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High expression of miR-532-5p, a tumor suppressor, leads to better prognosis in ovarian cancer both *in vivo* and *in vitro*

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Abstract

Ovarian cancer is the leading cause of death for gynaecological cancers, ranking fifth overall for cancer-related death among women. The identification of biomarkers and the elucidation of molecular mechanisms for improving treatment options have received extensive efforts in ovarian cancer research. MicroRNAs (miRNAs) have high potential to act as both ovarian cancer biomarkers and as critical regulators of ovarian tumor behavior. We comprehensively analyzed global messenger RNA (mRNA), miRNA expression, and survival data for ovarian cancer from the Cancer Genome Atlas (TCGA) to pinpoint miRNAs that play critical roles in ovarian cancer survival through their effect on mRNA expression. We performed miRNA overexpression and gene knockdown experiments to confirm mechanisms predicted in our bioinformatics approach. We established that overexpression of miR-532-5p in OVCAR-3 cells resulted in a significant decrease in cell viability over a 96-hour time period. In the TCGA ovarian cancer data set, we found 67 genes whose expression levels were negatively correlated with miR-532-5p expression and correlated with patient survival, such as *WNT9A*, *CSNK2A2*, *CHD4*, and *SH3PXD2A*. The potential miR-532-5p-regulated gene targets were found to be enriched in the Wnt pathway. Overexpression of miR-532-5p through miRNA mimic caused downregulation of *CSNK2A2*, *CHD4*, and *SH3PXD2A* in the OVCAR-3 cell line. We have discovered and validated the tumor-suppressing capabilities of miR-532-5p both *in vivo* through TCGA analysis and *in vitro* through ovarian cancer cell lines. Our work highlights the potential clinical importance of miR-532-5p expression in ovarian cancer patients.

Keywords

microRNA; ovarian cancer; the Cancer Genome Atlas; gene expression; therapeutic targets

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Introduction

Ovarian cancer is the leading cause of death for gynaecological cancers, and ranks fifth overall for cancer-related death among women. [1] Due to the presence of vague, mild symptoms in many ovarian cancer cases, more than half of patients that are diagnosed with ovarian cancer already have *stage 3* or *4* tumors, leading to higher mortality rates. [2] Furthermore, although patients initially respond well to chemotherapy, many ovarian cancer patients relapse and develop chemoresistance. [3, 4] Chemoresistance is believed to be a primary contributor to ovarian cancer death. [4] Overall, the 5-year survival rate for ovarian cancer starting from the time of diagnosis is low at only 45%. [5] In order to improve clinical outcomes in ovarian cancer patients, the elucidation of molecular mechanisms in disease initiation, progression, and prognosis as well as the identification of biomarkers for effective treatment are critical.

MicoRNAs (miRNAs) are 20–25 nucleotide RNA segments that regulate gene expression post-transcriptionally by either cleaving messenger RNA (mRNA) with the RNA-induced silencing complex (RISC) or degrading mRNA with the recruitment of GW- proteins. [6] miRNAs have been shown to regulate numerous cell processes such as apoptosis, migration, stress response, and differentiation. [7] Given the broad regulatory abilities of miRNAs, these non-coding RNA segments play an important role in cancer and are often dysregulated in tumors. [8] Knowledge of key miRNAs that influence ovarian cancer progression can help explicate ovarian cancer tumorigenesis and contribute to the development of improved methods of treating ovarian cancer. For example, one study has found that miR-130a and miR-374a regulate cisplatin-sensitivity in which the overexpression of these miRNAs in ovarian cancer A2780 cells reduced sensitivity to cisplatin while the inhibition of the two miRNAs resensitized cisplatin-resistant A2780 cells. [9] With rapid advancement of miRNA technology, including anti-miRs and miR-mimics, the elucidation of miRNA molecular mechanisms may be leveraged to develop new therapies in the future and improve patient outcomes. [10]

The Cancer Genome Atlas (TCGA) represents one of the largest efforts for the systematic collection of genomic and epigenomic information from large numbers of ovarian cancer patients. The project additionally collected patient survival information, allowing researchers access to prognostic data for ovarian cancer. [11] We comprehensively analyzed global mRNA, miRNA expression, and survival data for ovarian cancer from TCGA to pinpoint miRNAs that play important roles for ovarian cancer prognosis. Additionally, we performed validation experiments *in vitro* with the OVCAR-3 cell line. For this study, we propose and offer confirmation of novel mechanisms between miR-532-5p and cancer-related genes previously unknown in ovarian cancer research.

