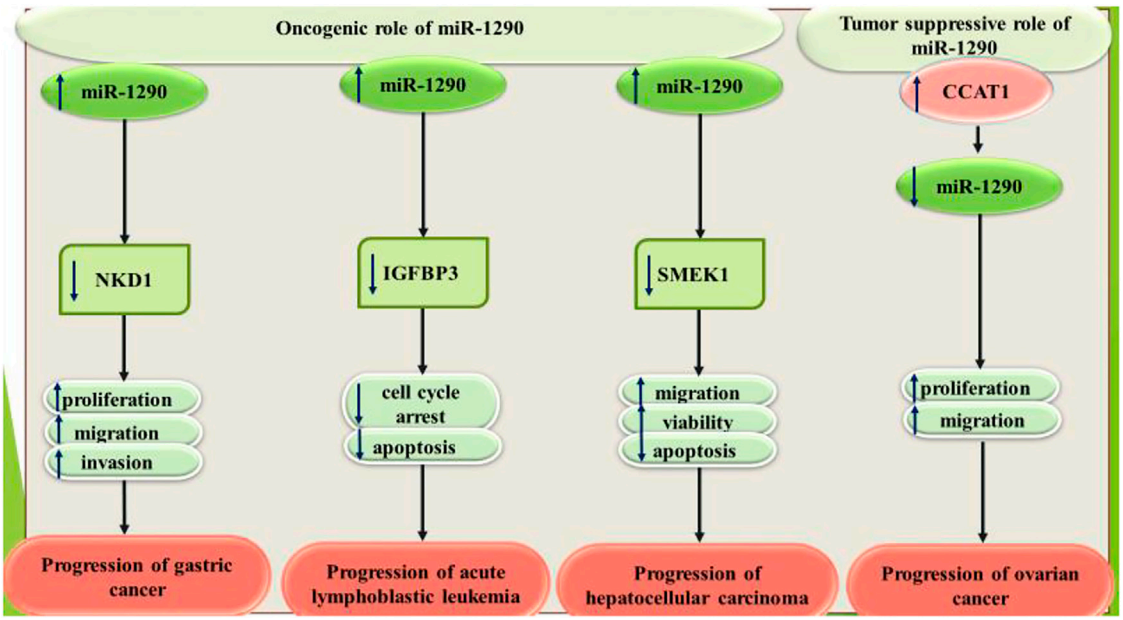

FIGURE 3 | miR-1290 has oncogenic roles in gastric cancer, acute lymphoblastic leukemia and hepatocellular carcinoma, while it has tumor suppressive roles in ovarian cancer.

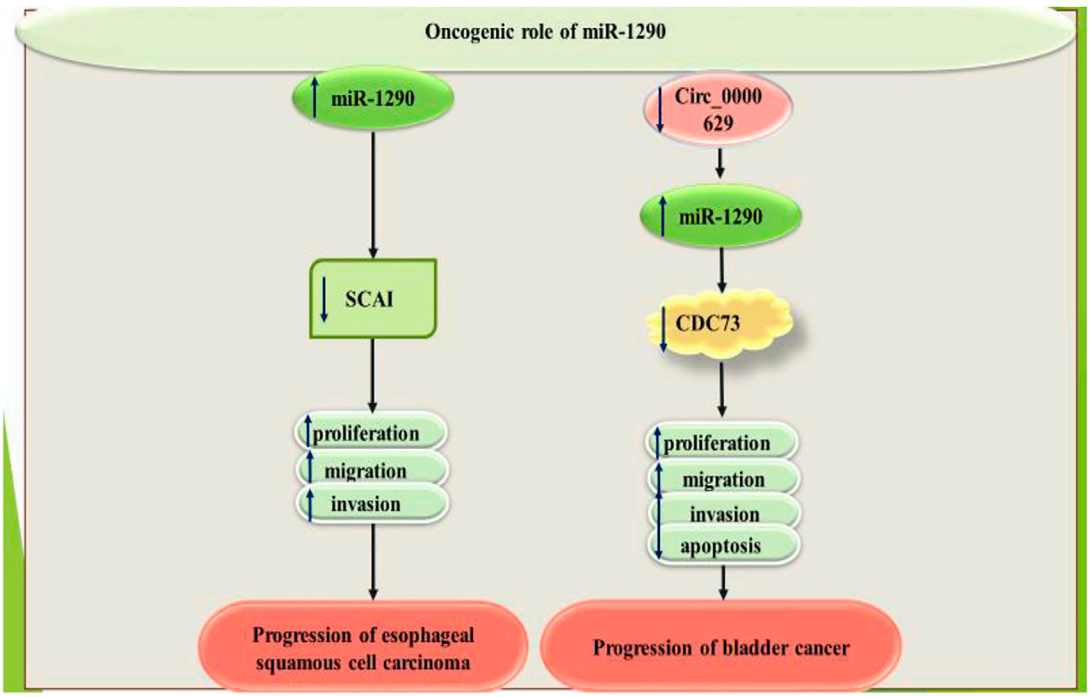

FIGURE 4 | Oncogenic role of miR-1290 in esophageal and bladder cancers.

IN VITRO STUDIES

Forced over-expression of miR-1290 in AsPC1 and Panc5.04 pancreatic cancer cell lines has led to enhancement of cell proliferation. Inhibition of miR-1290 in pancreatic cancer cells has the reverse effects. miR-1290 mimics have also enhanced invasive properties of these cells (Li et al., 2013).

Over-expression of miR-1290 has enhanced proliferation of proliferation of lung adenocarcinoma cells and induced cell cycle progression and invasiveness. Moreover, this miRNA has

TABLE 1 | Expression pattern of miR-1290 in cancer cell lines (Δ: knock-down or deletion, POL: Polygonatum odoratum lectin, 5-FU: 5-Fluorouracil).

Tumor type	Targets/Regulators and signaling pathways	Cell line	Function	References
Pancreatic cancer	—	Panc5.04, Panc8.13, Panc10.05, Panc198, HPDE	↑ miR-1290: ↑ proliferation, ↑ invasion	Li et al. (2013)
Lung cancer	SOCS4, JAK/STAT3 signaling pathway, PI3K/AKT signaling pathway	BEAS-2B, A549, SPC-A1	↑ miR-1290: ↑ proliferation, ↑ invasion, ↓ G1/G0 phase arrest, ↓ apoptosis	Xiao et al. (2018)
	GSK3, Wnt/-catenin pathway	A549	POL treatment: ↓ miR-1290 ↑ miR-1290 + POL treatment: ↓ POL-induced apoptosis Δ miR-1290 + POL treatment: ↑ POL-induced apoptosis ↑ miR-1290: did not affect proliferation, did not affect autophagy Δ miR-1290: did not affect proliferation, did not affect autophagy	Wu et al. (2016)
	BCL2	A549	asiatic acid treatment: ↑ miR-1290 ↑ miR-1290: ↑ acid-induced apoptosis	Kim et al. (2014)
Oral squamous cell carcinoma	CCNG2	NHOK, Cal-27, SCC-9, SCC-25, Tca-8113 c	Δ miR-1290: ↓ migration, ↓ invasion ↑ miR-1290: ↑ EMT process	Qin et al. (2019)
Laryngeal squamous cell carcinoma	KIF13B	UT-SCC-34	—	Janiszewska et al. (2015)
Colorectal cancer	INPP4B	FHC, and CRC cells SW480, HT-29, COLO205, SW403, KM202L, SW620	↑ miR-1290: ↑ proliferation Δ miR-1290: ↓ proliferation	Ma et al. (2018)
	—	Caco2, DLD1, HT29, LoVo, SW480	Δ miR-1290: ↓ proliferation, ↓ migration, ↓ invasion	Imaoka et al. (2016)
	hMSH2	RKO, SW480, HCT116, and LoVo	↑ miR-1290: ↑ viability, ↓ sensitivity to 5-FU Δ miR-1290: ↑ sensitivity to 5-FU, ↑ apoptosis	Ye et al. (2017)
	KIF13B, Akt and NF-kB pathways	SW620, 293T, SGC7901 c	↑ miR-1290: ↑ proliferation, ↑ reprogramming, ↓ cytokinesis	Wu et al. (2013)
Gastric cancer	NKD1	SGC7901, AGS, and BGC823, GES	↑ miR-1290: ↑ proliferation, ↑ invasion, ↑ migration	Huang et al. (2019)
	FOXA1	GES-1, SGC-7901	Δ miR-1290: ↓ proliferation, ↓ migration, no significant difference in apoptosis	Lin et al. (2016)
Acute lymphoblastic leukemia	IGFBP3	PBMCs	Δ miR-1290: ↑ cell cycle arrest, ↑ apoptosis	Zhou et al. (2017)
Ovarian cancer	CCAT1	OVCAR-8, SKOV-3 w, IOSE386, OMC685	Δ lncRNA CCAT1 (which sponges miR-1290): ↓ proliferation, ↓ migration	Lai and Cheng, (2018)
Breast cancer	FOXA1, NAT1	T47D, MCF-7	↑ miR-1290: ↓ expression levels of FOXA1 and NAT1 in ER-positive breast cancer cells	Endo et al. (2013)
Hepatocellular carcinoma	SMEK1	HUVECs, Hep3 B, HepG2, SMMC-7721, PLC/PRF/5, L-02	↑ miR-1290: ↑ migration, ↑ viability, ↑ capacity of HUVECs to form tube-like structures Δ miR-1290: ↓ migration, ↓ viability, ↑ apoptosis	Wang et al. (2021c)
Esophageal squamous cell carcinoma	SCAI	Eca109, TE13	↑ miR-1290: ↑ proliferation, ↑ invasion, ↑ migration	Li et al. (2015)
Chordoma	NONHSAT024778, Robo1	U-CH1	↑ NONHSAT024778 (which sponges miR-1290): ↑ proliferation, ↑ invasion, ↑ migration	Wang et al. (2021a)
Nasopharyngeal carcinoma	ZNF667-AS1, ABLIM1	NP69, c666-1, CNE-1, CNE-2, HNE1	↑ miR-1290: ↑ proliferation, ↑ invasion, ↑ migration, ↓ apoptosis	Chen et al. (2020)
Bladder cancer	Circ_0000629, CDC73	T24, SW780	↑ miR-1290: ↑ growth, ↑ invasion, ↑ migration, ↓ apoptosis	Wang et al. (2021b)

Studies in animal models.

suppressed cell apoptosis in this cell line. miR-1290 has been found to downregulate expression of SOCS4 to activate JAK/STAT3 and PI3K/AKT pathways (Xiao et al., 2018).

The anti-proliferative and apoptosis-inducing agent polygonatum odoratum lectin (POL) has been shown to decrease miR-1290 levels in A549 lung adenocarcinoma cells. Down-regulation of miR-1290 has been shown to increase POL-associated apoptosis in these cells. GSK3β has been found as the direct target of miR-1290 in A549 cells (Wu et al., 2016). Conversely, miR-1290 has been shown to sensitize A549 cells to the apoptosis-inducing agent asiatic acid through negatively regulating expression of BCL2. Expression of miR-1290 has been

TABLE 2 | Impact of miR-1290 in carcinogenesis based on investigations in animal models (Δ: knock-down or deletion).

Tumor type	Animal models	Results	References
Lung cancer	BALB/c-nu/nu nude mice	↑ miR-1290: ↑ tumor volume, ↑ tumor weight, ↑ invasion, ↑ metastasis	Xiao et al. (2018)
Colon cancer	male BALB/c nude mice	Δ miR-1290: ↑ 5-FU-induced apoptosis	Ye et al. (2017)
Hepatocellular carcinoma	male BALB/c and NOD-SCID mice	Δ miR-1290: ↓ tumor volumes, ↓ tumor weights, ↓ proliferation, ↑ apoptosis	Wang et al. (2021c)
Chordoma	male Balb/c NOD nude mice	Δ NONHSAT024778 (which sponges miR-1290): ↓ tumor volumes, ↓ tumor weights, ↓ tumor growth	Wang et al. (2021a)
Nasopharyngeal carcinoma	BALB/c nude mice	Δ miR-1290: ↓ tumor volumes, ↓ tumor weights	Chen et al. (2020)

up-regulated by asiatic acid. Most notably, the apoptosis-inducing effect of asiatic acid relies on miR-1290 activity. Taken together, miR-1290 has been shown to suppress viability and cell cycle progression of A549 cells (Kim et al., 2014). **Figure 1** summarizes the effects of miR-1290 in the pathoetiology of lung cancer.

In oral squamous cell carcinoma, miR-1290 has been shown to be up-regulated parallel with downregulation of CCNG2. miR-1290 silencing has inhibited metastatic ability and epithelial-mesenchymal transition (EMT). CCNG2 has been identified as the direct target of miR-1290 (Qin et al., 2019). Functional studies in laryngeal squamous cell carcinoma has shown that miR-1290 targets two tumor suppressor genes, namely ITPR2 and MAF (Janiszewska et al., 2015).

miR-1290 has oncogenic roles in colorectal cancer. miR-1290 silencing has suppressed proliferation of colorectal cancer cells. miR-1290 up-regulation has decreased expression of p27 and enhanced transcript and protein amounts of cyclin D1. NPP4B has been recognized as the target of miR-1290 (Ma et al., 2018). Moreover, miR-1290 silencing has improved cytotoxic effects of 5-fluouracil in colorectal cancer cells through targeting hMSH2 (Ye et al., 2017). **Figure 2** shows the oncogenic role of miR-1290 in squamous cell carcinoma and colorectal cancer.

Exosomal miR-1290 has been found to be high in gastric cancer cell lines. miR-1290-containing exosomes could promote proliferation, migratory aptitude, and invasiveness of gastric cancer cells. NKD1 has been identified as the direct target of miR-1290 in these cells (Huang et al., 2019). Moreover, miR-1290 has been revealed to increase proliferation and migratory aptitude of gastric cancer cells through targeting FOXA1 (Lin et al., 2016).

miR-1290 has also been shown to be overexpressed in B-acute lymphoblastic leukemia (ALL) cell line SUP-B15. The anticancer agent resveratrol has been found to down-regulate expression of miR-1290 and enhance IGFBP3 levels in the ALL cells. miR-1290 can target 3′ UTR of IGFBP3 (Zhou et al., 2017). Besides, exosomal miR-1290 has been demonstrated to promote angiogenic processes in hepatocellular carcinoma through influencing expression of SMEK1 (Wang et al., 2021c).

On the other hand, miR-1290 has been shown to exert tumor suppressive role in ovarian cancer. In fact, the oncogenic long non-coding RNA (lncRNA) CCAT1 facilitates ovarian carcinogenesis through decreasing miR-1290 levels (Lai and Cheng, 2018).

Figure 3 shows the roles of miR-1290 in gastric cancer, ALL, hepatocellular carcinoma and ovarian cancer.

Over-expression of miR-1290 has enhanced esophageal squamous cell carcinoma growth, migration and invasiveness through decreasing SCAI levels (Li et al., 2015). In bladder cancer cells, tumor suppressor Circular RNA circ_0000629 has been shown to exert its effects through suppressing miR-1290 levels and up-regulating CDC73 expression (Wang et al., 2021b). **Figure 4** shows the oncogenic role of miR-1290 in esophageal and bladder cancers.

Summary of *in vitro* studies regarding the role of miR-1290 in the carcinogenesis is provided in **Table 1**.

miRNA-1290 has important roles in determination of response of cancer cells to 5-fluouracil. miR-1290 silencing has improved cytotoxic effects of 5-fluouracil in xenografts models of this cancer via targeting hMSH2 (Ye et al., 2017). Other studies have shown oncogenic roles of miR-1290 in animal models of lung cancer (Xiao et al., 2018), hepatocellular carcinoma (Wang et al., 2021c) and nasopharyngeal carcinoma (Chen et al., 2020) (**Table 2**). On the other hand, animal studies have shown that the oncogenic lncRNA NONHSAT024778 acts through sponging miR-1290, thus revealing a tumor suppressor role for miR-1290 (Wang et al., 2021a).

STUDIES IN CLINICAL SAMPLES

In lung adenocarcinoma tissues, expression of miR-1290 has been negatively correlated with SOCS4 levels. Expression of SOCS4 has been inversely correlated with higher clinical stages and lymph node metastases (Xiao et al., 2018). Moreover, miR-1290 levels have been associated with clinicopathological landscapes and poor prognosis of patients with oral squamous cell carcinoma (Qin et al., 2019). In laryngeal squamous cell carcinoma, a high throughput miRNA profiling experiment has shown up-regulation of 33 miRNAs, among them being miR-1290 (Janiszewska et al., 2015).

Comparison of miRNA profiles between deficient and proficient mismatch repair colon cancer tissues has shown up-regulation of miR-1290 in deficient mismatch repair colorectal cancer tissues. Expression of miR-1290 has been correlated with poor prognoses of colon cancer in stages II and III patients who took 5-fluouracil-based chemotherapeutics regimens (Ye et al., 2017).

miR-1290 has also been exhibited to be up-regulated in serum exosomes of gastric cancer patients compared with healthy people (Huang et al., 2019). Another study in gastric cancer patients has

TABLE 3 | Dysregulation of miR-1290 in clinical specimens (DC: benign pancreatic disease controls, PFS: progression free survival, LUAD: Lung adenocarcinoma, ANCTs: adjacent non-cancerous tissues, OS: Overall survival, DFS: disease-free survival, TNM: tumor-node-metastasis, NSCLC: non-small-cell lung cancer, CRA: colorectal adenoma, HGSOC: high grade serous ovarian cancer, EOC: epithelial ovarian cancer, HGSOC: high grade serous ovarian carcinoma.).

Tumor type	Samples	Expression (tumor vs. Normal)	Kaplan-Meier analysis (impact of miR-1290 up-regulation)	Univariate/Multivariate cox regression	Association of miR-1290 expression with clinicopathologic characteristics	References
Prostate cancer	23 CRPC patients	up	Poor OS	—	—	Huang et al. (2015)
Pancreatic cancer (PC)	GEO datasets: (GSE113486 and GSE106817)	up	—	—	—	Wei et al. (2020)
	120 PC patients, 40 DC patients, and 40 healthy controls	up	—	miR-1290 expression was independent risk factors for PC.	gender (male), and stage III and IV	
	167 PC patients and 267 healthy subjects	up	shorter OS and DFS	miR-1290 was not found to be an independent negative prognostic factor for OS and DFS in PC patients	PC aggressiveness	Tavano et al. (2018)
	81 PDAC patients, 28 PNETs patients, 20 IPMN patients, 45 chronic pancreatitis patients, and 39 healthy controls	higher in patients with IPMNs than healthy controls, higher in patients with invasive pancreatic cancer than patients with IPMNs, higher in intermediate- and high-grade dysplasia than those with low-grade dysplasia	—	—	—	Li et al. (2013)
Lung cancer	70 LUAD patients and 40 healthy controls	up	shorter PFS	The level of miR-1290 was an independent prognostic factor in LUAD patients	gender (male), advanced TNM stage, tumor size, lymph node metastasis, distant metastasis, smoking, and drinking	Wu et al. (2020)
	32 pairs of LUAD tissues and ANCTs	up	—	—	—	Xiao et al. (2018)
	33 pairs of NSCLC tissues and ANCTs	up	shorter OS	—	stage IIIa, lymph node metastasis	Mo et al. (2015)
	serum samples from 73 NSCLC patients, 19 patients with various benign lung disease, 34 healthy controls	up	shorter OS	TNM stage and lymph node metastasis status and serum miR-1290 expression were found to be the independent prognostic factors for OS.	TNM stage, lymph node metastasis	
Oral squamous cell carcinoma (OSCC)	47 pairs of OSCC tissues and ANCTs	up	shorter OS	—	TNM stage and the lymph node metastasis	Qin et al. (2019)
	10 OSCC patients and 10 healthy volunteers	down	—	—	—	Nakashima et al. (2019)
	plasma samples from 55 OSCC patients	down	higher OS and DFS	Expression OF miR-1290 was found to be a significant prognostic factor for OSCC patients	tumor differentiation and response to CRT	
Laryngeal squamous cell carcinoma (LSCC)	50 LSCC patients and 5 epithelial no tumor controls	up	—	—	—	Janiszewska et al. (2015)
	5 pairs of LSCC tissues and ANCTs	up	—	—	—	Sun et al. (2013)
	48 LSCC patients	up	—	—	—	
Colorectal cancer (CRC)	GEO datasets: (GSE108153, GSE81581, GSE55139 and GSE41655)	up	—	—	—	Liu et al. (2019)
		up	—	—	—	

(Continued on following page)

TABLE 3 | (*Continued*) Dysregulation of miR-1290 in clinical specimens (DC: benign pancreatic disease controls, PFS: progression free survival, LUAD: Lung adenocarcinoma, ANCTs: adjacent non-cancerous tissues, OS: Overall survival, DFS: disease-free survival, TNM: tumor-node-metastasis, NSCLC: non-small-cell lung cancer, CRA: colorectal adenoma, HGSOC: high grade serous ovarian cancer, EOC: epithelial ovarian cancer, HGSOC: high grade serous ovarian carcinoma.).

Tumor type	Samples	Expression (tumor vs. Normal)	Kaplan-Meier analysis (impact of miR-1290 up-regulation)	Univariate/Multivariate cox regression	Association of miR-1290 expression with clinicopathologic characteristics	References
	15 CRC patients, 15 adenoma cases and 15 healthy controls					
	80 CRC patients, 50 adenoma cases, and 30 healthy controls	up	—	—	larger tumor size, advanced TNM stage, lymph node metastasis, and distant metastasis	
	8 pairs of CRC tissues and ANCTs	up	—	—	—	Ma et al. (2018)
	20 normal colon samples and 50 CRC samples	up	—	—	—	
	12 pairs of CRC tissues and ANCTs, and 12 colorectal adenomas tissues	up	poorer OS	High miR-1290 expression, large tumor size, lymphatic invasion, venous invasion, high T stage, lymph node metastasis, distant metastasis, and high carcinoembryonic antigen levels were associated with poor OS.	—	Imaoka et al. (2016)
	serum samples from 12 CRC patients,12 adenoma patients, and 12 healthy persons	up	worse OS	Increased serum miR-1290 level, poor differentiation, lymphatic invasion, venous invasion, high T stage, lymph node metastasis, distant metastasis, and high CEA levels were associated with poor OS.	—	
	serum samples from 211 CRC patients, 56 colorectal adenoma patients, and 57 healthy controls	up	—	—	stage IV, tumor size, serosal invasion, lymphatic and venous invasion, and metastasis	
	GEO database: GSE39833 (88 CRC patients and 11 healthy controls)	up	—	—	—	Li et al. (2016)
Colorectal cancer (CRC)	54 CRA patients	up	—	—	adenoma size	Handa et al. (2021)
Colon cancer	291 colon cancer tumor tissues	up	Lower OS and DFS	miR-1290 expression, N stage, AJCC stage, tumor differentiation, vascular invasion, miR-and MMR status were associated with decreased OS and DFS.	dMMR Status, tumor location, N stage, and tumor differentiation	Ye et al. (2017)
	25 pairs of colon cancer tissues and ANCTs	up	—	—	—	Wu et al. (2013)
Gastric cancer (GC)	serum samples from 20 GC patients and 10 healthy controls	up	—	—	—	Huang et al. (2019)
	20 pairs of GC tissues and ANCTs	up	—	—	advanced clinical staging and depth of tumor invasion	Lin et al. (2016)
Acute lymphoblastic leukemia (ALL)	15 ALL patients and 15 healthy controls	IGFBP3 (a target of miR-1290) expression is decreased	—	—	—	Zhou et al. (2017)

(Continued on following page)

TABLE 3 | (*Continued*) Dysregulation of miR-1290 in clinical specimens (DC: benign pancreatic disease controls, PFS: progression free survival, LUAD: Lung adenocarcinoma, ANCTs: adjacent non-cancerous tissues, OS: Overall survival, DFS: disease-free survival, TNM: tumor-node-metastasis, NSCLC: non-small-cell lung cancer, CRA: colorectal adenoma, HGSOC: high grade serous ovarian cancer, EOC: epithelial ovarian cancer, HGSOC: high grade serous ovarian carcinoma.).

Tumor type	Samples	Expression (tumor vs. Normal)	Kaplan-Meier analysis (impact of miR-1290 up-regulation)	Univariate/Multivariate cox regression	Association of miR-1290 expression with clinicopathologic characteristics	References
Ovarian cancer (OC)	sera samples from 70 EOC patients and 13 healthy controls	no significant difference	—	—	—	Kobayashi et al. (2018)
	30 HGSOC patients and 13 healthy controls	up	—	—	tumor burden	
	40 pairs of OC tissues and ANCTs	upregulation of lncRNA CCAT1 (which sponges miR-1290)	higher CCAT1 = shorter OS	—	tumor size and lymph node metastasis	Lai and Cheng, (2018)
Breast cancer	blood samples from 60 breast cancer patients and 20 healthy controls	up	—	—	lymph node metastasis and Stage II/III	Li et al. (2021)
	4 ER-high Ki67-low tumor tissues and 4 ER-low Ki67-high tumor tissues	down in ER-high Ki67-low tumors	—	—	tumor grade	Endo et al. (2013)
Hepatocellular carcinoma (HCC)	49 pairs of HCC tissues and ANCTs	Up	—	—	—	Wang et al. (2021c)
	serum samples of 49 HCC patients and serum samples of 28 healthy controls	Up	—	—	—	
Esophageal squamous cell carcinoma (ESCC)	24 pairs of ESCC tumor tissues and ANCTs	up	—	—	differentiation, N classification and tumor-node-metastasis stage	Li et al. (2015)
Chordoma	20 chordoma tissues and 10 FNP tissues	down	—	—	—	Wang et al. (2021a)
Nasopharyngeal carcinoma (NPC)	GEO database: (GSE70970)	up	—	—	—	Chen et al. (2020)
Cutaneous squamous cell carcinoma (cSCC)	8 cSCC patients and 8 controls	up	—	—	—	Geusau et al. (2020)
Cervical cancer	sera from 6 cervical cancer patients and 6 healthy persons	up	—	—	—	Nagamitsu et al. (2016)
	Sera of 20 cervical cancer patients 10 healthy persons	up	—	—	—	
	serum samples from 100 cervical cancer patients and 31 healthy controls	up	—	—	—	
	microarray analysis	up in cells with HPV infection upon 5-AZA treatment	—	—	—	Yao et al. (2013)

shown correlation between miR-1290 over-expression and clinical stage, deepness of invasion and lymph node positivity (Lin et al., 2016).

miR-1290 has also been shown to be upregulated in esophageal squamous cell carcinoma tissues compared with unaffected neighboring samples. Over-expression of miR-1290 has been associated with level of differentiation, N classification TNM stage in this type of esophageal cancer (Li et al., 2015).

On the other hand, in oral squamous cell carcinoma, levels of this miRNA has been reported to be decreased in blood samples of patients compared with control samples (Nakashima et al., 2019). Moreover, expression of miR-1290 has been reported to be decreased in chordoma samples (Wang et al., 2021a). **Table 3** summarizes the results of studies that reported dysregulation of miR-1290 in clinical samples.

