Supplementary information

Effects of Wnt Signaling on Epithelial-Mesenchymal Transition in Chronic Rhinosinusitis with Nasal Polyp

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Supplementary Methods

Murine model of chronic rhinosinusitis with polypoid lesions and tissue preparations

$Apc^{Min/+}$ mice on the C57BL/6J genetic background were purchased from the Jackson Laboratory (Bar Harbor, ME). Twenty female BALB/c mice (4 weeks of age, 13.5-15.5 g) were purchased from Orient Bio Inc. (Seongnam, Gyeonggi, Republic of Korea). The mice were maintained under specific pathogen-free conditions with a 12/12-hour light/dark cycle. This study was approved by the Institutional Animal Care at the Clinical Research Institute of Dankook University Hospital (No. DKU-16-027). Mice in the experimental groups were systemically sensitized with 25 μg of ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO) dissolved in 200 μl of phosphate buffered saline (PBS) in the presence of 2 mg of aluminum hydroxide gel as an adjuvant by intraperitoneal injection on days 0 and 5. One week after the second intraperitoneal injection, mice were challenged intranasally with 100 μl OVA diluted in 20 μl of PBS daily for one week. After that, continual local stimulation was maintained by the same procedure three times per week for 12 consecutive weeks. During the last eight straight weeks, 20 ng of SEB diluted in 20 μl of PBS was used as a challenge immediately after the administration of 3% OVA weekly. Additionally, Indocyanine green-001 (Selleckchem, Houston, TX; 2.5 mg/kg) or dexamethasone (1 mg/kg) were administered intraperitoneally each week from 5 weeks through 12 weeks before OVA and SEB administration, respectively. Mice were randomly assigned to control or experimental group. All mice gained 20.0-29.5 g in weight and they were healthy at the last challenge day. Mice were sacrificed 24 hours after the last OVA challenge and all mice included in the experiment were used for analysis (20/20 for first experiment and 20/20 for second experiment, respectively). The heads of 5 mice from each group were removed *en bloc* and then fixed in 4% paraformaldehyde for histopathologic analysis. After exposing the nasal cavity out of the head of the other mice, the nasal mucosa

was taken out meticulously using small curettes and micro-forceps under the microscopic vision.

RT2 Profiler PCR Array tests

A custom RT2 Profiler PCR Array (PAMM-090ZA; Qiagen, Germany) that could simultaneously detect 84 genes related to the Wnt signaling pathway was used. Genomic DNA contamination, reverse transcription, and positive PCR controls were included in each 96-well set on each plate. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as the assay reference gene. Total RNA was isolated from each tissue sample using TRIzol Reagent (Life Technologies, Carlsbad, CA) and quantified with Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA). The RT2 Profiler PCR Array tests were performed following the instructions of the manufacturer. Briefly, all cDNA in each sample (20 μl) was first diluted with RNase-free water. Then the diluted cDNA was mixed with RT2 SYBR Green qPCR Mastermix (catalog No. 330529; Qiagen) and RNase-free water. Of that mixture, 20 μl per well was added into the 96 wells of the array plate. The qPCR was carried out using the Applied Biosystems 7500 Real-Time PCR System (software ver. 2.0.6; Applied Biosystems, Foster City, CA) under the following thermal cycling conditions: 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The exported Ct values were input to a template Excel file provided by SABiosciences (Qiagen) and uploaded for the online analysis. After data review, qualified data from 35 CMT and 5 NMGT samples were analyzed by applying the $2^{-\Delta\Delta Ct}$ method.

Culture of human nasal epithelial cells, IF, and immunoblotting

Normal human nasal epithelial cells (hNECs, PromoCell, Heidelberg, Germany) were cultured

in airway epithelial cell growth medium (PromoCell). Antibiotics (1% penicillin and streptomycin) and an antifungal agent (fungizone, [Life technologies, Grand island]; 1 ml/1,000 ml of medium) were added to the medium (Clonetics Corp, Basel, Switzerland). hNECs were cultured in 1% fetal bovine serum (FBS)-supplemented medium for one hour before Wnt3a (R&D Systems, Minneapolis, MN) stimulation. Wnt3a (200 ng/ml) was added to the reservoirs in fresh medium supplemented with 10% FBS.