Materials and Methods

Identify miRNA-gene network that may act in conjunction to lead to variable survival rate in TCGA ovarian cancer patients

We chose to examine both the RNA-Seq (Illumina®) and RNA microarray (Agilent®) expression profiles to eliminate artifacts generated from the different technologies and to

increase our confidence in positive findings. The overall selection pipeline for selecting miRNAs of interest and predicting the regulatory mechanisms of the miRNAs can be found in Figure 1.

miRNA RNA-Seq data for 475 patients and miRNA microarray data for 569 patients as well as overall survival data were first downloaded from TCGA data portal (<https://tcga-data.nci.nih.gov/tcga/> [11]) using TCGA assembler (<http://health.bsd.uchicago.edu/yji/TCGA-Assembler.htm> [12]). We analyzed 270 mature miRNAs after applying a non-zero expression level in $\geq 80\%$ patients filter for 705 mature miRNAs in the RNA-Seq profile. From the microarray data, we analyzed a total of 724 mature miRNAs after removing all viral miRNAs from the microarray profile. Differential miRNA expression was related through linear regression analysis to overall survival in the two miRNA expression profiles independently. A candidate list of 10 miRNAs was obtained by selecting overlapping miRNAs that had miRNA-survival relationships (p -value < 0.05 in both RNA-Seq and microarray analyses). To be noted, microarray data differentiated between the miRNA expression of -5p and -3p miRNA forms, while RNA-Seq data did not differentiate between the two forms. The -5p and -3p forms were counted as distinct miRNAs in our study, but the p -value produced from RNA-Seq data applied to both the -3p and -5p forms in the microarray data.

The candidate list of 10 miRNAs was further narrowed down by testing whether miRNA expression in both microarray and RNA-Seq platforms possessed the same direction of correlation (negative or positive) to overall survival. This narrowed the list of candidates to seven miRNAs. All seven of these remaining miRNAs were associated with a protective phenotype in TCGA ovarian cancer, in which higher expression of the miRNA resulted in improved survival. Finally, hsa-miR-502-5p, hsa-miR-532-3p, and hsa-miR-532-5p were selected from the remaining candidate miRNAs due to the relatively unexplored nature of these miRNAs in ovarian cancer.

Since we suspected that the three miRNAs functioned by downregulating tumor-inducing genes, we analyzed miRNA expression microarray data, global messenger RNA (mRNA) expression profiles, and overall survival in ovarian cancer patients. As with the miRNA expression profiles, we utilized both the RNA-Seq and microarray data sets for mRNA expression to help eliminate artifact-driven results. The mRNA expression profiles for the RNA-Seq and microarray platforms contained 412 and 540 patients in TCGA, respectively. We first analyzed TCGA data to identify tumor-inducing genes that negatively correlated with survival and had $p < 0.05$ to survival for both the RNA-Seq and microarray profiles. We then analyzed the miRNA microarray and mRNA microarray expression data to identify genes whose expression negatively correlated with the expression of the three miRNAs with $p < 0.05$. The genes that had a negative correlation to overall survival and a negative correlation to miRNA expression were placed into miRNA-gene lists for each of the three miRNAs as potential target genes.

Obtaining 72, 57, and 64 genes as potential targets for miR-502-5p, -532-3p, and -532-5p respectively, we narrowed down the genes to the most promising target genes by 1) performing pathway analysis; 2) testing miRNA sequence compatibility; and 3) finding the

biologically relevant genes in TCGA ovarian cancer. The DAVID functional annotation tool was employed for the miRNA-gene relation lists to explore the top enriched pathways associated with the three miRNAs of interest. The miRNA target prediction tools DIANA (<http://diana.imis.athena-innovation.gr/DianaTools/index.php> [13]), TargetScan (<http://www.targetscan.org/> [14]), and ComiR (<http://www.benoslab.pitt.edu/comir/> [15]) were used to determine the compatibility of the miRNA sequences to gene sequences. cBioPortal (<http://www.cbioportal.org/> [16, 17]) was used to collect information about the specific number of amplifications, mutations, and deletions for a particular gene in TCGA ovarian cancer, informing us of biological relevant genes in TCGA ovarian cancer. With the information from pathway analyses, miRNA predicted targets, and cBioPortal statistics, miR-532-5p was selected to be investigated with ovarian cancer cell lines.

