ABSTRACT. Colorectal cancer is the third most common cancer in the United States, and the second leading cause of cancer-related deaths. As malignant cells adopt features of epithelial-mesenchymal-transition (EMT) their metastatic potential is increased. POPDC3 is a member of the POPDC family of transmembrane proteins. BVES, or POPDC1, has been shown to play an important role in regulating EMT. However, there have been no studies focusing on the related protein, POPDC3, in colorectal cancer. The goal of this study was to determine the role of POPDC3 in colorectal cancer. POPDC3 messenger RNA levels were downregulated in 153 human colorectal tumor samples as compared to the non-malignant control samples. RNA interference mediated POPDC3 gene silencing in the colorectal cancer cell line HCT116 resulted in pronounced differences in morphological phenotypes, suggesting a more mesenchymal state. These changes suggested that the loss of POPDC3 may further induce EMT in colorectal cancer cells. POPDC3 knockdown also caused a shift in the β-catenin localization from the membrane to the cytoplasm and nucleus, suggesting activation of the Wnt signaling pathway, which is an influential element in colorectal cancer development. These data suggest that POPDC3 could play a critical role in regulating EMT in colorectal cancer.

INTRODUCTION. Colorectal cancer (CRC) is the third most common cancer diagnosed in the United States, with approximately 1 in 20 adults being diagnosed with CRC in their lifetime [1]. Additionally, CRC is the second leading cause of cancer-related deaths, expected to cause 49,190 deaths in 2016 [2,3]. Early diagnosis and treatment is vital because CRC incidence rates remain high with an estimated 134,430 new cases and 49,190 related deaths, expected to cause 49,190 deaths in 2016 [2,3]. Therefore, it is vital to understand the molecular processes driving colorectal cancer pathogenesis in order to identify novel diagnostic assays and therapeutic strategies.

Late-stage colorectal tumor cells exhibit mesenchymal phenotypes, characterized by a loss of controlled cell motility, proliferation, and differentiation [7,8]. The process in which normal epithelial cells become mesenchymal cells is called epithelial-to-mesenchymal-transition (EMT). EMT is driven by a loss of cell junction molecules, such as those found at adherens and tight junctions [7]. Previous studies suggest that BVES (Blood Vessel Epicardial Substance), a member of the POPDC (Poppy Domain Containing) protein family, acts as a tumor suppressor in CRC [7,9] by regulating formation of tight and adherens-junctions.

The POPDC gene family consists of three novel protein-coding genes, of which BVES, also known as POPDC1, is the most studied [10]. BVES was discovered in 1999 as a highly expressed protein in the hearts of chicken embryos [11]. It has a unique three-pass transmembrane structure that is highly conserved among vertebrates, suggesting that the protein plays an integral role in cells [10]. BVES is known to regulate cell-to-cell adhesion by acting as a coordinator of tight junctions and adherens junctions, which connect adjacent cell membranes, and previous studies have shown that BVES expression is reduced in colon tumor cells compared to non-tumorous colon cells [7]. Restoring BVES expression in cancer cells attenuates CRC tumor growth and metastasis in mice [7], implicating BVES role in CRC pathogenesis. Taken together, these data are consistent with BVES acting as a tumor suppressor.

Previous studies demonstrate that another member of the POPDC family, the POPDC2 gene, plays a crucial role in heart and muscle development and differentiation [12,13]. However, POPDC3, the final member of the POPDC family, remains poorly characterized, especially concerning its role in colorectal cancer [10,14]. POPDC3 is 50% identical to POPDC2, and 25% conserved with BVES [10], suggesting POPDC3 may also play an important role in development and cancer pathogenesis. Reduced POPDC3 expression correlates to poor survival in gastric cancer patients [15], suggesting that POPDC3 may function in CRC development. To date, the role of POPDC3 in colorectal cancer has not been studied. Therefore, the objective of this study is to decipher the role of POPDC3 in colorectal cancer. We hypothesize that POPDC3 acts as an EMT regulator and has reduced expression in colorectal tumor cells compared to normal cells. To test this hypothesis, we compared the POPDC3 expression levels in nonmalignant and colorectal cancer cell lines. Additionally, we used RNA interference method to further reduce POPDC3 expression in cell lines in order to test for subsequent morphological and molecular differences. These differences can shed light onto the role of POPDC3 in CRC.

MATERIALS AND METHODS. TCGA Data Analysis.

