Dysregulation of WNT5A/ROR2 Signaling Charactezes the Progression of Barrett-Associated Esophageal Adenocarcinoma

Orestis Lyros1,2, Linghui Nie3, Tami Moore3, Rituparna Medda3, Mary Otterson3, Behnaz Behmaram4, Alexander Mackinnon4, Ines Gockel2, Reza Shaker1, and Parvaneh Rafiee3

Abstract

The mechanism underlying the progression of normal esophageal mucosa to esophageal adenocarcinoma remains elusive. WNT5A is a noncanonical WNT, which mainly functions via the receptor tyrosine kinase-like orphan receptor 2 (ROR2), and has an unclear role in carcinogenesis. In this study, we aimed to determine the role of WNT5A/ROR2 signaling in esophageal adenocarcinoma. Analysis of WNT5A and ROR2 expression patterns in healthy controls, Barrett and esophageal adenocarcinoma patients’ esophageal clinical specimens as well as in various esophageal cell lines demonstrated a ROR2 overexpression in esophageal adenocarcinoma tissues compared with Barrett and healthy mucosa, whereas WNT5A expression was found significantly downregulated toward esophageal adenocarcinoma formation. Treatment of esophageal adenocarcinoma OE33 cells with human recombinant WNT5A (rhWNT5A) significantly suppressed proliferation, survival, and migration in a dose-dependent fashion. rhWNT5A was found to inhibit TOPflash activity in ROR2 wild-type cells, whereas increased TOPflash activity in ROR2-knockdown OE33 cells. In addition, ROR2 knockdown alone abolished cell proliferation and weakened the migration properties of OE33 cells. These findings support an early dysregulation of the noncanonical WNT5A/ROR2 pathway in the pathogenesis of esophageal adenocarcinoma, with the loss of WNT5A expression together with the ROR2 overexpression to be consistent with tumor promotion.

Implications: The dysregulation of WNT5A/ROR2 noncanonical WNT signaling in Barrett-associated esophageal adenocarcinoma introduces possible prognostic markers and novel targets for tailored therapy of this malignancy. Mol Cancer Res. 14(7); 647–59. ©2016 AACR.

Introduction

Esophageal adenocarcinoma is noted by having both the fastest growing incidence of all cancers in the western world over the last several decades (1) and a high mortality rate that exceeds 80% (2). Despite improvements in chemotherapy regimens and the use of multimodal strategies (e.g., surgery, neoadjuvant therapy, and adjuvant therapy), esophageal adenocarcinoma remains a therapeutic challenge for oncologists (2) due to high rates of local recurrence and early regional and systemic metastases. Esophageal adenocarcinoma is often studied in conjunction with Barrett esophagus, the condition in which the normal squamous epithelium in the distal esophagus is replaced by a metaplastic columnar mucosa in response to chronic severe gastroesophageal reflux disease (GERD; ref. 3). The course of Barrett esophagus is mainly benign; however, this condition is the main predisposing factor for esophageal adenocarcinoma and is associated with an increased risk of progression to cancer (4). The molecular mechanisms underlying the development and the aggressive phenotype of esophageal adenocarcinoma remain largely undefined.

The embryologic WNT signaling pathway is important for development and tissue homeostasis, and its dysregulation has been associated with tumorigenesis (5). It has been established that WNT proteins regulate at least three distinct intracellular signaling pathways: β-catenin–dependent (canonical) and -independent (noncanonical) signaling pathways, the planar cell polarity/convergent extension pathway, and the Ca2+ pathway (summarized in ref. 6). The canonical WNT pathway regulates the cell cycle, growth, and proliferation (5), whereas the noncanonical WNT signaling is purported to have crucial functions in the regulation of cell migration and polarity (6). Cross-talk between canonical and noncanonical WNT pathways still remains elusive, but inhibitory interaction of the canonical by the noncanonical has been shown (7, 8).

Aberrant activation of the canonical WNT/β-catenin signaling has been associated with the development of esophageal adenocarcinoma (9). However, genetic alterations in components of the canonical WNT pathway such as β-catenin, APC, and Axin, which are observed in other human cancers and result in upregulated canonical signaling, are not frequently detected.
in esophageal adenocarcinoma (10, 11). Instead, esophageal adenocarcinoma is marked by changes, such as loss of the negative regulators of the pathway, Wnt inhibitory factor 1, and secreted frizzled receptor proteins, as well as induction of Wnt-2 expression, which are expected to increase signaling along the WNT axis (9, 12, 13). Thus, the mechanism of canonical WNT activation in esophageal adenocarcinoma is still unknown, whereas the involvement of the noncanonical WNT pathway has not been addressed yet.