After washing with PBS, hNECs were fixed in 4% paraformaldehyde (Biosesang, Seoul, South Korea) for 15 minutes at room temperature. Fixed cells were permeabilized for 30 minutes at 37°C with Triton X-100 in PBS and then blocked for one hour at room temperature with 3% bovine serum albumin (BSA) in PBST (PBS + 0.5% Tween 20). Cells were incubated overnight at 4°C with primary antibody (E-cad [610181; BD bioscience, Franklin Lakes NJ] and α-SMA [ab5694; Abcam, Cambridge, UK]). After washing with PBST, cells were then stained with the Alexa Flour 488 IgG anti-rabbit (for E-Cad) and Alexa Flour 555 IgG anti-mouse (for α-SMA), which were diluted in PBST with 1% BSA. F-actin was stained with Alexa Flour 647 phalloidin (Invitrogen, Carlsbad, CA) for 60 min at room temperature. After primary antibody incubations, cells were then incubated with immunofluorescence secondary antibodies (Invitrogen). To visualize the nucleus, cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO). The slides were covered with mounting medium (Dako, Santa Clara, CA). Cells were analyzed using an inverted laser-scanning microscope (Carl Zeiss Microscopy, Göttingen, Germany). The images were scanned under a ×40 oil immersion objective. Co-localization of target proteins was analyzed using Zeiss confocal software (ZEN Lite, Göttingen, Germany).

For immunoblotting, proteins were separated in 8-12% sodium dodecyl sulfate-polyacrylamide gels, transferred to Immobilon-P (Millipore, Billerica, MA). Membranes were incubated

sequentially with indicated primary and secondary antibodies. Membranes were incubated with HRP-conjugated secondary antibodies (1:5000) for one hour at room temperature and visualized by Luminata western HRP chemiluminescence substrates (Millipore), ECL Plus, or ECL Select detection reagents (GE Healthcare, San Diego, CA). Immunoblotting for GAPDH (sc-47724; Santa cruz, Dallas, TX) served as a protein loading control.

Supplementary Figure Legends

Figure S1. Epithelial to mesenchymal transition (EMT) markers in wild type and $Apc^{Min/+}$ mice. (A) Immunohistochemical intensity of β-catenin. (B) The signal intensity of Sox6. OVA, ovalbumin; PBS, phosphate-buffered saline; SEB, staphylococcal enterotoxin B; WT, wild type. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure S2. EMT is exaggerated in the polypoid lesions of $Apc^{Min/+}$ NP mice. Immunohistochemical expression is scored from 0 to 3 in a high-power field (HPF) which is measured in the epithelial layer of the phosphate-buffered saline (PBS)-instilled control group and non-polypoid or polypoid lesions from the ovalbumin (OVA)/staphylococcal enterotoxin B (SEB) induced NP group. (A and B) The expression score of epithelial markers (E-cadherin and β-catenin). (C) The expression score of mesenchymal marker (α-SMA). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure S3. EMT-related gene expression analyzed using PCR array. Heatmap with clustering dendrogram representing 96 EMT-related genes of littermate wild type mice (n = 3) and $Apc^{Min/+}$ mice (n = 3).

Figure S4. Epithelial changes and thickness of sinonasal mucosa from both animal experiments. (A) Epithelial changes defined as the ratio of inflamed mucosal tissue length and total epithelial length in the same location of each group. (B) Percentage of epithelial changes in both mice groups. (C) Epithelial thickness of wild type (WT) and $Apc^{Min/+}$ mice. (D) Epithelial thickness of BALB/c mice. DEX, dexamethasone; OVA, ovalbumin; PBS, phosphate-buffered saline;

SEB, staphylococcal enterotoxin B. *$P < .05$, **$P < .01$, ***$P < .001$.