Serum levels of miR-1290 have been shown to be higher in patients with intraductal papillary mucinous pancreatic cancer compared with healthy subjects. The ability of serum levels of miR-1290 in separation of patients with low-stage pancreatic cancer from controls has been higher than CA19-9. Notably, higher levels of miR-1290 has been predictive of poor outcome following pancreaticoduodenectomy (Li et al., 2013). In this type

TABLE 4 | Diagnostic value of miR-1290 in cancers (PC: pancreatic cancer; DC: benign pancreatic disease control; LUAD: Lung adenocarcinoma, EOC: epithelial ovarian cancer, HGSOC: high grade serous ovarian carcinoma).

Tumor type	Samples	Distinguish between	Area under curve	Sensitivity (%)	Specificity (%)	References
Pancreatic cancer (PC)	120 PC patients and 40 healthy controls	PC patients vs. healthy controls	0.93	75.0	97.5	Wei et al. (2020)
	120 PC patients and 40 DC	PC patients vs. DC	0.89	88.3	72.5	
	120 PC patients and controls	PC patients vs. all controls	0.91	74.2	91.2	
	81 PDAC patients and 39 healthy controls	PDAC patients vs. healthy controls	0.96	—	—	Li et al. (2013)
	81 PDAC patients and 45 chronic pancreatitis samples	PDAC patients vs. chronic pancreatitis samples	0.81	—	—	
	81 PDAC patients and 28 PNETs patients	PDAC patients vs. PNET samples	0.80	—	—	
	81 PDAC patients and all controlls	PDAC patients vs. all controls	0.85	—	—	
Lung cancer	70 LUAD patients and 40 healthy controls	LUAD patients vs. controls	0.937	80.0	96.7	Wu et al. (2020)
Colorectal cancer (CRC)	15 CRC patients, 15 colorectal adenoma patients and 15 healthy controls	CRC patients vs. healthy controls	0.96	78.79	93.33	Liu et al. (2019)
		colorectal adenoma patients vs. healthy controls	0.92	79.66	86.67	
	12 CRC patients,12 colorectal adenoma patients, and 12 healthy controls	CRC patients vs. healthy controls	1.000	100	100	Imaoka et al. (2016)
		colorectal adenoma patients vs healthy controls	0.722	50	100	
	211 CRC patients, 56 colorectal adenoma patients, and 57 healthy controls	CRC patients vs. healthy controls	0.830	70.1%	91.2	
		colorectal adenoma patients vs. healthy controls	0.718	46.4	91.2	
Ovarian cancer (OC)	sera samples from 70 EOC patients and 13 healthy controls	EOC patients vs. healthy controls	0.48	0.51	0.57	Kobayashi et al. (2018)
	30 HGSOC patients and 13 healthy controls	HGSOC patients vs. healthy controls	0.71	0.63	0.85	

of cancer, miR-1290 has been shown to appropriately distinguish neoplastic condition from both healthy condition and chronic pancreatitis (Wei et al., 2020). In colorectal cancer, levels of this miRNA could distinguish cancer status from healthy condition with up to ideal diagnostic power. Moreover, it can separate colorectal adenoma from healthy status with lower values (Imaoka et al., 2016). **Table 4** shows the diagnostic value of miR-1290 in cancers.

DISCUSSION

Several miRNAs have been found to influence the carcinogenesis. miR-1290 is an example of oncomiRs based on the bulk of relevant evidence. This miRNA has interactions with several cancer-related mRNAs such as SOCS4, GSK3, BCL2, CCNG2, KIF13B, INPP4B, hMSH2, KIF13B, NKD1, FOXA1, IGFBP3, FOXA1, NAT1, SMEK1, SCAI, ZNF667-AS1, ABLIM1, and CDC73.

Moreover, miR-1290 has interactions with a number of non-coding RNAs such as Circ_0000629, CCTA1 and NONHSAT024778. The interaction between lncRNAs/circRNAs and miRNAs has important implications in pathoetiology of cancers, thus future studies are needed to identify other non-coding RNAs that interact with miR-1290 in the context of neoplastic conditions. In fact, these lncRNAs and circRNAs can act as sponge for miRNAs to decrease its bioavalability, thus enhancing expression of targets of miR-1290. Therefore, they construct a competing endogenous RNA (ceRNA) network.

In addition to its role in the regulation of gene expression, miR-1290 can regulate activity of JAK/STAT3, PI3K/AKT, Wnt/β-catenin and NF-κB signaling pathways, thus influencing several cancer-related routes.

Most evidence indicates the oncogenic roles of miR-1290, yet controversial evidence also exists. Particularly, in the lung cancer, both oncogenic and tumor suppressor roles have been reported for miR-1290.

A number of anticancer agents such as POL, asiatic acid and resveratrol has been shown to affect expression of miR-1290. Moreover, this miRNA can influence response of neoplastic cells to the chemotherapeutic agent 5-fluouracil. Thus, one can deduce that miR-1290-targeting strategies can modulate response of cancer cells to a wide variety of antineoplastic modalities.

In addition to its therapeutic implications, the existence of miR-1290 in cancer-derived exosomes not only indicates its application in diagnostic approaches, but also shows the effect of these vehicles in conferring neoplastic features inside the tumor bulk.

The ceRNA networks constructed by circRNAs, miR-1290 and target mRNAs can be used as prognostic biomarkers and therapeutic targets in different cancers. These ceRNA networks are superior to single transcripts since they reflect a more comprehensive overview of dysregulated pathways. Theoretically, the ceRNA regulatory networks including lncRNAs or circRNAs-miR-1290-mRNAs can be applied as

prognostic biomarkers and therapeutic targets in different cancers. High throughput sequencing methods have facilitated applicability of these networks in diagnostic, prognostic and therapeutic fields. Moreover, these techniques have facilitated design of personalized therapeutic options based on the identified dysregulated networks in samples obtained from each patient. Application of this data can enhance survival of patients.

Cumulatively, miR-1290 is a cancer-related miRNA with possible application as diagnostic and prognostic marker in diverse types of cancers. Therapeutic applications of anti-miR-1290 modalities should be assessed in future. Moreover, future studies should address the possibility of targeting the miR1290-containg ceRNA networks.

AUTHOR CONTRIBUTIONS

SG-F wrote the draft and revised it. MT designed and supervised the study. TK and MS collected the data and designed the figures and tables. All the authors read and approved the submitted version.

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REVIEW
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A Review on the Role of miR-1246 in the Pathoetiology of Different Cancers

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miR-1246 is a microRNA firstly recognized through application of a high throughput sequencing technique in human embryonic stem cells. Subsequent studies have shown the role of this microRNA in the carcinogenesis. miR-1246 has been found to exert oncogenic roles in colorectal, breast, renal, oral, laryngeal, pancreatic and ovarian cancers as well as melanoma and glioma. In lung, cervical and liver cancers, studies have reported contradictory results regarding the role of miR-1246. miR-1246 has been reported to regulate activity of RAF/MEK/ERK, GSK3β, Wnt/β-catenin, JAK/STAT, PI3K/AKT, THBS2/MMP and NOTCH2 pathways. In addition to affecting cell cycle progression and proliferation, miR-1246 can influence stemness and resistance of cancer cells to therapeutics. In the current review, we describe the summary of *in vitro* and *in vivo* studies about the influence of miR-1246 in carcinogenesis in addition to studies that measured expression levels of miR-1246 in clinical samples.

Keywords: miRNA, MiR-1246, cancer, expression, biomarker, *in vivo*, *in vitro*, diagnosis

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INTRODUCTION

miR-1246 has been firstly recognized through application of a high throughput sequencing technique in human embryonic stem cells (Morin et al., 2008). Subsequent studies have mapped the human miR-1246-coding gene, i.e., *MIR1246* gene on chromosome 2q31.1 and reported the impact of p53 on the regulation of its expression (Zhang et al., 2011). Notably, the nucleotide sequence of the mature miR-1246 is identical to the central region of the RNU2-1 RNA (Xu et al., 2019), a small nuclear RNA which constructs the scaffold for establishment of the U2 complex in the spliceosome (Patel and Bellini, 2008).

Theoretically, the stem-loop TaqMan technique for detection of miR-1246 is expected to amplify both miR-1246 and RNA, U2 Small Nuclear 1 (RNU2-1). However, the poly-A tailing SYBR strategy can differentiate between miR-1246 and RNU2-1, since the sizes of the amplified fragments can be differentiated through assessment of their meting curves (Xu et al., 2019). Application of the latter strategy for assessment of miR-1246 expression in wild type and *MIR1246* knockout pancreatic adenocarcinoma cells and exosomes originated from these cells has led to identification of a variant of the mature miR-1246 in exosomes that is transcribed from cellular RNU2-1 in an independent manner from Drosha and Dicer miRNA processing enzymes (Xu et al., 2019).

Several researchers have assessed expression of miR-1246 in different cancer cell lines using a variety of miRNA-profiling assays. Subsequently, they have performed functional assays to find the effects of miR-1246 up-regulation or silencing on proliferation and invasive properties of these cells.

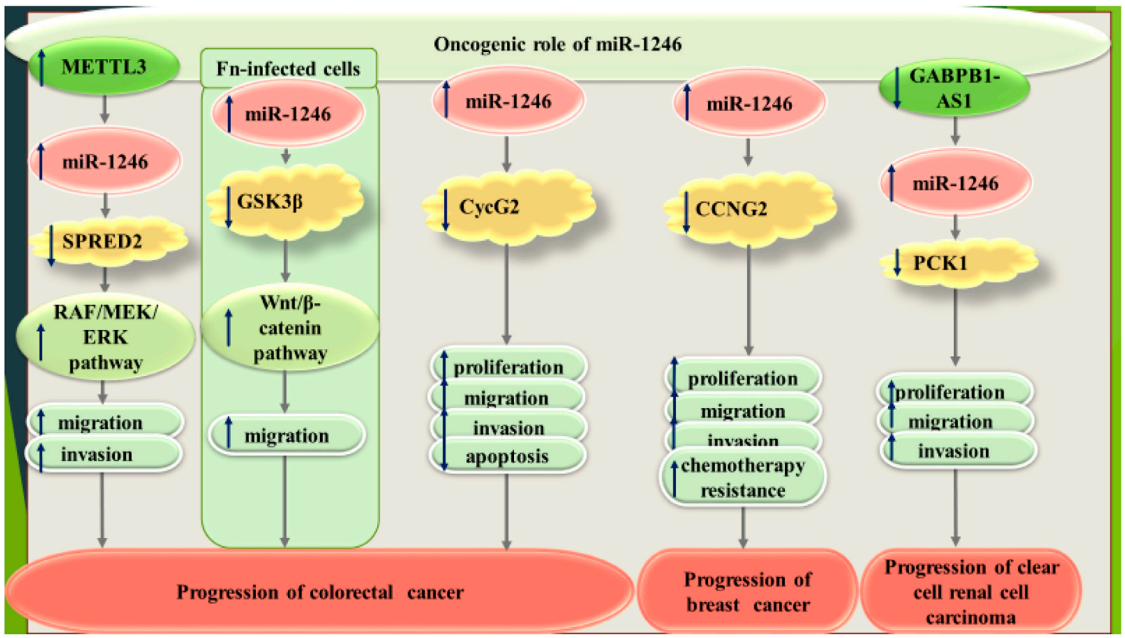

FIGURE 1 | Oncogenic role of miR-1246 in colorectal, breast and renal cancers.

Finally, the impact of this miRNA on tumor growth has been appraised in xenograft models constructed by injection of human cancer cell lines. In the current review, we describe the summary of these two types of studies in addition to those measured expression levels of miR-1246 in clinical samples.

CELL LINE STUDIES

Experiments in colorectal cancer cell lines have shown oncogenic role of miR-1246. In this type of cancer, the m (6) A methyltransferase METTL3 oncogene has been shown to increase methylation of pri-miR-1246 to enhance maturation of pri-miR-1246. Notably, miR-1246 has been predicted to suppress expression of the Sprouty Related EVH1 Domain Containing 2 (SPRED2) tumor suppressor, thus increasing activity of MAPK pathway (Peng et al., 2019).

Expression of miR-1246 has been found to be increased in exosomes derived from colorectal cancer cells infected with *Fusobacterium nucleatum*. In fact, this cancer-associated bacterium can enhance pro-metastatic behaviors through delivery of these exosomes into un-infected cells (Guo et al., 2021).

Expression of miR-1246 has also been reported to be surged in SW620, SW480, HCT116, HT29 and LOVO colorectal cancer cells, parallel with down-regulation of Cyclin G2 (CycG2). Experiments in HCT-116 and LOVO cells have verified CycG2 as the target of miR-1246. Up-regulation of miR-1246 has exerted pro-proliferative and pro-invasive effects in these cells, while its silencing has reversed these effects (Wang et al., 2016).

Exosomal and cellular levels of miR-1246 have been reported to be higher in organoid lines generated from colorectal cancer compared with organoid lines from colorectal adenomas. Consistent with this finding, miR-1246 up-regulation and down-regulation have enhanced reduced proliferation of an adenocarcinoma cell line, respectively (Nagai et al., 2021).

Another experiment in breast cancer cells has demonstrated high levels of miR-1246 in metastatic breast cancer cells compared with both non-metastatic cancer cells and non-neoplastic breast cells. miR-1246-containing exosomes from metastatic breast cancer cells can alter viability, migratory potential and chemoresistant phenotype of non-malignant breast cells. Functionally, miR-1246 suppresses expression of Cyclin G2 (Li et al., 2017).

In renal cell carcinoma cells, miR-1246 has an oncogenic effect through suppressing expression of PCK1. Notably, the tumor suppressor long non-coding RNA (lncRNA) GABPB1-AS1 has been shown to sponge miR-1246 in these cells (Gao et al., 2020).

Figure 1 shows the oncogenic role of miR-1246 in colorectal, breast and renal cancers.

miR-1246 has been demonstrated to increase the migration and invasive aptitudes of A549 adenocarcinomic human alveolar basal epithelial cells. In addition, miR-1246 could enhance epithelial-mesenchymal transition (EMT) of lung cancer cells. This miRNA could decrease levels of E-cadherin, while enhancing vimentin and TGF-β levels. Functionally, miR-1246 can target 3′-untranslated region of GSK-3β, thus regulating activity of Wnt/β-catenin pathway (Yang et al., 2019).

Yuan et al. have investigated the impact of ionizing radiation (IR)-induced extracellular miRNAs on proliferation and radioresistance of A549 adenocarcinomic cells. They have reported particular abundance of miR-1246 outside of cells compared with its levels inside the cells. Irradiation could increase expression levels of miR-1246 in A549 and H446 cells

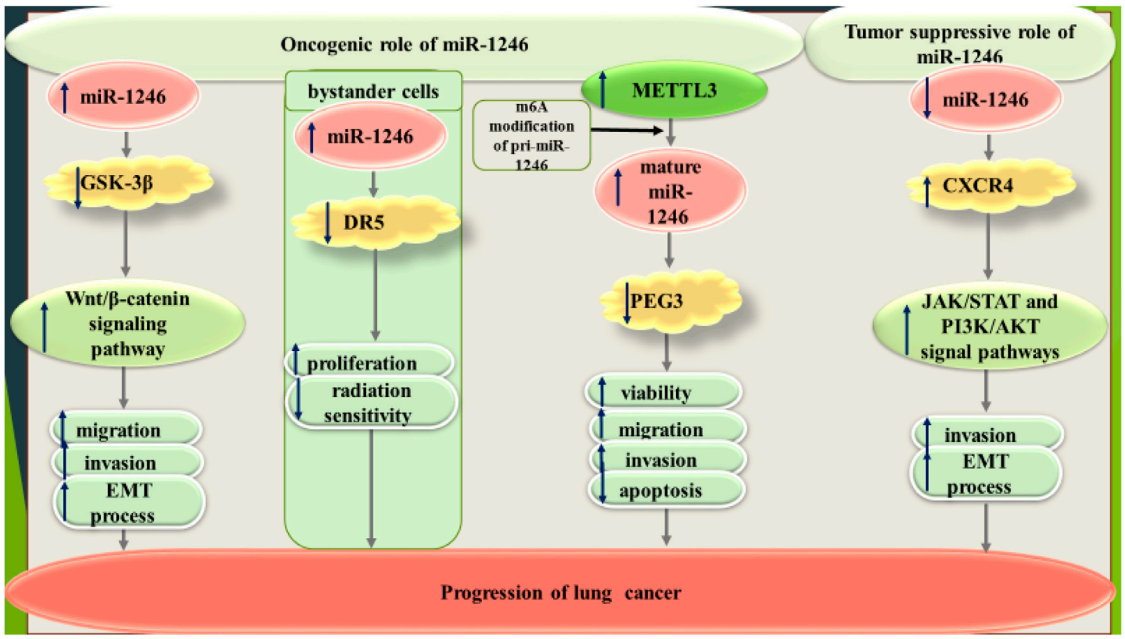

FIGURE 2 | Dual roles of miR-1246 in lung cancer.

in dose- and time-dependent manners. Extracellular miR-1246 has been shown to be transferred from donor cells to recipients through a non-exosome associated route enhancing proliferation and resistance of A549 cells to irradiation. Functionally, miR-1246 reduces expression of death receptor 5 (DR5) (Yuan et al., 2016).

miR-1246 has been among up-regulated miRNAs in the sphere-forming cells compared with the parental A549 and HCC1588 cells. Suppresion of miR-1246 has led to reduction of levels stemness and EMT markers in these cells. Moreover, anti-miR-1246 could suppress proliferation, sphere-formation, colony forming ability and invasiveness of lung cancer cells (Kim et al., 2016). Similarly, Huang et al. have reported up-regulation of miR-1246 and METTL3 in A549 and H1299 cells, parallel with down-regulation of PEG3. METTL3 has been shown to affect m6A marks of miR-1246, therefore increasing expression of miR-1246. Cumulatively, m6A methyltransferase METTL3 modifies the m6A marks of miR-1246 to up-regulates miR-1246 and subsequently increase progression of lung cancer (Huang et al., 2021).

Contrary to these studies, Xu et al. have reported down-regulation of miR-1246 in A549, H1650 and H1299 cell lines compared to a normal human bronchial epithelial cell line. MiR-1246 overexpression remarkably inhibited cell invasion as well as up-regulated E-cadherin expression and down-regulated N-cadherin, Vimentin, ZEB1 and Snail expressions in A549 cells. Further studies have confirmed CXCR4 as a target gene of miR-1246, and CXCR4 silence significantly abolished the promotion effect of miR-1246 suppression on cell invasion and EMT process in A549 cells. Besides, miR-1246 blocked JAK/STAT and PI3K/AKT signal pathways by regulation of CXCR4 (Xu et al., 2018). **Figure 2** shows dual roles of miR-1246 in lung cancer.

In SiHa HPV16-positive cervical cancer cell line, HPV16 E6 silencing has led to enhancement of miR-1246 expression, thus down-regulation of miR-1246 target DYRK1A. Meanwhile, overexpression of HPV16 E6 in HPV-negative C33A cell line has resulted in down-regulation of miR-1246 (Yang et al., 2015). Another study has shown that miR-1246 increases proliferation, invasiveness and migratory potential of SiHa cells through inhibition of expression of thrombospondin 2 (Chen et al., 2014). miR-1246 has also been among up-regulated miRNAs in radioresistant cervical cancer cells. Expression of this miRNA could be enhanced by irradiation of cervical cancer cells. Up-regulation of miR-1246 has increased survival of cervical cancer cells upon irradiation (Zhang et al., 2013). **Figure 3** shows dual roles of miR-1246 in cervical cancer.

Experiments in a co-culture model of hepatic stellate cells (HSCs) and hepatocellular carcinoma cells have shown that expression of miR-1246 is activated by HSCs. miR-1246 has been shown to target RORα. Up-regulation of miR-1246 or silencing of RORα has promoted proliferation, invasive properties, and metastatic aptitude of hepatocellular cancer cells through activation of Wnt/β-catenin pathway and enhancement of EMT (Huang J.-L. et al., 2020). Another study has shown that miR-1246 increases invasiveness of hepatocellular carcinoma cells via modulation of CADM1 expression (Sun et al., 2014). Moreover, miR-1246 has been reported to promote stemness features such as self-renewal, resistance to therapeutics, tumorigenic potential, and metastasis through enhancing activity of Wnt/β-catenin pathway. This effect is mediated through down-regulation of expression levels of AXIN2 and GSK3β. Oct4 has been identified as the direct regulator of miR-1246 expression which activates β-catenin in hepatic cancer stem cells (Chai et al., 2016).

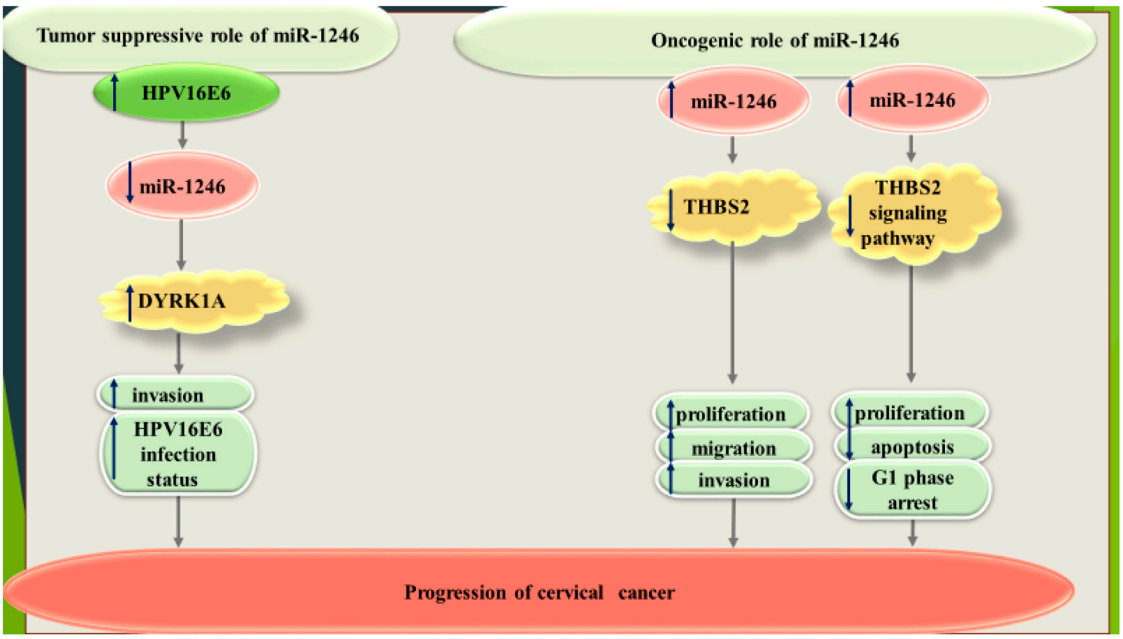

FIGURE 3 | Dual roles of miR-1246 in cervical cancer.

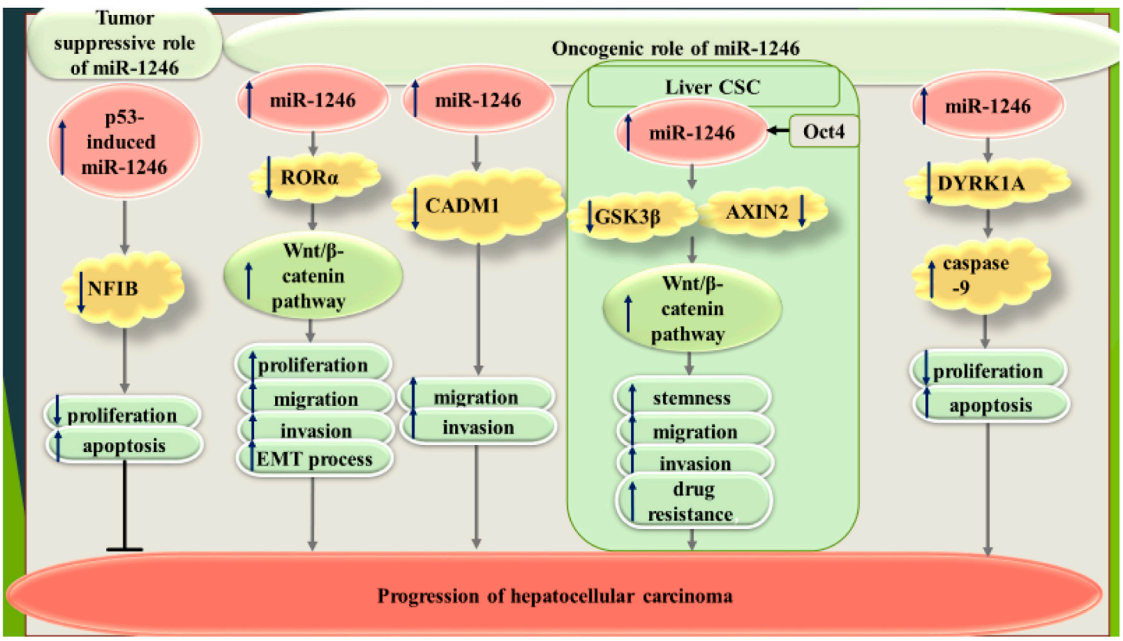

FIGURE 4 | Dual roles of miR-1246 in hepatocellular carcinoma.

On the other hand, Zhang et al. have shown that expression of miR-1246 is induced by p53. This miRNA has been shown to inhibit proliferation of hepatocellular carcinoma cells through influencing expression of NFIB (Zhang et al., 2015). **Figure 4** shows dual roles of miR-1246 in hepatocellular carcinoma.

In oral squamous cell carcinoma, miR-1246 has been shown to target CCNG2 to facilitate stemness properties and induce resistance to chemotherapy (Lin et al., 2018). Moreover, exosomal transfer of this miRNA has enhanced cell motility and invasiveness of oral squamous cell carcinoma cells through targeting DENND2D (Sakha et al., 2016). Consistent

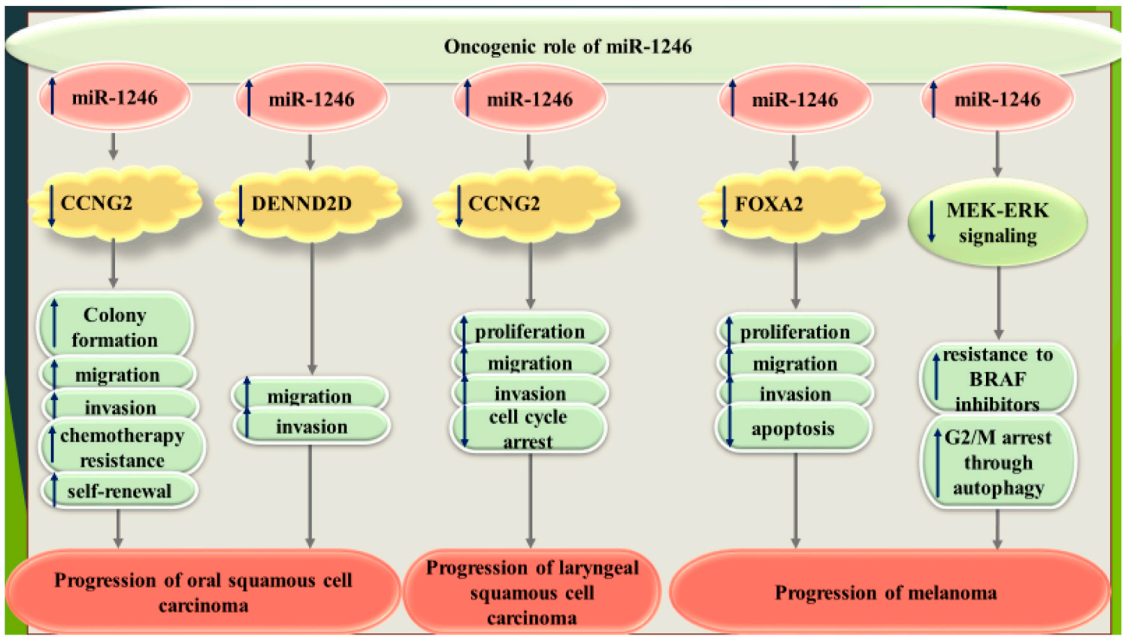

FIGURE 5 | Oncogenic role of miR-1246 in oral and laryngeal squamous cell carcinomas and melanoma.