WNT5A is classified as a nontransforming WNT family member that activates noncanonical WNT signaling by interacting with receptor tyrosine kinase–like orphan receptors (ROR; ref. 14). ROR2 is a member of the ROR family of receptor tyrosine kinases, acts as a receptor or coreceptor for WNT5A, and has shown to have essential functions in developmental morphogenesis (15). WNT5A/ROR2 signaling has been shown to inhibit the canonical WNT signaling, which is often activated in tumor cells, indicating that WNT5A might act as a tumor suppressor (16). Indeed, loss or low expression of WNT5A is associated with increased invasion and aggressiveness of many carcinomas (17, 18). Nevertheless, WNT5A has also demonstrated oncogenic properties based on findings depicting upregulation of WNT5A levels in melanoma, lung, and breast cancers (19–21). Although, the exact function of WNT5A is highly dependent on the receptor context, the ROR2 receptor on the other hand, appears to demonstrate oncogenic properties regardless the presence of ligand (22). Thus, the role of WNT5A/ROR2 signaling in carcinogenesis remains still ambiguous.

The current study was undertaken to explore the role of the noncanonical WNT5A/ROR2 signaling pathway in esophageal adenocarcinoma with respect to the canonical WNT/β-catenin signaling activation. We hypothesized that alteration in the WNT5A/ROR2 signaling pathway may underlie the aberrant activation of β-catenin transcriptional activity in esophageal adenocarcinoma cells. By analyzing human esophageal specimens and esophageal cell lines, we present evidence suggesting that the progression of esophageal adenocarcinoma is characterized by deregulation of the noncanonical WNT5A/ROR2 signaling pathway. The loss of WNT5A expression along with upregulation of ROR2 receptor appears to contribute to an aggressive phenotype of the esophageal adenocarcinoma cells. Our findings underscore the significance of the WNT5A/ROR2 signaling pathway in esophageal adenocarcinoma and provide rationale for the development of future target therapies along with new insights into the mechanisms of noncanonical WNT signaling in the context of tumor progression.

### Materials and Methods

#### Reagents and antibodies

Recombinant human WNT5A (rhWNT5A) was obtained from R&D Systems Inc. Antibodies against ROR2 and WNT5A were obtained from Abcam; active β-catenin antibody (ABC; unphosphorylated in Ser37 and Thr41 sites) was from Millipore, total β-catenin and phospho-GSK3β (Ser9) antibodies were obtained from Cell Signaling Technology. GSK3β and horseradish peroxidase secondary antibodies were obtained from Santa Cruz Biotechnology. β-Actin antibody, DNA demethylation drug: 5-azacytidine (5-Aza-CR), protease inhibitor cocktail, and all other reagents and chemicals were obtained from Sigma unless otherwise specified.

#### Human esophageal specimens

Archival paraffinized human esophageal specimens of esophageal adenocarcinoma patients (n = 11) who underwent esophagectomy in the Department of Thoracic Surgery at Froedtert Hospital and Medical College of Wisconsin from 2010 to 2012 were retrospectively analyzed (Table 1). Laser microdissection was undertaken to isolate RNA from tumor cells from paraffin blocks (purification up to 70%–80% was achieved). In addition, human esophageal biopsy specimens were collected from patients 18 years or older undergoing endoscopy for Barrett esophagus (n = 15) and healthy individuals (n = 10) undergoing endoscopy for nonesophageal indications (Table 2). Studies were approved by the Human Research Review Committee of the Medical College of Wisconsin, and study participants gave written informed consent prior to their studies.

#### Cell lines

Human squamous esophageal telomerase-immortalized cells, EPC1 (EPC1-hTERT) and EPC2 (EPC2-hTERT) with intact p53 and p16 genes (23) were the generous gifts of Dr. Hiroshi Nakagawa (Gastroenterology Division, University of Pennsylvania, Philadelphia, PA). CP-A cells are a nondysplastic columnar cell line derived from Barrett esophagus that was obtained from the ATCC. OE33 cells are poorly differentiated esophageal adenocarcinoma cancer cells (Sigma). Cells have been recently tested and authenticated by immunofluorescence staining for cytokeratin expression and were grown according to a standard protocol as described previously (24).