Cells and materials

Human ovarian serous cystic adenocarcinoma cell line OVCAR-3 [OVCAR3] (ATCC® HTB-161™) and the cell line SK-OV-3 [SKOV-3; SKOV3] (ATCC® HTB-77™) were obtained from ATCC in August 2015. ATCC performs short tandem repeat profiling, cell morphology monitoring, and cytochrome C oxidase I testing to ensure the correct identity of cell lines. The cells in this study were used within six months of receiving the lines from ATCC.

The cryopreserved cells were thawed according to ATCC procedures. SKOV-3 cells were incubated at 37°C and 5% CO₂ in McCoy's 5a Medium Modified (ATCC cat # 30-2007) supplemented with 15% fetal bovine serum and 1% penicillin/streptomycin. OVCAR-3 cells were incubated at 37°C and 5% CO₂ in RPMI-1640 Medium (ATCC cat # 30-2001) supplemented with 20% fetal bovine serum and 1% penicillin/streptomycin. Cell cultures for the ovarian cancer cell lines were passaged 2–3 times per week.

miRNA mimic for hsa-miR-532-5p was obtained from Qiagen (cat # MSY0002888). miRIDIAN microRNA Mimic Negative Control #1 was obtained from GE Healthcare Dharmacon (cat # CN-001000-01-05). miRNA mimic and negative control scramble were diluted to 20 nM as a master stock, aliquoted, and stored at –20°C. *WNT9A*, *CSNK2A2*, *SH3PXD2*, and *CHD4* primers for quantitative PCR (qPCR) were obtained from Life Technologies as TaqMan® Gene Expression Assays (cat # 4331182). siRNA for *CSNK2A2* and *CHD4* were obtained from Life Technologies as part of the Silencer® Select Class (cat # 4390824).

Effect of miR-532-5p overexpression on cell proliferation

OVCAR-3 and SKOV-3 cells were plated in 96-well plates at 7.5×1000 cells per well in triplicates. After 16 hours, the cells were transfected with 5 nM miR-532-5p mimic, miRNA negative control scramble, or water independently using DharmaFECT Transfection Reagents according to the manufacturer's instructions. DharmaFECT Transfection Reagent 1 (Thermo Fisher Scientific cat # T-2001-03) was used to treat SKOV-3 cells and DharmaFECT Transfection Reagent 3 (cat # T-2003-02) was used to treat OVCAR-3 cells. After incubation for six hours, the transfection media was removed and replaced with 200 µL of complete culture media with fetal bovine solution. To measure cell proliferation,

CellTiter-Glo Luminescent Cell Viability Assay (Promega # G7570) was performed at 0, 24, 48, 72, and 96 hours. Luminescence for the cell viability assays were measured using a BioTEK Synergy HT plate reader (model # SIAFRT). Media was replaced at 48 hours for assays that were measured at 72 and 96 hours.

Effect of miR-532-5p overexpression on selected genes

qPCR was performed to obtain the relative expression of specific messenger mRNAs in the cancer cell lines. Cells were first plated in 24-well plates at 0.25×10^6 cells per well in triplicates. After 16 hours, the cells were transfected with 5 nM miR-532-5p mimic, or miRNA negative control scramble using DharmaFECT Transfection Reagents. After 6 hours, the transfection media was removed and replaced with complete media. Viable cells were harvested at hours 24, 48, 72, and 96 hours for RNA isolation. The Qiagen QIAshredder (cat # 79654) was used to homogenize the cells, and the Qiagen miRNeasy Mini Kit (cat # 217004) was used to extract total RNA. The Applied Biosystems High Capacity cDNA Reverse Transcription Kit (cat # 4368814) for mRNA and miRNA were used to generate complementary cDNA. The ViiATM 7 Real-Time PCR System from Life Technologies was used to run qPCR. The standard curve method with relation to housekeeping gene beta-2-microglobulin (*B2M*) was used.

Effect of silencing the genes CSNK2A2, and CHD4

OVCAR-3 cells were plated in 96-well plates at 7.5×1000 cells per well in triplicates. After 16 hours, the cells were transfected with 25 nM siRNA for either *CSNK2A2* or *CHD4*, Silencer Negative Control No. 1 siRNA (Life Technologies cat # AM4611), or water using DharmaFECT Transfection Reagent 1 according to the manufacturer's instructions. After incubation for six hours, the transfection media was removed and replaced with 200 μ L of complete culture media. To measure cell proliferation, CellTiter-Glo Luminescent Cell Viability Assay (Promega # G7570) was performed at 0, 24, 48, 72, and 96 hours. Luminescence for the cell viability assays were measured using a BioTEK Synergy HT plate reader.