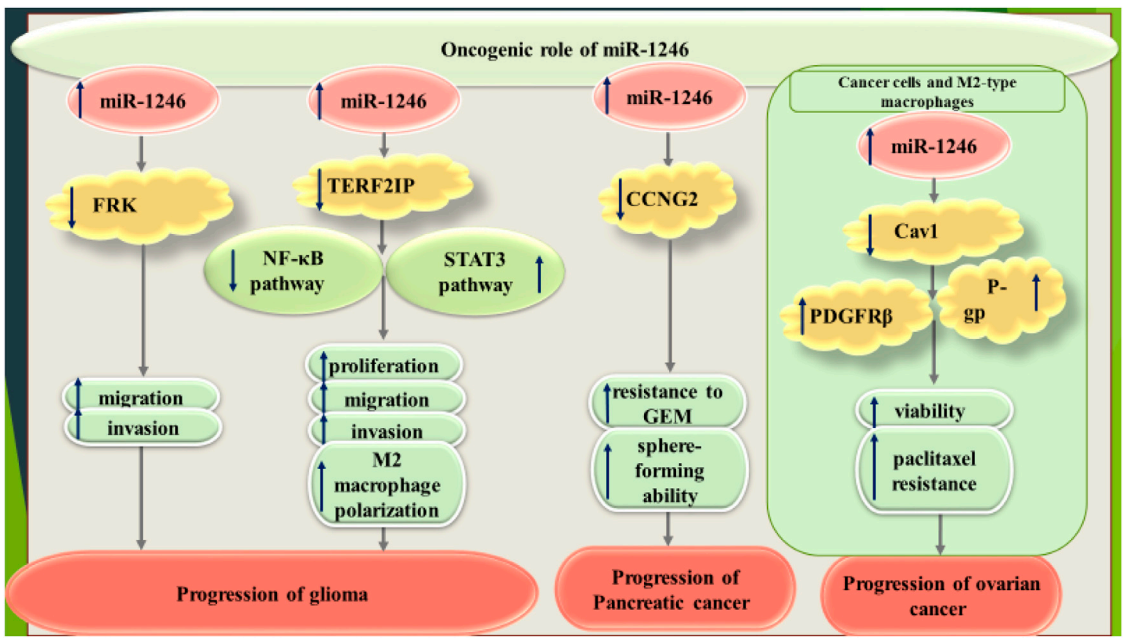

FIGURE 6 | Oncogenic role of miR-1246 in glioma, pancreatic cancer and ovarian cancer.

with this finding, small extracellular vesicles originated from laryngeal squamous cell carcinoma cells have been shown to enter into neighboring cells. Lack of miR-1246 in these vesicles abolished development of this kind of cancer. miR-1246 content of small vesicles could participate in the pathoetiology of laryngeal squamous cell carcinoma through suppressing CCNG2 expression (Huang Q. et al., 2020). miR-1246 is involved in the progression of melanoma via changing expression levels FOXA2 (Yu et al., 2020). Moreover, miR-1246 has been shown to increase resistance of melanoma cells to BRAF inhibitors (Kim et al., 2017). **Figure 5** shows oncogenic role of miR-1246 in oral and laryngeal squamous cell carcinomas and melanoma.

TABLE 1 | Outlines of *in vitro* studies about function of miR-1246 (Δ: knock-down or deletion, FN: Fusobacterium nucleatum, sEV: Small extracellular vesicle, GEM: gemcitabine).

Tumor type	Targets/Regulators and signaling pathways	Cell line	Function	References
Colorectal cancer	m6A, METTL3, SPRED2, RAF/MEK/ERK pathway	LoVo, HCT116, CaCo2, DLD-1, HT-29, NCM460	Δ METTL3: ↓ migration, ↓ invasion ↑ METTL3: ↑ migration, ↑ invasion	Peng et al. (2019)
	GSK3β, Wnt/β-catenin pathway	HCT116, SW480	Fn infection: ↑ secretion of exosomes Fn-Ex treatment: ↑ migration, ↑ wound closure ↑ miR-1246: ↑ migration, ↑ wound closure	Guo et al. (2021)
	CycG2	SW620, SW480, HCT116, HT29, LOVO, IECs	Δ miR-1246: ↓ proliferation, ↓ migration, ↓ invasion, ↑ apoptosis ↑ miR-1246: ↑ proliferation, ↑ migration, ↑ invasion, ↓ apoptosis	Wang et al. (2016)
	—	HT-29	Δ miR-1246: ↓ proliferation ↑ miR-1246: ↑ proliferation	Nagai et al. (2021)
Lung cancer	GSK-3β, Wnt/β-catenin signaling pathway	A549	Δ miR-1246: ↓ migration, ↓ invasion, ↓ EMT process ↑ miR-1246: ↑ migration, ↑ invasion, ↑ EMT process	Yang et al. (2019)
	DR5	A549, SK-MES-1, H446	Δ miR-1246: ↓ proliferation, ↑ radiation sensitivity ↑ miR-1246: ↑ proliferation, ↓ radiation sensitivity	Yuan et al. (2016)
	—	A549, HCC1588	Δ miR-1246: ↓ proliferation, ↓ stemness, ↓ EMT process, ↓ sphere-formation, ↓ colony formation, ↓ invasion	Kim et al. (2016)
	CXCR4, JAK/STAT and PI3K/AKT signal pathways	A549, H1650, H1299, 16HBE14o	↑ miR-1246: ↓ invasion, ↓ EMT process	Xu et al. (2018)
	METTL3, m6A, PEG3	A549, H1299, H520, H1975	Δ METTL3: ↓viability, ↓ colony formation, ↓ migration, ↓ invasion, ↑ apoptosis ↑ miR-1246: ↑ migration, ↑ invasion, ↓ apoptosis	Huang et al. (2021)
Cervical cancer	HPV16E6, DYRK1A	HeLa, SiHa, Caski, C33A	Δ miR-1246:↑ invasion↑ miR-1246: ↓ invasion	Yang et al. (2015)
	THBS2	SiHa	↑ miR-1246: ↑ proliferation, ↑ migration, ↑ invasion Δ miR-1246: ↓ proliferation, ↓ migration, ↓ invasion	Chen et al. (2014)
	THBS2, THBS2/MMP signaling pathway	SiHa	Δ miR-1246: ↓ proliferation, ↑ apoptosis, ↑ G1 phase arrest	Du et al. (2019)
	—	Siha, Hela	radiation treatment: ↑ miR-1246 ↑ miR-1246: ↓ radiosensitivity	Zhang et al. (2013)
Prostate cancer	—	RWPE-1, LNCaP, Du145, PC3	↑ miR-1246: ↓ proliferation, ↓ migration, ↓ invasion, ↓ EMT process, ↑ apoptosis	Bhagirath et al. (2018)
Breast cancer	CCNG2	MCF-7, MDA-MB-231, MCF-10A, HMLE	↑ miR-1246: ↑ proliferation, ↑ migration, ↑ invasion, ↑ chemotherapy resistance	Li et al. (2017)
Hepatocellular carcinoma	RORα, Wnt/β-catenin pathway	HSCs, PLC, MHCC97H, HCCLM3	↑ miR-1246: ↑ proliferation, ↑ migration, ↑ invasion, ↑ EMT process	Huang et al. (2020a)
	CADM1	HepG2, SMMC7721 and BEL7402	Δ miR-1246: ↓ migration, ↓ invasion ↑ miR-1246: ↑ migration, ↑ invasion	Sun et al. (2014)
	p53, NFIB	HepG2, Hep3B, Huh7, C3A, PLC, LO2, SUN387	Δ miR-1246: ↓ Proliferation ↑ miR-1246: ↑ proliferation	Zhang et al. (2015)
	Oct4, AXIN2, GSK3β, Wnt/β-catenin pathway	Hep3B, Huh7	Δ miR-1246: ↓ invasion, ↓ migration, ↓ ability to initiate hepatosphere formation, ↓ self-renewal, ↑ sensitization to 5-fluorouracil, cisplatin and sorafenib	Chai et al. (2016)
	Galectin-9, DYRK1A, caspase-9	Li-7, Huh7, HLE	↑ galectin-9: ↓ Proliferation, ↑ apoptosis, ↑ miR-1246 ↑ miR-1246+ galectin-9 treatment in Li-7 cells: ↓ Proliferation, ↑ apoptosis	Fujita et al. (2015)
	—	BEL-7402	bafilomycin A1-treatment: ↓ Proliferation, ↓ invasion, ↑ miR-1246	Lu et al. (2015)

(Continued on following page)

TABLE 1 | (*Continued*) Outlines of *in vitro* studies about function of miR-1246 (Δ: knock-down or deletion, FN: Fusobacterium nucleatum, sEV: Small extracellular vesicle, GEM: gemcitabine).

Tumor type	Targets/Regulators and signaling pathways	Cell line	Function	References
Oral squamous cell carcinoma	CCNG2	OC3, FaDu	Δ miR-1246: ↓ migration, ↓ invasion, ↓ self-renewal, ↓ colony formation, ↓ chemoresistance ↑ miR-1246: ↑ invasion, ↑ colony formation, ↑ number of spheres, ↑ stemness	Lin et al. (2018)
	DENND2D	HOC313-P, TSU, HeLa	↑ miR-1246: ↑ migration, ↑ invasion, did not affect growth	Sakha et al. (2016)
Laryngeal squamous Cell carcinoma	CCNG2	Hep-2, AMC-HN-8	Δ miR-1246 in sEV: ↓ Proliferation, ↓ migration, ↓ invasion, ↑ cell cycle arrest	Huang et al. (2020b)
Melanoma	FOXA2	HEM, A375, A2058	Δ miR-1246 in sEV: ↓ Proliferation, ↓ migration, ↓ invasion, ↑ apoptosis ↑ miR-1246: ↑ proliferation, ↑ migration, ↑ invasion, ↓ apoptosis	Yu et al. (2020)
	MEK-ERK signaling	A375P BRAF V600E, A375P/Mdr, SK-MEL-2 BRAF-WT	↑ miR-1246: ↑ resistance to BRAF inhibitors, ↑ G2/M arrest through autophagy	Kim et al. (2017)
	IL-6, STAT3, Akt	HMVECs, A375, A375SM	↑ miR-1246: ↑ resistance to 5-FU	Torii et al. (2021)
Glioma	FRK	H-GDEs	↑ miR-1246: ↑ migration, ↑ invasion	Qian et al. (2021a)
	TERF2IP, STAT3 pathway, NF-κB pathway	U87MG, U251, U937	↑ miR-1246: ↑ proliferation, ↑ migration, ↑ invasion, ↑ M2 macrophage polarization	Qian et al. (2020)
Pancreatic cancer	CCNG2	Panc1, Panc1-P	↑ miR-1246: ↑ resistance to GEM, ↑ sphere-forming ability	Hasegawa et al. (2014)
Ovarian cancer	Cav1, PDGFRβ, P-gp	eyA8, SKOV3-ip1, A2780, HeyA8-MDR, SKOV3-TR, A2780-CP20, HIO180	Δ miR-1246: ↓ paclitaxel resistance, ↓ viability	Kanlikilicer et al. (2018)
	—	HO-8910	bafilomycin A1-treatment: ↓ Proliferation, ↓ invasion, ↑ miR-1246	Lu et al. (2015)
Clear cell renal cell carcinoma	GABPB1-AS1, PCK1	786-o and caki-1	↑ GABPB1-AS1: ↓ Proliferation, ↓ migration, ↓ invasion	Gao et al. (2020)
Acute myeloid leukemia	LRIG1, STAT3 pathway	LSCs	↑ miR-1246 + LSCs co-cultured with EVs: ↑ viability, ↑ colony formation, ↓ apoptosis, ↓ differentiation	Chen et al. (2021a)
	Raptor/mTOR pathway	Molm-14, HL-60, U-937, LT-HSC	↑ miR-1246: ↓ protein synthesis, ↑ quiescence	Abdelhamed et al. (2019)
Leukemia	AXIN2, GSK-3β, Wnt/β-catenin pathway, P-gp	K562, HL-60 cells and drug-resistant K562/ADM, HL-60/RS	Δ miR-1246: ↓ Proliferation, ↑ apoptosis, ↑ chemo-sensitivity	Xie et al. (2021)
T cell acute lymphoblastic leukemia (T-ALL)	NOTCH2 Pathway	T-ALL	Δ miR-1246: ↓ Proliferation ↑ miR-1246: ↑ Proliferation	Luo et al. (2018)
Gastric cancer	Oxidative stress response, axon guidance mediated by netrin, salvage pyrimidine deoxyribonucleotides	NCI-N87	cisplatin treatment: ↑ miR-1246	Yin et al. (2019)
Gallbladder cancer	—	G415	Δ miR-1246: ↓ Proliferation, ↓ invasion, ↑ apoptosis ↑ miR-1246: ↑ Proliferation, ↑ invasion, ↓ apoptosis	Ueta et al. (2021)
Sarcoma	—	LP6, LPS12	↑ miR-1246: ↑ Proliferation	Kohama et al. (2021)

Exosomes originated from glioma cell cultures under hypoxic conditions could shuttle miR-1246 to normoxic glioma cells and enhance their migratory potential and invasiveness (Qian M. et al., 2021). Another study has shown the impact of these exosomes in induction of polarization of macrophages into M2 phenotype through targeting TERF2IP and subsequent influence on the activities of STAT3 and NF-κB signaling (Qian et al., 2020).

In pancreatic cancer, miR-1246 could increase chemoresistance and stemness through modulation of CCNG2 (Hasegawa et al., 2014).

Finally, in ovarian cancer, miR-1246 can confer resistance to chemotherapeutics through influencing Cav1/p-gp/M2-type macrophages (Kanlikilicer et al., 2018).

Figure 6 shows the oncogenic role of miR-1246 in glioma, pancreatic cancer and ovarian cancer.

TABLE 2 | Outline of studies about the function of miR-1246 in animal models (Δ: knock-down or deletion, PDX: derived orthotopic xenograft, NOD-SCID: non-obese diabetic/severe combined immunodeficiency, NSG: NOD Cg-Prkdcscid Il2rgtm1Wjl/SzJ).

Tumor type	Animal models	Results	References
Colorectal cancer	Male BALB/c nude mice	Δ METTL3: ↓ metastasis	Peng et al. (2019)
	BALB/c nude mice	Δ miR-1246 in Fn-Ex group: ↓ metastasis	Guo et al. (2021)
Lung cancer	Female BALB/c nude mice	Δ METTL3: ↓ tumor volume, ↓ tumor weight ↑ miR-1246: ↑ tumor volume, ↑ tumor weight	Huang et al. (2021)
Cervical cancer	Athymic BALB/c nude mice	Δ miR-1246: ↓ tumor volume, ↓ tumor growth	Du et al. (2019)
Prostate cancer	Nude mice	↑ miR-1246 in xenograft tissues: ↓ tumor growth	Bhagirath et al. (2018)
Breast cancer	Plasma of a PDX mouse	miR-1246 was higher in the plasma exosomes of patient-PDX mice compared to control mice	Hannafon et al. (2016)
Hepatocellular carcinoma	Male BALB/c-nu/nu mice	↑ miR-1246: ↑ tumor growth, ↑ metastasis	Huang et al. (2020a)
	Male BALB/c nude or NOD-SCID mice	Δ miR-1246: ↓ tumor initiation, ↓ tumor volume, ↓ metastasis	Chai et al. (2016)
	Female athymic BALB/c-nu/nu mice	↑ galectin-9: ↓ tumor growth of Li-7 cells, ↑ apoptosis, ↑ miR-1246	Fujita et al. (2015)
Oral squamous cell carcinoma	BALB/c nude mice	Δ miR-1246: ↓ tumor size ↑ miR-1246: ↑ tumor growth	Lin et al. (2018)
Glioma	Male nude mice	↑ miR-1246: ↑ proliferation, ↑ M2 macrophage polarization	Qian et al. (2020)
Pancreatic cancer	Female non-obese mice with diabetes/severe combined immunodeficiency	↑ miR-1246 in Panc1-P-I-OE: ↑ tumourigenicity	Hasegawa et al. (2014)
Ovarian cancer	Nude mice	Δ miR-1246 + chemotherapy: ↓ tumor weight, ↓ macrophages recruited by tumors	Kanlikilicer et al. (2018)
Acute myeloid leukemia (AML)	NOD/SCID mice	↓ EVs-miR-1246: ↓ tumor volume, ↓ tumor weight	Chen et al. (2021a)
	NSG and C57BL/6J mice	↑ miR-1246: ↑ quiescence	Abdelhamed et al. (2019)
Leukemia	Male BALB/c nude mice	Δ miR-1246: ↓ tumor volume, ↓ tumor weight, ↓ chemotherapy resistance	Xie et al. (2021)

Table 1 shows the outlines of *in vitro* studies focusing on the function of miR-1246 in cancer.

ANIMAL STUDIES

Most of animal studies have indicated an oncogenic role for miR-1246, since its silencing has led to reduction of tumor size and attenuation of tumor growth (**Table 2**). Moreover, expression of miR-1246 has been found to be elevated in the plasma exosomes of patient-originated orthotopic xenograft animals compared to control animals (Hannafon et al., 2016). However, in prostate cancer, miR-1246 up-regulation has significantly inhibited tumor growth in the xenograft models, suggesting its tumor suppressive role. Moreover, in miR-1246 overexpressing xenograft models, exosomal levels of this miRNA has been reduced. Taken together, miR-1246 has been identified as a tumor suppressor miRNA being selectively packaged in prostate cancer exosomes, resulting in its high abundance in serum yet low concentrations inside the cells (Bhagirath et al., 2018). In the xenograft model of leukemia, miR-1246-containing extracellular vesicles have been shown to confer quiescence on residual hematopoietic stem cells (Abdelhamed et al., 2019).

CLINICAL STUDIES

Serum levels of miR-1246 have been found to be higher in the sera of colorectal cancer patients compared to healthy subjects (Salah et al., 2020). Similarly, miR-1246 has been found as the most up-regulated miRNA in the sera of patients with lung cancer (Yang et al., 2019). Levels of miR-1246 have been found to be higher in laryngeal squamous cell carcinoma tissues and plasma small extracellular vesicles. This miRNA has been more enriched in small extracellular vesicles instead of being in soluble form (Sakha et al., 2016). Almost all studies in clinical settings have reported up-regulation of miR-1246 in neoplastic tissues and sera of patients compared with controls (**Table 3**).

However, Yang et al. have shown down-regulation of miR-1246 in cervical cancer tissues compared with normal controls. Notably, down-regulation of miR-1246 has been inversely correlated with clinical stage and HPV16 E6 infection. Yet, its levels have not been correlated with age, tumor diameters, invasion deepness, lymph node involvement, or vascular invasion (Yang et al., 2015).

Table 3 Results of studies that reported dysregulation of miR-1246 or other genes that interact with miR-1246 in clinical samples (OS: Overall survival, DFS: disease-free survival, TNM: tumor-node-metastasis, ANCTs: adjacent non-cancerous tissues, FN: Fusobacterium nucleatum, CD44v6: a CSC population with increased resistance to chemotherapeutic agents, NMRD: non-malignant respiratory diseases, NSCLC: non-small cell lung cancer, PTGs: potential target genes, LUAD: lung adenocarcinoma, ESCC: esophageal squamous cell carcinoma, miR-1246real and miR-1246pred: real and predicted miR-1246 expression levels, BPH: benign prostate hyperplasia, EFS: event-free survival, PFS: progression-free survival, LC: liver cirrhosis, CH: chronic hepatitis, HC: healthy controls, UICC: Union for International Cancer Control, GBM: glioblastoma, LGG: low-grade glioma, PDAC: pancreatic ductal

TABLE 3 | Results of studies that reported dysregulation of miR-1246 or other genes that interact with miR-1246 in clinical samples.

Tumor type	Samples	Expression of miR-1246 or other genes (tumor vs. normal)	Kaplan-Meier analysis (impact of miR-1246 dysregulation or other genes dysregulation)	Univariate/ Multivariate cox regression	Association of expression of miR-1246 or expression of other genes with clinicopathologic characteristics	Method for assessment of miR-1246 expression	References
Colorectal cancer (CRC)	60 pairs of CRC tissues and ANCTs	Up-regulation of m6A Up-regulation of METTL3	—	—	lymph node invasion, and distant metastasis	SYBR Premix Ex Taq Kit	Peng et al. (2019)
	GEO database: GSE17536	Up-regulation of METTL3	Lower OS	—	—		
	Serum samples from 82 patients and blood samples from 102 healthy controls	Up-regulation of miR-1246	—	—	—	Mir-X miRNA RT-qPCR TB Green Kit	Guo et al. (2021)
	40 CRC Patients and 40 healthy controls	Up-regulation of exosomal miR-1246	—	—	—		
	82 fecal samples	Up-regulation of miR-1246	—	—	Abundance of Fn		
	sera of 37 CRC patients and 30 healthy controls	Up-regulation of miR-1246	—	—	—	miScript syber green PCR kit (Qiagen)	Salah et al. (2020)
	10 pairs of CRC tissues and ANCTs	Up-regulation of miR-1246	—	—	—	mirVana™ qRT-PCR microRNA detection kit	Wang et al. (2016)
	Serum samples from 43 CRC patients	Up-regulation of miR-1246 in chemoresistant patients	—	—	—	TaqMan miRNA Assay	Jin et al. (2019)
	26 CRC patients	Up-regulation of miR-1246 (lower in post-treatment sera)	—	—	—	Taqman miRNA Assay	Handa et al. (2021)
	181 pairs of CRC tissues and ANCTs	Up-regulation of miR-1246	—	—	CD44v6 status	SYBR Green	Toden et al. (2019)
	150 pairs of CRC tissues and ANCTs	Up-regulation of miR-1246	worse OS and DFS	miR-1246 was found to be an independent prognostic factor for OS and DFS	stage IV		
Colorectal cancer (CRC) and Colorectal adenomas (CRA)	6 CRC and 8 CRA patients	Up-regulation of miR-1246 in both cellular compartments and exosomes (higher in CRC-derived organoids than CRA-derived organoids)	—	—	—	TaqMan™Advanced miRNA assays	Nagai et al. (2021)
Colon cancer	88 primary CRC patients and 11 healthy controls	Up-regulation of miR-1246	—	—	—	TaqMan microRNA kits	Ogata-Kawata et al. (2014)
	Serum from 29 of the patients after surgical resection	Down-regulation of miR-1246	—	—	—		
	13 CRC patients and 8 healthy controls	Up-regulation of miR-1246	—	—	—		
Lung cancer	serum from 11 lung cancer patients and 5 healthy control	Up-regulation of miR-1246	—	—	metastasis	SYBR Green	Yang et al. (2019)
	105 NSCLC patients, 50 NMRD patients, and 50 healthy controls	Up-regulation of miR-1246 in NSCLC patients than in patients with NMRD and healthy controls	Worse OS and DFS	Serum exosomal miR-1246, TNM stage, and lymph node metastasis were found as independent prognostic factors for OS.	advanced clinical stage and with lymph node metastasis	miScript SYBR-Green PCR Kit	Huang and Qu (2020)
	GEO database: GSE137140 and GSE69732	Up-regulation of miR-1246	High expression of UBE2C, UCHL1, TRAIP, TNNT1, TNNI3, RAC3 (PTGs of cmiRNA-1246) = poor OS High expressions of PITX2, NRAS, ENFA4, DNAJA3,	—	—	—	Huang et al. (2020c)

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TABLE 3 | (*Continued*) Results of studies that reported dysregulation of miR-1246 or other genes that interact with miR-1246 in clinical samples.

Tumor type	Samples	Expression of miR-1246 or other genes (tumor vs. normal)	Kaplan-Meier analysis (impact of miR-1246 dysregulation or other genes dysregulation)	Univariate/ Multivariate cox regression	Association of expression of miR-1246 or expression of other genes with clinicopathologic characteristics	Method for assessment of miR-1246 expression	References
			TBCE, and TGIF1(PTGs of cmiRNA-1246) = longer OS in LUAD patients				
Lung cancer	86 pairs of NSCLC tissues and ANCTs	Up-regulation of METTL3 (that upregulates miR-1246)	—	—	Lymph node metastasis, tumor size, and TNM stage	TaqMan MicroRNA Assays	Huang et al. (2021)
	86 pairs of NSCLC tissues and ANCTs	Up-regulation of m6A and Up-regulation of miR-1246	—	—	—		
	52 NSCLC patients and 45 healthy controls	Up-regulation of miR-1246	—	—	—	QIAGEN SYBR green Master Mix	Zheng et al. (2021)
Cervical cancer	68 cervical cancer patients and 52 healthy controls	Down-regulation of miR-1246	—	—	Advanced clinical stage, invasive cervical wall N1/2, HPV positivity	Stem-loop primers using SYBR® Premix Ex Taq™ II kit	Yang et al. (2015)
	18 pairs of cervical cancer tissues and ANCTs	Down-regulation of miR-1246	—	—	—		
	26 cervical cancer patients and 16 healthy controls	Up-regulation of miR-1246	—	—	—	TaqMan miRNA RT-Kit with stem-loop RT-primer	Nagamitsu et al. (2016)
Esophageal cancer	Serum from 55 ESCC patients and 39 healthy controls	Up-regulation of miR-1246	—	—	—	miScript SYBR®-Green PCR Kit (Qiagen)	Hoshino et al. (2020a)
	Serum from 101 ESCC patients and 34 healthy controls	Up-regulation of miR-1246	Worse 5-years OS and DFS	_	Tumor depth, positive lymph node metastasis, stage, and survival of patients		
	101 ESCC patients and 35 healthy controls	Up-regulation of miR-1246	—	miR-1246real was found to be an independent factor for N stage and miR-1246real, and miR-1246pred was found to be an independent factor for N stage and miR-1246pred	—	miScript SYBR®-Green PCR Kit (Qiagen	Hoshino et al. (2020b)
	Serum from 101 ESCC patients and 46 healthy controls	Up-regulation of miR-1246	lower 2-years OS	Expression of miR-1246 was the strongest independent risk factor for a poor survival with a hazard ratio of 4.032	T3–4, lymph node metastasis, distant metastasis, stage III–IV	TaqMan MicroRNA Assays	Takeshita et al. (2013)
	22 pairs of ESCC tissues and ANCTs	No significant differences	—	—	—		
	32 lymph nodes	Higher in proximal lymph nodes than abdominal lymph nodes, thoracic lymph nodes, and cervical lymph nodes	—	—	—		
	Serum from 72 ESCC patients and 50 healthy controls	Up-regulation of miR-1246	worse OS	—	Tumor invasion and positive lymph node metastasis, albeit insignificantly	miScript SYBR® Green PCR kit (Qiagen)	Hoshino et al. (2021)
	Urine from 72 ESCC patients and 50 healthy controls	Up-regulation of miR-1246	—	—	—	—	
Prostate cancer (PCa)	Serum from 6 PCa patients, 3 BPH patients and 3 healthy controls	Up-regulation of miR-1246 in PCa than BPH and healthy controls	—	—	Advancing PCa stage, lymph node metastasis	TaqMan MicroRNA Assays	Bhagirath et al. (2018)
			—	—			

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TABLE 3 | (*Continued*) Results of studies that reported dysregulation of miR-1246 or other genes that interact with miR-1246 in clinical samples.