#### Cellular assays

Cell proliferation was assessed either by [3H] thymidine uptake, as described previously (25), or by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as

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### Table 1. Demographic and histologic findings in esophageal adenocarcinoma patients

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suggested by the manufacturer (Roche Diagnostics). Migration of OE33 cancer cells was assayed with Wound Scratch Assay and cell transmigration by using 24-well HTS FluoroBlok inserts in triplets (8-μm pore size; Becton Dickinson). Later, cells were serum starved for 24 hours before initiation of the assay. Cells (4 × 10^5) were resuspended in serum-free RPMI and added to the top chamber. Consecutively, RPMI with or without rhWNT5A was added to the bottom chamber. Chambers were incubated either for 24 hours or 48 hours at 37°C in 5% CO₂. After incubation, the number of migrated cells in the bottom chamber was determined by luminescence assay according to the recommendations of the manufacturer (CellTiter-Glo Cell Viability assay, Promega Corporation) following incubation with Calcein AM Fluorescent Dye (Bio-Rad), with 250 nmol/L primer and 1 μL of cDNA per 20-μL reaction as described previously (24). 5-Aza-CR was administrated to the OE33 cells at the concentration of 0.5 μmol/L and RNA was collected after 6, 24, 48, and 72 hours and analyzed as described above. Gene expression was analyzed with iQ5 software (Bio-Rad). The primers used are; ROR2: (F: 5'-ATC TCT TCC AGC CTT CCT TC-3', R: 5'-GAT CAA TGT CCT TCA AGT TCT T-3'), WNT5A: (F: 5'-GGG CAA TGT CCT TCA AGT TCT T-3', R: 5’-ATG AGC CGG ACA CCC CAT GGC A-3'), GAPDH: (F: 5'-ATG AGC CGG ACA CCC CAT GGC A-3', R: 5'-TACT GCG AGG ATG CTG AAG GC-3', R: 5'-CCA CIGG CAT TCT TCT CT-3'), Ki67: (F: 5’-ATT ATG GCT TCA GGG TAT ATG AA-3', R: 5’-TCA GGG TCA GAA GAG AA-3').

### RNA isolation and qRT-PCR

RNA was isolated from biopsies and OE33 cell using Arcturus PicoPure RNA (Life Technologies) and the RNeasy Kit (Qiagen), respectively. cDNA was synthesized from 1 μg of total RNA using the iScript cDNA synthesis kit according to the manufacturer's protocol (Bio-Rad). qRT-PCR was performed with Taqman EvaGreen Supermix (Bio-Rad), with 250 nmol/L primer and 1 μL of cDNA per 20-μL reaction as described previously (24). 5-Aza-CR was administrated to the OE33 cells at the concentration of 0.5 μmol/L and RNA was collected after 6, 24, 48, and 72 hours and analyzed as described above. Gene expression was analyzed with iQ5 software (Bio-Rad). The primers used are; ROR2: (F: 5'-ATC TCT TCC AGC CTT CCT TC-3', R: 5'-GAT CAA TGT CCT TCA AGT TCT T-3'), WNT5A: (F: 5'-GGG CAA TGT CCT TCA AGT TCT T-3', R: 5’-ATG AGC CGG ACA CCC CAT GGC A-3'), GAPDH: (F: 5'-ATG AGC CGG ACA CCC CAT GGC A-3', R: 5'-TACT GCG AGG ATG CTG AAG GC-3', R: 5'-CCA CIGG CAT TCT TCT CT-3'), Ki67: (F: 5’-ATT ATG GCT TCA GGG TAT ATG AA-3', R: 5’-TCA GGG TCA GAA GAG AA-3').
Immunohistochemical analysis

Mucosal esophageal biopsy specimens were embedded in paraffin blocks and cut into 5-μm sections. The sections were routinely stained with hematoxylin and eosin for histologic diagnosis, and additional sequential sections were subjected to IHC using antibodies against WNT5A and ROR2, as described previously (24). Expression of ROR2 immunohistochemical staining was quantified by both, determination of the intensity score of ROR2 expression in all biopsy samples using a 0–4 simple scoring system by a specialized pathologist as well as using an Automated Cellular Imaging System III (ACIS III, DAKO) in a single case as described previously (27). This case was representative of the entire cohort and contained areas of normal squamous mucosa, Barrett mucosa, and adenocarcinoma, and each of these histologic areas were analyzed independently and graphed. The percentage of ROR2-positive tissue and the intensity of ROR2 were measured independently and used to create a region score. Obvious artifacts including tissue folding, edge effect, nonspecific chemical precipitation, and dust or debris artefacts were excluded.

WNT5A and ROR2 gene silencing

OE33 and EPC2-hTERT cells were transfected with predesigned short interfering RNA (siRNA) targeting human WNT5A and/or ROR2 gene and a control negative siRNA (Dharmacon) according to the manufacturer’s protocol and described previously (24). The ratio of siRNA to DharmaFECT reagent was 25 nmol/L siRNA to 1.5 μL of transfection reagent.

