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Intramucosal Distribution of WNT Signaling Components in Human Esophagus

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Abstract

Goals—To determine expression and localization of Wnt signaling molecules across the esophageal mucosal thickness.

Background—The molecular mechanisms governing the biology and pathobiology of esophageal squamous mucosa in health and disease are not completely understood. Earlier genome-wide expression study of normal-looking esophageal squamous mucosa has shown differential expression of the Wingless-type MMTV integration site family (Wnt) modulators Dickkopf (Dkk) homologs among healthy individuals and patients with reflux esophagitis and Barrett metaplasia suggesting that the Wnt pathway may be involved in esophageal mucosal biology.

Study—Seven full-thickness human donor esophagi were cryosectioned for immunohistochemical analysis, and lamina propria (LP), basal (BC), intermediate (IC), and superficial (SC) cells were also dissected by laser-capture microdissection for real-time polymerase chain reaction.

Results—Wnt1, 2b, and 3a were expressed primarily in BC, Wnt3, and 5b in LP, and Wnt5a in IC. Frizzled 1, low-density lipoprotein receptor-related protein 6, secreted frizzled-related protein 1, T-cell-specific transcription factor 3, and dishevelled 3 were expressed highest in LP decreasing precipitously medially toward SC. Dkk1 predominantly expressed in SC was more than 100-folds greater than other layers ($P < 0.001$). Dkk4 was expressed primarily in SC but Dkk3 was opposite with greatest expression in LP. Immunohistochemical analysis showed Wnt1 and 3a in BC, Wnt5a in IC and SC, Dkk1 predominantly in SC, Dkk4 in SC and IC, and Dkk3 and SFRP1 in LP and BC.

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The authors declare no conflict of interest.

Conclusions—Signaling components are expressed in a differential manner within the different layers of esophageal squamous mucosa suggesting their involvement in mucosal cell biology locally.

Keywords

wingless-type MMTV integration site family members; Dickkopf homologs; frizzled; secreted frizzled-related protein 1; T-cell-specific transcription factor 3; squamous mucosa

The molecular genetic mechanisms governing the biology and pathobiology of the squamous mucosa of the esophagus have received little attention compared with other areas of the digestive system. Available studies have mostly focused on malignant tissues^{1–5} and Barrett metaplasia,^{6–10} consequently the mechanisms involved in homeostasis of the esophageal squamous tissue remain for the most part unexplored. The stratified squamous epithelium of the human esophagus consists of 3 morphologically different cell types^{11,12} organized in 3 distinct medially transforming strata of basal, prickle, and corneum/squamous, respectively. The ability of the cells to replicate as they move from the basal cell (BC) layer toward the esophageal lumen is significantly reduced such that the superficial cells (SCs) at or near the lumen are terminally differentiated and no longer able to replicate.¹³ The mechanisms governing and factors influencing these transformations in health and disease have not been systematically studied.

Earlier genome wide expression study¹⁴ of normal-looking esophageal squamous mucosa has shown differential expression of the Wnt modulators, DDK homologs among healthy individual and patients with reflux esophagitis and Barrett metaplasia, suggesting involvement of Wnt signaling in the biology of the esophageal squamous mucosa. In this previous study, analysis was performed on squamous mucosal cells dissected by laser-capture microdissection (LCM) from mucosal tissues obtained by biopsy and therefore the regional differences within the mucosal layers were not studied.

The Wnt pathway is an intricate ensemble of signaling molecules, which perform several functions in cells. The Wnt pathway consists of the canonical and the noncanonical pathways. The canonical pathway is activated by the binding of the Wnt ligand to the frizzled (FZD) receptors which results in cytosolic stabilization and nuclear localization of β -catenin.¹⁵ β -catenin then associates with T-cell-specific [lymphoid enhancer-binding factor (TCF/LEF) transcription factors] resulting in the induction of Wnt-related gene expression. The canonical Wnt pathway-related gene induction leads to cell proliferation.¹⁵ The noncanonical pathway consisting of the PCP, Wnt/JNK, and the Wnt/calcium pathways, which do not use the β -catenin/TCF-mediated gene expression is nonproliferative and stimulates cell differentiation.^{16,17} The noncanonical Wnt pathways have been linked to cell polarity, cell differentiation, cell motility, and appropriate distribution of cells within an organ.^{16,17} The Wnt pathways are controlled by various modulators which include Dickkopf (Dkk) proteins, secreted frizzled-related proteins (SFRPs) and Wnt inhibitory factor.^{18,19} Whether the canonical or the noncanonical pathway is activated is dependant on the type of FZD receptors, Wnt ligands, modulatory molecules, and transcription factors present in the tissues.^{15,18,19}

Recent study has shown that components of the Wnt pathway are distributed differentially in the mucosa of small bowel and colon suggesting that they may be instrumental in cell homeostasis of these tissues.²⁰ Finally, studies using overexpression of Dkk1 to inhibit the Wnt pathway indicate that it may be instrumental in the maintenance of the cell lineages of the mucosa of gastrointestinal tract.^{21,22} In these studies, Dkk1 overexpression lead to a substantial reduction of the villus structure in the small bowel and decreased crypts in the colon concomitant with a reduction of the progenitor cells.^{21,22}

Despite the fact that the involvement of the Wnt pathway has been shown in esophageal squamous cell carcinoma and adenocarcinoma^{23,24} its role in homeostasis of the esophageal squamous mucosa has not been investigated directly. The present study was therefore undertaken to determine the expression and localization of Wnt signaling components in the lamina propria (LP), BC, intermediate cell (IC), and the SC layers of the human esophagus.