Results

TCGA ovarian cancer analysis identified miR-532-5p and its regulatory gene network as potential prognostic markers

Our first application of TCGA ovarian cancer data was to find clinically relevant miRNAs through independent analysis of survival and miRNA expression data from the RNA-Seq and RNA microarray expression profiles (Fig. 1A). Linear regression analysis using TCGA ovarian cancer data sets identified 28 and 150 miRNAs whose expression levels may be correlated with patient survival ($p < 0.05$) from the RNA-Seq and microarray data sets, respectively. The 10 overlapping miRNAs between the two analyses were hsa-miR-150, hsa-miR-505, hsa-miR-877, hsa-miR-199a, hsa-miR-660, hsa-miR-199b, hsa-miR-502-5p, hsa-miR-146a, hsa-miR-532-3p, and hsa-miR-532-5p. After matching for the same consistent direction between miRNA expression and survival in both two analyses, we narrowed the candidate miRNAs to seven (miR-150, -505, -660, -502-5p, -146a, -532-3p, and -532-5p). Interestingly, all seven of these remaining miRNAs were associated with a

protective phenotype in TCGA ovarian cancer, meaning higher miRNA expression correlated with longer patient survival. Subsequently, we selected to perform functional analyses for miR-502-5p, -532-3p, and -532-5p as these miRNAs were relatively understudied in ovarian cancer.

Since miRNAs are known to negatively regulate gene expression to achieve their biological effects and our three selected miRNAs were associated with a protective phenotype, we reasoned that the selected miRNAs would act as tumor-suppressors by downregulating what we termed tumor-inducing genes in ovarian cancer. To this end, we correlated overall survival with global mRNA profiles. 1419 genes and 2568 genes were identified using the RNA-Seq and microarray data sets, respectively ($p < 0.05$). When limited to only those genes whose expression levels were negatively correlated to survival, the overlap of genes between the two independent analyses produced a list of 250 tumor-inducing genes.

The 2568 survival-related genes from the mRNA microarray profile were then correlated with the microarray expression profiles for miR-502-5p, -532-3p, and -532-5p. 317 genes for miR-502-5p, 293 genes for miR-532-3p, and 389 genes for miR-532-5p were identified as genes whose expression correlated with miRNA expression. Finally these three lists of genes related to miRNA expression were overlapped with the list of 250 tumor-inducing genes. The overlap comparison identified 72 genes for miR-502-5p, 57 genes for miR-532-3p, and 64 genes for miR-532-5p (Fig. 1B). We noticed that there was a heavy overlap of genes among the three lists. For example, the miR-532-3p gene list ($n=57$) and miR-532-5p gene list ($n=64$) had 53 overlapping genes. In total, 47 genes were shared among all three miRNA-gene lists, suggesting that miR-502-5p, -532-3p, and -532-5p may be regulating similar gene networks.

Employing the DAVID annotation tool for pathway analysis, we found 26 pathways that were considered enriched in the gene list for miR-532-5p. The Wnt pathway ($p=0.0098$) was also enriched as miR-532-5p expression correlated with the expression of four genes in the Wnt pathway (*WNT9A*, *CSNK2A2*, *FZD1*, and *SFRP4*). A total of four genes were tested in subsequent *in vitro* validation experiments. Two of these genes (*WNT9A* and *CSNK2A2*) are part of the Wnt pathway. One gene, *SH3PXD2A*, was predicted by three miRNA target prediction tools to be bounded by miR-532-5p. The fourth gene, *CHD4*, shows a high alteration rate in TCGA ovarian cancer samples (12.2%) according to cBioPortal.

miR-532-5p overexpression decreased cell viability in OVCAR-3 cells and decreased proliferation in SKOV-3 cells

miR-532-5p overexpression in OVCAR-3 cells not only led to decreased cell proliferation, but to decreased cell viability overall (Two-way ANOVA test, $p < 0.05$). As shown in Figure 2, OVCAR-3 cell viability decreased significantly over a 96-hour time frame after miR-532-5p mimic treatment (Student's t-test, $p < 0.05$ at all time points measured). The cell viability in the miR-532-5p mimic treated cells decreased to 45.67%, 26.02%, 18.27%, and 13.12% at 24, 48, 72, and 96 hours respectively, when compared to the zero hour population. In comparison, the cell viability of the scramble control cells increased to 106.01%, 117.51%, 160.90%, and 197.29% at the same time points.