To examine the mRNA expression of POPDC3 in colon cancer tissue as compared to normal tissue, data were obtained from The Cancer Genome Atlas (TCGA) Research Network on the website: http://cancergenome.nih.gov/. This data contained the mRNA expression levels of several genes in 153 patients in tumor/normal-matched samples, which means that the tumor sample expression is already calculated relative to the normal sample. Tumor samples were of adenocarcinoma tissue and normal samples were from a normal pool. From each patient, the mRNA expression levels of POPDC3 were graphed using GraphPad Prism.

Cell Lines.

In order to find out which cell lines to use for the POPDC3 knockdown experiments, seven human colorectal cancer cell lines (Caco-2, HCT116, SW480, HCA7, RKO, DLD1, and HT29), one non-malignant human embryonic kidney cell line (HEK293T), and one non-malignant human breast cell line (MCF10A) were used to test for their POPDC3 mRNA levels. All the cells used in this study were procured from American Type Culture Collection (ATCC, Manassas, VA) and were cultured in ATCC-recommended media.

RNA Expression Analysis.

RNA was isolated from the following cell lines: Caco-2, HCT116, SW480, HCA7, RKO, DLD1, HT29, MCF10A, and HEK293T. RNA was isolated using Zymo Trizol kit. The RT-qPCR was performed using BVES and POPDC3 TaqMan probes. Relative expression was normalized to GAPDH, calculated using the comparative Ct method (2−ΔΔCt), and normalized to MCF10A, the adult human cell line.

POPDC3 Knockdown.

POPDC3 was knocked down in the HCT116 cell line using POPDC3 siRNA. The siRNA was purchased from Santa Cruz Biotechnology. The control samples had non-target scrambled siRNA. Cells were plated to the same confluence. To test if the knockdown was successful, RT-qPCR was performed. Relative expression was normalized to GAPDH, calculated using 2−ΔΔCt, and normalized to the control.
**Microscopy.**

Pictures of the HCT116 cells were taken using a phase-contrast light microscope approximately 72 hours after the gene knockdown using siRNA in order to examine changes in cell morphology.

**Immunofluorescence.**

The colorectal cancer HCT166 cells were plated on a cover slip and incubated with formaldehyde and ZO-1 and β-catenin antibodies were used to check for localization of these proteins. DAPI was used to stain nuclei. A fluorescent microscope was used to take the immunofluorescence photographs.

**Statistical Analysis.**

The results are expressed as the mean ± standard error of the mean. Student’s t-test was used for the knockdown, and p-values less than 0.05 are considered to be statistically significant.

**RESULTS.**

**Colon Cancer Tissue Samples and Cell Lines Demonstrate Reduced POPDC3 Expression.**

Data from the TCGA was analyzed for POPDC3 mRNA expression in 153 adenocarcinoma tumor samples as compared to the non-malignant matched samples. In all 153 patients, the POPDC3 mRNA levels were lower in the tumor samples than the normal samples (Figure 1A). The tumor samples had an average of 2.98 (±0.08) fold lower POPDC3 mRNA level than the normal controls.

After determining that POPDC3 was underexpressed in human samples from TCGA, several cultured cell lines were used to compare the POPDC3 mRNA levels in malignant and non-malignant cells. For a reference point, the non-malignant human breast cell line MCF10A was used. The remaining seven cell lines were colorectal cancer cell lines (Caco-2, HCT116, SW480, HCA7, RKO, DLD1, and HT29) as well as the one noncancerous human embryonic kidney cell line HEK293T. All the colorectal cancer cell lines tested had low POPDC3 mRNA levels compared to the control cell line, MCF10A (Figure 1B).

**POPDC3 Knockdown Induces Mesenchymal Morphology.**

Based on the mRNA expression data, the HCT 116 cell line was chosen for the POPDC3 knockdown experiment. HCT 116 cells are well characterized in literature and easy to manipulate in laboratory conditions. We used scrambled non-target siRNA as a control and pooled siRNA for POPDC3 knockdown. POPDC3 mRNA levels were significantly decreased in the knockdown as compared to the control (Figure 2A).

The POPDC3 knockdown cells were examined by phase-contrast light microscopy and found to be morphologically distinct from the control (scrambled non-target) cells. Specifically the POPDC3 knockdown cells had a spindle-like morphology, resembling the appearance of mesenchymal cells (Figure 2B). These data suggest that POPDC3 may play a role in regulating cell morphology.