MATERIALS AND METHODS

Tissue Procurement

Full thickness esophagi were obtained from organ donors according to the Institutional Review Board approved protocol and the study was also approved by the human research review committee of the Medical College of Wisconsin. The esophagus was cut longitudinally and the mucosa was exposed. Observations were made of the squamous mucosa for lesions and ulcerations and none were found. The esophagi were from the distal end as determined by the smooth muscle composition of muscularis externa attached to the specimens but the exact location could not be determined. The esophagus was cut into 1×4-cm full thickness pieces and flash-frozen in liquid nitrogen, and maintained at -80°C until further processing.

Tissue Processing and LCM

The specimens were prepared for cryo-sectioning by embedding in Histo Prep Frozen Tissue embedding media (Fisher Scientific, Pittsburgh, PA). Tissue sections (10 µm) were prepared and placed on Fisherbrand Superfrost/Plus microscope precleaned glass slides (Fisher Scientific). The slides were kept on dry ice, and LCM was performed on them within 1 to 2 hours after sectioning.

The tissue sections were hematoxylin and eosin-stained and the squamous mucosal epithelial cells were sectioned by LCM technique using the PixCell II LCM System (Arcturus, Mountain View, CA) and CapSure HS LCM caps (Arcturus). Cells from the LP, BC layer, and the intermediate and superficial layers representing the prickle cell and stratum corneum layers, respectively, were carefully removed (Fig. 1). LCM caps that captured unwanted cells were discarded.

RNA from the LCM-generated samples was extracted using Arcturus PicoPure RNA extraction kit (Arcturus, Mountain View, CA) according to the manufacturer's protocol.

Real-time Polymerase Chain Reaction

LCM extracted RNA from the different cell layers was put through one round of amplification as described previously.¹⁴ Briefly, T7-(dT)24 primer (Sigma Proligo, St Louis, MO) was used for the first-strand synthesis followed by the second-strand synthesis. In vitro transcription for cRNA amplification was done using MEGAscript T7 Kit (Ambion, Austin, TX). cRNA was cleaned up and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The amplified cRNA was then used for first-strand cDNA synthesis using Oligo (dT) primers according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). This first-strand cDNA was then used for real-time polymerase chain reaction (PCR).

Temperature gradients were run to determine the optimal annealing temperatures, and various concentrations of cDNA were also used to optimize the real-time PCR reaction. The real-time PCR efficiency was 90% to 100%. Nontemplate blanks with primers were run to rule out the formation of primer dimers. Melt-curve analysis was also performed to determine the number of products. Only a single peak was observed indicating a single product. Furthermore, gel electrophoresis was performed, which confirmed the presence of one product of the appropriate size.

Real-time PCR was performed on the iCycler iQ Real-Time PCR Detection System using SYBR Green Supermix (BioRad, Hercules, CA). Primers used for real-time PCR are provided in Table 1. A 2-step amplification and thermal denaturation protocol was used for real-time PCR. The protocol was as follows: 1 step of 95°C for 3 minutes; 40 to 50 cycles of 95°C for 10 seconds and 54.5 to 58.6°C (depending on gene) for 45 seconds; 1 step of 95°C for 1 minute; 1 step of 55°C for 1 minute; and thermal denaturation protocol from 55 to 95°C at 5°C/cycle for 80 cycles. ICycler iQ optical system software (version 3.0a, BioRad) was used to calculate the relative amounts of genes of interest. Standard curves for genes of interest and GAPDH were constructed using four 10-folds serial dilution of sample cDNA. These Ct values were then used to determine the relative quantity of the genes of interest. Each sample was run in triplicate and the means were normalized with GAPDH to obtain the expression ratios. The means of the expression ratios of genes/GAPDH $\pm$ SEM of 6 or 7 samples are displayed in the figures.

Immunohistochemistry

Immunohistochemical analysis (IH) of Wnt signaling components was performed on full thickness frozen esophageal sections. Esophageal sections (10 μ m) were fixed in 4% paraformaldehyde for 30 minutes at room temperature. Then washed in phosphate-buffered saline (PBS) 3 times for 10 min/wash followed by incubation in peroxide solution (10% methanol, 2% H₂O₂ in PBS) for 20 minutes. The slides were then incubated in blocking solution [4% of appropriate normal serum (donkey or mouse)+0.2% Triton X-100 in PBS] for 30 minutes. After the blocking step, the sections were incubated overnight at 4°C with the appropriate primary antibodies. Wnt 1 and Wnt 5a were raised in goats against a peptide mapping near the N-terminus of Wnt1 of human origin and a peptide mapping within an internal region of Wnt 5a of human origin, respectively (Santa Cruz Biotechnology Inc, Santa Cruz, CA). Wnt 3a antibody was raised in rabbit to synthetic peptide corresponding to