Tumor type	Samples	Expression of miR-1246 or other genes (tumor vs. normal)	Kaplan-Meier analysis (impact of miR-1246 dysregulation or other genes dysregulation)	Univariate/ Multivariate cox regression	Association of expression of miR-1246 or expression of other genes with clinicopathologic characteristics	Method for assessment of miR-1246 expression	References
	Serum form 44 PCa patients, 4 BPH patients and 8 healthy controls	Up-regulation of ex-miR-1246 in PCa than BPH and healthy controls			Advancing PCa stage, lymph node metastasis		
	36 pairs of PCa tissues and ANCTs	Down-regulation of miR-1246	—	—	—		
Breast cancer	Circulating exosomal miRNA from 4 trastuzumab-resistant and 4 sensitive patients	Up-regulation of miR-1246 in trastuzumab-resistant HER2-positive breast cancer patients	poorer EFS	Expression of miR-1246 strongly showed poor EFS for early-stage patients, and poor PFS for metastatic patients	—	miScript SYBR Green PCR Kit (Qiagen)	Zhang et al. (2020)
	Plasma from 16 breast cancer patients and 16 healthy controls	Up-regulation of miR-1246 in plasma exosomes	—	—	—	Stem-loop primer using TaqMan microRNA Reverse Transcription Kit	Hannafon et al. (2016)
	Serum from 56 breast cancer patients and 19 healthy controls	Up-regulation of miR-1246	—	—	—	Taqman assay	Li et al. (2017)
	11 studies with 921 breast cancer patients	Up-regulation of miR-1246	—	—	—	—	Wang et al. (2018a)
Breast cancer	Serum from 100 breast cancer patients and 40 healthy controls	Up-regulation of miR-1246	—	—	—	miScript SYBR Green PCR kit (Qiagen GmbH)	Fu et al. (2016)
	GEO database: (GSE73002) (1,288 BC patients and 2,686 healthy controls)	Up-regulation of miR-1246	—	—	—	—	Cui et al. (2018)
	GEO database: (GSE73002) (429 BC patients and 895 healthy controls.)	Up-regulation of miR-1246	—	—	—		
Hepatocellular carcinoma (HCC)	Serum from 33 primary HCC patients, 22 metastatic liver tumor patients, 30 healthy controls	Up-regulation of miR-1246 in metastatic liver tumors	—	—	Females, patients ≤60 years old, and patients with cirrhosis and low level of serum AFP	miScript SYBER Green PCR kit (Qiagen)	Ahmed et al. (2019)
	7 HCC patients, 21 cirrhosis patients and 14 healthy controls	Up-regulation of miR-1246	—	—	—	QX200 EvaGreen ddPCR protocol	Moshiri et al. (2018)
	Plasmas from 9 HCC and 6 cirrhotic patients	Up-regulation of miR-1246	—	—	—		
	Plasmas from 22 HCC patients and 11 healthy controls	Up-regulation of miR-1246	—	—	—		
	Plasmas from 24 HCC and 14 cirrhotic patients	Up-regulation of miR-1246	—	—	—		
	Serum from 50 HCC patients and 50 healthy controls	Up-regulation of miR-1246	shorter OS	—	—	miScript SYBR-Green PCR Kit (Qiagen GmbH)	Chen et al. (2021b)
	50 pairs of HCC tissues and ANCTs	Up-regulation of miR-1246	—	—	TNM staging, differentiation, and metastasis		
	31 pairs of HCC tissues and ANCTs	Up-regulation of miR-1246	—	—	—	An Agilent oligonucleotide microarray system (Agilent Gene Spring GX11.51, Agilent Technologies)	Huang et al. (2020a)
	Serum from 121 HCC patients, 48	Up-regulation of miR-1246 in HCC	shorter OS and DFS	Serum miR-1246, Albumin, AFP-L3,	UICC-TNM classification, tumor differentiation, and	TaqMan Advanced miRNA Assays	Chuma et al. (2019)

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TABLE 3 | (*Continued*) Results of studies that reported dysregulation of miR-1246 or other genes that interact with miR-1246 in clinical samples.

Tumor type	Samples	Expression of miR-1246 or other genes (tumor vs. normal)	Kaplan-Meier analysis (impact of miR-1246 dysregulation or other genes dysregulation)	Univariate/ Multivariate cox regression	Association of expression of miR-1246 or expression of other genes with clinicopathologic characteristics	Method for assessment of miR-1246 expression	References
	CH patients, 25 LC patients and 15 healthy controls	compared to CH, LC, HC		tumor differentiation, and were independently correlated with poor prognosis	pathological portal vein invasion		
	38 liver cancer patients	Up-regulation of miR-1246	shorter DFS	—	—	miScript SYBR Green PCR kit (Qiagen)	Sun et al. (2014)
	28 pairs of HCC tissues and ANCTs	Expression of miR-1246 was consistent with p53 levels	—	—	—	SYBR PremixEx Taq™	Zhang et al. (2015)
	62 HCC patients received liver transplantation	Up-regulation of miR-1246 in HCC recipients with HCC recurrence after liver transplantation than those without tumor recurrence	Poor OS and DFS after liver transplantation	Early-phase circulating miR-1246 was found to be significant predictor for predicting OS and DFS of HCC recipients	The serum AST level from day 0 to day 3, serum ALT level from day 0 to day 6 after liver transplantation, and expression of TNF-a	TaqMan MicroRNA Assays	Ng et al. (2016)
	114 pairs of HCC tissues and ANCTs	Up-regulation of miR-1246	Worse OS and DFS	miR-1246 was an independent prognostic factor for both OS and DFS.	Serum alpha fetoprotein (AFP) level	—	Chai et al. (2016)
	5 LC and 5 HCC patients	Higher in HCC than in LC	—	—	—	TaqMan microRNA Reverse Transcription Kit	Wang et al. (2018b)
	10 CH, 13 LC, 18 HCC patients and 14 healthy controls	Higher in HCC than in LC and CH groups, not different from NC group	—	—	—		
	40 CH, 40 LC, 50 HCC patients and 50 healthy controls	Up-regulation of miR-1246 in HCC than in LC and NC groups	—	—	—		
Oral squamous cell carcinoma (OSCC)	30 pairs of OSCC tissues and ANCTs	Up-regulation of miR-1246	poor OS	—	T category, stage, and lymph node metastasis	TaqMan miRNA assays	Lin et al. (2018)
	106 pairs of OSCC tissues and ANCTs	Up-regulation of miR-1246	poor OS	miR-1246 expression, tumor grade and TNM stage were independent prognostic factors for OSCC.	TNM stage, nodal status, and tumor grade	SYBR PrimeScript miRNA RT-PCR kit	Liao et al. (2015)
	Plasma from 10 advanced OSCC patients and 10 healthy controls	Up-regulation of miR-1246	—	—	—	miScript SYBR Green PCR kit (QIAGEN)	Nakashima et al. (2019)
	55 advanced OSCC patients	Up-regulation of miR-1246	—	—	Tumor stage		
Laryngeal squamous cell carcinoma (LSCC)	Plasma from 61 LSCC patients, 26 healthy controls	Up-regulation of miR-1246	poor OS	—	—	miScript SYBR Green PCR Kit (QIAGEN)	Huang et al. (2020b)
	61 pairs of LSCC tissues and ANCTs	Up-regulation of miR-1246	poor OS	—	—		
	GEO database: (GSE124678, GSE70289, GSE62819) (14 LSCC tissues and 49 ANCTs)	Up-regulation of miR-1246	—	—	—	—	Jing et al. (2020)
Pancreatobiliary tract cancer	12 pancreatobiliary tract cancer patients and 13 healthy controls	Up-regulation of miR-1246	—	—	—	TaqMan MicroRNA Assays	Machida et al. (2016)
Melanoma	43 pairs of melanoma tissues and ANCTs	Up-regulation of miR-1246	—	—	—	SYBR-Green Premix Ex Taq II	Yu et al. (2020)
	42 melanoma patients and 20 healthy controls	Up-regulation of miR-1246 in melanoma patients' EVs	—	—	metastatic tumor EVs	KAPA SYBR Fast qPCR Kit	Torii et al. (2021)
Glioma	26 glioma patients		—	—	—	SYBR Premix Ex Taq™ Kit	Qian et al. (2020)

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TABLE 3 | (*Continued*) Results of studies that reported dysregulation of miR-1246 or other genes that interact with miR-1246 in clinical samples.

Tumor type	Samples	Expression of miR-1246 or other genes (tumor vs. normal)	Kaplan-Meier analysis (impact of miR-1246 dysregulation or other genes dysregulation)	Univariate/ Multivariate cox regression	Association of expression of miR-1246 or expression of other genes with clinicopathologic characteristics	Method for assessment of miR-1246 expression	References
		Up-regulation of miR-1246 in GBM than LGG patients					
	CGGA, GEO Databases: (GSE25632, GSE104554) (311 glioma patients)	Up-regulation of miR-1246	Worse prognosis	miR-1246 was an independent risk factor for OS.	Tumor recurrence	—	Ji et al. (2020)
Pancreatic cancer (PC)	Plasma from 15 PC patients and 15 healthy controls	Up-regulation of miR-1246	—	—	—	qScript miRNA cDNA Synthesis Kit	Xu et al. (2017)
	7 PDAC patients, 4 IPMN patients, 4 NET patients	Up-regulation of miR-1246 in patients with IPMN	—	—	—		
	GEO datasets (GSE113486, GSE106817, GSE59856)	Up-regulation of miR-1246	—	—	—	Hairpin-itTM microRNA RT-PCR Quantitation Kit	Wei et al. (2020)
	120 PC patients, 40 benign pancreatic disease controls (DC) and 40 healthy controls	Up-regulation of miR-1246 in PC than DC and HC group	—	miR-1246 was significant and independent risk factors for PC	Tumor size		
	Serum from 34 pairs of pre- and post-operation PC patients	Down-regulation of miR-1246 after surgical resection of malignancies	—	—	—		
Ovarian cancer (OC)	15 OC tissues and 7 normal ovarian surface epithelium tissues	Up-regulation of miR-1246 in OC exosomes	—	—	Paclitaxel-resistant	PerfeCTa microRNA Assay Kit	Kanlikilicer et al. (2018)
	Serum from 110 HGSOC patients and 52 Healthy controls	Up-regulation of miR-1246	—	—	—	Rotor-Gene Thermal Cycler (Qiagen	Todeschini et al. (2017)
	serum from 58 HGSOC patients and 13 Healthy controls	Up-regulation of miR-1246	—	—	—		
	59 high-grade OSC patients	Up-regulation of miR-1246	—	—	—	TaqMan microRNA reverse transcription kit	Cha et al. (2017)
Clear cell renal cell carcinoma (ccRCC)	48 pairs of ccRCC tissues and ANCTs	Down-regulation of GABPB1-AS1 (that sponges miR-1246)	better OS	—	inversely associated with tumor size, TNM stage, and Furhman stage	Roche PCR system	Gao et al. (2020)
Gastric cancer (GC)	urine from and 7 GC patients and 3 healthy controls	Up-regulation of miR-1246	—	—	—	Illumina NextSeq 500 SE50 (20M) sequencing	Qian et al. (2021b)
Gallbladder cancer (GBC)	Serum EVs from 3 patients with GBC, 3 with Benign and 10 healthy controls	Up-regulation of miR-1246 in GBC than Benign and healthy controls	—	Serum EV miR-1246 was significant independent prognostic factor	Advanced-stage GBC	—	Ueta et al. (2021)
	GEO database: GSE104165, GSE112408	Up-regulation of miR-1246 in GBC tissues	—	—	—		
Sarcoma	22 Sarcoma patients, 17 DDLPS patients, and 3 EWS patients	Up-regulation of miR-1246 in DDLPS	—	—	—	miScript®SYBR®Green PCR kit	Kohama et al. (2021)

adenocarcinomas, IPMN: intraductal papillary mucinous neoplasms, NET: well differentiated neuroendocrine tumors, HGSOC: High-grade serous ovarian carcinoma, OSC: ovarian serous carcinoma, EVs: extracellular vesicles, DDLPS: dedifferentiated liposarcoma, EWS: Ewing's sarcoma).

Diagnostic value of miR-1246 has been validated in different neoplastic disorders (**Table 4**). The most promising results have been revealed in breast cancer. This miRNA could separate breast cancer patients from healthy controls with area under receiver operating characteristic curve (AUC) of 0.967 (Cui et al., 2018).

TABLE 4 | Diagnostic value of miR-1246 in cancers (NMRD: non-malignant respiratory diseases, NSCLC: non-small cell lung cancer, ESCC: esophageal squamous cell carcinoma, ETR: Early tumor recurrence, HGSOC: High-grade serous ovarian carcinoma).

Tumor type	Numbers of clinical samples	Distinguish between	Area under curve	Sensitivity (%)	Specificity (%)	Accuracy (%)	References
Colorectal cancer (CRC)	Sera of 37 CRC patients and 30 healthy controls	37 CRC patients vs. healthy controls	—	100	80	—	Salah et al. (2020)
	Serum samples from 43 CRC patients	Chemoresistant CRC patients vs. chemosensitive group	0.749	—	—	—	Jin et al. (2019)
Lung cancer	105 NSCLC patients, 50 NMRD patients, and 50 healthy controls	NSCLC patients vs. healthy controls	0.827	—	—	—	Huang and Qu (2020)
		NSCLC patients vs. NMRD patients	0.757	—	—	—	
	52 NSCLC patients and 45 healthy controls	NSCLC patients vs. healthy controls	0.6761	—	—	—	Zheng et al. (2021)
Esophageal cancer	Serum from 55 ESCC patients and 39 healthy controls	ESCC patients vs. healthy controls	0.816	72.7	69.2	—	Hoshino et al. (2020a)
	Serum from 101 ESCC patients and 34 healthy controls	ESCC patients vs. healthy controls	0.779	71.3	70.6	—	
	101 ESCC patients and 35 healthy controls	ESCC patients vs healthy controls	0.754	71.29	73.91	—	Hoshino et al. (2020b)
	101 ESCC patients and 46 healthy controls	ESCC patients vs. healthy controls	0.754	71.3	73.9	—	Takeshita et al. (2013)
	serum 72 ESCC patients and 50 healthy controls	ESCC patients vs. healthy controls	0.912	91.7	76.0	—	Hoshino et al. (2021)
	urine from 72 ESCC patients and 50 healthy controls	ESCC patients vs. healthy controls	0.823	90.3	62.0	—	
Prostate cancer (PCa)	26 lymph node metastatic PCa, 43 non-metastatic PCa, and 8 healthy controls	Non-metastatic vs. localized metastatic PCa patients	0.648	81	~59	—	Bhagirath et al. (2018)
	43 metastatic castration-resistant PCa cases	Normal and aggressive PCa patients and normal controls	0.933	88.37	100	—	
Breast cancer	32 trastuzumab-resistant patients and 36 trastuzumab sensitive patients	Trastuzumab-resistant patients vs. trastuzumab sensitive patients	0.750	78.1	75	—	Zhang et al. (2020)
	Plasma from 16 breast cancer patients and 16 healthy controls	Breast cancer patients vs. healthy controls	0.69	—	—	—	Hannafon et al. (2016)
	serum from 100 breast cancer patients and 40 healthy controls	Breast cancer patients vs. healthy controls	0.904	93.0	75.0	—	Fu et al. (2016)
	Plasma from 146 breast cancer patients and 90 healthy controls	Breast cancer patients vs. healthy controls	0.95	85.0	93.0	88.0	Jang et al. (2021)
	Plasma from 80 breast cancer patients and 56 healthy controls	Breast cancer patients vs. healthy controls	0.963	86.0	96.0	90.0	
	859 BC patients and 1,791 healthy controls	Breast cancer patients vs. healthy controls	0.967	89.8	91.7	—	Cui et al. (2018)
Hepatocellular carcinoma (HCC)	Serum from 33 primary HCC patients, 22 metastatic liver tumor patients	HCC patients vs. metastatic liver tumor patients	0.708	72.2	67.8	—	Ahmed et al. (2019)
	16 HCC patients and 27 cirrhosis patients	HCC patients vs. cirrhotic patients	0.97	86.7	84.6	85.7	Moshiri et al. (2018)
	29 HCC patients and 25 healthy controls	HCC patients vs. healthy controls	0.83	57.1	78.6	71.4	
	Serum from 50 HCC patients and 50 healthy controls	HCC patients vs. healthy controls	0.865	82.0	80.0	—	Chen et al. (2021b)
	37 HCC patients with ETR and 84 HCC patients without ETR	HCC patients with ETR vs. HCC patients without ETR	0.762	54.1	77.4	—	Chuma et al. (2019)
	62 HCC patients received liver transplantation	High group vs. low group for tumor recurrence	0.775	88.9	66.0	—	Ng et al. (2016)
Pancreatobiliary tract cancer	12 pancreatobiliary tract cancer patients and 13 healthy controls	Pancreatobiliary tract cancer patients vs. healthy controls	0.814	0.667	1.000	—	Machida et al. (2016)
Ovarian cancer (OC)	Serum from 168 HGSOC patients and 65 Healthy controls	HGSOC patients vs. Healthy controls	0.89	87	77	84	Todeschini et al. (2017)

In hepatocellular carcinoma, miR-1246 could be used as a diagnostic marker for differentiation of cancer status from cirrhosis and healthy controls with AUC values of 0.97 and 0.83, respectively (Moshiri et al., 2018). Expression level of miR-1246 in serum samples have been shown to distinguish colorectal cancer patients from healthy subjects with sensitivity of

100% and specificity of 80% (Salah et al., 2020). This miRNA could separate lung cancer patients from healthy controls with AUC value of 0.82 (Huang and Qu, 2020). Moreover, serum and urine levels of miR-1246 could be used as diagnostic markers for esophageal cancer with AUC values of 0.91 and 0.82, respectively (Hoshino et al., 2021).

DISCUSSION

miR-1246 is a miRNA with essential impact on carcinogenic events in different tissues. It exerts oncogenic roles in colorectal, breast, renal, oral, laryngeal, pancreatic and ovarian cancers as well as melanoma and glioma. However, in lung, cervical and liver cancers, studies have reported contradictory results regarding the role of miR-1246. Although several targets have been found for miR-1249 using bioinformatics tools and luciferase assay, CCNG2 is the most appreciated target of this miRNA in the context of cancer. miR-1246/CCNG2 axis not only regulates cell proliferation and cell cycle progression, but also is involved in chemoresistant phenotype.

The main mechanism of dysregulation of miR-1246 in cancer is methylation of pri-miR-1246 by methyltransferase METTL3 and modulation of maturation of pri-miR-1246. Unlike other miRNAs, the role of sponging lncRNAs on its expression is less studied.

miR-1246 has been reported to regulate activity of RAF/MEK/ERK, GSK3β, Wnt/β-catenin, JAK/STAT, PI3K/AKT, THBS2/MMP and NOTCH2 pathways. The role of miR-1246 in response to therapeutic modalities has been verified in different settings, indicating its crucial roles in determination of response to targeted therapies, radiotherapy as well as chemotherapy. In fact, miR-1246 can facilitate evolution of cancer through conferring stemness and EMT as well as induction of cell cycle progression and proliferation.

Diagnostic role of miR-1246 has been vastly appraised in different clinical settings, revealing nearly ideal AUC values, particularly in esophageal, prostate, breast, lung, liver, pancreatobiliary tract and ovarian cancers. The AUC, sensitivity and specificity values obtained for miR-1246 in different cancers are far superior to conventional biomarkers in these cancers. Thus, this miRNA represents an appropriate diagnostic biomarker for neoplastic conditions. Since its levels have been decreased following therapeutic interventions, it has additional advantage in patients' follow-up. Although miR-1246 can be a putative therapeutic target for cancer, there is no tissue-specific therapeutic approach designed based on miR-1246 until now.

Taken together, miR-1246 is mostly regarded as an oncogenic miRNA in human cancers, albeit some inconsistencies exist for some types of cancers. The interactions of miR-1249 with other types of non-coding RNAs such as lncRNAs and circular RNAs have not been completely assessed. Identification of such interactions has implications in design of diagnostic panels for different cancers.

CONCLUSIONS AND FUTURE PERSPECTIVES

miR-1246 is an oncogenic miRNA in several tissues. Therapeutic intervention with its expression or methylation pattern can be regarded as a novel modality. However, it is necessary to design tissue-specific therapeutic approaches.

AUTHOR CONTRIBUTIONS

SG-F wrote the draft and revised it. MT supervised and designed the study. MS and TK collected the data and designed the figures and tables. All the authors read and approved the submitted version.

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Exosome-Transmitted *miR-128* Targets CCL18 to Inhibit the Proliferation and Metastasis of Urothelial Carcinoma

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Objective: To investigate the regulatory function of exosome-transmitted *miR-128* and chemokine (C-C motif) ligand 18 (CCL18) on urothelial carcinomas (UCs).

Methods: Tumor tissues, paracancerous tissues, and serum were collected from 20 patients with UCs (diagnosed at Beijing Friendship Hospital, Capital Medical University). CCL18 was detected by immunohistochemistry and ELISA. PCR was used to measure the expression levels of CCL18 and *mir-183*, *miR-128*, *mir-33a* in UCs. We acquired exosomes from mesenchymal stem cells and synthesized exosomes overexpressing *miR-128* (HMSC-128-EV). The effects of *miR-128* on the migration and invasion abilities, apoptosis and epithelial-mesenchymal transition of BUC T24 cells were investigated by co-culturing HMSC-128-EV. The therapeutic potential of *miR-128* on disease models was explored by injecting HMSC-128-EV into nude mice.

Results: The expression of CCL18 in UCs was significantly higher than that in normal tissues ($p < 0.05$), and the serum level of CCL18 in patients with UC was significantly increased compared with those in healthy controls ($p < 0.05$). CCL18 overexpression or downregulation enhanced or suppressed the proliferation, migration and invasion of BUC T24 cells, resectively ($p < 0.05$). The exosome-transmitted miR-128 can inhibit cell proliferation ($p < 0.05$), invasion ($p < 0.05$), and migration ($p < 0.05$) in UCs, and these effects can be reversed by CCL18. In terms of apoptosis, *miR-128* was able to promote the occurrence of BUC T24 apoptosis ($p < 0.05$), which can also be reversed by CCL18. In addition, *miR-128* can inhibit the proliferation ($p < 0.05$) and metastasis ($p < 0.05$) of UCs in nude mice.

Conclusion: The *miR-128* inhibits the proliferation, invasion, migration of UCs, and promotes its apoptosis by regulating CCL18 secretion.

Keywords: exosome, urothelial carcinoma, miR-128, chemokine (C-C motif) ligand 18, proliferation, metastasis

INTRODUCTION

Urothelial carcinomas (UCs) are the ninth most common form of manignancies, killing over 165,000 patients annually around the world (Rosenberg et al., 2016). Urinary bladder cancer accounts for 90–95% of all UCs and is the most common malignancy of UCs (Rouprêt et al., 2021). In terms of the histological type, urothelial and squamous carcinoma account for about 90 and 5% of all bladder cancers, respectively (Sjödahl et al., 2012). UCs of the bladder can be classified as nonmuscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) based on pathologic stage. For the NMIBC, the recurrence rate is more than 50%, and disease progression will develop for 10–15% of patients (Têtu, 2009; Hedegaard et al., 2016). Currently, cisplatin-based combination chemotherapy has been used as the standard treatment for unresectable and metastatic/advanced UC, but patients who relapse after first-line treatment or have progression while receiving first-line treatment have a particularly poor prognosis, and second-line chemotherapy also shows only moderate efficacy, with an ORR of 12% and a median OS of 5–7 months (Kim and Seo, 2018). Thus, it is of great practical significance to develop drugs that can inhibit the proliferation and metastasis of UCs of the bladder.

The development and metastasis of tumors are the results of the interaction between tumor cells and the microenvironment. Exosomes are newly discovered extracellular vesicles in the tumor microenviroment, which play important roles in the cell proliferation, apoptosis, metastasis and other biological behaviors of tumor (Kahroba et al., 2019). These vesicles are 30–150 nm in diameter (Maia et al., 2018), and can be detected in various body fluids including blood, interstitial fluid, and urine (Harding et al., 2013). The contents of exosomes are proteins, DNAs, micro-RNAs (miRNAs), long non-coding RNAs, circular RNAs and other molecules (Rajagopal and Harikumar, 2018). Exosome-transmitted miRNAs have been proved to participate in regulating the activity of bladder cancer cells through different signaling pathways (Cai et al., 2020). The *miR-128* is a kind of miRNA enriched in the brain. Previous studies have shown that *miR-128* plays an important role in the development of the nervous system and the maintenance of its normal function (Persengiev et al., 2012). miR-128 can also be found to be abnormally expressed in the serum of some patients with malignant tumors (Roth et al., 2011), and is involved in regulating the occurrence and development of various cancers. The CCL18 is a target gene of *miR-128*, and by regulating the expression of CCL18, *miR-128* can regulate tumor invasion and metastasis (Song et al., 2018; Korbecki et al., 2020). However, the effect and mechanism of exsome-transmitted *miR-128* on the proliferation and metastasis of UCs has not been studied. Further exploration of the therapeutic potential of *miR-128* will provide entirely new options for the treatment of metastatic/advanced UC.

The chemokine (C-C motif) ligand 18 (CCL18) plays an important role in the progression of cancers, and this chemokine is mainly produced by tumor-associated macrophages (TAMs) (Lin et al., 2015; Ma et al., 2019). Previous studies have found that CCL18 is up-regulated in malignant tumors, and can promote the invasion and metastasis of tumor cells, which is a potential pathogenic molecule of urothelial carcinoma (Bo et al., 2018; Liu T. et al., 2019). The target genes of CCL18 were predicted by miRanda and Targetscan online software, and it was found that CCL18 had binding sites with *miR-128*, miR-183 and miR-33a. Only *miR-128* was negatively correlated with the expression of CCL18 in UCs cells, and CCL18 was not correlated with the expression of the other two miRNAS in UCs.