Figure S5. Proliferation and differentiation markers and TGF-β1 signaling in the $Apc^{Min/+}$ NP models. (A-C) The signal intensity of Ki-67, cilia of epithelial cells stained with acetyl-α-tubulin, and the number of goblet cells stained with Periodic Acid Schiff in $Apc^{Min/+}$ mice. (D and E) Immunohistochemical intensities of TGF-β1 and pSmad-3 (a downstream molecule of TGF-β1)-positive cell counts in the epithelial layer of wild type C57BL/6J and $Apc^{Min/+}$ mice. HPF, high-power field. OVA, ovalbumin; PBS, phosphate-buffered saline; SEB, staphylococcal enterotoxin B; WT, wild type. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure S6. Local and systemic inflammation is increased in $Apc^{Min/+}$ mice. (A and B) IL-4 and IFN-γ-positive cell counts per HPF. (C) Total IgE and specific IgE levels in serum analysis. (D) Comparing spleen size between WT and $Apc^{Min/+}$ mice. HPF, high-power field; Ig, Immunoglobulin; OD, optical density; OVA, ovalbumin; PBS, phosphate-buffered saline; SEB, staphylococcal enterotoxin B. *$P < .05$, **$P < .01$, ***$P < .001$

Figure S7. EMT markers in the Wnt signaling inhibition experiments with BALB/c mice. (A) Immunohistochemical intensity of β-catenin. (B) The signal intensity of Sox6. DEX, dexamethasone; OVA, ovalbumin; PBS, phosphate-buffered saline, SEB, staphylococcal enterotoxin B. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure S8. EMT is promoted in the polypoid lesions of BALB/c mice. (A-C) The expression

score (0/1/2/3) of E-cadherin, β-catenin, and α-SMA of PBS-instilled control group and OVA/SEB-induced NP group. OVA, ovalbumin; PBS, phosphate-buffered saline, SEB, staphylococcal enterotoxin B. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure S9. The effect of ICG-001 treatment on **p**roliferation and differentiation markers, and TGF- β1 signaling. (A) The intensity of Ki-67 in the epithelial layer. (B) The intensity of cilia of epithelial cells stained with acetyl-α-tubulin. (C) The number of goblet cells stained with Periodic Acid Schiff. (D and E) TGF-β1 and pSmad-3 expression of BALB/c mice. DEX, dexamethasone; HPF, high-power field; OVA, ovalbumin; PBS, phosphate-buffered saline, SEB, staphylococcal enterotoxin B. *$P < .05$, **$P < .01$, ***$P < .001$.

Figure S10. Wnt target genes are overexpressed in CRSwNP. (A) The mRNA expression levels of vascular endothelial growth factor (VEGF), angiogenin, tissue inhibitor of metalloproteinase (TIMP)1, and TIMP2. (B) Cyclin D1-positive cell counts in a HPF (x400). (C) TGF-β1 intensity in the epithelial layer of control and NP tissues. (D) pSmad-3-positive cell counts in the sinonasal epithelium. CRSsNP, chronic rhinosinusitis without nasal polyp; CRSwNP, chronic rhinosinusitis with nasal polyp; HPF, high-power field; UP, uncinate process. *$P < .05$, **$P < .01$, ***$P < .001$.