residues surrounding Asp294 of human Wnt 3a (Cell signaling, Danvers, MA). Dkk and SFRP1 primary antibodies were raised in goat against recombinant human Dkk homolog 1, 3, 4, and SFRP1 proteins (R&D Systems, Minneapolis, MN). The antibody dilutions used for Wnt 1, 3a, and 5a were 1:25, 1:25, and 1:10, respectively. Dkk1 and Dkk3 were diluted 1:50 and Dkk4 was used at 1:10 dilution. Antibody dilution for SFRP 1 was 1:25. Then after washing in PBS the sections were incubated with secondary antibody (peroxidase-conjugated mouse antigoat antibody) at a 1:500 dilution in PBS (Jackson Immune Research, Westgrove, PA) for 2 hours at room temperature. After 3 washings with PBS (10-min each), the slides were developed with DAB (3,3'-diaminobenzidine) (Vector Laboratories Inc, Burlingame, CA) and counter-stained with hematoxylin-2 (Sigma). Negative IgG-isotype-matched controls on serial sections were prepared by incubating without primary antibody followed by incubation with secondary antibody and further processed as above. After dehydrating and mounting, images were captured using a Spot II high-resolution digital camera (Diagnostic Instruments Inc, Sterling Heights, MI) mounted on microscope (Nikon Eclipse 50i) and processed with Adobe Photoshop program.

Statistical Analysis

Statistical analysis was done using a commercially available package (SigmaStat 2.03 for Windows, SPSS Inc, San Rafael, CA). Statistical comparison was done using 1-way analysis of variance (ANOVA) to determine differences among all layers. Pair-wise comparison using *t* statistics or Mann-Whitney Rank Sum test for nonparametric data was used subsequently to determine differences between the layers.

RESULTS

Real-time PCR Analysis

Real-time PCR analysis of the LCM-generated (Fig. 1) samples, demonstrated differential expression of the Wnt signaling components throughout the thickness of the squamous mucosa. Different magnitudes of expression of Wnt ligands (Wnt 1, 2b, 3, 3a, 5a, 5b), receptors [FZD 1, low-density lipoprotein receptor-related protein 6 (LRP 6)], modulating proteins (Dkk 1, 3, 4, SFRP 1), and intracellular components [TCF 3, dishevelled (DVL) 3] were detected in all layers.

Wnt Ligand Expression

Wnt 1—Wnt 1 expression was significantly different between the different layers ($P<0.02$; Fig. 2A). It was expressed predominantly in the BC layer and its expression level was 3-folds greater than the IC layer ($P<0.04$), and more than 5-folds greater than the SC layer ($P<0.02$) but not significantly different from the LP. Wnt 1 expression in the LP was more than 3-folds greater than the SC layer ($P<0.03$).

Wnt 2b—Wnt 2b expression in the different layers was also statistically significant ($P<0.05$; Fig. 3A). Highest expression was observed in the BC layer and was similar to the LP. Lowest expression was observed in the IC layer. Expression in the BC layer was more than 6-folds greater than the IC layer ($P<0.02$). Wnt 2b expression in the LP was more than 4-folds greater than that observed in the IC layer ($P<0.025$).

Wnt 3—Wnt 3 expression was also significantly different between the different layers ($P<0.03$, Fig. 3B). It was expressed primarily in the LP and was more than 11-folds greater than the BC layer ($P<0.02$) and more than 9-folds greater than the IC layer ($P<0.04$).

Wnt 3a—There was also a significant difference in Wnt 3a expression between the different layers ($P<0.02$; Fig. 4A). It was expressed highest in the BC layer and was more than 7-folds greater than the IC layer ($P<0.05$) and more than 9-folds greater than the SC layer ($P<0.04$).

Wnt 4—Wnt 4 expression was lowest at the LP and increased gradually toward the SC layer. The difference in expression between the different layers however did not reach statistical significance (Fig. 5A).

Wnt 5b—Wnt 5b level of expression was also significantly different between the layers ($P<0.005$; Fig. 5B). It was highest in the LP and was more than 15-folds and 6-folds greater than the IC layer ($P<0.005$) and SC layers ($P<0.02$), respectively. Expression in the BC layer was not different from the LP but was more than 8-folds ($P<0.008$) and 3-folds ($P<0.03$) greater than the intermediate and SC layers, respectively.

Wnt 5a—Wnt 5a expression was also determined to be statistically different between the different layers ($P<0.03$; Fig. 6A) but its expression pattern was different from Wnt 5b. Expression in the IC layer was highest and more than 4-folds greater than the BC layer ($P<0.002$). Pair-wise comparison between the other layers did not reach statistical significance.

Wnt Receptor Expression

FZD 1—Comparison of the expression of Wnt receptor, FZD 1 revealed significant differences between the different layers ($P<0.04$, Fig. 7A). FZD 1 was expressed greatest in the LP and lowest in the SC layer. Expression in the LP was more than 8-folds greater than the SC layer ($P<0.02$). Comparison between the other layers did not reach statistical significance.