Luciferase reporter assay

The assay was performed as described previously (24). In summary, OE33 cells in 60% confluence in 24-well plates were transfected with DNA plasmids of β-catenin-LEF/TCF-sensitive (TOP-flash) or β-catenin-LEF/TCF-insensitive (FOP-flash) reporter vectors (Addgene) using Lipofectamine 2000 (Invitrogen) for 48 hours. One microgram of TOPflash as well as 1 μg of FOPflash were used per well of a 24-well plate. phRL-TK plasmid (Promega) was cotransfected as control for transfection efficiency. Reporter assay was performed using dual luciferase reporter system (Promega). Luciferase activity was measured via GLOMAX 20/20 Luminometer (Promega). Values for each reporter were normalized to phRL-TK values.

Statistical analysis

IBM SPSS Statistics 19 software was used for statistical analysis. All experiments were performed in triplicate, and values are means ± SE. Expression patterns between squamous mucosa, Barrett metaplasia, and esophageal adenocarcinoma tissue were compared within esophageal tissue sections obtained from esophageal adenocarcinoma patients (n = 11) undergoing esophagectomy, without previous treatment. High levels of ROR2 were detected in esophageal adenocarcinoma tissues, whereas ROR2 expression was weak in the corresponding Barrett esophagus and even weaker and isolated in the proliferative basal layer of the corresponding squamous esophageal mucosa (Fig. 1A). A simple grading system (0–4; Fig. 1B) as well as an image analysis software were used to quantify the intensity and region score (%; Fig. 1C) of ROR2 staining in the different tissues as described in Materials and Methods. Statistical analysis, using Friedmann–Kendall W test to analyze the overall results of intensity score between the three related conditions yielded a Kendall coefficient of concordance of 0.363, which approached statistical significance (P = 0.079). Finally, significance between two-group comparisons was determined by Wilcoxon signed-rank tests, followed by correction for multiple comparisons. Overall, ROR2 expression was significantly higher in esophageal adenocarcinoma compared with squamous mucosa and Barrett esophagus tissues.

WNT5A expression is downregulated in esophageal adenocarcinoma

Next, we examined the level of WNT5A mRNA expression in esophageal biopsy specimens from three independent patients’ groups, healthy controls (n = 10), Barrett (n = 15), and esophageal adenocarcinoma patients (n = 5). To ensure tissue homogeneity in esophageal adenocarcinoma tissues, laser microdissection was utilized to isolate tumor lesions. qRT-PCR analysis demonstrated significantly lower level of WNT5A mRNA in esophageal adenocarcinoma samples compared with squamous mucosa and Barrett esophagus biopsy samples (Fig. 2A). We compared mRNA expression of WNT5A gene between groups by using a global nonparametric Kruskal–Wallis test, which showed a significant overall difference (P = 0.0069). Subsequent comparisons between groups (squamous mucosa vs. Barrett esophagus, squamous mucosa vs. esophageal adenocarcinoma, and Barrett esophagus vs. esophageal adenocarcinoma) was done by the nonparametric Mann–Whitney test. Corresponding with the gene expression, Western blot analysis of biopsy samples from esophageal adenocarcinoma patients showed a lower level of WNT5A protein in tumor site compared with the corresponding healthy esophageal mucosa (Fig. 2B). Conversely, the level of ROR2 protein expression was higher in tumor biopsy compared with healthy mucosa. Immunohistochemical analysis of WNT5A expression in healthy esophageal mucosa revealed a nuclear staining throughout all mucosal layers, whereas ROR2 expression was detected mainly in cell cytoplasm and membrane of the basal mucosal layer (Fig. 2C). Further immunohistochemical analysis confirmed the negative correlation of ROR2 and WNT5A expression in esophageal adenocarcinoma tissue (Fig. 2D).

RESULTS

ROR2 is overexpressed in human esophageal adenocarcinoma

To demonstrate whether ROR2 expression alters in the course of progression from normal squamous epithelium to esophageal adenocarcinoma, ROR2 expression was evaluated by IHC in esophageal tissue sections obtained from esophageal adenocarcinoma patients (n = 11) undergoing esophagectomy, without previous treatment. High levels of ROR2 were detected in esophageal adenocarcinoma tissues, whereas ROR2 expression was weak in the corresponding Barrett esophagus and even weaker and isolated in the proliferative basal layer of the corresponding squamous esophageal mucosa (Fig. 1A). A simple grading system (0–4; Fig. 1B) as well as an image analysis software were used to quantify the intensity and region score (%; Fig. 1C) of ROR2 staining in the different tissues as described in Materials and Methods. Statistical analysis, using Friedmann–Kendall W test to analyze the overall results of intensity score between the three related conditions yielded a Kendall coefficient of concordance of 0.363, which approached statistical significance (P = 0.079). Finally, significance between two-group comparisons was determined by Wilcoxon signed-rank tests, followed by correction for multiple comparisons. Overall, ROR2 expression was significantly higher in esophageal adenocarcinoma compared with squamous mucosa and Barrett esophagus tissues.