In this paper, we found that CCL18 was highly expressed in UCs, and inhibition of CCL18 could inhibit the biological activity of UCs. Also, as a downstream molecule of *miR-128*, CCL18 participates in the regulation of the biological process of UCs. CCL18 and *miR-128* are of great significance as new targeting molecules for UCs treatment.

MATERIALS AND METHODS

Materials

Tumor tissues, paracancerous tissues, and serum were obtained from 20 patients with UCs diagnosed in Department of Urology, Beijing Friendship Hospital. The inclusion criteria were patients who were diagnosed with UCs and had not received any treatment such as chemotherapy, radiotherapy and biological drugs (monoclonal antibodies) before sampling. The human urothelial carcinoma cell line BUC T24 was purchased from Gai Ning Biological Company. Human bone marrow-derived mesenchymal stem cells (HMSCs) were purchased from Shanghai Yaji Biotechnology Co., Ltd. This study conformed to the Declaration of Helsinki and was reviewed and approved by the Ethics Committee of Beijing Friendship Hospital, Capital Medical University, and all patients signed written informed consent.

Immunohistochemistry

Paraffin sections were deparaffinized with xylene for 10 min and rehydrated with graded alcohol. Antigen retrieval was performed by boiling the sections in 0.1 M citric acid buffer (pH 6.0) at 120°C for 10 min in a decloaking chamber (Biocare Medical, Walnut Creek, CA). After natural cooling, the sections were washed 3 times with PBS for 5 min each time. Then, the sections were stained with rabbit anti-CCL18 IgG Ab (20 μg/ml, Abcam) and rabbit anti-Ki67 (2 μg/ml, Abcam). Stayed overnight on a shaker at 4°C. After incubation, the slices were washed with PBS for 3 times, 5 min each time, then added hypersensitivity two-step immunohistochemical detection reagent, incubated at room temperature for 20 min; washed 3 times with PBS, 5 min each time, DAB (Sigma-Aldrich) was used for color development. Then hematoxylin complex dyeing, gradient alcohol dehydration, xylene transparent and sealed. The sections were observed, photographed, and counted under a light microscope.

The apoptosis of tumor was detected by TUNEL staining (Abcam), and the operation procedure was referred to the instruction manual.

Enzyme-Linked Immunosorbent Assay

Serum CCL18 levels were determined using Duoset ELISA assays (RandD Systems, Minneapolis, MN, United States) consulting the

manufacturers instructions. The serum sample was centrifuged and the supernatant was added into the well plate. Each well was sealed with blocking solution for 2 h, washed with PBST solution, and incubated with antibodies for 2 h. Finally, the substrate TMB-hydrogen peroxide urea solution was added, and the color was developed at room temperature for 10 min. The OD_{450} value was detected by a microplate reader.

Reverse Transcription-Polymerase Chain Reaction

BUC T24 cells were incubated until cell confluency reached 50%, and then transfected separately with each group using Lipofectamine 2000 (ThermoFisher). Group A was the control group. Cells of group B was transfected with 2 μg/μL PCDNA3.1-CCL18 (GenePharma). Cells of group C was transfected with 50 nM siRNA (GenePharma) for knockdown of CCL18. After 24 h, the cells were collected to perform RT-PCR. According to the manufacturer's instruction, total RNA was reversely transcribed into cDNA by using the ReverTra Ace qPCR-RT Kit (Toyobo, Osaka, Japan) in line. PCR was carried out on CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, United States) by using SYBR-Green RealMastcrMix (Bio-Rad) to conduct amplified detection.

CCL18 primer sequences:
Forward: 5′-AAACTCGAGCTGCCCAGCATCATGAAGG-3′,
Reverse: 5′-TTTGGATCCCCTCAGGCATTCAGCTTCAG-3′.
miR-183 primer sequences:
Forward: 5′- CGTTGGATTCCTATGGCACTGGT-3′,
Reverse: 5′- TTCAAGCAGGGTCCGAGGTATTC-3′.
miR-128 primer sequences:
Forward: 5′- TCACAGTGAACCGGTCTCTTT-3′,
Reverse: 5′- GCTGTCAACGATACGCTACG-3′.
miR-33a primer sequences:
Forward: 5′- GGTGCATTGTAGTTGCATTGC-3′,
Reverse: 5′- GTGCAGGGTCCG AGGTATTC-3′.

Western Blotting

Cell lysates were collected by digesting with RIPA buffer (Beyotime, Nanjing, China) and protease inhibitors (Sigma-Aldrich, United States). BCA kit determined protein concentration (Thermo Fisher, United States). Thirty μg protein were loaded on 10% or 15% SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were incubated with specific first antibodies and corresponding second antibody. Primary antibodies purchased from Abcam (Cambridge, United Kingdom) included rabbit anti-CCL18 (2 μg/ml), rabbit anti-Bim (1: 500); rabbit anti-β-catenin, rabbit anti-Ecadherin, rabbit anti-N-cadeherin, rabbit anti-Bax (1:1,000); rabbit anti-Vimentin, rabbit anti-Bad, rabbit anti-Bcl2, rabbit anti-caspase 3, rabbit anti-caspase 9 (1:2,000). Secondary antibodies included goat anti-mouse IgG (ab6789, 1:5,000; Abcam) and goat ant-rabbit IgG (ab6721, 1:5,000; Abcam).

Cell Viability Detection

Cell viability was measured using Cell Counting kit-8 (RandD). A single cell suspension (5×10^3/ml, 100 μL) was seeded into a 96-well plate. Subsequently, 10 μL CCK-8 reagent was added to each well and the plates were incubated for 2 h at 37 ˚C. Finally, the absorbance was measured at 450 nm using a scanning microplate reader (ThermoFisher).

Transwell Cell Migration and Invasion Assay

Transwell holes were pretreated with 1:8 diluted Matrigel (BD), then the above cells were inoculated in 24-well plates with 2.5×10^5/well, culturing for 8 h. The number of migration cells was detected by crystal violet staining. For the invasion assay, Matrigel diluted with 100 μL was added in the bottom center of the upper Transwell invasive chambers, incubating for 4 h at 37˚C to form a gel. Then, the cells were added and treated in the same steps as the migration experiment. After that, the cells on the lower surface were fixed with 100% methanol for 30 min and then the cells were stained with 0.05% crystal violet for 30 min. Images of the invasion and migration cells were taken under a microscope. All of the experiments were performed in triplicate.

Prediction of miRNA Targeting 3′UTR of Human CCL18 Gene

The miRanda and Targetscan online softwares were used for prediction of target genes.

Firstly, log in to miRanda (http:/www. Microrna.org/microrna/home.do). Search for miRNAs whose target gene was human CCL18mRNA (NM002988). The evaluation indexes of the interaction between miRNA and target gene mRNA included MIRSVR score and Phastcons score. Then login to Targetscan (http://www.targetscan.org/vert_80/) online searching for miRNAs whose target gene is human CCLI8 (ENST0000921.3). The evaluation index of the combination of miRNA and target gene mRNA included Total context++ score or Aggregat PCT.

Preparation and Identification of Exosomes Overexpressing *miR-128* (HMSC-128-EV)

HMSCs were plated into six well culture-plates with 1×10^6/well for 24 h, and then the culture medium was replaced using the mixture including fresh medium and adenovirus with *miR-128* overexpression for 24 h. The supernatant was collected and used to extract exosomes overexpressing *miR-128* (HMSC-128-EV) according to Invitrogen Kit (Invitrogen 4478359 Total Exosome Isolation Reagent) for total exosome isolation reagent. To confirm that the extracts were indeed exosomes, extracts were examined for CD9 and CD63 expression using Western blotting and flow cytometry, electron microscopy was used to visualize extract morphology, dynamic light scattering (DLS) was used to measure the size of the extracts, and laser light scattering was used to measure the zeta potential of the extracts. Further, exosome RNA was extracted using the Exosome DNA Extraction Kit (Biovision) and detected the expression of *miR-128* by the RT-PCR.

TABLE 1 | Co-culture of exosomes grouping.

Group	Tab
BUC T24	Control
BUC T24 treated with HMSC-EVs	HMSC-EV
BUC T24 treated with HMSC-128-EVs	HMSC-128-EV
BUC T24 treated with HMSC-128-EVs and CCL18	HMSC-128-EV + CCL18

Co-culture of UCs and Exosomes

DiL dye was added to HMSC-EVs and HMSC-128-EV. Exosomes were washed with PBS after staining for 30 min, and the pellet was taken after centrifugation at 10,000 g for 60 min. After resuspending exosomes, they were cocultured at a concentration of 20 μg/ml with BUC T24 for 0.5, 1, and 4 h, respectively. Finally, the uptake of exosomes by cells was observed by confocal microscopy and flow cytometry.

After determining that exosomes could be absorbed by UCs, BUC T24 were divided into four groups (**Table 1**). After 24 h of treatment, the expression of *miR-128* was detected by RT-PCR, and the content of CCL18 in the supernatant was detected by ELISA.

Colony Formation Assay

The grouping of cells was the same with **Table 1**. Cells were trypsinized and washed twice with phosphate-buffered saline. The number of cells was counted following staining with trypan blue at room temperature and the cells were prepared into a suspension with a density of 0.5×10^3 cells/ml. The cell suspension (2 ml) was inoculated into 6-well plates, followed by incubation under normal conditions at 37°C. Half the medium was replenished on day 5. The medium was discarded on day 14 and cells were washed once with phosphate-buffered saline. Cells were fixed with methanol at room temperature for 10 min and stained with crystal violet at room temperature for 5 min. Following extensive washing with phosphate-buffered saline and the cells were observed under a microscope (Leica). Five fields were selected for colony counting. The colony formation rate was then calculated using the following equation: colony formation rate = (number of clones)/(number of seeded cells) ×100%.

Wound Healing Assay

The grouping of cells was the same with **Table 1**. The cells inoculated in 6-well plates were culture to achieve 60% cell fusion, discarding the culture medium and washed in PBS for 3 times. The sterilized micropipette tips were used to quickly draw a straight line at the bottom of the culture dish, washed with PBS for 3 times. After incubation for 0, 6, 12, and 24 h, respectively, the images were collected.

Flow Cytometry

The grouping of cells was same with **Table 1**. After treatment for 24 and 48 h, Annexin/PI staining was used to detect cell apoptosis by flow cytometry.

Tail-Vein Injection of Exosomes in Nude Mice

The BUC T24 cells were inoculated subcutaneously in nude mice. Then, 15 nude mice with similar tumor sizes were selected and randomly divided into three groups with five in each group. PBS (Control), HMSC-EV and HMSC-128-EV were injected intravenously, respectively, every 2 days (200 μg per mice). The weight of nude mice and tumor volume were measured to draw the weight curve of mice and the tumor growth curve. On the 16th day, the tumors were taken out and weighed, performed with TUNEL and Ki67 staining.

Fluorescent Imaging of Nude Mice

The experimental grouping was the same as mentioned above. Female nude mice, aged 6–8 weeks, were injected with luciferase labeled BUC T24-Luc cells via tail-vein with 2×10^7 cells in 200 μL PBS. Nude mice were treated in the way described above and scanned with fluorescence imaging weekly. Before imaging, the mice were anesthetized by intraperitoneal injection of 0.7% pentobarbital sodium with 10 μL/g and then injected with luciferase substrate (150 mg/kg). After 10 min, the mice were placed in a fluorescence imager for fluorescence observation and quantitative analysis. Fluorescence images were obtained and average fluorescent value in select regions of interest (ROI) were quantified with IVIS® software. On the 28th day, the nude mice were killed and their lungs were taken out for imaging.

Statistics

SPSS 17.0 for Windows (IBM Corporation, Armonk, NY) and GraphPad Prism (GraphPad Software, Inc., San Diego, CA, United States) software were used for statistical analyses. Normally distributed measurement data were expressed as mean ± SD and compared with Student t-test, while non-normally distributed measurement data were expressed as median (interquartile range) and compared with Mann-Whitney test (non-parametric distribution). One-way analysis of variance (ANOVA) was followed by post-hoc analysis by Tukey's test for multiple comparisons. Two-way repeated-measures ANOVA was followed by post-hoc analysis by LSD test for multiple comparisons of repeated measurement data. Survival rates were calculated using the Kaplan–Meier method and comparisons were performed using the Log-rank test. $p < 0.05$ was considered as statistically significant.

RESULTS

High Expression of CCL18 in UCs

We first investigated the expression of CCL18 in UCs. The expression of CCL18 in UCs was significantly higher than that in normal tissues ($p < 0.05$), as shown in **Figures 1A–C**. Besides, compared with healthy volunteers, the serum level of CCL18 was significantly increased in patients with UCs ($p < 0.05$), (**Figure 1D**), indicating that CCL18 is closely related to UCs. Baseline characteristics are shown in **Table 2**.

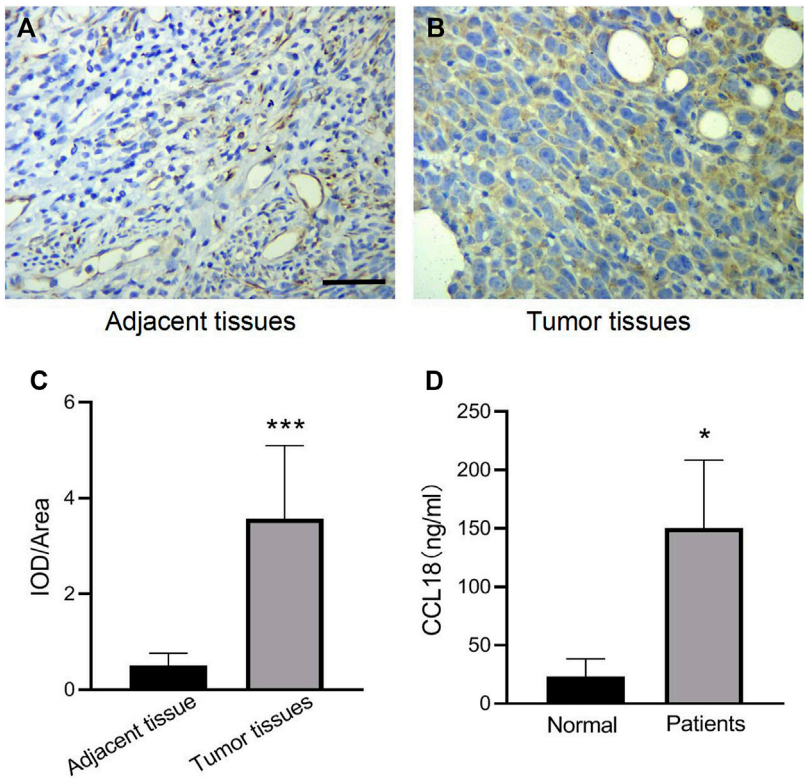

FIGURE 1 | The expression of CCL18 in UCs patients was higher than that in normal subjects. **(A,B)** is the result of CCL18 immunohistochemistry in UCs tissues and adjacent cancers (400 x). **(C)** is the statistical result of the optical density of immunohistochemistry. **(D)** is the expression of CCL18 in the serum of UCs patients and healthy people. *$p < 0.05$ vs. Normal people, ***$p < 0.001$ vs. Adjacent tissue. Scale bars: 25 μm.

TABLE 2 | Baseline characteristics.

Characteristic	Normal (n = 20)	Patient (n = 20)
Age—yr		
Median	66	67
Range	28–87	27–83
Weight—kg		
Median	71.5	70.6
Range	65.4–75.8	63.2–74.1
Male sex—no. (%)	15/20 (75.0)	14/20 (70.0)
Current or former smoker—no./total no. (%)	12/20 (60)	13/20 (65)
Visceral disease — no./total no. (%)	11/20 (55)	12/20 (60)

CCL18 Promotes Proliferation, Invasion, and Migration of UCs

To further confirm the effect of CCL18 on UCs, we manipulated CCL18 expression in BUC T24 cells. The proliferation of BUC T24 cells with CCL18 overexpression was significantly enhanced compared with untreated cells ($p < 0.05$). In contrast, the proliferation of cells with downregulated expression of CCL18 was inhibited ($p < 0.05$), as shown in **Figure 2**. In addition, CCL18 overexpression also significantly promoted the migration and invasion of BUC T24 cells ($p < 0.05$), and the opposite effect of CCL18 downregulation was observed, as shown in **Figure 2**.

The results of wound healing assays also showed that the wound healing degree of the CCL18 overexpression group was significantly higher than that of the untreated group and the CCL18 knockdown group ($p < 0.05$), after 12 h (Si.1). After 24 h, all the scratches were healed just in the CCL18 overexpression group.

Prediction and Verification of miRNA Targeting CCL18

Previous studies have confirmed that miRNAs can regulate the biological processes of a variety of tumors, and CCL18 may also

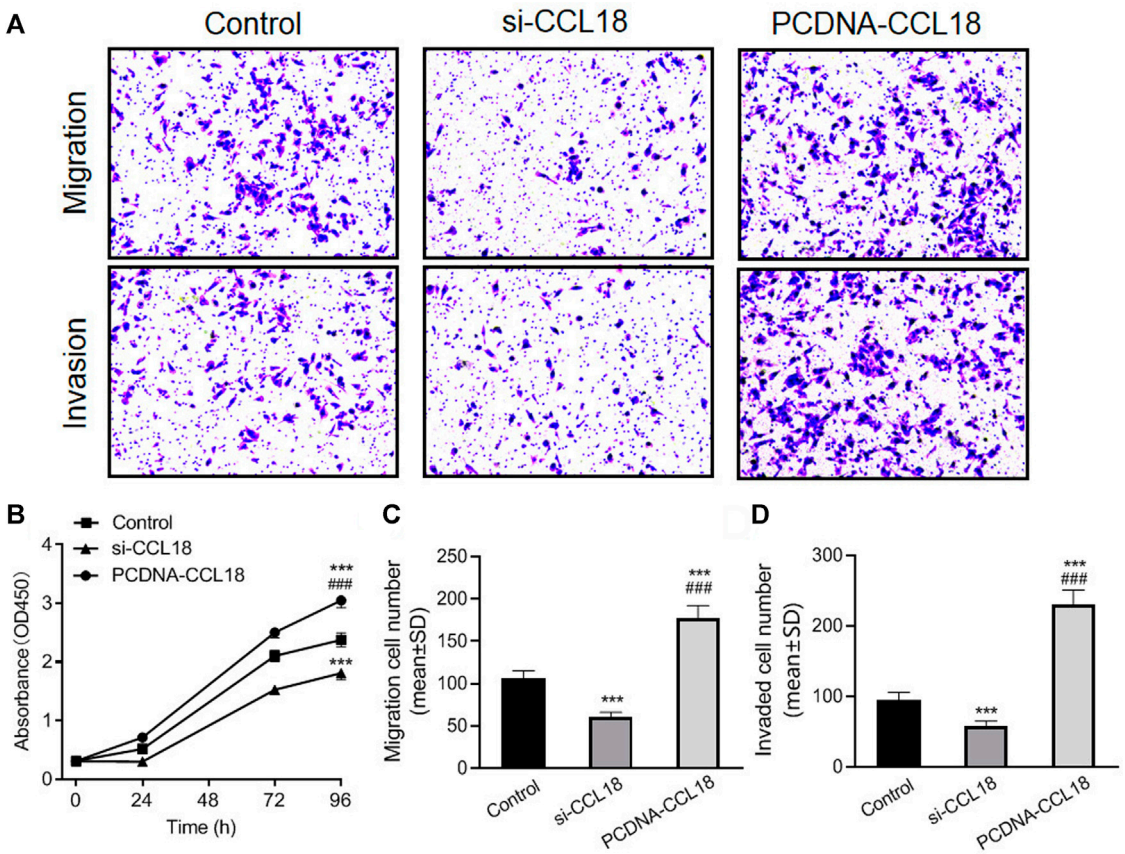

FIGURE 2 | CCL18 promotes proliferation, migration, and invasion of BUC T24 cells. **(A)** is picture of Transwell cell migration and invasion assay (stained with crystal violet). The migration and invasion ability of BUC T24 was significantly enhanced after CCL18 overexpression and suppressed after CCL18 silencing. **(B)** is the statistical result of cell proliferation ability detected by CCK-8 assay. The proliferative capacity of BUC T24 was promoted by CCL18 overexpression and restricted by CCL18 silencing at 96 h time point. **(C,D)** are the statistical results of migration and invasion, respectively. ***$p < 0.001$ vs. Control, ###$p < 0.001$ vs. Si-CCL18.

TABLE 3 | mirSVR score and PhastCon score.

miRNA	mirSVR score	Phastcon score
miR-183	−0.9208	0.5013
miR-374a	−0.9214	0.5265
miR-374b	−0.9157	0.5272
miR-33a	−0.8308	0.5084
miR-33b	−0.8289	0.5084
miR-128	−0.5164	0.5486
miR-138	−0.2031	0.5399
miR-410	−0.1158	0.5271

function as a target protein of miRNAs (Song et al., 2018; Korbecki et al., 2020). To confirm whether CCL18 was regulated by miRNAs in UCs, we screened for possible types of miRNAs. The miRanda detected 37 conservative miRNAs targeting human CCL8 mRNA, further screening out eight miRNAs with mirSVR score ≤ -0.1 and Phastcons score ≥0 (**Table 3**).

A total of 30 conservative miRNAs targeting human CCL18 mRNA were detected by total context++ score from the Targetscan. Combined mirSVR score, Phastcons score, and total context++ score, three miRNAs with high targeting, including miR-183, miR-128, and miR-33a, were screened out. The interaction between miRNAs and CCL18-3′UTR was shown in **Figure 3**. Furthermore, the contents of CCL18, *miR-183, miR-128 and miR-33a* in urothelial carcinoma tissues were detected, indicating a negative correlation between the content of CCL18 and *miR-128* (r_s = -0.521, $p < 0.05$), as shown in **Figure 3**. No correlation between CCL18 mRNA expression and miR-183 and miR-33a was detected ($p > 0.05$).

HMSC-128-EV Inhibits the CCL18 Secretion of UCs

Mesenchymal stem cells (MSCs) can migrate to tumor sites and perform complex functions during tumor progression. Exosome vectors were prepared by HMSC, and exosome miRNAs were transferred into tumor cells, which have been widely used in cancer research. To further confirm the regulatory effect of *miR-128* on CCL18, we chose exosomes as the carrier of *miR-128*. Firstly, HMSC-EV was prepared and identified by CD9 and

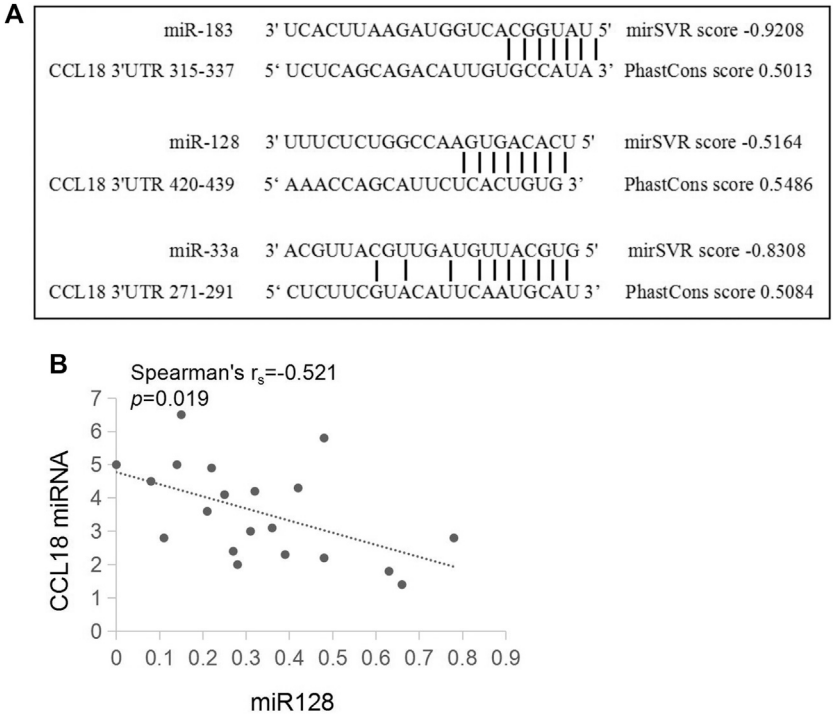

FIGURE 3 | There is a pairing relationship between ccl18 and the three types of miRNAs and a negative correlation with the amount of miR128 expressed. **(A)** is a schematic of CCL18-3′UTR and miR-183, miR-128, and miR-33a sequence binding. **(B)** is the relationship between CCL18 and the relative expression amount of miR-128.

CD63 surface-specific markers of exosome (Si.3 A-C). The TEM showed that the HMSC-EVs were spherical and homogeneous in shape and size, with a diameter of about 100 nm (Si.3 D-E). Besides, the diameter and Zeta potential of the HMSC-EVs were 105.4 ± 11.4 nm and −23.7 ± 1.2 mv, respectively, indicating the surface of exosomes is negatively charged (Si.3 F). Further, HMSCs were infected with adenovirus to overexpress the *miR-128*. Then the HMSC-128-EV was produced. The expression levels of *miR-128* in the above two exosomes were detected by RT-PCR, demonstrating that *miR-128* was highly expressed in HMSC-128-EV but not expressed in HMSC-EV (Si.3 G).

We used HMSC-EV and HMSC-128-EV to co-culture with BUC T24 and found that exosomes could be effectively endocytosed by BUC T24 (**Figures 4A–C**). Meanwhile, HMSC-128-EV could significantly increase the level of *miR-128* in BUC T24 ($p < 0.001$) (**Figure 4D**).

To confirm the effect of *miR-128* on the synthesis of CCL18 by BUC T24, we treated BUC T24 with HMSC-DC, HMSC-128-EV. By ELISA, we found that compared with the control group, the content of CCL18 released by BUC T24 in HMSC-Ev group was not different ($p > 0.05$), while the content of CCL18 released in HMSC-128-EV group was significantly decreased ($p < 0.05$) (**Figure 4E**). It suggested that overexpression of *miR-128* could inhibit the synthesis and secretion of CCL18 by UCs.

The *miR-128* can Inhibit Cell Proliferation, Migration and Invasion in UCs, Which can Be Reversed by CCL18

Considering that HMSC-128-EV were able to affect CCL18 expression of BUC T24, we further investigated the effect of HMSC-128-EV on the tumor process. CCK8 results showed that the cell viability of HMSC-EV groups were not different from the control group ($p > 0.05$), but it was significantly reduced in the HMSC-128-EV group ($p < 0.001$) (**Figure 5A**). Similarly, the numbers of clone formation in HMSC-Ev group was not different from the control group ($p > 0.05$), and was significantly reduced in HMSC-128-EV group ($p < 0.001$) (**Figures 5B,C**).

There was no significant differencein tumor cell migration and invasion ability between HMSC-EV group and control group ($p > 0.05$), while those in HMSC-128-EV group was significantly reduced than those in the control group ($p < 0.001$) (**Figures 5D–F**).