FZD 6—The expression of FZD 6 was higher in the LP and BCs compared with the IC layer and SC layer. The difference in expression between these layers did not reach statistical significance (Fig. 7B).

LRP 6—The expression level of LRP 6 was significantly different between the different layers ($P<0.02$, Fig. 7C). Expression in the LP was highest followed by the BC layer and lower levels of expression were observed in the IC and SC layers. The expression in the LP was more than 11-folds greater than the IC layer ($P<0.01$). Pair-wise comparison between the other layers did not reach statistical significance.

Wnt Modulating Protein Expression

Dkk1—The expression of Dkk1 was predominantly in the SC layer and decreased abruptly in the other layers (Figs. 8A, B). ANOVA showed significant differences in the expression

of Dkk1 among cell layers ($P<0.002$). Pair-wise comparison demonstrated that the expression of Dkk1 in SC layer was more than 100-fold greater than all of the other layers ($P<0.001$). Comparison between other cell layers did not reach statistical significance.

Dkk3—In contrast to Dkk1, the expression of Dkk3 decreased precipitously from the LP to the SC layer (Fig. 9A). ANOVA showed significant differences in expression of Dkk3 among cell layers ($P<0.001$). Pair-wise comparison demonstrated that the expression of Dkk3 in the LP was more than 25-folds greater than the IC layer and 7-folds greater than SC layer ($P<0.003$). Dkk3 expression in the BC layer was more than 3-folds greater than that determined in the SC layer and the IC layers ($P<0.005$). Expression in the LP was also significantly greater than the BC layer ($P<0.03$).

Dkk4—The expression of Dkk4 on the other hand increased precipitously and significantly from the LP to the SC layer (Fig. 10A). ANOVA showed significant differences in the expression of Dkk4 among the cell layers ($P<0.001$). Pair-wise comparison demonstrated that Dkk4 expression in the SC layer was more than 30-folds greater than the LP and the BC layer ($P<0.001$) and more than 4-folds greater than the IC layer ($P<0.02$). Expression in the IC layer was 7-folds greater than the LP and the BC layer ($P<0.05$).

SFRP 1 Expression—SFRP 1 was expressed differentially among the different layers of the esophageal mucosa ($P<0.005$, Fig. 11A). Its expression in the LP was more than 12-folds greater than the SC and the IC layers ($P<0.04$). Expression in the BC layer was also significantly greater than the IC layer ($P<0.04$).

Wnt Signaling (Intracellular) Components

TCF 3—Comparison of TCF 3 expression between the different mucosal layers also reached statistical significance ($P<0.001$, Fig. 12A). TCF 3 was expressed more than 18-folds greater in the LP compared with the IC layer and SC layer ($P<0.015$) and greater than 5-folds compared with the BC layer ($P<0.03$). Expression in the BC layer was more than 6-folds ($P<0.001$) and 3-folds ($P<0.02$) greater than the IC layer and SC layer, respectively.

DVL 3—The differences in DVL 3 levels between the different layers of the squamous mucosa was determined to be statistically significant ($P<0.01$, Fig. 12B). DVL 3 expression was highest in the LP and was more than 9-folds greater than the IC layer ($P<0.05$). Pair-wise comparison between the other layers did not reach statistical significance.

Immunolocalization of Wnt Signaling Components

The expression and localization of Wnt 1, Wnt 3a, Wnt 5a, Dkk1, Dkk3, Dkk4, and SFRP 1 proteins was also determined by IH.

Wnt Immunolocalization—Wnt1-specific staining was localized primarily in the BC layer (Figs. 2C, D). Wnt 3a-specific staining was also localized primarily in the BC layer (Figs. 4B, C). Wnt 5a-specific staining, however, is observed in both the superficial and the IC layers (Figs. 6B, C). Microphotographs are representative of IH performed on 6 individual esophagi (Wnt1 $\times 100$, 3a $\times 200$, Wnt5a $\times 100$ magnification).

Dkk Immunolocalization—As demonstrated in Figures 8C and D, Dkk1-specific staining was observed predominantly in the SC layer. Dkk3-specific staining was observed in the LP and the BC layer (Figs. 9B, C) whereas Dkk4-specific staining was observed in the SC layer and the IC layer (Figs. 10B, C). Microphotographs are representative of IH performed on 6 individual esophagi (Dkk1, 4 ×100, Dkk3 ×200 magnifications).

SFRP1 Immunolocalization—SFRP 1-specific staining was observed in the LP and the BC layers (Figs. 11B, C). Microphotographs are representative of IH analysis performed on 6 individual esophagi (×100 magnification).

DISCUSSION

The esophageal mucosa consists of stratified squamous epithelium with 3 distinct populations of cells. The layer of cells adherent to the basement membrane that is, the BCs, differentiate into the prickle cells which in turn transform into the stratum corneum cells.¹² Although the BCs are proliferative and give rise to the other cell types, the cells in the stratum corneum are terminally differentiated and can no longer replicate.¹³ A better understanding of the control mechanism of differentiation and proliferation processes in these cells and the influences of various stressors such as acid exposure is crucial for a better understanding of the pathobiology of the esophageal squamous mucosa in disease states such as gastroesophageal reflux disease.

