Effects of *Fusobacterium nucleatum* on disruption of tight junction in human bronchial epithelial cell layer and mouse lung

Yukihiro Karahashi

Nihon University Graduate School of Dentistry,

Major in Periodontology

(Directors: Prof. Shuichi Sato and Prof. Kenichi Imai)

Contents

This doctoral thesis was prepared using the original article "*Fusobacterium nucleatum* putatively affects the alveoli by disrupting the alveolar epithelial cell tight junction, enlarging the alveolar space, and increasing paracellular permeability" (Yukihiro Karahashi, Marni E. Cueno, Noriaki Kamio, Yuwa Takahashi, Ikuko Takeshita, Kaori Soda, Shuichiro Maruoka, Yasuhiro Gon, Shuichi Sato, and Kenichi Imai. Biochem Biophys Res Commun 682 (2023), 216-222.) with new unpublished data (Fig. 2C).

Abstract

The global population is aging, and elderly people have a higher incidence of lower airway diseases owing to decline in swallowing function, airway ciliary motility, and overall immunity associated with aging. Furthermore, lower airway diseases in the elderly tend to have a high mortality rate. *Fusobacterium nucleatum* (Fn) is abundant in the human oral cavity and has been associated with periodontal disease, which in-turn has been linked to respiratory disease development. Tight junctions (TJs) line the airway and alveoli surfaces serving as a first line of defense against multiple pathogens. Fn has already been linked to respiratory diseases, however, how Fn affects the alveolar TJ was not fully elucidated. Here, a TJ network was designed and analyzed, Fn cells were grown and inoculated *in vitro* (16HBE and primary cells) and *in vivo* (mice lung), measured transepithelial electrical resistance, performed RT-PCR, checked for *in vitro* cell and mice lung permeability, and determined air space size through morphometric measurements. Results show that Fn can potentially affect TJs proteins that are directly exposed to the alveolar surface. Additionally, Fn could possibly cause neutrophil accumulation and an increase in alveolar space. Moreover, Fn putatively may cause an increase in paracellular permeability in the alveoli.

Keywords: Periodontal diseases; *Fusobacterium nucleatum*; Respiratory diseases; Tight junction; Bronchial epithelial cell

Introduction

The oral cavity is the entry point for bacteria and viruses to enter the body, and it is also the entrance to the lower airway, including the bronchi and lungs, in which inflammation occurs during lower airway diseases. Therefore, if aspiration of oral bacteria has an adverse effect on lower airway diseases, it is not difficult to imagine such an effect. In fact, a number of clinical studies have shown that "chronic periodontitis and oral bacteria" are associated with the onset, progression, and exacerbation of the following diseases: pneumonia, influenza, and chronic obstructive pulmonary disease [1]. On the other hand, it has been reported that oral health management, including oral care, is effective in preventing the onset of lower airway diseases such as aspiration pneumonia and influenza [1]. Therefore, active oral health care is now being provided mainly to elderly people who need nursing care.

Fusobacterium species are anaerobic opportunistic pathogens commonly found in mammalian mucosal sites [2]. Among the known *Fusobacterium* species, *Fusobacterium nucleatum* (Fn) is abundant in the human oral cavity and often associated with periodontal disease and implicated in several systemic diseases, including respiratory diseases [3, 4, 5]. The surfaces of both the airways and alveoli have an epithelial cell layer that has a barrier function serving as a first line of defense against multiple pathogens and is dependent on tight junctions (TJs) [6]. Briefly, TJs are heteromeric protein complexes that comprise the sealing interface between adjacent epithelial cells and TJ damage has been linked to epithelial barrier breakdown which often is associated with multiple airway diseases [6, 7]. Moreover, TJs are mainly comprised of two integral protein families, namely: occludin which has extracellular loops of the same size and claudin which has an extracellular loop larger than the other [8, 9]. Considering Fn has already been

associated with respiratory diseases, it would be interesting to determine the effects of Fn on an alveoli (in particular alveolar TJs). However, this was not fully elaborated.