To explore the mechanisms underlying the effects of *miR-128* on the proliferation, invasion, and migration functions of UCs, we used CCL18 (0.5 ng/ml) and HMSC-128-EV to coculture with UCs. We found a significant recovery in the proliferation, invasion, and migration functions of the UCs compared with the HMSC-128-EV group ($p < 0.001$).

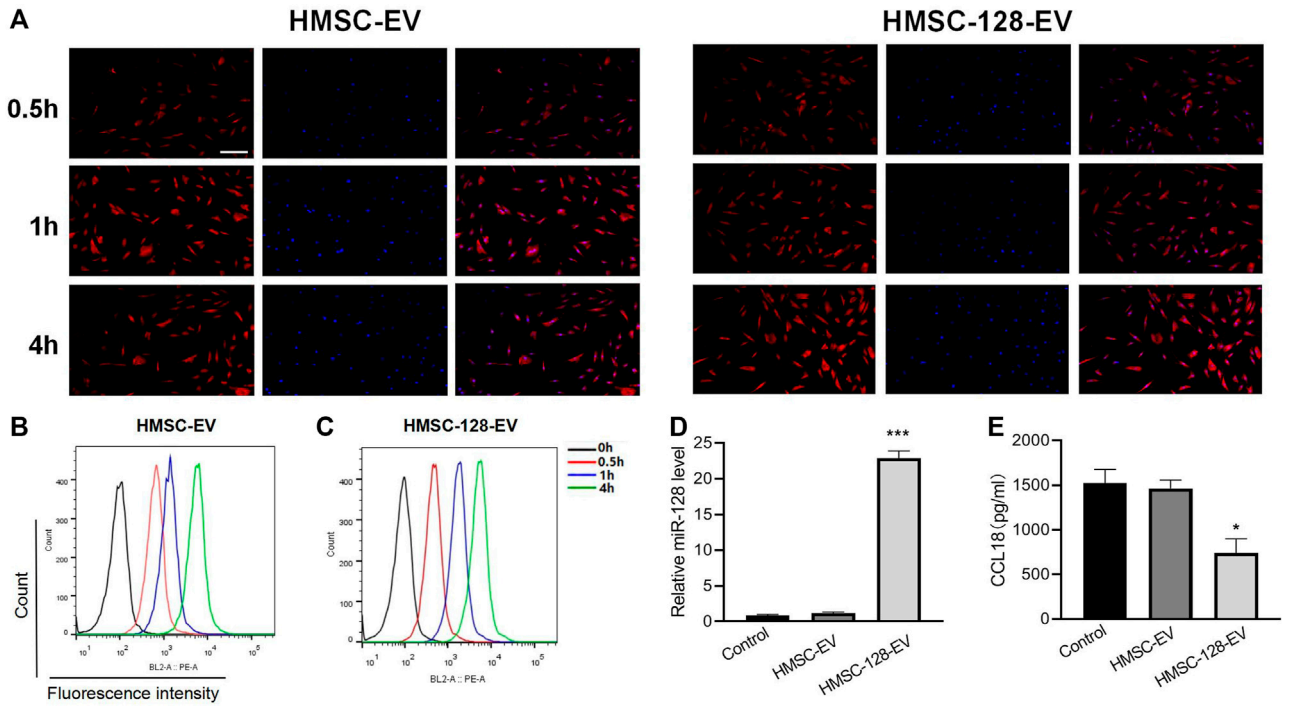

FIGURE 4 | HMSC-128-EV is able to significantly increase miR-128 within BUC T24 while reducing CCL18 expression. **(A–C)** are the results of endocytosis of exosomes detected by confocal microscopy and flow cytometry, and both HMSC-EV and HMSC-128-EV could be endocytosed by BUC T24. **(D)** is the level of miR-128 within BUC T24 of each group as determined by RT-PCR, and HMSC-128-EV was able to significantly increase intracellular miR-128. **(E)** is CCL18 within BUC T24 of each group determined by ELISA, and HMSC-128-EV was able to inhibit CCL18 expression within the cells. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. Control. Scale bars: 30 μm.

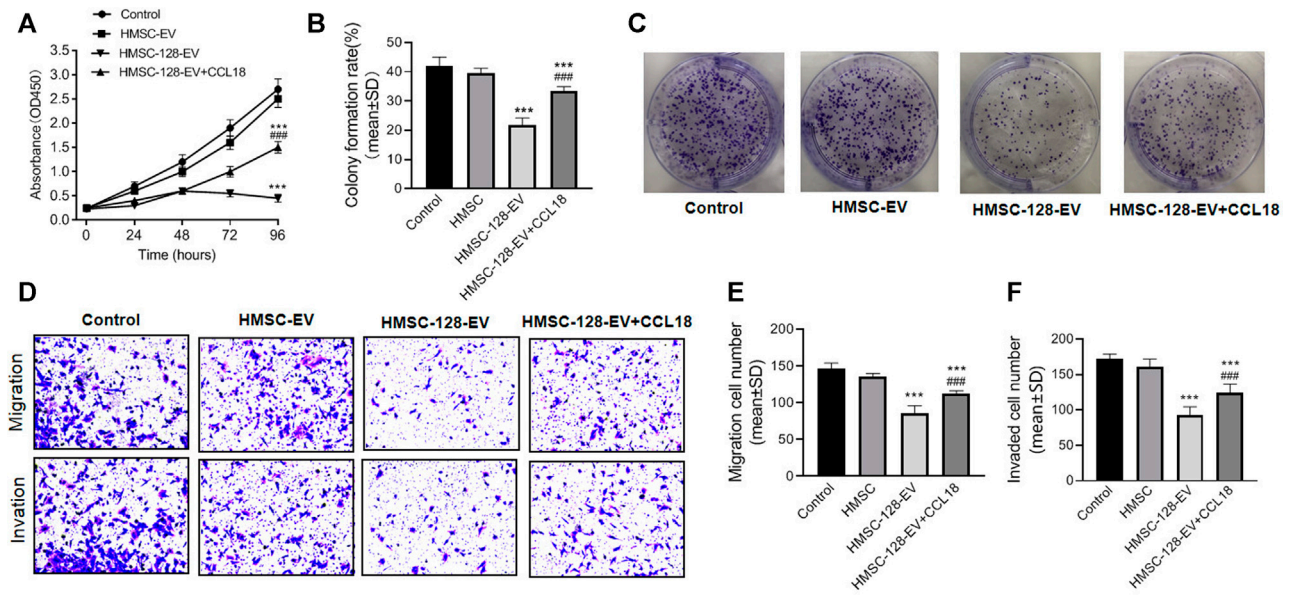

FIGURE 5 | HMSC-128-EV inhibited BUC T24 proliferation, migration, invasion. **(A)** is the result of cell viability assays by CCK8, which showed that HMSC-128-EV could significantly inhibit the viability of BUC T24 when co-cultured for 96 h. **(B, C)** are the results of clonogenic assay testing cell proliferation, and HMSC-128-EV could significantly inhibit the proliferation capacity of BUC T24. **(D–F)** are the results of Transwell cell migration and invasion assays, and HMSC-128-EV could significantly inhibit the migration and invasion abilities of BUC T24. $^{***}p < 0.001$ vs. Control, $###p < 0.001$ vs. HMSC-128-EV.

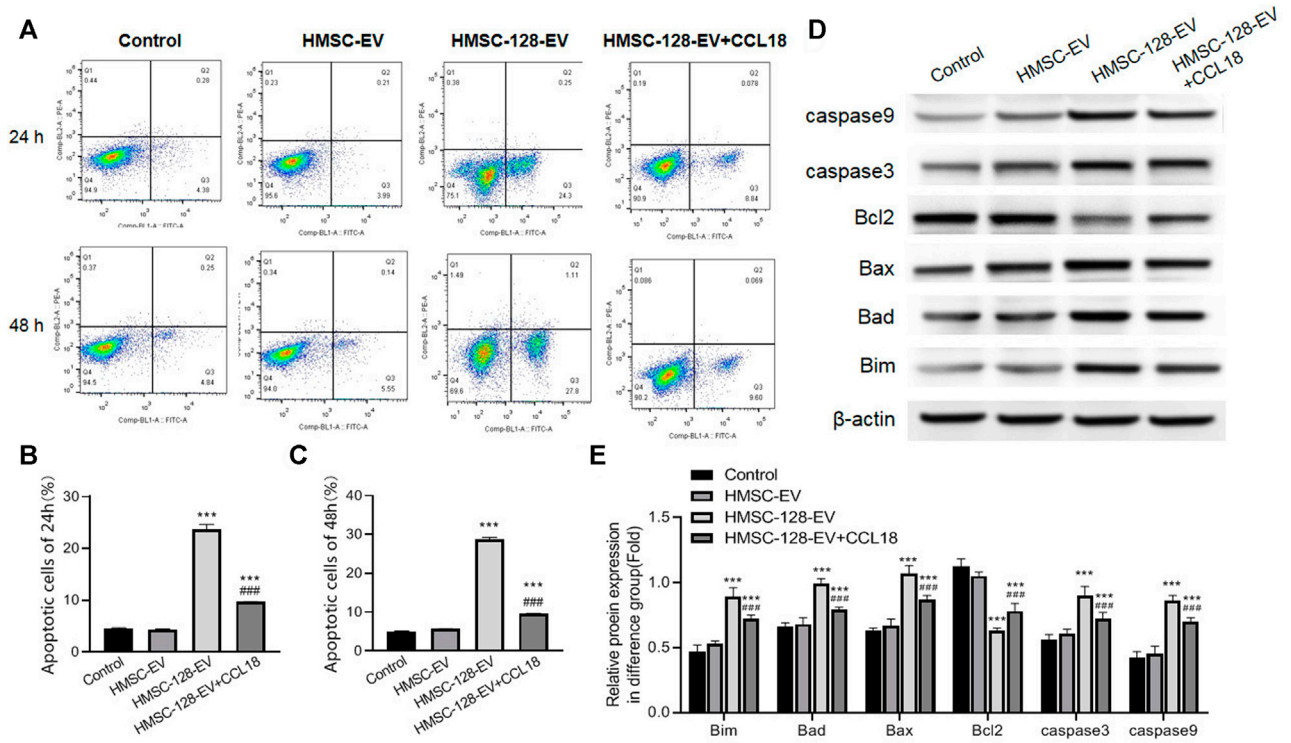

FIGURE 6 | HMSC-128-EV promoted BUC T24 apoptosis. **(A)** is the result of apoptosis detection using flow cytometry (stained by annexin/PI). **(B, C)** are the apoptosis rates after co-culture for 24 and 48 h. **(D, E)** are the western blotting typical picture and statistical results of Bim, Bad, Bax, caspase 3, caspase 9 and Bcl2 in each group, respectively. HMSC-128-EV was able to promote the expression of BUC T24 apoptotic protein and inhibit the expression of survival factors. ***$p < 0.001$ vs. Control, ###$p < 0.001$ vs. HMSC-128-EV.

The *miR-128* can Promote Apoptosis of UCs Cells, Which can be Reversed by CCL18

Besides, we also explored the apoptosis situation of tumor cells. The apoptosis rates of tumor cells in HMSC-EV groups was not different from the control group ($p > 0.05$), while the apoptosis rate in the HMSC-128-EV group was significantly higher than that of the control group ($p < 0.001$) (**Figures 6A–C**).

In terms of a series of protein markers associated with tumor cells apoptosis and survival, on the one hand, the pro-apoptotic proteins including Bim, Bad, Bax, caspase 3, and caspase 9 in each group were also detected, indicating that compared with the control group, the protein expressions of HMSC-128-EV group were significantly higher ($p < 0.05$) (**Figures 6D,E**). On the other hand, the level of cell survival-promoting factor Bcl2 was the lowest in the HMSC-128-EV group ($p < 0.001$). Taken together, *miR-128* silencing CCL18 may promote apoptosis of UCs cells.

In addition, we found that exogenous supplementation with CCL18 (0.5 ng/ml) resulted in a significant reduction ($p < 0.001$) in the proportion of cells undergoing apoptosis and in the detection of apoptosis related proteins (Bim, Bad, Bax, caspase 3, and caspase 9) compared with the HMSC-128-EV group. The cell survival promoting factor Bcl2 was significantly elevated ($p < 0.001$).

The *miR-128* can Inhibit Epithelial-Mesenchymal Transition of UCs, Which can Be Reversed by CCL18

EMT is a major feature of UCs, we further explored on the effect of HMSC-128-EV on the EMT of UCs. Compared with the control group, after treatment of *miR-128*, the intracellular E-cadherin expression was significantly increased ($p < 0.001$), and the expression of N-cadherin, β-catenin, and Vimentin was significantly decreased ($p < 0.001$) (**Figures 7A,B**).

After exogenous supplementation with CCL18, N-cadherin, β-catenin, and Vimentin significantly increased ($p < 0.001$) and E-cadherin significantly decreased ($p < 0.001$) compared with the HMSC-128-EV group. It is suggested that blocking CCL18 by *miR-128* may inhibit the EMT of UCs cells, thereby regulating tumor invasion.

The *miR-128* can Significantly Inhibit the Growth of UCs in Nude Mice

To confirm the therapeutic effect of HMSC-128-EV *in vivo*, we injected HMSC-128-EV into the tail vein of nude mice transplanted with UCs. For monitoring the growth of xenograft tumors, tumor volume and weight were measured in all mice. The results showed that the tumor volume and weight of mice treated with HMSC-128-EV decreased significantly, compared with

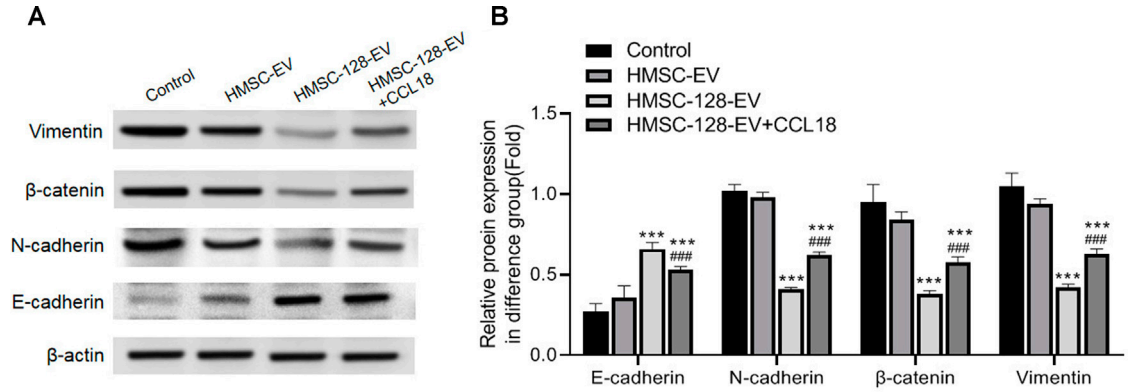

FIGURE 7 | HMSC-128-EV inhibited EMT of BUC T24. **(A)** is the typical picture of western blotting of E-cadherin, N-cadherin, beta-catenin, and Vimentin. **(B)** is the statistical results of western blotting of E-cadherin, N-cadherin, Beta-catenin, and Vimentin. HMSC-128-EV significantly inhibited N-cadherin, Beta-catenin, and Vimentin expression and promoted E-cadherin expression. ***p < 0.001 vs. Control, ###p < 0.001 vs. HMSC-128-EV.

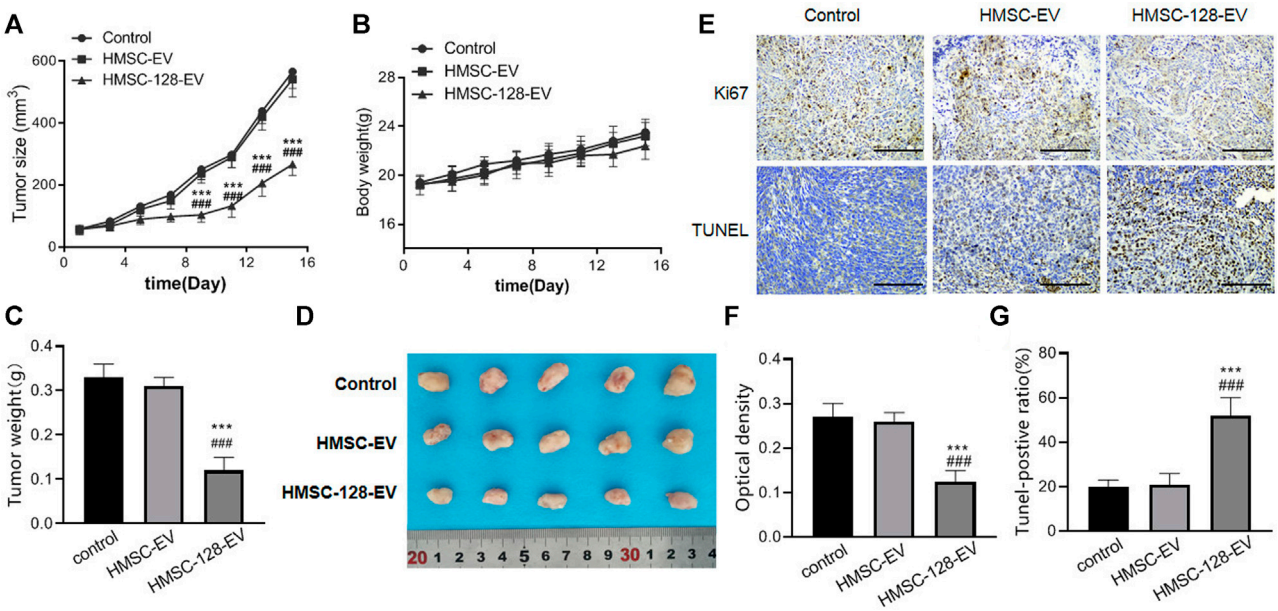

FIGURE 8 | HMSC-128-EV inhibited the growth of UCs in nude mice. **(A, B)** are line charts of the tumor volume and the body weight of nude mice in each group over time, respectively. **(C)** is the weight of tumors in each group after 16 days of treatment. **(D)** is a typical diagram of tumors from each group after 16 days of treatment. **(E)** is the immunohistochemical staining results of Ki67 and TUNEL of tumor tissues in each group. **(F, G)** are the statistical results of Ki67 and TUNEL, respectively. HMSC-128-EV significantly inhibited the proliferation and promoted the apoptosis of UCs in nude mice. *p < 0.05, **p < 0.01, ***p < 0.001 vs. Control. #p < 0.05, ##p < 0.01, ###p < 0.001 vs. HMSC-EV. Scale bars: 100 μm.

control and HMSC-EV groups (p < 0.05) (**Figures 8A,C,D**). Meanwhile, during the administration, the weight of mice increased steadily, and no weight loss and other adverse symptoms were observed (**Figure 8B**). Furthermore, in order to reflect the status of proliferation and apoptosis, Ki67 and TUNEL staining were performed on the transplanted tumor cell. Compared with the control and HMSC-EV groups, the mean optical density value of Ki67 positive was significantly reduced in the HMSC-128-EV group (p < 0.001), while the proportion of Tunel-positive tumor cells was significantly increased (p < 0.001) (**Figures 8E–G**).

The *miR-128* can Inhibit the Metastasis of UCs and Extend the Lifespan of Nude Mice

To evaluate the inhibitory effect of HMSC-128-EV on the metastasis of UCs, we monitored lung metastases in nude mice. For the control and HMSC-EV groups, the fluorescence intensities in the lungs of mice increased gradually with time (**Figure 9A**). On the 21st day, compared with the control and HMSC-EV groups, the fluorescence brightness, the weight of lung tissue, and the number of metastatic nodules in mice of the

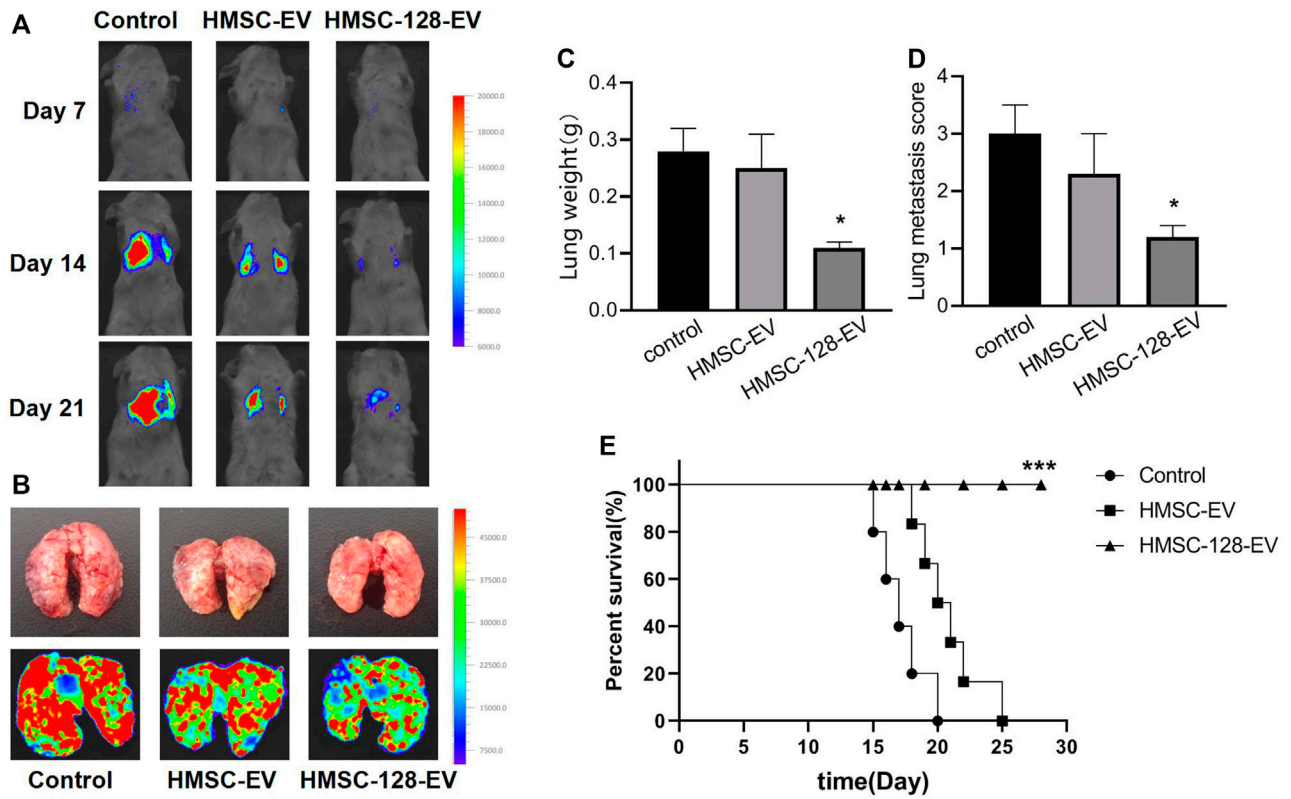

FIGURE 9 | HMSC-128-EV inhibited the metastasis of UCs and prolonged the survival time of nude mice. **(A,B)** typical images of live nude mice and lung fluorescence imaging, respectively. **(C,D)** are the statistical results of lung tissue weight and the number of lung metastatic nodules in nude mice of each group, respectively. HMSC-128-EV could significantly reduce the lung metastasis of UCs in nude mice. **(E)** is the survival curve of nude mice in each group. HMSC-128-EV can significantly prolong the survival time of nude mice with UCs. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. Control.

HMSC-128-EV group were significantly decreased ($p < 0.05$) (**Figures 9B–D**). Moreover, the survival rate of mice in the HMSC-128-EV group was significantly increased ($p < 0.05$) (**Figure 9E**).

DISCUSSION

In this study, we found that CCL18 was highly expressed in UCs, and down-regulation of CCL18 can inhibit proliferation, migration, and invasion of UCs. CCL18 may be regulated by *miR-128*. Exosome delivered *miR-128* can significantly inhibit CCL18 synthesis of UCs cells. DNA sequence alignment also detected a binding site between CCL18 and *miR-128*, and the expression of the two was negatively correlated in UCs cells. Further studies found that *miR-128* significantly inhibited the proliferation, migration, and invasion, and promoted apoptosis of UCs cells. On the animal model, we also confirmed that *miR-128* can inhibit the proliferation and metastasis of UCs in nude mice, and prolong their survival time. Our findings shed new light on the therapeutic innovation of UCs. The *miR-128*, loaded in exosomes, has the potential to become a new therapeutic agent for UCs.

CCL18 is a kind of chemokine, predominantly expressed by monocyte-derived cells with M2 phenotype and dendritic cell (Kodelja et al., 1998; Schutyser et al., 2005). CCL18 is not only a constitutive product under normal conditions but also an inducible chemokine under inflammatory conditions (Schutyser et al., 2005). Previous studies have demonstrated a strong correlation between CCL18 expression and various malignancies, such as ovarian cancer, gastric cancer (Schutyser et al., 2002; Leung et al., 2004). Our study found that CCL18 was highly expressed in the UCs. This finding is the same as previous studies (Liu X. et al., 2019). Inhibition of CCL18 expression can significantly inhibit the proliferation, invasion, and migration of BUC T24, indicating that the high expression of CCL18 plays an important role in the maintenance of UCs tumor process. Therefore, artificially regulating CCL18 expression may have therapeutic effects on UCs.

Many studies have proved that miRNA can regulate the growth, differentiation, and apoptosis of tumor cells (Tong and Nemunaitis, 2008; Shimono et al., 2009). Altered expression of multiple miRNAs including *miR-21, miR-155* was associated with tumorigenesis (Zhang et al., 2008; Mattiske et al., 2012). In addition, some miRNAs, such as let-7, *miR-34a,* et al. possess tumor suppressor functions (Akao et al., 2006; Liu et al., 2011). The *miR-128* has been demonstrated to play an important

role in the occurrence, development, and targeted therapy of colorectal, prostate, and ovarian cancers (Khan et al., 2010; Li et al., 2014; Liu T. et al., 2019). For the UCs, multiple miRNAs have been confirmed to participate in the biological process (Song et al., 2010; Fujii et al., 2015). But the role of *miR-128* in the tumor process of UCs has not been reported. In this study, we found that *miR-128* played an important role in the proliferation and metastasis of UCs. In this study, we found that *miR-128* was functionally linked to the secretion of CCL18 of UCs. It was able to significantly inhibit CCL18 synthesis of BUC T24. Meanwhile, *miR-128* loaded in exosomes was able to obviously inhibit cell proliferation, metastasis, and invasion of UCs. This suggests that *miR-128* may regulate the tumorigenic process of UCs by regulating CCL18 secretion from BUC T24 in the tumor microenvironment. This may be the mechanism by which miR-128 has therapeutic potential for UCs.

By bioinformatics analysis, we found that CCL18 was a target protein of *miR-128*. The *miR-128*, loaded in exosome, was able to inhibit CCL18 secretion from BUC T24. Our study also found that the effect of *miR-128* on the proliferation, invasion and migration of UCs can be reversed by CCL18. In addition, we also found that *miR-128* promoted apoptosis and inhibited EMT of UCs could be reversed by CCL18. These indicated that the effect of *miR-128* on the biological process of UCs was directly related to CCL18. This pathway was first identified in UCs and enriched the regulatory mechanism of the tumor microenvironment of UCs, and its significance as an avenue for targeted therapy of UCs warranted further investigation.