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ROR2/WNT5A expression in esophageal cell lines

The baseline level of WNT5A and ROR2 gene and protein expression in esophageal cell lines derived from normal esophagus (EPC1, EPC2), nondysplastic Barrett esophagus (CP-A), and esophageal adenocarcinoma (OE33) were evaluated by RT-PCR and Western blotting. The level of WNT5A mRNA expression in EPC1 and EPC2 cells was higher than Barrett esophagus–derived cells (CP-A), and WNT5A expression was
further reduced in OE33 cells (Fig. 3A). Conversely, the ROR2 mRNA expression was significantly higher in CP-A and OE33 cells compared with EPC1 and EPC2 cells (Fig. 3B). In agreement with the gene expression, ROR2 protein expression in CP-A and OE33 cells was higher than in normal epithelial cells, whereas WNT5A protein expression analysis demonstrated a reverse pattern of expression (Fig. 3C). To investigate whether WNT5A loss of expression could be caused by promoter methylation based on previous reports (28), OE33 cells were treated with 5-Aza-CR, a drug capable of demethylating WNT5A's promoter (29). WNT5a mRNA expression was increased following 0.5 μmol/L 5-Aza-CR treatment (Fig. 3D), suggesting that promoter hypermethylation may contribute to the downregulation of WNT5A.

In all, the data derived from the in vitro model verify a high expression of ROR2 receptor together with a simultaneous loss of WNT5A ligand, underlying the deregulation of the WNT5A/ROR2 axis toward esophageal adenocarcinoma progression.

Recombinant WNT5A suppresses OE33 cells' tumor growth

Next, we examined the functional consequences of the downregulated WNT5A expression in the esophageal adenocarcinoma tumor growth. We evaluated the role of WNT5A by treating OE33 cells with exogenous recombinant WNT5A protein (rhWNT5A). Application of rhWNT5A drastically decreased OE33 cell proliferation (Fig. 4A), cell survival (Fig. 4B), invasion (Fig. 4C), and cell migration (Fig. 4D) in a dose- and time-dependent manner, as shown by 3[H] thymidine uptake, MTT assay, transmigration, and wound scratch assays, respectively. These findings suggest that WNT5A functions as tumor suppressor in esophageal adenocarcinoma.

ROR2 overexpression induces tumor growth in OE33 cells

Given the overexpression of ROR2, despite the loss of WNT5A, we aimed to assess the functional role of ROR2 receptor with respect to the tumor growth by siRNA-mediated gene silencing. After 48 hours of siRNA transfection, ROR2 gene expression in OE33 cells was effectively decreased as estimated 24 and 48 hours posttransfection by qRT-PCR (Fig. 5A). The knockdown of ROR2 gene expression was further confirmed by Western blotting (Fig. 5B). Remarkably, treatment with rhWNT5a for 24 hours resulted in similar downregulation of ROR2 protein expression, suggesting that the overexpression of ROR2 receptor may be compensatory to the loss of WNT5A ligand (Fig. 5C). Similarly, WNT5A gene expression was found upregulated following ROR2 silencing, supporting a compensatory feedback loop between ligand and receptor (Fig. 5D). Functional assays revealed that silencing of ROR2 decreased OE33 cell proliferation (Fig. 5E) and...
transmigration (Fig. 5F), signifying that ROR2 overexpression induces tumor growth.

**WNT5A suppresses canonical Wnt/β-catenin signaling in OE33 cells**

To determine the nature of tumor suppressor properties of WNT5A, we investigated whether WNT5A inhibits the canonical Wnt/β-catenin signaling in OE33 cells. Treatment with rhWNT5A for 24 hours significantly decreased TOPFlash activity in a dose-dependent manner (Fig. 6A). In agreement with a reduced β-catenin transcriptional activity, the gene expression of the β-catenin target gene, AXIN2, was suppressed by rhWNT5A (Fig. 6B). At the molecular level, rhWNT5A was found to reduce the level of phosphorylated GSK3β (Ser 9; Fig. 6C), indicating activation of GSK3β and subsequent inhibition of β-catenin-mediated signaling (30). Of note, the WNT5A-mediated dephosphorylation of GSK3β was not rescued by the canonical rhWNT3A, suggesting independent downstream signaling transduction between the two WNTs.