Materials and Methods

Network design and analyses of the tight junction pathway

TJ network design was based on the tight junction reference pathway registered in the KEGG Pathway Database (http://www.genome.jp/kegg/pathway.html). The Cytoscape software was used to design and connect the different components that comprise the TJ pathway into one holistic and comprehensible framework [10]. Briefly, nodes represent the different components associated in the TJ pathway, particularly those involved in cell polarity and paracellular permeability, whereas, edges represent interaction between the TJ components. Similarly, network analyses were established using the Cytoscape software via centrality measurements [10]. For this study, the following centrality measurements were performed: (1) closeness centrality to find the TJ component that has a functional relevance to other TJ components; (2) betweenness centrality to determine the TJ component crucial to maintain coherence and functionality; (3) stress centrality to elucidate the importance of a TJ component; (4) eccentricity centrality to highlight how easily influenced the TJ component can be affected; and (5) edge betweenness centrality to emphasize the degree of connection between two TJ components [11]. Briefly, for each centrality measurement made, the threshold was first established and, afterwards, the significance of a centrality measurement was based on whether the nodal or edge values were higher than the threshold established. Additionally, common significant TJ components were made based on the results from all centrality measurements that were combined into one network design (unified network).

Bacterial strains and culture

Fn ATCC 25586 was grown in brain-heart infusion (BHI) broth supplemented with 5 μg/mL hemin and 0.5 μg/mL menadione. The bacterial culture was incubated under anaerobic conditions (80% N_2, 10% H_2, and 10% CO_2) at 37ºC using an anaerobic chamber (ANX-3; Hirasawa, Tokyo, Japan) for 24 h. The bacterial cell density was adjusted to 1.0×10^{10} colony-forming units (CFU)/mL, and the bacterial suspension was heat-killed at 60ºC for 1 h and subsequently stored at -80ºC until use.

Cell culture

Human bronchial epithelial cell line, 16HBE cells, was maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37ºC in a 5% CO_2 atmosphere.

Primary human bronchial epithelial cells (ScienCell Research Laboratories, Carlsbad, CA, USA) were cultured in bronchial epithelial cell medium with growth supplement following the manufacture's recommendations. Primary cell growth medium and growth supplement were purchased from ScienCell Research Laboratories. During culturing, primary cells were incubated at 37ºC in an atmosphere of 5% CO_2 for 48 h.

Transepithelial electrical resistance (TER) measurements

Transepithelial electrical resistance (TER) measurements were performed as previously described [11, 12]. Briefly, 16HBE cells (1×10^5 cells/well) and primary cells (5×10^5 cells/well) were seeded into Transwell chambers (Corning, Kennebunk, ME, USA) and treated with Fn (0.75×10^8, 1.0×10^8, 1.25×10^8, 1.5×10^8 CFU/mL). TER was evaluated using Millicell ERS-2 (Millipore, Burlington, MA, USA)

according to manufacturer's instruction. TER (ohms × cm^2) was calculated following the equation: (TER sample - TER blank) × surface area (cm^2).

Cell permeability assay

Cell monolayer permeability was assessed by fluorescein isothiocyanate (FITC)-dextran fluxes across this layer. A solution of 4 kDa FITC-dextran (1 mg/mL) (Sigma-Aldrich) was added to the apical compartment of Transwell insert. Afterwards, the supernatant was harvested from the basal component at 60 min after addition of FITC-dextran. FITC-dextran intensity in the supernatant was measured using a fluorescent microplate reader 6 for excitation at 485 nm and emission at 535 nm.

Real-time polymerase chain reaction

Cells were treated with Fn (1.0×10^8 CFU/mL) for 12 h. Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA synthesis from total RNA was performed using PrimeScript RT Master Mix (Takara Bio, Kusatsu, Japan). The following primers were used in this study: E-cadherin (E-cad), forward (5′ -AAG GTG ACA GAG CCT CTG GAT AGA-3′) and reverse (5′ -CAT TCC CGT TGG ATG ACA CA-3′); Occludin, forward (5′-CGA GGA GTG GGT TAA AAA TGT GT-3′) and reverse (5′ -ACT GTC AAC TCT TTC CAC ATA GTC AGA-3′); ZO-1, forward (5′ -CGG TCC TCT GAG CCT GTA AG-3′) and reverse (5′ -GGA TCT ACA TGC GAC GAC AA-3′); ZO-2, forward (5′ -GAG GAG TAG GAG CAG GAG CA-3′) and reverse (5′ -GGA GCC AAC CTG ACA GCT C-3′); JAM-A, forward (5′ -CCG TGT GGA GTG GAA GTT TGA-3′) and reverse (5′ -TCA CCC GGT CCT CAT AGG AA-3′); CLDN1, forward (5′ -GGA TTT ACT CCT ATG CCG GCG ACA

ACA-3′) and reverse (5′ -CTC TGC GAC ACG CAG GAC ATC CA-3′); GAPDH, forward (5′ -ACC AGC CCC AGC AAG AGC ACA AG-3′) and reverse (5′ -TTC AAG GGG TCT ACA TGG CAA CTG-3′). cDNA amplification and detection were accomplished using a TP-800 Thermal Cycler Dice Real-Time System (Takara Bio) with TB Green Premix Ex Taq (Takara Bio). Relative quantification of gene expression was determined by using the ΔΔCt method.