In SKOV-3 cells, miR-532-5p mimic treatment also produced tumor-suppressing effects over a 96-hour time period (Two-way ANOVA test, $p < 0.05$). The phenotype produced in SKOV-3 cells treated with miR-532-5p mimic was moderate, but results support the overall tumor-suppressing abilities of miR-532-5p in ovarian cancer.

miR-532-5p overexpression significantly downregulated CSNK2A2, SH3PXD2A, and CHD4 gene expression in OVCAR-3 cells

In OVCAR-3 cells treated with miR-532-5p mimic, *CSNK2A2* expression was significantly downregulated compared to controls at all time points over a 96-hour time frame (Student's t-test, $p < 0.05$, Fig 3A). Compared to scramble controls, miR-532-5p caused a 5.523-fold, 2.345-fold, 2.928-fold, and 1.749-fold decrease in *CSNK2A2* expression at 24, 48, 72, and 96 hours, respectively.

WNT9A expression levels were below the qPCR detection limit in both OVCAR-3 and SKOV-3 cells. Therefore, we were unable to conclude the effect of miR-532-5p overexpression on *WNT9A* in either ovarian cancer cell lines.

miR-532-5p overexpression significantly downregulated *SH3PXD2A* gene expression in OVCAR-3 cells (Fig 3B). *SH3PXD2A* gene expression decreased significantly at 24, 72, and 96 hours by 2.719-fold, 2.289-fold, and 1.704-fold compared to scramble treated cells (Student's t-test, $p < 0.05$).

When overexpressing miR-532-5p in OVCAR-3 cells, we observed a significant and consistent downregulation of *CHD4* as well (Fig 3C). Specifically, miR-532-5p overexpression led to 1.445-fold, 1.368-fold, 1.374-fold, and 1.131-fold decreases in *CHD4* expression at 24, 48, 72, and 96 hours post-transfection, respectively (Student's t-test, $p < 0.05$).

Silencing CSNK2A2 and CHD4 significantly decreased cell proliferation in OVCAR-3 cells

To confirm that downregulation of *CSNK2A2* and *CHD4* indeed produces a phenotypic decrease in cell proliferation, we performed siRNA gene knockdown experiments on *CSNK2A2* and *CHD4* separately. When we knocked down *CSNK2A2* in OVCAR-3, there was significant reduction in cell proliferation that led to decreased cell viabilities at 24, 48, 72, and 96 hours (Two-way ANOVA test, $p < 0.0001$, Fig 4A). When we knocked down *CHD4* in OVCAR-3 cells through siRNA, we observed that *CHD4* also decreased cell proliferation (Two-way ANOVA test, $p = 0.0016$, Fig 4B).

Discussion

The aim of this study was to identify miRNAs that are clinically relevant to ovarian cancer patient survival and to discover the functional roles these miRNAs possess in tumors. To these ends, we analyzed *in vivo* data from TCGA ovarian cancer patients and performed *in vitro* experiments to validate our proposed miRNA mechanisms. We selected miR-532-5p as our primary miRNA of interest after filtering miRNAs through a rigorous selection process. In our TCGA analysis, miR-532-5p conferred a protective phenotype to patients in which higher expression of miR-532-5p predicted longer overall survival in patients. When we

overexpressed miR-532-5p in the OVCAR-3 cell line, a significant decrease in cell viability was observed, supporting the notion that miR-532-5p is a tumor-suppressor in ovarian cancer cells. By further analyzing TCGA data, we identified a set of genes for which miR-532-5p may regulate in ovarian tumors. Indeed, we offer confirmation that overexpression of miR-532-5p downregulates specific genes such as *CSNK2A2*, *CHD4* and *SH3PXD2A*. Furthermore, our data support that the downregulation of *CSNK2A2* and *CHD4* leads to decreased ovarian cancer cell proliferation.