Earlier studies have demonstrated the overexpression of Wnt modulators Dkk1 and Dkk4 in the disease-free squamous mucosa of the esophagitis patients compared with that of healthy controls and patients with Barrett's suggesting the involvement of the Wnt pathway. The present study was therefore undertaken to determine the presence and distribution of Wnt pathway components in the esophageal squamous mucosa in humans. The study findings indicate that within the layers of the human esophageal squamous mucosa various components of the Wnt signaling pathway are distributed in a location-dependent manner.

The Wnt pathway consists of the canonical and the noncanonical pathways. The activation of the canonical pathway leads to cytosolic stabilization and nuclear localization of β -catenin, which in turn associates with TCF/LEF transcription factors resulting in the induction of Wnt-related gene expression.¹⁵ The canonical Wnt-related gene induction leads to cell proliferation.¹⁵ The noncanonical pathway consisting of the PCP, Wnt/JNK, and the Wnt/calcium pathways, does not use the β -catenin/TCF-mediated gene expression and is believed to be nonproliferative and stimulates cell differentiation.^{16,17} Our findings of greater expression of canonical Wnt 1, 2b, and 3a in the BC compared with other layers is consistent with the proposed role of these molecules in stimulating cell proliferation as has been observed in other cells and organs.^{25–27} The presence of canonical Wnt 3²⁵ in the LP in juxtaposition to the BC layer found in this study may also contribute to proliferation of this layer.

In contrast to the preponderance of expression of the canonical Wnt ligands primarily in the BC layer and LP of the esophagus, the expression of noncanonical ligands is distributed differently throughout various layers. In that Wnt 5a is expressed mostly in the differentiated

IC layer but the greatest level of expression of Wnt 5b is observed in the LP and the BC layer. The expression of Wnt 4 on the other hand is not significantly different between the layers. The noncanonical ligand may perform various functions in the squamous mucosa including stimulation of differentiation and modulation of the canonical pathway as observed in other systems.^{15,28,29}

The expression of canonical Wnt signal transducer TCF 3^{25,30} is significantly greater in the BC layer compared with the intermediate and SC layers. The expression of the other membrane bound and intracellular components of the Wnt pathway including FZD 1, LRP 6, and DVL 3 however is similar between the BC, IC, and the SC layers suggesting that TCF 3 may play an important role in the BC layer. As transduction of canonical Wnt signaling through FZD 1 leads to TCF 3-mediated gene induction^{15,25,30} which can stimulate cell proliferation,^{15,16} TCF 3 could be involved in canonical Wnt signaling in BC layer proliferation. The similar expression of FZD 6, a member of noncanonical pathway³¹ throughout the different layers, in conjunction with the noncanonical ligands mentioned above may play various roles including modulation of canonical signaling and stimulation of cell differentiation^{20,31,32} in the squamous mucosal layers.

The function of the Dkk family of proteins has not been directly studied in the esophageal mucosa but Dkk1 has been shown to inhibit the canonical Wnt pathway by binding to LRP 5/6 and not allowing its association with the FZD receptor.^{18,19} The presence of a relatively high concentration of Dkk1 and Dkk4 in the terminally differentiated SCs and their relatively low expression in the BC layer is consistent with their previously described^{33–35} role of inhibiting proliferation and promoting differentiation.

Dkk1 is a downstream target of the canonical Wnt pathway and its induction controls Wnt activity by a negative-feedback mechanism. It can also be induced by pathways not related to Wnt³⁵ such as in a p53-dependent manner and by proapoptotic agents.^{36,37} Apoptosis has been shown to increase at the squamous layer of the esophageal mucosa as a result of excessive exposure to gastroesophageal refluxate.³⁸ Such a role is also suggested by earlier studies showing that the expression of Dkk1 was significantly higher in the normal appearing mucosa of the esophagitis patients compared with Barrett's patients and healthy controls.¹⁴ In this regard, the localization of the Dkk1 molecules at SC layer may be part of an intricate mechanism of cell homeostasis for the removal of damaged cells owing to exposure to various luminal contents not only from the gastroesophageal refluxate but also from oral intake. Increased acid exposure as observed in gastroesophageal reflux disease patients may disrupt this homeostatic mechanism and result in overexpression of Dkk1 in SC layers leading to apoptosis and development of mucosal breaks.

The current study also showed that the secreted Wnt pathway modulators Dkk3 and SFRP 1 are primarily localized at the lamina propria and the BC layers. The role of Dkk3 is not well understood but its levels have been shown to be suppressed in malignant tissues³⁹ and its ectopic expression has been reported to lead to suppressed growth of cultured cells⁴⁰ suggesting that it may be involved in cell proliferation. SFRPs have N-terminal cysteine-rich domains similar to those found in the membrane bound FZD receptors which form complexes with Wnt ligands thereby modulating their activities.⁴¹ As demonstrated for

Dkk3, suppression of SFRP 1 may also lead to malignancy as observed in breast cancer and esophageal adenocarcinoma.^{23,42} These secreted Wnt antagonists therefore may modulate the proliferative activity of the canonical Wnt ligands in the BC layer.