In addition, our innovative use of embryonic stem cell exosomes as a delivery vehicle for *miR-128* confirmed its ability to efficiently elevate *miR-128* levels in BUC T24, and affected its function. Exosomes are saucer-shaped vesicles released by cells to carry membrane and cytosolic components, with a diameter of 30–100 nm (Simons and Raposo, 2009). Exosome could bear combinations of ligands, bind to target-cell membranes, and fuse with target cells (Théry et al., 2002). Thus, exosomes can be used as drug delivery vehicles and have a good delivery effect (Batrakova and Kim, 2015; Haney et al., 2015). In this study, we confirmed that exosomes not only could deliver *miR-128* to BUC T24 but also had a promising therapeutic effect in animal experiments. These findings further support the possibility that *miR-128* in combination with exosomes could be used to treat the UCs. This innovation in miRNA delivery modality was the first reported in UCs. It provided a new way for the application of miRNAs in the treatment of UCs.

The present study also has limitations. UCs are capable of secreting a variety of cytokines in the tumor microenvironment, we only explored the role of CCL18 and did not investigate whether other cytokines also contribute to UCs. We will further refine it in the future studies.

CONCLUSION

Our study innovatively found that *miR-128* could influence the tumor process of the UCs by regulating CCL18 secretion. Exosomes have the potential to function as a carrier of *miR-128* in the treatment of UCs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Beijing Friendship Hospital, Capital Medical University, 2021-P2-159-02. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

DS and YL conceived the article, DS write the manuscript, DS, YL and ZC finish the experiemnt, DS, YL, and ZC analyze the data, YL and ZC revised the manuscript, YL proved funding support.

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FAT10 is a Prognostic Biomarker and Correlated With Immune Infiltrates in Skin Cutaneous Melanoma

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Background: Skin Cutaneous Melanoma (SKCM) is the deadliest cutaneous neoplasm. Previous studies have proposed ubiquitin-like protein FAT10 plays key roles in the initiation and progression of several types of human cancer, but little is known about the interrelation between *FAT10* gene expression, tumor immunity, and prognosis of patients with SKCM.

Methods: Here, we first performed pan-cancer analysis for FAT10's expression and prognosis using the Cancer Genome Atlas and the Genotype-Tissue Expression data. Subsequently, we investigated the mRNA expression level, prognostic value, and gene-gene interaction network of FAT10 in SKCM using the Oncomine databases, GEPIA, TIMER, UALCAN, and starBase. The relationship between FAT10 expression and tumor immune invasion was studied by using the TIMER database. Additionally, the expression and functional status of FAT10 in SKCM were evaluated by the single-cell RNA sequencing and CancerSEA databases.

Results: In this study, we found that FAT10 expression was increased in SKCM and was correlated with a better survival rate in patients with SKCM. Moreover, we identified FAT10 level was significantly positively associated with immune infiltrates, biomarkers of immune cells, and immune checkpoint expression, and negatively correlated with tumor cell invasion and DNA damage, indicating that increased FAT10 expression in SKCM was a favorable response to immune checkpoint inhibitors.

Conclusion: Our findings suggest that upregulation of FAT10 correlated with better prognosis and tumor immune infiltration in SKCM.

Keywords: FAT10, bioinformatics analysis, tumor-infiltrating, prognosis, skin cutaneous melanoma, competing endogenous RNA

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INTRODUCTION

Skin Cutaneous Melanoma (SKCM) is a primary malignant tumor that originates from benign moles (Cramer, 1991). It is derived from neural crest stem cells, which can produce melanin in the skin (Ding et al., 2021). Nevis will not develop into melanoma in most cases (Damsky and Bosenberg, 2017). However, certain factors increase the possibility of malignant transformation, such as Sun exposure, fair skin type, constant friction, or physical irritation to the affected skin (Bloethner et al., 2009; Darmawan et al., 2019). SKCM accounts for about 5% of the total number of skin cancers, but its death toll accounts for more than 75% of skin cancer deaths (Rebecca et al., 2020). The 5 years

relative survival rate of local SKCM patients was 98%, the 5 years relative survival rate of regional SKCM patients was 64%, while the 5 years survival rate of patients with metastatic SKCM dropped to 23% (Rebecca et al., 2020). Therefore, we urgently need to clarify the molecular mechanism of SKCM, which may lead to the development of new treatments to improve long-term survival.

Fan *et al.* first discovered the ubiquitin-like protein FAT10 in 1996 (Fan et al., 1996). FAT10 shares many similarities with ubiquitin, while ubiquitin recycled from the degraded target proteins, FAT10 degraded along with its target, resulting in a relatively short half-life (Hipp et al., 2005; Liu et al., 2018). It is a protein belonging to the immune system, which can be strongly upregulated by pro-inflammatory cytokines (Aichem and Groettrup, 2016). Some reports described that FAT10 is involved in cardioprotection and regulates IRE1α/c-Jun N-terminal kinase protein-dependent apoptosis in pancreatic β cells (Brozzi et al., 2016; Zhang et al., 2020). Animal studies have found that FAT10 "knockout" mice showed no distinct phenotypic changes, even if they do, they are relatively small. But these mice became more sensitive to endotoxin attack, and compared with wild-type mice, their lymphocytes were more prone to spontaneous apoptosis (Canaan et al., 2006). The description of the role of FAT10 in cancer in the published literature is contradictory. Several clinical studies have shown that FAT10 could confer malignant characteristics to non-tumorigenic cells and enhance the malignant-related characteristics of cancer cells (Gao et al., 2014; Zhang et al., 2020). However, the colony formation and transformation ability of FAT10 was not observed in the experiment of S Lukasiak *et al.*, which contradicts the function of FAT10 as a proto-oncogene (Lukasiak et al., 2008).

Despite these previous studies, there is still a lack of systematic research on the expression, prognosis, and mechanism of FAT10 in SKCM. In addition, the association between FAT10 and tumor immune infiltration in SKCM has not yet been determined. This study conducted expression analysis and survival analysis of FAT10 in various types of human cancers. Furthermore, the noncoding RNA (ncRNA)-associated regulation of FAT10 was explored in SKCM, including microRNA (miRNA) and long noncoding RNA (lncRNA). We then determined the relationship between FAT10 expression and immune cell infiltration, biomarkers of immune cells, or immune checkpoints in SKCM. Together, our findings corroborate that FAT10 may play a key role in the prognosis of SKCM while indicating a potential mechanism by which the FAT10 expression might adjust tumor immunity by regulating the infiltration of immune cells in SKCM patients.

MATERIALS AND METHODS

Oncomine Database Analysis

Oncomine (https://www.oncomine.org/resource/login.html) is a comprehensive data mining platform, as well as the world's largest cancer-related gene microarray database. It contains the most complete cancer mutation profile, related gene expression profile, and related clinical information, which can be used to discover new biomarkers or therapeutic targets. In this study, the *FAT10* gene was selected as the research object to compare its expression level in cancer tissues and normal tissues. When the folding change>1.5, with a p-value>0.001, the expression levels in different tissues were considered to be significantly different. We set the data type to "all" and the threshold value of gene rank to "top 10%" (Rhodes et al., 2007).

Gene Expression Profiling Interactive Analysis Database Analysis

Gene Expression Profiling Interactive Analysis (GEPIA, www.gepia.cancer-pku.cn) is an interactive web server for cancer and normal gene-expression profiling and interactive analyses based on the Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) data, with 9,736 tumors and 8,587 normal samples, respectively (Tang et al., 2017). GEPIA is used to analyze the expression of FAT10 and lncRNA in various types of cancer based on a given TCGA and GTEx expression data set, under the settings of the p-value of 0.01 and fold change of 1. The impact of FAT10 expression on survival rates was evaluated using the GEPIA, including overall survival (OS) and disease-free survival (RFS). GEPIA was also employed to conduct the prognostic values of candidate lncRNAs in FAT10. A log-rank p-value <0.05 was considered statistically significant. Moreover, FAT10 expression correlation with immune checkpoints in SKCM was perform using the GEPIA database. Items with IRI >0.1 and p-value <0.05 were set as selection criteria and determined to be significant.

Candidate miRNA Prediction

The upstream binding miRNA of FAT10 was predicted by multiplel target gene prediction programs (PITA, RNA22, miRmap, microT, miRanda, PicTar, and TargetScan). When predictive miRNAs appeared in more than two programs at the same time, they were used for subsequent analysis. These predicted miRNAs were considered candidate miRNAs of FAT10.

UALCAN Database Analysis

The UALCAN portal (http://ualcan.path.uab.edu/analysis-prot.html) is an interactive network resource that can be used to analyze cancer omics data (Chen et al., 2019). We used this online tool to assess the expression level of the upstream binding miRNAs of FAT10 of SKCM located in metastases, primary tumors, and normal tissues.

starBase Database Analysis

starBase (http://starbase.sysu.edu.cn/), a database for exploring miRNA-related research (Li et al., 2014). In our study, starBase was introduced to perform survival analysis on hsa-miR-3127-5p in SKCM. A log-rank p-value <0.05 was

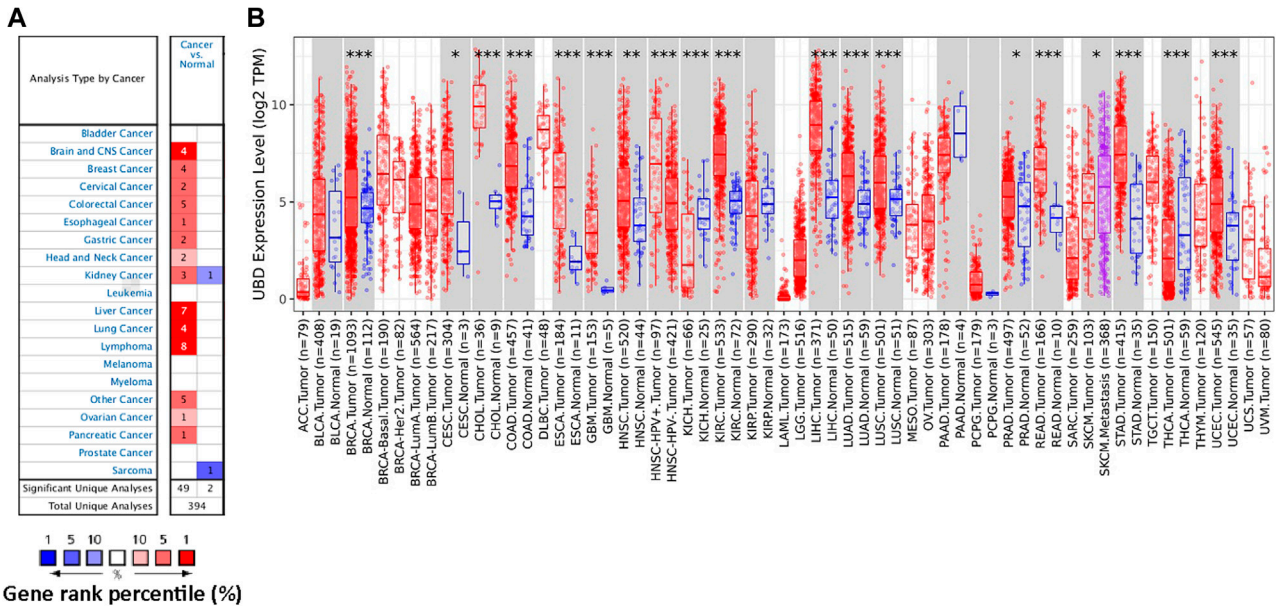

FIGURE 1 | The expression level of *FAT10* in various cancer tissues or tumor cells. **(A)** Through Oncomine analysis, the expression level of *FAT10* in various normal or cancer tissues. **(B)** Through TIMER analysis, the expression level of *FAT10* in various normal or cancer tissues (*$p < 0.05$, ***$p < 0.001$).

considered statistically significant. In addition, we predicted candidate lncRNAs that might bind to hsa-miR-3127-5 based on starBase.

TIMER Database Analysis

TIMER (https://cistrome.shinyapps.io/timer/) is a web server for determining the abundance of tumor infiltrates based on gene expression analysis (Li et al., 2017). The gene name *FAT10* under the DiffExp module has default parameters was used for obtaining the different expression levels in normal or tumor tissues. We used TIMER to analyze the level of immune cell infiltration in SKCM and the correlation between FAT10 and tumor-infiltrating immune cell biomarker gene expression, including B cells, $CD8^+$ T cells, $CD4^+$ T cells, M1 macrophages, M2 macrophages, neutrophils, and dendritic cells. p-value <0.05 was considered statistically significant.

Human Protein Atlas and CancerSEA Database Analysis

To confirm our outcome, The Human Protein Atlas (www.proteinatlas.org/) was utilized to determine the expression of FAT10 at the translational level (Thul et al., 2017). The expression and distribution of FAT10 in SKCM tissues was clarified by the single-cell RNA sequencing (scRNA-seq), which was obtained from CancerSEA (Yuan et al., 2019).

Statistical Analysis

All data analyses was automatically calculated by the above-mentioned online database. Log-rank p-value <0.05 or p-value <0.05 were considered statistically significant.

RESULTS

Expression of FAT10 in SKCM and Other Cancers

In order to study the possible role of FAT10 in cancer, we first analyzed its expression in various cancer types through Oncomine database analysis. Except for sarcoma cancer, compared with normal tissues, the expression level of *FAT10* gene in all other available cancer tissues was significantly up-regulated, including brain and central nervous system cancer, breast, cervical, colorectal, esophageal, gastric, head and neck, liver, lung, lymphoma, ovarian, kidney, and pancreatic tissues (**Figure 1A**). Similarly, the RNA-seq data we collected from the TCGA database showed that significantly up-regulated *FAT10* was detected in 19 types of cancers, including SKCM (**Figure 1B**). We also collected RNA-seq data from the TCGA and GTEx expression data sets, and the results showed that *FAT10* was significantly up-regulated in 21 cancer types (**Supplementary Figure S1**).

The Prognostic Values of FAT10 in Human Cancer

We investigated whether the expression level of FAT10 affected the prognosis of cancer patients (BRCA, CESC, COAD, GBM, KICH, LGG, OV, SKCM, and THYM). Two prognostic indices, consisting of OS and RFS, were included. For OS, highly expressed FAT10 in OV and SKCM patients had a better prognosis, but LGG patients with higher expression of FAT10 indicated an unfavorable prognosis (**Figure 2**). For RFS, high FAT10 expression levels were associated with better prognosis in BRCA, OV, and SKCM patients, but THYM patients with higher expression of the *FAT10* gene were related to poor

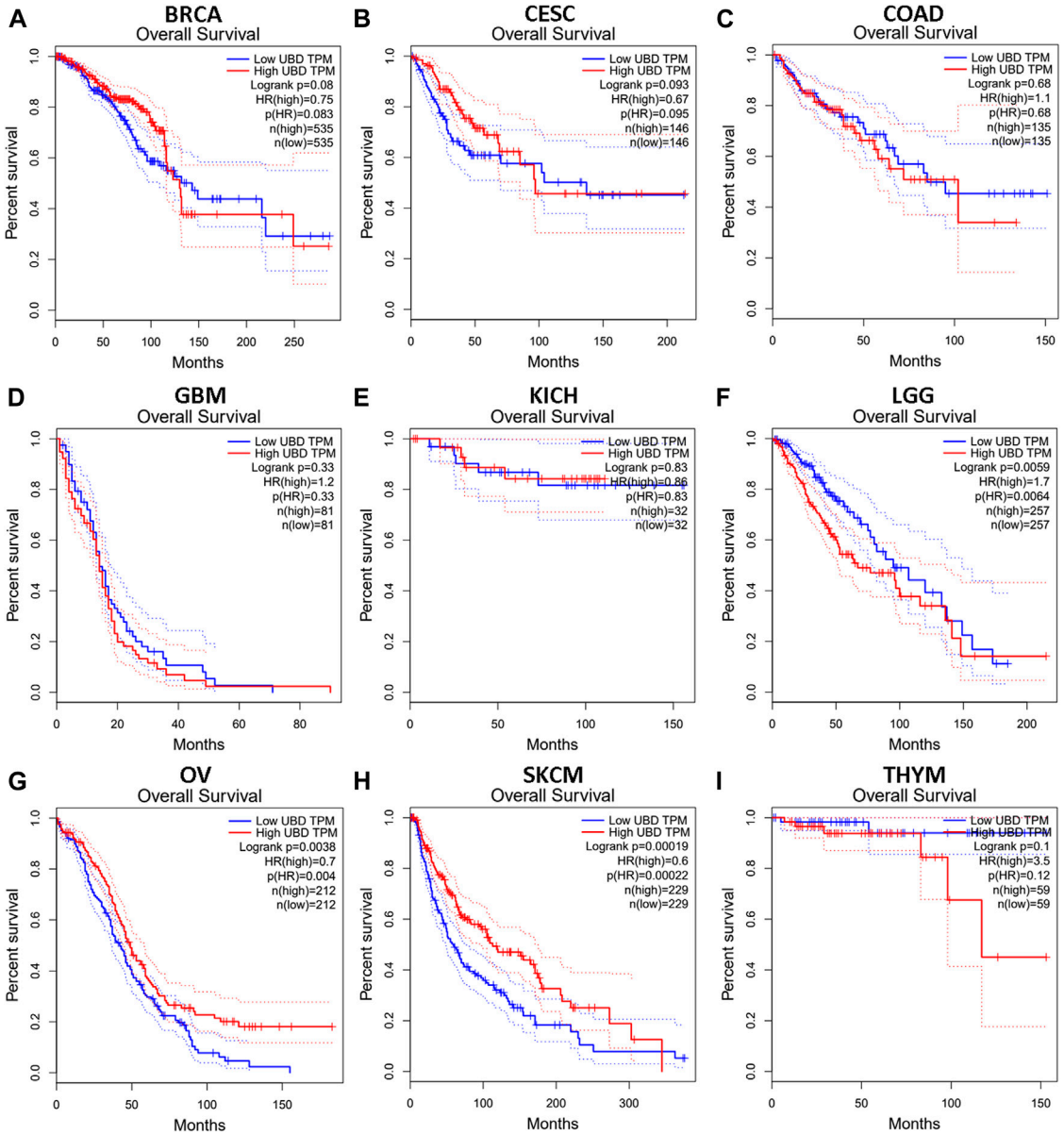

FIGURE 2 | The OS analysis for FAT10 in various human cancers via GEPIA database. **(A–I)** The OS plot of FAT10 in BRCA **(A)**, CESC **(B)**, COAD **(C)**, GBM **(D)**, KICH **(E)**, LGG **(F)**, OV **(G)**, SKCM **(H)**, THYM **(I)**.

prognosis (**Figure 3**). We have not observed that FAT10 was statistically significant in predicting the prognosis of patients in other types of cancer. These results confirmed the prognostic value of FAT10 in certain types of cancer, and the increase or decrease of FAT10 expression has different prognostic values, depending on the type of cancer.

Prediction and Analysis of Upstream miRNAs of FAT10

ncRNA is responsible for the regulation of gene expression, which has been widely recognized. In order to determine whether FAT10 was regulated by some ncRNAs, we first predicted the upstream miRNAs that might bind to FAT10 and found 7 miRNAs.

To visualize the results, we used cytoscape software to establish a miRNA-FAT10 regulatory network. According to the mechanism of miRNA regulating target gene expression, there should be a positive correlation between miRNA and FAT10. Therefore, we conducted a related expressive analysis. As shown in **Figure 4**, there is a significant positive correlation between FAT10 and hsa-miR-3127-5p in SKCM. No statistical expression relationship between FAT10 and the other six predicted miRNAs was observed. We finally determined the

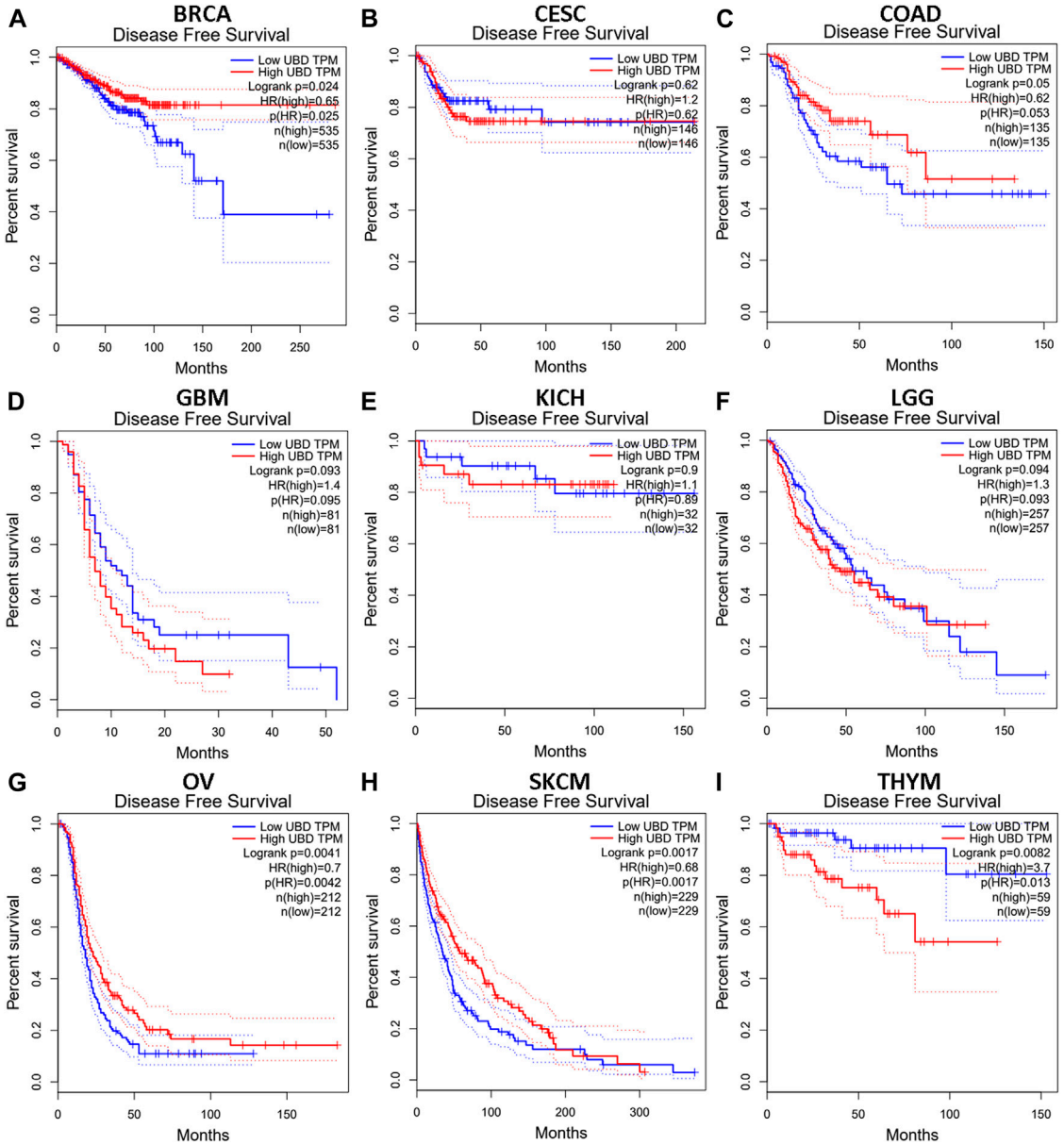

FIGURE 3 | The RFS analysis for FAT10 in various human cancers via GEPIA database. **(A–I)** The RFS plot of FAT10 in BRCA **(A)**, CESC **(B)**, COAD **(C)**, GBM **(D)**, KICH **(E)**, LGG **(F)**, OV **(G)**, SKCM **(H)**, THYM **(I)**.

expression and prognostic value of hsa-miR-3127-5p in SKCM.

Prediction and Analysis of Upstream lncRNAs of Hsa-miR-3127-5p

Next, we used the starBase database to predict the upstream lncRNA of hsa-miR-3127-5p. There were 115 possible lncRNAs discovered. Futuremore, GEPIA was used to determine the expression level of these lncRNAs in SKCM. We found that among all 115 lncRNAs in SKCM, 20 lncRNAs were significantly down-regulated compared with the normal control, including HCP5 and N4BP2L2-IT2. Subsequently, we evaluated the prognostic value of 20 lncRNAs in SKCM. As suggested in **Figure 5**, SKCM patients with higher expression of HCP5 or N4BP2L2-IT2 possessed better OS. The competitive endogenous RNA (ceRNA) hypothesis suggests that lncRNA can increase mRNA expression through competitive binding with shared miRNAs. Thus, there should be a positive correlation between lncRNA and mRNA or a negative correlation between lncRNA and miRNA. We also used the starBase database to detect the expression correlation between lncRNA and hsa-miR-3127-5p/

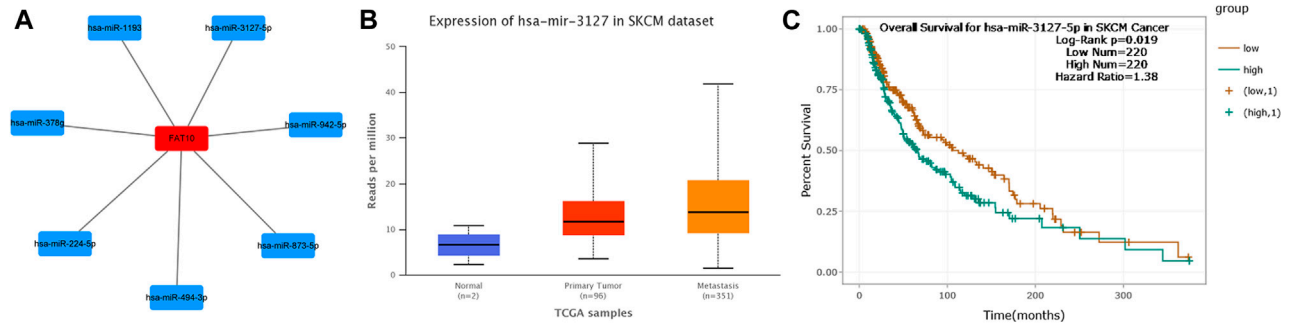

FIGURE 4 | Identify hsa-miR-3127-5p as a potential upstream miRNA of FAT10 in SKCM. **(A)** The miRNA-FAT10 regulatory network was established via cytoscape software. **(B)** The expression of hsa-miR-3127-5p in SKCM and control normal samples via UALCAN analysis. **(C)** The prognostic value of hsa-miR-3127-5p in SKCM assessed by starBase database.