In ovarian cancer, miR-532-5p expression has been found to be downregulated in borderline ovarian neoplasms compared to benign ovarian neoplasms. [18] The function of miR-532-5p, however, appears to be tissue-specific depending on the type of cancer. In a miRNA study of clear cell renal cell carcinoma, miR-532-5p was observed to be downregulated in clear cell renal cell carcinoma tissues when compared to adjacent non-tumorous tissues. [19] For retinoblastoma (RB), a primary pediatric intraocular tumor, miR-532-5p-induced expression in retinoblastoma cell lines decreased cell viability, suggesting that miR-532-5p in retinoblastoma possesses tumor-suppressing abilities. [20] In another study, miR-532-5p has been suggested to downregulate the tumor-suppressing gene *RUNX3* gene in malignant melanoma, implicating that the miRNA acts as a tumor-inducer. [21,22] Additionally, miR-532-5p was observed to be an upregulated miRNA in triple-negative breast cancer tissues. [23] The results from our study indicate that miR-532-5p in ovarian cancer confers a protective phenotype to patients and acts as a tumor-suppressor. The novelty of our research is that we offer confirmation of the tumor-suppressing functionality of miR-532-5p through an analysis of a large collection of ovarian cancer patient samples provided by TCGA and through *in vitro* experiments with ovarian cancer cell lines. We also propose miR-532-5p regulatory gene networks that have not been elucidated in ovarian cancer previously.

The Wnt pathway is a crucial pathway that regulates cell proliferation, cell fate determination, and cell migration. [24] The canonical Wnt pathway controls intracellular levels of β -catenin, a protein independently involved in cell adhesion and co-transcriptional activity for Wnt signal transduction [25,26] Dysregulation of the Wnt pathway has been heavily implicated in multiple human diseases, especially in cancers such as colorectal cancer, [27] chronic lymphocyte leukemia [28], gastric cancer [29], hepatocellular cancer [30], prostate cancer [24, 31], and breast cancer [24,31]. In ovarian cancer, activation of the Wnt pathway plays an important role in ovarian cancer chemoresistance to drugs such as cisplatin. [32] Abnormal activation of the Wnt pathway has also been implicated in epithelial ovarian cancer to promote epithelial-to-mesenchymal transition, which drastically increases tumor aggressiveness. [33] Upregulation of another miRNA, miR-1207, has been shown to suppress *SFRP4*, a negative regulator of the Wnt pathway. The overexpression of miR-1207 consequently increases tumorigenicity of ovarian cancer cells by the activation of the Wnt pathway. [34] From the DAVID annotation tool in our TCGA analysis, the Wnt pathway was the second strongest associated pathway for the list of genes correlated with miR-532-5p expression. With support from our TCGA analysis and our findings regarding the gene *CSNK2A2*, a member of the Wnt pathway, we strongly suspect that miR-532-5p is involved in Wnt pathway activation in ovarian tumors.

CSNK2A2, also known as *CK2*, is considered a master regulatory gene for its role in regulating protein behavior, which is performed mostly through the phosphorylation of target proteins. [25] *CSNK2A2* acts as a multisite regulator of the Wnt pathway, and is best-known for phosphorylating the transcriptional co-factor β -catenin. [25] Increased *CSNK2A2* expression has been observed in breast cancer tissue and breast cancer cell lines [25,35] Additionally, *CSNK2A2* upregulation has been associated with colon cancer progression. [36,37] Increased expression of *CSNK2A2* has also been observed in villous colon adenomas when compared to normal or tubular colon adenomas. [37] In addition to the Wnt pathway, *CSNK2A2* is believed to regulate cell behavior through other functions, including NF- κ B activation. NF- κ B is a transcriptional regulator involved in apoptotic resistance and the promotion of proliferation. [25] In our current study, we found that *CSNK2A2* expression was strongly negatively correlated with miR-532-5p expression in TCGA ovarian cancer. When we overexpressed miR-532-5p in OVCAR-3 cells, *CSNK2A2* expression was dramatically downregulated over the entire 96-hour time period. This indicates that miR-532-5p negatively regulates a master regulatory gene that controls Wnt pathway activation among other cell functions. In addition, knockdown experiments for *CSNK2A2* confirmed that *CSNK2A2* is essential for OVCAR-3 cell proliferation, which demonstrates that regulation of *CSNK2A2* is a critical functional mechanism for miR-532-5p.