In summary, the present study determined that within the different layers of the esophageal squamous mucosa components of the Wnt signaling pathway including ligands, receptors, transcription factors, intracellular signaling components, and modulating proteins are expressed in a differential manner. Although not studied directly the differential expression of these molecules in the proliferative BC layer and the differentiated IC and SC layers can be speculated to have local effects on the biology and pathobiology of the esophageal squamous mucosa.

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FIGURE 1.

LCM of hematoxylin and eosin-stained full thickness human esophagus. A, Esophageal mucosa before LCM showing the SC layer, the IC layer, the BC layer, and the LP. Cells from each layer were carefully dissected (indicated by the asterisk) using LCM caps. Those LCM caps which captured unwanted cells were discarded. The images after LCM of SC (B), IC (C), BC (D), and LP (E) are shown (100× magnification).

FIGURE 2.

Real-time PCR and IH of Wnt 1 in human esophageal mucosa. A, B, Real-time PCR data for Wnt 1 in laser-capture microdissected SC layer, the IC layer, the BC layer, and the LP. Each sample was run in triplicate and the means were normalized with GAPDH to obtain the expression ratio. The means of the expression ratios of Wnt 1/GAPDH $\pm$ SEM of 7 samples are displayed in A. A representative real-time PCR trace from 7 analyses is also displayed (B). The expression of Wnt 1 was significantly different between the mucosal layers (ANOVA, $P < 0.02$). Wnt 1 expression in the BC layer was significantly greater than the IC layer ($\text{¶}P < 0.04$) and the SC layer ($\text{*}P < 0.02$). Expression in the LP was also significantly greater than the SC layer ($\text{§}P < 0.03$, $n = 7$). C and D, IH of cryosections (10 μ m) of full thickness human esophagus. Wnt 1-specific staining ($\rightarrow$) was observed primarily in the BC layer with lower intensity detectable in the LP (D). Negative IgG-isotype-matched controls on serial sections are also shown (C). Microphotographs are representative of IH performed on 6 esophagi ($\times 100$ magnification).

FIGURE 3.

Real-time PCR analysis of Wnt 2b and Wnt 3 in human esophageal mucosa. Real-time PCR data for Wnt 2b (A) and Wnt 3 (B) in laser-capture microdissected SC layer, the IC layer, the BC layer, and the LP. Each sample was run in triplicate and the means were normalized with GAPDH to obtain the expression ratio. The means of the expression ratios of Wnt 2b/GAPDH $\pm$ SEM (A) and Wnt 3/GAPDH $\pm$ SEM (B) of 7 samples are displayed in the figure. The expression of Wnt 2b was significantly different between the mucosal layers (ANOVA, $P<0.05$). Wnt 2b expression in the BC layer was significantly greater than the IC layer ($*P<0.02$). Expression in the LP was also significantly greater than the IC layer (§ $P<0.025$, $n = 7$). The expression of Wnt 3 was significantly different between the mucosal layers (ANOVA, $P<0.03$). Wnt 3 expression in the LP layer was significantly greater than the BC layer ($*P<0.02$) and the IC layer (§ $P<0.04$, $n = 7$).

FIGURE 4.

Real-time PCR and IH of Wnt 3a in human esophageal mucosa. A, Real-time PCR data for Wnt3a in laser-capture microdissected SC layer, the IC layer, the BC layer, and the LP. Each sample was run in triplicate and the means were normalized with GAPDH to obtain the expression ratio. The means of the expression ratios of Wnt 3a/GAPDH $\pm$ SEM of 7 samples are displayed in A. The expression of Wnt 3a was significantly different between the mucosal layers (ANOVA, $P < 0.02$). Wnt 3a expression in the BC layer was significantly greater than the IC layer ($*P < 0.05$) and the SC layer ($\$P < 0.04$, $n = 7$). B and C, IH of cryosections (10 μ m) of full thickness human esophagus. Wnt 3a-specific staining ($\rightarrow$) was observed predominantly in the BC layer (C). Negative IgG-isotype-matched controls on serial sections are also shown (B). Microphotographs are representative of IH performed on 6 esophagi ($\times 200$ magnification).

FIGURE 5.

Real-time PCR analysis of Wnt 4 and Wnt 5b in human esophageal mucosa. Real-time PCR data for Wnt 4 (A) and Wnt 5b (B) in laser-capture microdissected SC layer, the IC layer, the BC layer, and the LP. Each sample was run in triplicate and the means were normalized with GAPDH to obtain the expression ratio. The means of the expression ratios of Wnt 4/GAPDH $\pm$ SEM of 6 samples are displayed in A. The means of the expression ratios of Wnt 5b/GAPDH $\pm$ SEM of 7 samples are displayed in B. Comparison of Wnt 4 expression between the different layers did not reach statistical significance (ANOVA, $n = 6$). The expression of Wnt 5b was significantly different between the mucosal layers (ANOVA, $P < 0.005$). Wnt 5b expression in the LP layer was significantly greater than the IC layer ($*P < 0.005$) and the SC layer ($§P < 0.02$). Expression in the BC layer was significantly greater than the IC layer ($¶P < 0.008$) and SC layer ($£P < 0.03$, $n = 7$).