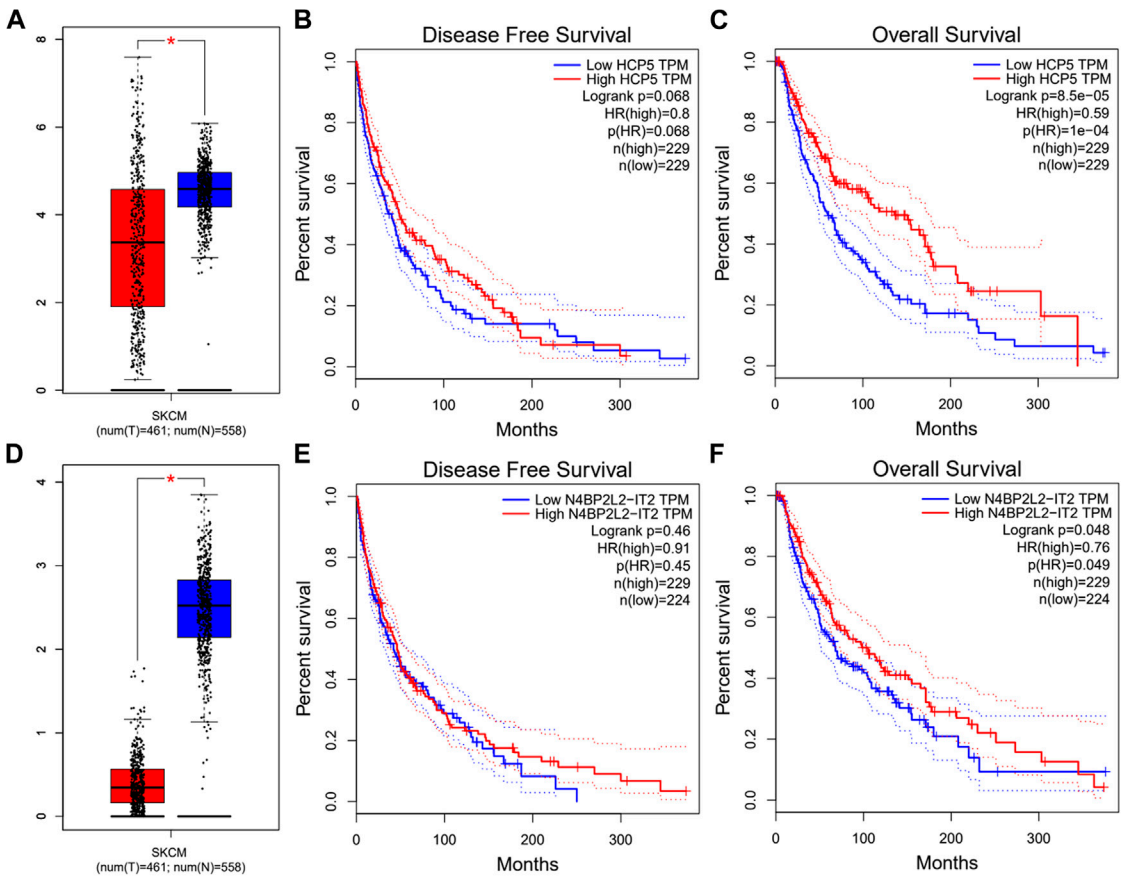

FIGURE 5 | Expression analysis and survival analysis of lncRNA upstream of hsa-miR-3127-5p in SKCM. **(A,D)** The expression of HCP5 **(A)** and N4BP2L2-IT2 **(D)** in TCGA SKCM compared with "TCGA and GTEx normal" data. **(B,C,E,F)** The RFS analysis for HCP5 **(B)** and N4BP2L2-IT2 **(E)** in HCC. The OS for HCP5 **(C)** and N4BP2L2-IT2 **(F)** in SKCM. *p-value < 0.05.

FAT10 in SKCM, as shown in **Table 1**. Considering the results of expression analysis, survival analysis, and correlation analysis, HCP5 might be the most potential upstream lncRNA of the hsa-miR-3127-5p/FAT10 axis in SKCM.

FAT10 Positively Correlates With Immune Cell Infiltration in SKCM

FAT10 is a member of ubiquitin-like proteins, which is involved in the inflammatory response and immune cell infiltration. We

TABLE 1 | Correlation analysis between lncRNA and hsa-miR-3127-5p or lncRNA and FAT10 in SKCM via starBase database.

lncRNA	miRNA	R value	*p* value
HCP5	hsa-miR-3127-5p	–0.220[a]	2.49E-06[***,a]
N4BP2L2-IT2	hsa-miR-3127-5p	0.100[a]	3.02E-02[*,a]
lncRNA	mRNA	R value	*p* value
HCP5	FAT10	0.690[a]	4.70E-67[***,a]
N4BP2L2-IT2	FAT10	0.120[a]	1.00E-02[*,a]

[a]*These results are statistically significant.*
*p *value < 0.05; ***p *value < 0.001.*

found that higher expression levels of FAT10 were related to better prognosis and high immune infiltration in SKCM. There were significant changes in the level of immune cell infiltration under different copy numbers of FAT10 (**Figure 6A**). The study of the correlation between the expression level of FAT10 and the level of immune cell infiltration could provide useful clues for studying the function and mechanism of FAT10. We then assessed the correlation between the expression level of FAT10 and the level of immune cell infiltration. As shown in **Figures 6B–G**, FAT10 expression was significantly positive correlation with all analyzed immune cells in SKCM (B cell, $CD8^+$T cell, $CD4^+$T cell, macrophage, neutrophil, and dendritic cell). These findings strongly indicate that FAT10 play a specific role in the immune infiltration of SKCM.

Expression Correlation of FAT10 and Biomarkers of Immune Cells in SKCM

To investigate the role of FAT10 in tumor immunity, we used the GEPIA database to determine the correlation between FAT10 and the expression of immune cell biomarkers in SKCM. We found that FAT10 expression was positively correlated with B cell's biomarkers (CD19 and CD79A),

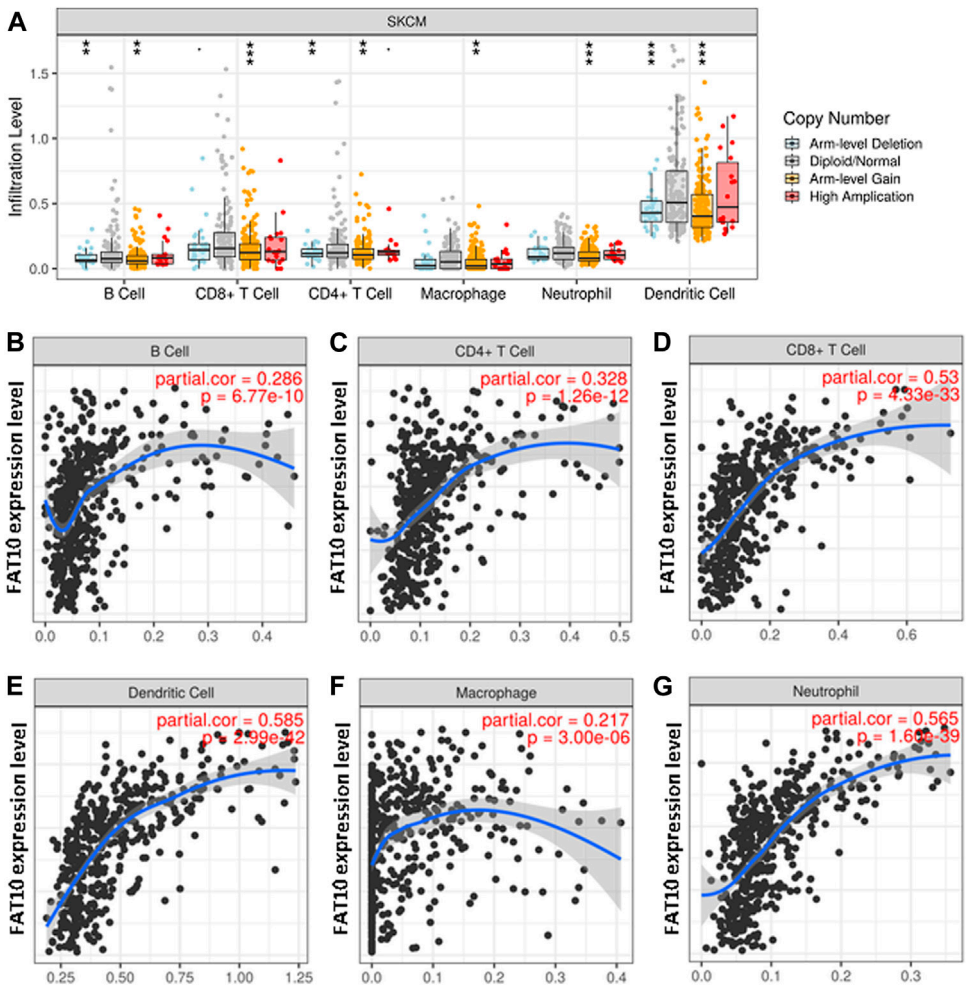

FIGURE 6 | The relationship between immune cell infiltration and FAT10 level in SKCM. **(A)** The infiltration level of various immune cells in SKCM with different copy numbers of FAT10. **(B–G)** FAT10 expression level and B cells **(B)**,$CD4^+$ T cells **(C)**, $CD8^+$ T cells **(D)**, dendritic cells **(E)**, macrophages **(F)**, or neutrophils **(G)** correlation of infiltration level in SKCM.

TABLE 2 | Correlation analysis between FAT10 and biomarkers of immune cells in SKCM via GEPIA database.

Immune cell	Biomarker	R Value	*p* value
B cell	CD19	0.61[a]	7.8E-48[***,a]
	CD79A	0.66[a]	1.4E-58[***,a]
$CD8^+$ T cell	CD8A	0.83[a]	1.5E-119[***,a]
	CD8B	0.82[a]	4.5E-115[***,a]
$CD4^+$ T cell	CD4	0.68[a]	9.2E-63[***,a]
M1 macrophage	NOS2	–0.033	4.8E-01[a]
	IRF5	0.46[a]	6.9E-26[***,a]
	PTGS2	0.013	7.8E-01
M2 macrophage	CD163	0.54[a]	4.4E-36[***,a]
	VSIG4	0.53[a]	1.8E-34[***,a]
	MS4A4A	0.57[a]	2.6E-40[***,a]
Neutrophil	CEACAM8	-0.07	1.3E-01
	ITGAM	0.57[a]	1.3E-41[***,a]
	CCR7	0.71[a]	5.9E-72[***,a]
Dendritic cell	HLA-DPB1	0.78[a]	1.7E-94[***,a]
	HLA-DQB1	0.60[a]	1.3E-46[***,a]
	HLA-DRA	0.81[a]	1.8E-107[***,a]
	HLA-DPA1	0.79[a]	4.3E-100[***,a]
	CD1C	0.51[a]	2E-32[***,a]
	NRP1	0.14[a]	2.9E-03[**,a]
	ITGAX	0.49[a]	3.8E-29[***,a]

[a]*These results are statistically significant.*
***p value < 0.01; ***p value < 0.001.*

$CD8^+$T cell's biomarkers (CD8A and CD8B), $CD4^+$T cell's biomarker (CD4), M1 macrophage's biomarkers (IRF5), M2 macrophage's biomarkers (CD163, VSIG4, and MS4A4A), neutrophil's biomarkers (ITGAM and CCR7), and dendritic cell's biomarkers (HLA-DPB1, HLA-DQB1, HLA-DRA, HLA-DPA1, CD1C, NRP1, and ITGAX) in SKCM (**Table 2**). These results further indicate that there is a positive relationship between FAT10 and immune cell infiltration.

Relationship Between FAT10 and Immune Checkpoints in SKCM

PD1 and CTLA-4 are important immune checkpoints, as well as negative regulators of T cell immune function. PD-L1 expression is present in many different tumor types, which is associated with a poor prognosis. Inhibition of these targets can increase activation of the immune system *in vivo*. Considering that FAT10 may play an important role in the development of SKCM, we evaluated the relationship between FAT10 and PD1, PD-L1, or CTLA-4. After purity adjustment, FAT10 in SKCM was significantly positively correlated with PD1, PD-L1, or CTLA-4 (**Figures 7A–C**). Similar to TIMER data analysis, GEPIA data analysis showed that FAT10 had a significant positive correlation with PD1, PD-L1, or CTLA-4 in SKCM (**Figures 7D–F**). These results indicate that the rational application of CTLA-4 and PD-1 or its ligand PD-L1 inhibitors can help restore the anti-tumor immune response, thereby bringing long-term benefits to patients.

Validation of FAT10 Expression

The results illustrated that the protein level of FAT10 was higher in SKCM tissues than in normal skin tissues (**Figures 8A–B**). T-SNE describes the expression profles of FAT10 in the single cells obtained from SKCM tissues. Each point represents a single cell (**Figure 9A**). We used CancerSEA to predict the FAT10-associated functional states in SKCM by scRNA-seq datasets, The result shows that FAT10 expression is negatively associatered with tumor cell invasion and DNA damage (**Figure 9B**).

DISCUSSION

Melanoma is a fatal skin cancer that affects many people around the world every year (Owens, 2014). In addition, due to the high resistance of metastatic melanoma to radiotherapy and chemotherapy drugs, its prognosis is extremely poor (Liu et al., 2012). Elucidating the molecular mechanism and process of SKCM may provide new ideas and insights for the development of effective therapeutic targets or the search for promising prognostic biomarkers. There is increasing evidence that has demonstrated that the ubiquitin-like modifier FAT10 is directly involved in the development of a variety of cancers, including SKCM. Nevertheless, the knowledge of FAT10 in SKCM is still inadequate, and further research is needed.

In this study, we first used the Oncomine database to perform pan-cancer analysis on the expression of FAT10, and then further verified the expression of FAT10 using the TIMER, GEPIA, and Human Protein Atlas database. The results of our FAT10 survival analysis for cancer types of interest show that FAT10 expression was increased in SKCM and correlated with a better survival rate in patients with SKCM. In addition, FAT10 expression is negatively associatered with tumor cell invasion and DNA damage. The research of Canaan *et al.* showed the pro-survival effect of FAT10 (Canaan et al., 2006). This report, together with our analysis, reveals the pro-survival effect of FAT10 in SKCM.

Although ncRNAs cannot encode proteins, they are ubiquitous in organisms (Yamamura et al., 2018). In the last 2 decades, a large number of studies have shown that ncRNA (including miRNA, lncRNA, and circular RNA) communicate with each other through the ceRNA mechanism and play a critical role in the regulation of gene expression (Gao et al., 2020; Ghafouri-Fard et al., 2020; Lou et al., 2020). To explore the upstream regulatory miRNAs of FAT10, seven prediction programs (PITA, RNA22, miRmap, microT, miRanda, PicTar, and TargetScan) were introduced to predict miRNAs that may bind to FAT10. In the end, we obtained seven miRNAs. The specific mechanisms of most of these miRNAs in SKCM were unknown or controversial. Previous studies have shown that hsa-miR-873-5p inhibits the translation of endogenous growth differentiation factor 15 (GDF15) in melanoma cell lines (Teng et al., 2017). However, the role of GDF-15 is still controversial, depending on the tumor entity and model studied (Baek and Eling, 2019). Based on the results of correlation

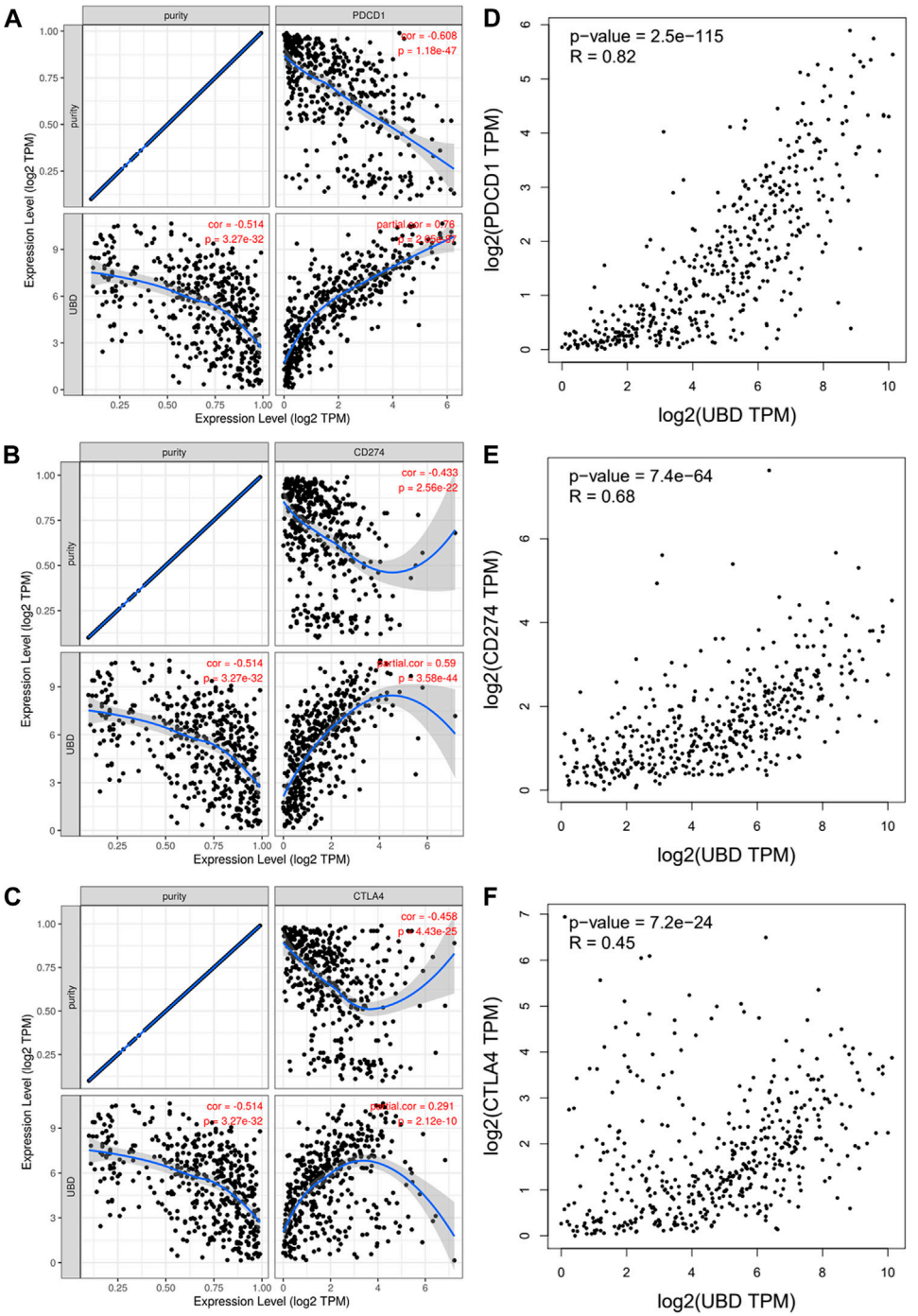

FIGURE 7 | The correlation between FAT10 expression and PD-1, PD-L1 and CTLA-4 expression in SKCM. **(A)** Use TIMER to adjust the spearman correlation between FAT10 and PD-1 expression in SKCM through purity. **(B)** Use TIMER to adjust the spearman correlation between FAT10 and PDL1 expression in SKCM through purity. **(C)** Use TIMER to adjust the spearman correlation between FAT10 and CTLA-4 expression in SKCM through purity. **(D)** The expression correlation of FAT10 and PD1 in SKCM *via* GEPIA analysis. **(E)** The expression correlation of FAT10 and PD-L1 in SKCM via GEPIA analysis. **(F)** The expression correlation of FAT10 and CTLA-4 in SKCM *via* GEPIA analysis.

analysis, expression analysis and survival analysis, hsa-miR-3127-5p was identified as the most potential upstream oncogenic miRNA of FAT10.

According to the ceRNA hypothesis theory (Salmena et al., 2011), the potential lncRNA of hsa-miR-3127-5p/FAT10 axis should be the tumor suppressor lncRNA in SKCM. Then, we

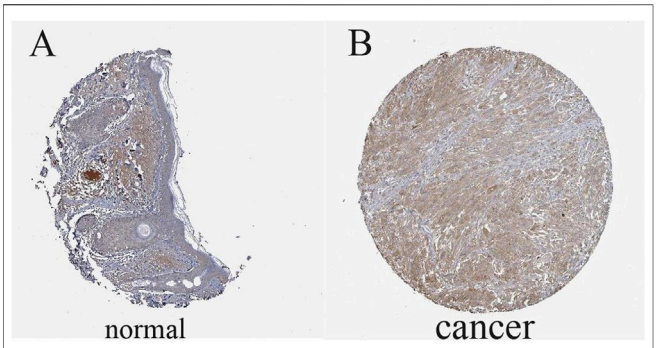

FIGURE 8 | The level of FAT10 in normal skin tissues **(A)** and SKCM tissues **(B)**.

predicted the upstream lncRNA of the hsa-miR-3127-5p/FAT10 axis. A total of 115 possible lncRNAs were discovered. Combining the results of expression analysis, survival analysis and correlation analysis, we finally determined the two most potential down-regulated lncRNAs (HCP5 and N4BP2L2-IT2). The two lncRNAs were involved in multiple immune-related processes, and cell proliferation, apoptosis (Zhou et al., 2021; Zou and Chen, 2021). The high level of HCP5 expression was related to the better survival of SKCM patients, could significantly inhibit the proliferation, colony formation, and invasion of primary SKCM cells and promote cell apoptosis. In contrast, low-level expression of HCP5 was associated with poor survival of SKCM patients (Zou and Chen, 2021). Finally, the HCP5 and N4BP2L2-IT2/hsa-miR-3127-5p/FAT10 axis were identified as potential regulatory pathways in SKCM.

A large number of studies have shown that tumor immune cell infiltration can affect the prognosis of cancer patients and the efficacy of radiotherapy, chemotherapy, or immunotherapy (Waniczek et al., 2017; Zhang et al., 2018; Lyu et al., 2020). To gain further insight into the mechanism of FAT10 in SKCM tumorigenesis, we performed the correlation between *FAT10* gene expression and immune cell infiltration. Our work suggested that the transcription levels of FAT10 was closely correlated with various levels of immune infiltration in SKCM. There is a moderate positive relationship between FAT10 expression level and infiltration level of macrophages, and significantly positive correlations between infiltration level of B cells, $CD8^+$ T cells, $CD4^+$ T cells, neutrophils, and dendritic cells. The *FAT10* gene structurally belongs to the major histocompatibility complex locus, which is composed of multiple genes that play different key roles in immune surveillance against cancer diseases effect (Gruen and Weissman, 1997; Canaan et al., 2006). At the tissue level, high-level expression of human FAT10 was found in lymphoid organs like the thymus, spleen, and lymph nodes(Lee et al., 2003; Lukasiak et al., 2008). FAT10 mRNA is expressed in organs where lymphocytes develop into mature and active, indicating that FAT10 protein may play a key role in lymphocyte maturation (Bates et al., 1997; Canaan et al., 2006). At the same time, in the process of pro-inflammatory immune response in the tumor microenvironment, the expression of FAT10 increases under the action of factors such as the release of pro-inflammatory factors by infiltrating macrophages (Lukasiak et al., 2008). FAT10 may in turn promotes the maturation of more lymphocytes. This may be the reason why the expression level of FAT10 is positively correlated with the infiltration of immune cells. Another important aspect of this study was the significant positive correlation between FAT10 and these biomarkers of infiltrating immune cells. Together, our findings suggested FAT10 might be involved in the regulation of SKCM tumor immunity.

In recent years, people have increasingly realized the role of the immune system in the occurrence and development of cancer, and at the same time, tumor immunotherapy has developed rapidly (Xie et al., 2021). Both sufficient immune cells infiltrate the tumor microenvironment and adequate expression of immune checkpoints were required for tumor immunotherapy to achieve curative effect (Chae et al., 2018). The CTLA-4 and PD-1 immune checkpoint pathways can maintain peripheral tolerance by down-regulating T cell activation, which can be used by tumors to grow and develop rather than being eliminated by the immune system. The use of

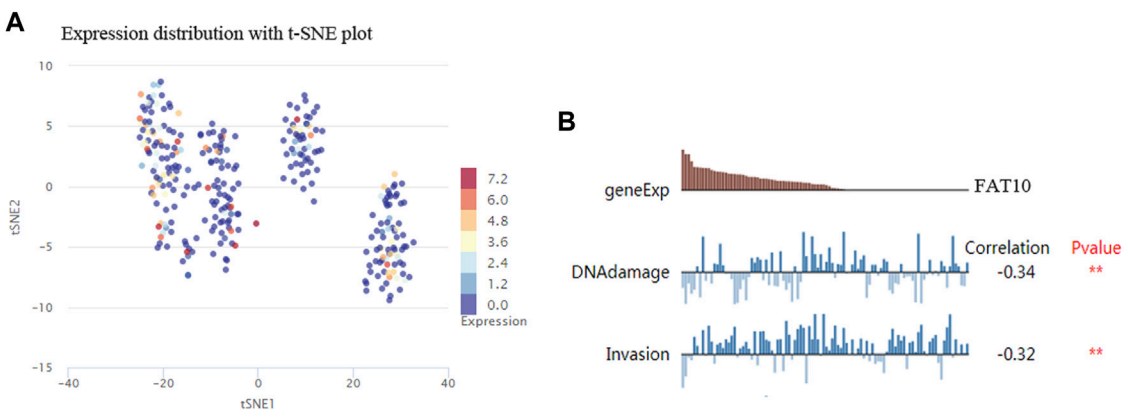

FIGURE 9 | T-SNE describes the expression profile of FAT10 in a single cell obtained from SKCM tissue. Each dot represents a cell **(A)**. The CancerSEA database determines the correlation between FAT10 expression and two functional states **(B)**. $^{**}p < 0.01$.

CTLA-4 and PD-1 or its ligand PD-L1 inhibitor can relieve the immunosuppressive state and restore the anti-tumor immune response. Moreover, combined use of an anti-CTLA-4 immune-checkpoint inhibitor with an anti-PD-1/PD-L1 monoclonal antibody may have a complementary effect(Curran et al., 2010), leading to long-term benefit in a substantial proportion of patients receiving treatment (Buchbinder and Desai, 2016). In addition, when tumor-infiltrating immune cells express PD-L1, patients have the strongest response to anti-PD-L1 blockers (Herbst et al., 2014). To date, three immune checkpoint inhibitors (Ipilimumab, Nivolumab, and Pembrolizumab) have been approved for use in SKCM. The main limitation of the use of these immune checkpoint inhibitors is the low response rate (Burtness et al., 2019). We assessed the relationship between FAT10 and immune checkpoints. The results show that the high expression of FAT10 was closely related to PD1, PD-L1, or CTLA-4 in SKCM, suggesting that patients with high expression of FAT10 were more likely to benefit from immunotherapy in SKCM.

In conclusion, our research indicated that FAT10 was highly expressed in multiple types of human cancer (including SKCM) and found to be related to a better prognosis of SKCM. We constructed a reverse mRNA prediction model to reflect the upstream regulatory mechanism of FAT10 in SKCM, namely HCP5/hsa-miR-3127-5p axis. In addition, our current research results showed that FAT10 increased tumor immune cell infiltration and immune checkpoint expression, which was beneficial to tumor immunotherapy, indicating that FAT10 may play its anti-cancer effect in SKCM. However, more basic experiments and clinical trials are needed to confirm our results.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

HZ designed the study, collected and cleaned the data. YW helped in data collection and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2022.805887/full#supplementary-material

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