To further demonstrate the role of the ROR2 receptor in the WNT5A-induced inhibition of the canonical WNT/β-catenin signaling, the level of TOPFlash activity was evaluated by treating OE33 cells with rhWNT5a for 24 hours after sufficient siRNA-mediated ROR2 gene silencing. TOPFlash activity was inhibited by rhWNT5A treatment in wild-type OE33 cells; however, it was markedly increased in ROR2-knockdown cells (Fig. 6D). We further confirmed this finding by increased level of ABC by rhWNT5A stimulation in the ROR2-knockdown cells (Fig. 6E). These findings signify that the presence of ROR2 receptor dictates the function of WNT5A regarding inhibition or induction of the canonical WNT/β-catenin signaling.

**WNT5A knockdown induces proliferation in normal EPC2 epithelial cells**

Finally, to reversely address, whether WNT5A silencing in normal epithelial cells could induce a tumorigenic phenotype, we investigated the proliferative properties of EPC2 normal epithelial cells following by knocking down the WNT5A gene expression using siRNA transfection. Sufficient WNT5A gene silencing in EPC2 cells was demonstrated after 48 hours of siRNA transfection and was found to result in significant increase of Ki67 expression (Fig. 7A) as well as in marked induction of cell proliferation, mainly 72 hours posttransfection (Fig. 7B) as shown by qRT-PCR and 3[H]
thymidine uptake, respectively. Further analysis with qRT-PCR demonstrated an increase in AXIN2 gene expression following WNT5A silencing in EPC2 cells, indicating an enhanced canonical WNT signaling, whereas ROR2 gene expression remained unchanged (data not shown). Those findings are consistent with a tumorigenic phenotype induced by WNT5a loss of expression in the context of normal epithelial esophageal cells.

**Discussion**

In the current study, we examined the role of noncanonical WNT5A/ROR2 signaling pathway in the Barrett-associated esophageal adenocarcinoma. Loss of WNT5A expression along with ROR2 overexpression was found to characterize esophageal adenocarcinoma compared with Barrett and healthy esophageal tissues. Similarly, in vitro analysis demonstrated low level of WNT5A expression and ROR2 overexpression in OE33 cells compared with the normal squamous (EPC1, EPC2) and Barrett metaplastic (CP-A) cell lines. Treatment of OE33 cells with drug 5-Aza-CR an epigenetic modiﬁer which results in DNA demethylation (29) restored the WNT5A expression, suggesting the epigenetic nature of WNT5a loss in esophageal adenocarcinoma. Moreover, treatment of OE33 cells with recombinant WNT5A inhibited cell proliferation, migration, and invasion, whereas, at the molecular level, signiﬁcantly reduced TOPflash luciferase activity, AXIN2 gene expression, and GSK3β phosphorylation at
serine 9, indicating antagonism of the canonical WNT signaling. This antagonism required the presence of ROR2 receptor, as exogenous WNT5A, in contrast to wild-type OE33 cells, and was found to induce TOPflash activity and to increase the transcriptional level of active β-catenin in ROR2-knockdown OE33 cells. Notably, ROR2 knockdown itself resulted in decreased cell
proliferation and migration, pointing to a WNT5a ligand–
-independent role in tumor progression. Together, our findings high-
light a deregulation of WNT5A/ROR2 signaling pathway in the pathogenesis of esophageal adenocarcinoma, with its exact role, however, to be dictated by the ligand/receptor context.

Altered expression patterns of the noncanonical WNT5A and its receptor ROR2 have already been reported in various malignancies, indicating the critical roles of WNT5A-ROR2 axis in tumor progression (31, 32). In this study, we report a gradual loss of WNT5A expression along the esophageal adenocarcinoma
progression and attribute it to WNT5A promoter methylation, proposing that deactivation of WNT5a-induced downstream signaling may induce tumorigenesis in esophageal adenocarcinoma. This notion was further supported in vitro, as rhWNT5A significantly inhibited OE33 cell proliferation, migration, and invasion. Indeed, loss or low expression of WNT5A is associated with increased invasion and aggressiveness of many solid tumors, such as thyroid cancer (16), breast cancer (18), neuroblastoma (17) and colon cancer (19, 28). Furthermore, loss of WNT5A expression has been shown to correlate with cell transformation (33), whereas ectopic expression of human WNT5A reversed the phenotype of a mouse epithelial cell line from tumorigenic to