FIGURE 6.

Real-time PCR and IH analysis of Wnt 5a in human esophageal mucosa. A, Real-time PCR data for Wnt 5a in laser-capture microdissected SC layer, the IC layer, the BC layer, and the LP. Each sample was run in triplicate and the means were normalized with GAPDH to obtain the expression ratio. The means of the expression ratios of Wnt 5a/GAPDH $\pm$ SEM of 7 samples are displayed in A. The expression of Wnt 5a was significantly different between the mucosal layers (ANOVA, $P < 0.03$). Wnt 5a expression in the IC layer was significantly greater than the BC layer ($*P < 0.002$, $n = 7$). B and C, IH of cryosections (10 μ m) of full thickness human esophagus. Wnt 5a-specific staining ($\rightarrow$) was observed in the IC layer and the SC layer (C). Negative IgG-isotype-matched controls on serial sections are also shown (B). Microphotographs are representative of IH performed on 6 esophagi ($\times 100$ magnification).

FIGURE 7.

Real-time PCR analysis of FZD 1, FZD 6, and LRP 6 in human esophageal mucosa. Real-time PCR data for FZD 1, 6 and LRP 6 in laser-capture microdissected SC layer, the IC layer (IC), the BC layer, and the LP. Each sample was run in triplicate and the means were normalized with GAPDH to obtain the expression ratio. The means of the expression ratios of FZD 1/GAPDH $\pm$ SEM (A) and FZD 6/GAPDH $\pm$ SEM (B) of 7 samples are displayed. The means of the expression ratios of LRP 6/GAPDH $\pm$ SEM of 6 samples are also displayed in C. The expression of FZD 1 was significantly different between the mucosal layers (ANOVA, $P < 0.04$). FZD 1 expression in the LP layer was significantly greater than the SC layer ($*P < 0.02$, $n = 7$). Comparison of FZD 6 expression between the different layers did not reach statistical significance (ANOVA, $n = 7$). The expression of LRP 6 was significantly different between the mucosal layers (ANOVA, $P < 0.02$). LRP 6 expression in the LP layer was significantly greater than the IC layer ($*P < 0.01$, $n = 6$).

FIGURE 8.

Real-time PCR and IH of Dkk1 in human esophageal mucosa. A, B, Real-time PCR data for Dkk1 in laser-capture microdissected SC layer, the IC layer, the BC layer, and the LP. Each sample was run in triplicate and the means were normalized with GAPDH to obtain the expression ratio. The means of the expression ratios of Dkk1/GAPDH $\pm$ SEM of 7 samples are displayed in A. A representative real-time PCR trace from 7 analyses is also displayed (B). The expression of Dkk1 was significantly different between the mucosal layers (ANOVA, $P < 0.002$). Dkk1 expression in the SC layer was significantly greater than LP, BC layer, and the IC layer ($*P < 0.001$, $n = 7$). C and D, IH of cryosections (10 μ m) of full thickness human esophagus. Dkk1-specific staining ($\rightarrow$) was localized predominantly in the SC layer (D). Negative IgG-isotype-matched controls on serial sections are also shown (C). Microphotographs are representative of IH performed on 6 esophagi (Dkk1 $\times 100$ magnification).

FIGURE 9.

Real-time PCR and IH analysis of Dkk3 in human esophageal mucosa. A, Real-time PCR data for Dkk3 in laser-capture microdissected SC layer, the IC layer, the BC layer, and the LP. Each sample was run in triplicate and the means were normalized with GAPDH to obtain the expression ratio. The means of the expression ratios of Dkk3/GAPDH $\pm$ SEM of 6 samples are displayed in A. The expression of Dkk3 was significantly different between the mucosal layers (ANOVA, $P < 0.001$). Dkk3 expression in the LP was significantly greater than the BC layer (* $P < 0.03$), the IC layer (§ $P < 0.003$), and the SC layer (£ $P < 0.003$). Expression in the BC layer was significantly greater than the IC layer (¶ $P < 0.001$) and the superficial layer (# $P < 0.005$, $n = 6$). B and C, IH of cryosections (10 μ m) of full thickness human esophagus. Dkk3-specific staining ($\rightarrow$) was localized primarily in the LP and BC layer (C). Negative IgG-isotype-matched controls on serial sections are also shown (B). Microphotographs are representative of IH performed on 6 esophagi ($\times 200$ magnification).

FIGURE 10.