Animal study

All animal experimentation was approved by the Nihon University Animal Care and Use Committee (AP16D047, AP18D049, AP22DEN049). Male C57BL/6J mice were obtained from CLEA Japan (Tokyo, Japan). All mice were allowed free access to food and water, while kept in a temperature-controlled room (23ºC) on a 12 h light/dark cycle. The mice were euthanized by CO_2 asphyxiation before harvesting lung specimens or whole blood.

Morphometric measurements of air space size

Mice were anesthetized with isoflurane and intratracheally inoculated with 50 μL Fn (1.0×10^8 CFU) once per day for 3 days. To measure air space size, lung specimens were harvested 1 day after the final Fn inoculation and fixed with 4% paraformaldehyde, embedded in paraffin, cut out as thin sections with 4 μm thickness, and stained with both hematoxylin and eosin. The liner intercept of 50 alveoli were measured.

Mice lung permeability assay

The mice were anesthetized with isoflurane and intratracheally inoculated with 50 μL Fn (1.0×10^8

CFU) once per day for 3 days. At 1 day after the final Fn inoculation, mice were euthanized after 1 h inoculation of FITC-dextran. Subsequently, whole blood was collected from the abdominal aorta. Serum was separated by centrifuging blood samples and stored at -80ºC until use. FITC-dextran intensity in the serum was measured using a fluorescent microplate reader for excitation at 485 nm and emission at 535 nm.

Statistical analysis

All statistical analyses were performed using KALEIDAGRAPH (Synergy Software, Reading, PA, USA). Student's *t*-test was used for comparing the means of two groups, and one-way ANOVA with Tukey's post *hoc* analysis was used for comparisons of more than three groups. Significance level was defined by at least $p < 0.05$. All data are expressed as mean ± standard deviation (SD).

Results

ZO-1 and ZO-1-associated TJ proteins are significant

TJs are important structures giving epithelial tightness and preventing both exudate formation and inflammatory perturbations on airway epithelium function [6]. To elucidate the significant airway TJs that could potentially affect cell polarity and permeability, TJ network design and centrality measurements were performed. The results identified significant TJ components based on stress (Fig. 1A), betweenness (Fig. 1B), closeness (Fig. 1C), eccentricity (Fig. 1D), and edge betweenness (Fig. 1E). Similarly, based on the unified network (Fig. 1F), the results found that ZO-1, cingulin, GEF-H1, and RhoA are significant when considering the whole TJ network. Interestingly, the results show that ZO-1 is a common significant component in the TJ network. ZO-1 is a TJ-linked protein in several epithelia [14] and has been associated to decreased barrier function in different airway disease models [7] highlighting its importance in the airway epithelium. Moreover, considering eccentricity measurements (Fig. 1D), significant ZO-1-associated TJ components (occludin, claudin, JAM-A) found in the airway epithelium that has the potential to be easily affected by external factors likewise emphasizing the importance of these TJ components in maintaining the barrier function of the airway epithelium [6, 7, 15]. Taken together, the results at this point would suggest that the following components of the TJ network: occludin, ZO-1, JAM-A, and claudin are putatively affected during an oral disease.

Fn affects TJ barrier function in both immortalized and primary alveoli epithelial cells

TJs play a part in maintaining paracellular resistance in the interstitial space between epithelial cells [16]. To determine the effects of varying Fn concentration on the TJ of alveoli epithelial cells, TER measurements

were done and, similarly, qRT-PCR was performed to determine gene expression levels of certain TJ components, namely: occludin, ZO-1, JAM-A, and claudin [particularly claudin 1 (CLDN1) since it is ubiquitously expressed along airway epithelium [17]] in-line with earlier results (Fig. 1) and ZO-2 since it is a scaffold protein necessary for TJ formation [18]. Additionally, a representative adherens junction (E-cad) was likewise included in qRT-PCR assay for comparison against the selected TJs. Moreover, both immortalized (16HBE) and human primary alveoli epithelial cells were used. For the 16HBE cells, results show that Fn presence (regardless of concentration) resulted in lower TER measurements (Fig. 2A, *left panel*), whereas, for the primary cells, only higher Fn concentrations resulted to significantly lower TER measurements (Fig. 2B, *left panel*). Furthermore, Fn-treated 16HBE cells also had increased paracellular permeability (Fig. 2C). These results would suggest that Fn can putatively affect TJ in both the 16HBE and primary alveoli epithelial cells.