The *WNT9A* gene encodes for a secreted signaling protein that is involved in the canonical Wnt pathway. *WNT9A* has been found to be overexpressed in multiples cancers such as chronic lymphocytic leukemia [38], colon [39–41], gastric [39,40], mammary [42], and cervical carcinomas [42]. However, *WNT9A* also has been suggested to suppress cellular proliferation in breast cancer for one study [43]. In ovarian cancer, it has been observed that *WNT9A* expression was unchanged between biopsies between normal ovarian tissue and malignant tissue. [44] Our TCGA analysis indicated that *WNT9A* was among the top candidates for miR-532-5p targeting because of its strong correlation in gene expression to miR-532-5p expression. Additionally, *WNT9A* was classified by us as a highly biologically-relevant gene to TCGA ovarian cancer due to the high number of gene alterations in patients. Interestingly, we could not reliably measure *WNT9A* expression with conventional qPCR procedures because the gene's endogenous expression was extremely low in the cells we tested. Despite our inconclusive results, *WNT9A* may still interact with miR-532-5p, but the consequences of this regulation remain unclear.

SH3PXD2A (also known as Tps5) plays an important role in underlying networks of adhesion, signaling, and scaffolding in cell systems. [45] In particular, *SH3PXD2A* has been discovered as instrumental for the formation of invadopodia, actin-based protrusions of the plasma membrane of metazoan cells. Invadopodia increase the metastatic potential of carcinomas by acting as sites for extracellular degradation, promoting epithelial-to-mesenchymal transition. [45–48] Invadopodia is increasingly recognized as one of the major avenues of how cancers metastasize. [49] The prognostic relevance of *SH3PXD2A* expression has been suggested in gliomas where *SH3PXD2A* expression was found to be predictive of poorer clinical outcomes in glioma patients, but most clearly for low-grade gliomas. [50] Among the genes we checked with miRNA target prediction tools (DIANA, TargetScan, ComiR), *SH3PXD2A* received the highest scores for compatibility with the sequence of miR-532-5p. We found from overexpressing miR-532-5p in OVCAR-3 cells

that miR-532-5p indeed downregulates *SH3PXD2A* expression for the most part. To be noted, we observed that many genes correlated with miR-532-5p expression in ovarian cancer were involved in cell adhesion and the extracellular matrix. The confirmation that miR-532-5p regulates *SH3PXD2A* expression, which plays a critical role in invadopodia and extracellular degradation, supports the argument that miR-532-5p may be an important regulator of cell adhesion and the extracellular matrix for ovarian tumor cells.

The gene *CHD4* acts as a vital component of the nucleosome remodeling and deacetylase (NuRD) complex, an important epigenetic regulatory network. *CHD4* is suspected to play a significant epigenetic role in carcinogenesis by controlling the transcription of genes involved in differentiation [51–54]. In a study involving glioblastoma tumor initiating cells, suppression of *CHD4* led to pro-differentiation effects such as inhibition of neurosphere formation, and decreased expression of genes such as *SOX2* and *EGFR*. [51] In serous endometrial tumors, *CHD4* was discovered to be frequently possess somatic mutations in *CHD4* (17%). [54] Another ovarian cancer study has found that *CHD4* inactivation in *BCRA2* mutant cancers confers chemoresistance, and that reduced *CHD4* expression in *BCRA2* mutant ovarian cancers correlates with poorer overall survival. [55] *CHD4* was found to be strongly, negatively correlated with miR-532-5p expression in the TCGA ovarian cancer data set and was observed to have a negative correlation with overall survival in patients. Additionally, *CHD4* possessed a high gene alteration rate among TCGA ovarian cancer patients when we queried the tool cBioPortal. We found that *CHD4* is consistently downregulated in miR-532-5p overexpressed OVCAR-3 cells. The results of our *CHD4* knockdown in OVCAR-3 cells indicate that *CHD4* downregulation suppresses ovarian cancer cell proliferation. Taken together, we confirm in this study that miR-532-5p acts through *CHD4* and *CSNK2A2* to regulate cell proliferation in OVCAR-3 cells, but more research is required to better understand the tumor-suppressing role of miR-532-5p in ovarian cancer.