**β-catenin Mislocalization after POPDC3 Knockdown in HCT116.**

In order to find the localization of ZO-1, an epithelial-phenotype related protein, and β-catenin, a mesenchymal-phenotype related protein, immunofluorescence was used. The POPDC3 knockdown caused a change in the localization of ZO-1 and β-catenin from the cell membrane towards the cell cytoplasm and nucleus (Figure 3).
Studying the molecular mechanisms driving colorectal cancer is essential to develop new drugs and techniques to combat the disease. Due to a correlation between reduced POPDC3 expression and poor gastric cancer patient outcome, we hypothesized that POPDC3 could also have a role in CRC development. POPDC3 has not been characterized in CRC development, therefore examining the gene function may allow for an enhanced understanding of colorectal cancer. This study found that the POPDC3 mRNA is reduced in colorectal cancer samples compared to noncancerous samples in clinical samples, indicating a correlation between colorectal cancer and POPDC3 mRNA levels (Figure 1A). No past research has examined POPDC3 expression in colorectal cancer clinical samples.

This study also reports that in seven specific colon cancer cell lines, POPDC3 expression was reduced in comparison to a nonmalignant cell line (Figure 1B). A previous research study also examined the POPDC3 expression in six of the seven colorectal cancer cell lines, with the exception being the RKO cell line [7]. These findings support this study’s results, with the colorectal cancer cell lines having a marked decrease of POPDC3 and Bves expression.

No known studies have determined the effect of knocking down POPDC3 expression in the colorectal cancer cell line HCT116, making this the first study to do so.

The knockdown of POPDC3 in the colorectal cancer cell line HCT116 caused a pronounced difference in cell morphology (Figure 2). There was a more spindle-like morphology in POPDC3 deficient cells. The physical characteristics of these cells resembled more of a mesenchymal cell, suggesting that knocking down POPDC3 could be causing an increased change from the epithelial phenotype to a mesenchymal phenotype. This indicates that POPDC3 could be playing a significant role in EMT regulation in colorectal cancer.

The knockdown of POPDC3 also caused a shift in the localization of the β-catenin protein from the cell membrane to the cytoplasm and specifically to the nucleus (Figure 3). This change in localization from the cell membrane to nucleus is indicative of an increase in the activation of the Wnt signaling pathway [16]. An activation of the Wnt signaling pathway has previously been associated with cancer growth, including colorectal cancer growth [17]. Therefore, POPDC3 could be playing a key role in suppressing the activation of the Wnt signaling pathway, a known inducer of tumorigenesis.

Overall, this study concluded that POPDC3 may be an essential gene in suppressing the development of colorectal cancer. However, this study is not without limitations. It is suggested that the POPDC3 gene could be regulating the process of EMT in the HCT116 cell line, but a future test to confirm this correlation is necessary. One example of such test would be to include the protein vimentin as a marker in the immunofluorescence experiment. Vimentin is a mesenchymal cell marker, and it can be used as further evidence that a knockdown of POPDC3 induces EMT [18]. Testing the cell migration and invasion, which are some of the characteristics of EMT, could also verify the POPDC3 role in colorectal cancer. In addition, future studies could expand on the colorectal cancer cell lines tested to ensure that they all follow this pattern. With the limited time frame of this study, only the effects of underexpressing POPDC3 could be tested. However, a future study could examine the effects of the overexpression of POPDC3, which may reveal more information about this gene. POPDC3 could also be an essential gene for suppressing the activation of the Wnt signaling pathway in the HCT116 cell line, but a Wnt signaling pathway assay, such as a TOP-flash assay, should be performed to confirm these results. If the hypothesis is correct, this test would show that knocking down POPDC3 activates or increases WNT signaling. An analysis of POPDC3 expression in the precancerous colon polyps would provide additional insight into when POPDC3 plays a role in CRC pathogenesis.

CONCLUSION.

The POPDC3 gene has been one of the least studied members of the POPDC gene family. However, this research and previous studies suggest that it may also play an essential role in cancer development. In colorectal cancer, POPDC3 gene expression is underepressed. Knocking down the POPDC3 gene appears to induce EMT in malignant cell lines e.g. HCT 116 and nonmalignant cell lines, e.g. HEK 293T (data not shown). In addition, the POPDC3 gene could be associated with the Wnt signaling pathway, which has been known to influence cancer growth. All of these results suggest that the POPDC3 gene plays a significant role in colorectal cancer development. This understanding of the relationship between genes and malignant cancer can improve our insight of how to effectively treat and diagnose colorectal cancer.

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REFERENCES.


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