Figure 6.
WNT5A inhibits canonical WNT/β-catenin signaling in OE33 cancer cells. Recombinant WNT5A inhibits the canonical WNT/β-catenin signaling in OE33 cancer cells. A, luciferase assay demonstrated that treatment of OE33 cells with rhWNT5A for 24 hours significantly decreased TOPFlash activity in a dose-dependent manner. Treatment with rhWNT5A (500 ng/mL) for 24-hour suppressed AXIN2 gene expression as measured by qRT-PCR (B) and reduced the phosphorylated levels of GSK3β (Ser 9), despite the costimulation with rhWNT3A (C). D, rhWNT5A decreased TOPFlash activity in wild-type OE33 cells, but increased the TOPFlash activity in ROR2-knockdown OE33 cells. E, rhWNT5a increased the expression of active β-catenin (ABC) in ROR2-knockdown OE33 cells.
nontumorigenic (34). Consistent with these observations, we
demonstrated that WNT5A silencing induces cell proliferation
in normal epithelial EPC2 cells, indicating a protective role for
WNT5A against tumorigenesis. However, WNT5A has also been
shown to possess oncogenic properties, based on findings depict-
ing upregulation of WNT5A levels in melanoma, lung, and gastric
cancers (20, 21, 35). These diverse functions of WNT5a toward
tumor progression could reflect differences in receptor context or
cell context of cancer cells (14, 31).

On the other hand, the receptor of WNT5A, ROR2 was found
overexpressed, suggesting a ROR2-mediated, however WNT5A-
independent, downstream signaling activation in esophageal ade-
ocarcinoma progression. On the basis of its high levels of expres-
sion, ROR2 has already been reported to promote tumorigenesis in
various malignancies, such as osteosarcoma, melanoma, renal cell
carcinoma (RCC), as well as gastrointestinal stromal tumor and
oral cancers (36). Especially, strong correlation between absence of
ROR2 and decreased cell migration has been shown in siRNA
studies of osteosarcoma cells (37), whereas ROR2 knockdown in
RCC cells decreased cell migration and inhibited tumor growth in an
orthotopic xenograft mouse model (22). Furthermore, ROR2
silencing inhibited invasion of melanoma cells in a murine in vivo
tumor model (38). In this study, we confirmed similar tumor-
promoting profile for ROR2 in esophageal adenocarcinoma by
showing that ROR2 knockdown results in significant decrease of
both, cell proliferation and migration, in OE33 cancer cells. Intrigu-
ingly, these effects were observed in wild-type OE33 cells, which are
characterized by WNT5A loss, signifying that the ROR2 receptor
activates a downstream signaling independently of WNT5A. In all,
these observations imply the tumorigenic role of ROR2 regardless
the presence of WNT5A ligand.

Cross-talk between WNT5A/ROR2 signaling and the canonical
WNT signaling has been proposed (36). Particularly,
in vivo studies
in mice have shown that when ROR2 or WNT5A expression is
knocked down, WNT/β-catenin signaling is enhanced (39). Given
that β-catenin signaling induces tumor progression, these observa-
tions justify the WNT5A-mediated tumor suppression, in which
ROR2 appears to have an essential role as receptor (37, 39, 40).
Indeed, in ovarian cancer, WNT5A-mediated inhibition of β-catenin
transcriptional activity has been associated with inhibition of tumor
progress via cellular senescence (41). Taking into consideration that
the canonical WNT/β-catenin signaling is activated in esophageal
adenocarcinoma cancer cells (24), we could attribute the suppres-
sion of OE33 cell proliferation by WNT5A to the inhibition of
β-catenin transcriptional activity, as shown by WNT5A-mediated
decrease in TOPFlash activity and AXIN2 gene expression. Interest-
ingly, following ROR2 knockdown, rhWNT5A activated β-catenin-
mediated gene transcription instead of inhibiting it as shown in
ROR2 wild-type cells, pointing to the essential role of ROR2
receptor in dictating WNT5A’s function; whether it will suppress

![Figure 7](http://example.com/figure7.jpg)

**Figure 7.**
WNT5A silencing in EPC2 normal epithelial cells. Transfection of EPC2 cells with WNT5A siRNA. A, qRT-PCR analysis of WNT5A (top) and Ki67 (bottom) gene expression following transfection of WNT5A siRNA for 48 hours. B, [H]3 thymidine uptake assay in EPC2 cells as assessed 24, 48, and 72 hours posttransfection. * P < 0.05 (NT siRNA vs. WNT5A siRNA).
the β-catenin axis or enhance it. The most possible explanation for this notion is that in absence of ROR2, WNT5A activates β-catenin via Frizzled receptors binding (14). Someone would expect WNT5A to induce the canonical WNT signaling in normal epithelial cells, due to their low ROR2 expression. However, WNT5A silencing in EPC2 cells resulted in increased canonical WNT signaling as dictated by induction of AXIN2 gene expression, indicating that in normal epithelial cells, WNT5A might antagonize other WNTs. Thus, the function of WNT5A regarding the activation of canonical WNT signaling is not only determined by the receptor context but also the presence of other WNTs.