Table S1. List of primary antibodies for immunohistochemistry and immunofluorescence

Primary antibody (Target species)	Type	Source	Cat. Number	Dilution
Ki-67 (Mouse)	Rabbit IgG polyclonal	Invitrogen (Carlsbad, CA)	PA5-19462	1:200
Acetyl-α-tubulin (Mouse)	Rabbit IgG Polyclonal	Cell Signaling (Danvers, MA)	5335	1:1500
Sox6 (Mouse)	Rabbit IgG polyclonal	Novus Biologicals (Centennial, CO)	NBP185811	1:200
Twist (Mouse)	Rabbit IgG polyclonal	Abcam (Cambridge, UK)	ab49254	1:100
TGF-β1 (Mouse)	Rabbit IgG polyclonal	Abcam (Cambridge, UK)	ab92486	1:1000
WNT3A (Mouse)	Rabbit IgG Monoclonal	Cell Signaling (Danvers, MA)	2721	1:100
Nuclear β-catenin (Mouse)	Rabbit IgG polyclonal	Santa Cruz (Dallas, TX)	SC-7963	1:200
Cyclin D1 (Mouse)	Rabbit IgG Monoclonal	Abcam (Cambridge, UK)	ab134175	1:200
Vimentin (Mouse)	Chicken IgY Polyclonal	Novus Biologicals (Centennial, CO)	NB300-223	1:200
IL-17 (Mouse)	Rat IgG1 Monoclonal	LSBio (Seattle, WA)	LS-B4912	1:200
α-smooth muscle actin (Mouse)	Rabbit IgG Polyclonal	Abcam (Cambridge, UK)	ab5964	1:200
β-catenin (Mouse)	Rabbit IgG Monoclonal	Cell signaling (Danvers, MA)	8480	1:100
E-cadherin (Mouse)	Rabbit IgG Polyclonal	Cell signaling (Danvers, MA)	4065	1:100
Neutrophil (Mouse)	Rat IgG2b Monoclonal	Abcam (Cambridge, UK)	ab2557	1:50

IL-4 (Mouse)	Rat IgG1 Monoclonal	R&D systems (Minneapolis, MN)	MAB404R	1:100
IFN-γ (Mouse)	Rat IgG2a Monoclonal	R&D systems (Minneapolis, MN)	MAB785	1:100
p-Smad3 (Mouse and human)	Rabbit IgG monoclonal	Abcam (Cambridge, UK)	ab52903	1:100
Cyclin D1 (Human)	Rabbit IgG Monoclonal	Abcam (Cambridge, UK)	ab134175	1:500
TGF- β1 (Human)	Rabbit IgG Polyclonal	Abcam (Cambridge, UK)	Ab92486	1:1000
Wnt3a (Human)	Rabbit IgG Monoclonal	Cell signaling (Danvers, MA)	2721	1:100
Frizzled-1 (Human)	Rabbit IgG Polyclonal	Novus Biologicals (Centennial, CO)	NLS4150	1:200
Frizzled-3 (Human)	Rabbit IgG Polyclonal	Novus Biologicals (Centennial, CO)	NLS4454	1:250
Nuclear β-catenin (Human)	Rabbit IgG Monoclonal	Cell Signaling (Danvers, MA)	8814	1:100
E-cadherin (Human)	Mouse IgG2a Monoclonal	BD (Franklin Lakes, NJ)	610181	1:200
α-smooth muscle actin (Human)	Rabbit IgG Polyclonal	Abcam (Cambridge, UK)	ab5694	1:200
F-actin, Alexa 647-phalloidin		Invitrogen (Carlsbad, CA)	A-22287	1:200
IF 2nd Ab-Green	Mouse IgG Polyclonal	Invitrogen (Carlsbad, CA)	DI-2488	1:400
IF 2nd Ab-Red	Rabbit Polyclonal	Invitrogen (Carlsbad, CA)	A21207	1:400
DAPI	-	Sigma-Aldrich (St. Louis, MO)	D9542	1:1000

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172 **Table S2.** List of mouse gene-specific TaqMan probes for real-time qPCR analysis

Genes TaqMan Primer	Assay ID
IL-4	Mm00445258_g1
IL-5	Mm01290072_g1
IL-17A	Mm00439618_m1
IFN-γ	Mm99999071_m1
GAPDH	Mm03302249_g1