One of the surprising aspects of our experimentation was the discrepancy of our results between the OVCAR-3 cell line and SKOV-3 cell lines. While we found that miR-532-5p overexpression reduced cell viability as a whole in OVCAR-3 cells, we observed only a modest decrease in cell proliferation in SKOV-3 cells with the same procedure. We decided to rely on the OVCAR-3 cell line more than the SKOV-3 cell line due to recent research that has cast doubt on the utilization of SKOV-3 as a model system for ovarian serous carcinoma. It is important to note that TCGA collected only serous cystadenocarcinoma samples for ovarian cancer. Two papers have found that the SKOV-3 cell line fares poorly in resembling high-grade ovarian serous carcinomas in terms of molecular biomarkers. [56,57] Another study has observed that SKOV-3 xenografts produce tumors that resemble more the clear cell subtype than serous subtype for ovarian cancer. [58] For these reasons, we believed that the OVCAR-3 cell line was a better model system that was more representative of the clinical population studied in TCGA.

In summary, we have discovered that miR-532-5p acts as a significant tumor-suppressor in ovarian cancer and that miR-532-5p expression is connected to ovarian cancer patient survival outcomes. Additionally, we have found that overexpression miR-532-5p downregulates *CSNK2A2*, *SH3PXD2A*, and *CHD4*, possibly regulating the Wnt pathway as

well. The validation of the tumor-suppressing capabilities of miR-532-5p confirms that our integrative approach of utilizing *in vivo* data through TCGA to inform our *in vitro* experiments can yield important insights for ovarian cancer. With this approach, we propose novel mechanisms between miRNAs and cancer-related genes that have not been elucidated previously. The immediate application of our findings is that miR-532-5p may be used as prognostic tools in ovarian cancer, which has already been proposed for miR-532-5p in colorectal cancer [59]. The better outcome is that our research with miR-532-5p in ovarian cancer will stimulate more extensive investigations into the molecular mechanism of miR-532-5p and be leveraged for the development of improved clinical therapies for ovarian cancer.

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Figure 1. Selection pipeline for miRNAs of interest and finding miRNA-gene pairs in TCGA ovarian cancer dataset

A) Selection pipeline and specific criteria to narrow down the list of possible mature miRNAs to three miRNAs of interest. Expression data for miRNA and overall survival data (oval shapes) were obtained from TCGA ovarian cancer. Intermediate lists (trapezoid shapes) and findings (diamond shapes and outlined boxes) derived from our analysis of TCGA data sets. B) Selection pipeline and specific criteria to find possible miRNA-gene candidate pairs. Survival, mRNA, and miRNA expression data (oval shapes) were obtained from TCGA. Intermediate lists (trapezoid shapes) and findings (diamond shapes and outlined boxes) derived from our analysis of TCGA data sets. Linear regression analysis was employed between the gene expression data and overall survival data, as well as between the gene expression data and miRNA expression data.

Figure 2. Effects of miR-532-5p mimic treatment on cell viability in OVCAR-3 cells

The solid line represents the percent cell viability of the scramble control as compared to the zero hour cell viability. The dashed line represents the percent cell viability of the miR-532-5p mimic treated cells. Results were obtained through cell viability assays at 0, 24, 48, 72, and 96 hours post-transfection. $P < 0.0001$ is the result of a two-way ANOVA test between the scramble and miR-532-5p mimic experiments.

Figure 3. Fold changes in relative *CSNK2A2*, *SH3PXD2A*, and *CHD4* expression after miR-532-5p mimic treatment on OVCAR-3 cells

A) The results of *CSNK2A2* gene expression in the scramble control cells (open bars) against miR-532-5p mimic treated cells (shaded bars). B) The results of *SH3PXD2A* gene expression in the scramble control cells (open bars) against miR-532-5p mimic treated cells (shaded bars). C) The results of *CHD4* gene expression in the scramble control cells (open bars) against miR-532-5p mimic treated cells (shaded bars). All results were obtained at 24, 48, 72, and 96 hours post-transfection through qPCR. Expression is normalized to *B2M*. The symbol * signifies a p-value < 0.05 from the Student's t-test.

Figure 4. Effects of gene knockdown experiments on cell viability in OVCAR-3 cells

A) The solid line represents the percent cell viability of the scramble control treated cells as compared to the zero hour cell viability. The dashed line represents the percent cell viability of the *CSNK2A2* siRNA knockdown cells. $P < 0.0001$ is the result of a two-way ANOVA test between the scramble and siRNA knockdown experiments. B) The solid line represents the scramble control. The dashed line represents the percent cell viability of the *CHD4* siRNA knockdown cells. $P = 0.0016$ is the result of a two-way ANOVA test between the scramble and siRNA knockdown experiments. All results were obtained through cell viability assays at 0, 24, 48, 72, and 96 hours post-transfection.