Real-time PCR and IH of Dkk4 in human esophageal mucosa. A, Real-time PCR data for Dkk4 in laser-capture microdissected SC layer, the IC layer, the BC layer, and the LP. Each sample was run in triplicate and the means were normalized with GAPDH to obtain the expression ratio. The means of the expression ratios of Dkk4/GAPDH $\pm$ SEM of 7 samples are displayed in A. The expression of Dkk4 was significantly different between the mucosal layers (ANOVA, $P < 0.001$). Dkk4 expression in the SC layer was significantly greater than the LP layer (* $P < 0.001$), the BC layer (§ $P < 0.001$), and the IC layer (£ $P < 0.02$). Expression in the intermediate cell layer was significantly greater than the BC layer (¶ $P < 0.05$) and the LP layer (# $P < 0.008$, $n = 7$). B and C IH of cryosections (10 μ m) of full thickness human esophagus. Dkk4-specific staining ($\rightarrow$) was localized primarily in the SC layer and the intermediate cell layer (C). Negative IgG-isotype-matched controls on serial sections are also shown (B). Microphotographs are representative of IH performed on 6 esophagi ($\times 100$ magnification).

FIGURE 11.

Real-time PCR and IH of SFRP 1 in human esophageal mucosa. A, Real-time PCR data for SFRP 1 in laser-capture microdissected SC layer, the intermediate cell layer (IC), the BC layer, and the LP. Each sample was run in triplicate and the means were normalized with GAPDH to obtain the expression ratio. The means of the expression ratios of SFRP 1/GAPDH $\pm$ SEM of 7 samples are displayed in A. The expression of SFRP 1 was significantly different between the mucosal layers (ANOVA, $P < 0.005$). SFRP1 expression in the LP was significantly greater than the intermediate cell layer ($*P < 0.005$) and the SC layer ($\$P < 0.04$). Expression in the BC layer was significantly greater than the intermediate cell layer ($\P P < 0.04$, $n = 7$). B and C, IH of cryosections (10 μ m) of full thickness human esophagus. SFRP 1-specific staining ($\rightarrow$) was localized primarily in the LP and the BC layer (C). Negative IgG-isotype-matched controls on serial sections are also shown (B). Microphotographs are representative of IH analysis performed on 6 esophagi ($\times 100$ magnification).

FIGURE 12.

Real-time PCR analysis of TCF 3 and DVL 3 in human esophageal mucosa. A and B, Real-time PCR data for TCF 3 and DVL 3 in laser-capture microdissected SC layer, the intermediate cell layer (IC), the BC layer, and the LP. Each sample was run in triplicate and the means were normalized with GAPDH to obtain the expression ratio. The means of the expression ratios of TCF 3/GAPDH $\pm$ SEM (A) and DVL 3/GAPDH $\pm$ SEM (B) of 6 samples are displayed in the figure. The expression of TCF 3 was significantly different between the mucosal layers (ANOVA, $P < 0.001$). TCF 3 expression in the LP layer was significantly greater than the BC layer (* $P < 0.025$), intermediate cell layer (§ $P < 0.003$), and the SC layer (£ $P < 0.015$). Expression in the BC layer was significantly greater than the intermediate cell layer (¶ $P < 0.001$) and SC layer (# $P < 0.02$, $n = 6$). The expression of DVL 3 was significantly different between the mucosal layers (ANOVA, $P < 0.01$). DVL 3 expression in the LP layer was significantly greater than the intermediate cell layer (* $P < 0.05$, $n = 6$).

Table 1

Primers for Real-time PCR

Gene	Sense	Antisense
Dkk1	AGCGTTGTTACTGTGGAGAAG	GTGTGAAGCCTAGAAGAATTACTG
Dkk3	GCTTCTTCCTACATCTTCTTCC	AACCCTGCCTGACTCTCC
Dkk4	GGAGGTGCCAGCGAGATG	GGTGCCCAAGTTGTTCCCTTC
DVL 3	CGCCTGATGAGAAGACACAAG	GAGAGTGACCGTGATGATGTTG
FZD 1	GGCACCAAGACCGAGAAG	TAGAAGTAGCAGGCGATGAC
FZD 6	AGCAGCATCCATCTCCAG	GACTAATCCTTCCTTCACTCC
GAPDH	GTCCACTGGCGTCTTCAC	CTTGAGGCTGTTGTCATACTTC
LRP 6	CCACCTTCTCCATACACAG	GCCTCATCCTTCTCTAATAGC
SRRP 1	GGTCTTAGTTCTGGTTGATTC	CCTGCTCTTCCGTATTCTG
TCF 3	CTGTCTCCTGTCTCTTCTC	AAACTTGGGCTGGGTAAC
Wnt 1	CCCCACCAGTGTAGCATCTT	GATTCAAGGAAAAGCCACCA
Wnt 2b	TGTCCTGGCTGGTTCCTTAG	CTTCTACTCTCCCTTCAAATCTCC
Wnt 3	ATGAACAAGCACAACAAC	CTTCTCTACTACCATCTCC
Wnt 3a	ATGAACCGCCACAACAAC	TTCTCCACCACCATCTCC
Wnt 4	GTCTTCGCCGTCTTCTCAG	CTTCCAGGTTCCGCTTGC
Wnt 5a	GCACCAGAGCAGACAACC	TCACAACACGGAGGAATCAG
Wnt 5b	GAGAAGTCCAAGGTCATC	TTCAGTTTAGGGCTTTCC