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174 **Table S3.** List of human gene primers used for real-time qPCR analysis

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
WNT1	CTTCGGCAAGATCGTCAACC	GTGCAGGATTCGATGGAACC
WNT3A	GGCTGTTGGGCCACAGTATTCC	GCTGGGCATGATCTCCACGTAG
WNT5B	TCAAGAGAGCGAGAAGACTGG	GTCTGTCAGAAGCTGAGCCC
WNT8A	CTGATAACAGGTCCCAAGGC	ACTTCTCAGCCTGTTGTGGG
β-catenin	CCAGGTGGTGGTTAATAAGG	CTGAGGAGAACGCATGATAG
FZD1	AGCGCCGTGGAGTTCGT	CGAAAGAGAGTTGTCTAGTGAGGAAAC
FZD2	CACGCCGCGCATGTC	ACGATGAGCGTCATGAGGTATTT
FZD3	GGTGTTCCTTGGCCTGAAGA	CACAAGTCGAGGATATGGCTCAT
VEGF	CTACCTCCACCATGCCAAGT	GCAGTAGCTGCGCTGATAGA
Angiogenin	CATCATGAGGAGACGGGG	TCCAAGTGGACAGGTAAGCC
TIMP1	AGACCTACACTGTTGGCTGTGAG	GACTGGAAGCCCTTTTCAGAG
TIMP2	ATGCACATCACCCTCTGTGA	CTCTGTGACCCAGTCCATCC
GAPDH	ACTCATCAACCCTCCCCCG	GGCTGAGTTCCTGCTGTCTT

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176 **Table S4.** List of antibodies for immunoblotting

Antibody	Type	Source	Cat. Number	Dilution
E-cadherin	Rabbit IgG Monoclonal	Cell signaling (Danvers, MA)	#3195	1:2000
N-cadherin	Rabbit IgG Polyclonal	Santa Cruz (Dallas, TX)	sc-7939	1:2000
β-catenin	Mouse IgG1 Monoclonal	Santa Cruz (Dallas, TX)	sc-7963	1:2000
α-smooth muscle actin	Rabbit IgG Polyclonal	Abcam (Cambridge, UK)	ab5694	1:2000
GAPDH	Mouse IgG Monoclonal	Santa Cruz (Dallas, TX)	sc-47724	1:2000
Rabbit IgG HRP	Goat IgG Polyclonal	Invitrogen (Carlsbad, CA)	G21234	1:5000
Mouse IgG HRP	Goat IgG Polyclonal	Invitrogen (Carlsbad, CA)	G21040	1:5000

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178 **Table S5.** Over-expressed genes for epithelial to mesenchymal transition in the $Apc^{Min/+}$ mice
179 relative to the control mice

Gene symbol	Gene name	Fold change
Bmp1	Bone morphogenetic protein 1	2.56
Cdh2	Cadherin 2	6.65
Fn1	Fibronectin 1	2.38
Foxc2	Forkhead box C2	2.88
Gng11	Guanine nucleotide binding protein (G protein), gamma 11	6.64
Gsc	Goosecoid homeobox	8.00
Itga5	Integrin alpha 5 (fibronectin receptor alpha)	5.90
Mmp3	Matrix metallopeptidase 3	2.76
Mmp9	Matrix metallopeptidase 9	2.92
Serpine1	Serine (or cysteine) peptidase inhibitor, clade E, member 1	5.74
Snai1	Snail homolog 1	6.48
Snai2	Snail homolog 2	2.00
Snai3	Snail homolog 3	11.10
Sox10	SRY-box containing gene 10	5.05
Tmeff1	Transmembrane protein with EGF-like and two follistatin-like domains 1	2.32
Tmem132a	Transmembrane protein 132A	6.62
Twist1	Twist homolog 1	3.13
Vcan	Versican	4.28
Vps13a	Vacuolar protein sorting 13A	2.32
Wnt5b	Wingless-related MMTV integration site 5B	6.45

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