It remains questionable, how rhWNT5A impaired the invasive properties of OE33 wild-type cells, when WNT5A is known to induce cell migration and cancer metastasis by promoting epithelial–mesenchymal transition and activating transcription of matrix metalloproteases (MMP; ref. 42). Notably, exogenous WNT5A decreased ROR2 expression in OE33 cells indicating a possible reduction of receptor levels in the presence of ligand, highly likely via receptor-mediated endocytosis and degradation. Given that cell motility regulation via WNT5A requires ROR2 receptor (43), we can speculate at this point that the subsequent ROR2 downregulation in OE33 cells following rhWNT5A stimulation may underlay the impaired invasiveness. In favor of this notion, ROR2 knockdown induced WNT5A gene expression and was also found to inhibit invasion and migration. However, the complex role of ROR2 receptor in esophageal adenocarcinoma invasiveness, in absence of WNT5A ligand, remains unclear and merits further study.

Our current findings highlight that deregulation of WNT5A/ROR2 signaling may contribute to the high invasive properties and subsequently to the poor prognosis of esophageal adenocarcinoma. Because of the limited number of patients in this study, we were unable to provide a powerful correlation between expression patterns and tumor stage or prognosis in this study, which is an acknowledged limitation. However, we observed a stronger ROR2 staining together with a further decreased WNT5A gene expression in the esophageal adenocarcinoma tissues classified in pT3/N1 stage compared with those in pT1/N0 stage (data not shown), which allows us, at this point, to speculate at the prognostic potential of ROR2 and WNT5A expressions in esophageal adenocarcinoma. Similar correlations, which allow conclusions on the prognostic value of WNT5A and ROR2, have been performed in other malignancies (31). Intriguingly, in these studies, a poor outcome was associated with the overexpression of both WNT5A and ROR2 (38). Our report, however, indicates that is WNT5A loss-of-expression in combination with ROR2 gain-of-expression that contributes to the tumor growth and metastatic potential of esophageal adenocarcinoma cells. On the basis of our in vitro data, we can speculate that high WNT5A levels may be associated with a better prognosis, whereas ROR2 overexpression with a poor one. Undoubtedly, our data make apparent that in the case of esophageal adenocarcinoma, the prognostic value of WNT5A cannot be evaluated independently of ROR2 and vice versa. With respect to WNT5A’s receptors, the expression ROR1 is yet not assessed in this study. ROR1 has been shown to be highly expressed in several types of cancer, including mainly hematopoietic malignancies (44) and might play a role in the esophageal adenocarcinoma tumor progression. However, in contrast to the well-established function of ROR2, it remains unclear whether ROR1 acts as a coreceptor for WNT5A-mediated signal in tumor biology (31) and such an investigation was beyond the scopes of this study.

In conclusion, we have examined for the first time that the WNT5A/ROR2 axis in the pathogenesis of esophageal adenocarcinoma and we have shown that its deregulation is significantly involved to esophageal adenocarcinoma tumor progression. Loss of WNT5A with concurrent activation of WNT5A-independent ROR2 signaling appears to mark the progression of esophageal adenocarcinoma (Fig. 8). Although already speculated in other malignancies, we confirmed that WNT5A ligand could function in two discreet pathways in esophageal adenocarcinoma based on ROR2 receptor availability. It becomes evident that whether WNT5A contributes to promote or suppress tumor progression is partly dependent on the cellular context, the receptor’s context, and the balance of the canonical WNT signaling and noncanonical WNT signaling. Thus, it is of great importance to understand the respective molecular mechanisms governing WNT5A-mediated tumor-progressive and tumor-suppressive functions, to develop novel and proper diagnostic and therapeutic strategies targeting WNT5A signaling for esophageal adenocarcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): O. Lyros, T. Moore, A. Mackinnon, I. Gockel, P. Raﬁee
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): O. Lyros, T. Moore, A. Mackinnon, I. Gockel, P. Raﬁee
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