Moreover, for the 16HBE cells, results show that occludin, ZO-2, and JAM-A gene expression were significantly affected by Fn presence while CLDN1 has a tendency to be similarly affected by Fn presence (Fig. 2A, *right panel*). Interestingly, for the primary cell, only JAM-A and CLDN1 were significantly affected by Fn presence (Fig. 2B, *right panel*). These results would imply that ZO-1 gene expression was not significantly affected by Fn presence and only TJ proteins exposed to the surface were affected. Similarly, E-cad was unaffected by Fn presence insinuating that only TJs are affected by Fn. ZO-1 is necessary for proper TJ strand formation [18], however, ZO-1 is dispensable for barrier function [19] while ZO-2 is necessary for barrier function [20]. In this regard, the results suggest the following: (1) ZO-1 (a cytoplasmic protein) was unaffected by Fn presence since ZO-1 was not directly exposed to Fn; and (2) possible direct Fn exposure to alveolar epithelial cells can negatively affect gene expression of TJ exposed on the alveolar surface.

It is worth mentioning that TJ gene expression between 16HBE and primary cells differed with Fn presence. This observation was attributed to the difference of using immortalized and primary cells in *in vitro* experimentation related to airway pathophysiology [21, 22]. In particular, primary cells maintain normal cellular response under a given condition, whereas, immortalized cells lose this ability [22]. Thus, the results obtained using 16HBE cells demonstrate how Fn may affect TJ gene expression in the absence of a cellular response (Fn affects all TJs involved in barrier function), whereas, results obtained using primary cells show how Fn may influence TJ gene expression with a normal cellular response (Fn only affects JAM-A and CLDN1).

Fn affects mice alveolar tissue

One key diagnostic marker for respiratory distress and injury is the breakdown of airway epithelial function which is correlated to TJ breakdown [6, 23]. To elucidate Fn effects on alveolar tissue, Fn inection was simulated on mice alveolar tissue *in vivo* and checked for TJ gene expression, alveoli distance, and paracellular permeability. For TJ gene expression, based on earlier results, both CLDN1 and JAM-A were likewise checked (Fig. 2B, *right panel*). Results show that both CLDN1 (Fig. 3A) and JAM-A (Fig. 3B) gene expression were likewise decreased with Fn presence consistent with earlier results. Both CLDN1 and JAM-A are essential to maintain airway barrier function with CLDN1 providing TJ sealing properties [24] while JAM-A promote TJ assembly [25]. These results would suggest that Fn presence along the alveolar tissue is capable of disrupting TJ barrier function. Moreover, histological staining of mice alveolar tissues shows the difference in alveolar space before Fn-treatment (Fig. 3C, *left panel*) and after Fn-treatment (Fig. 3C, *right panel*). Noticeably, the results only observed neutrophil accumulation among Fn-treated mice alveolar tissues (Fig. 3C, *upper right lower panel*). Neutrophils are critical for host defense in the lungs and

neutrophil accumulation is a hallmark of bacterial infection within the lungs which in-turn may lead to lung disease development [26]. Additionally, in the Fn-treated mice alveolar tissue, increase in alveolar distance measurements was observed (Fig. 3D). Increase in alveolar space is consistent with lung infection [27]. Furthermore, Fn-treated mice also had increased paracellular permeability (Fig. 3E) indicating less restriction in airway epithelial response which coincidentally is also consistent with lung infection [28]. It is worth mentioning that neutrophil accumulation has been associated with paracellular permeability [29]. Considering that Fn presence has resulted to neutrophil accumulation (Fig. 3C), increased alveolar space (Fig. 3D), and increased paracellular permeability (Fig. 3E), it was likewise postulated that Fn-related increase in alveolar space and paracellular permeability may putatively be associated with neutrophil accumulation owing to the tissue-destructive potential of neutrophils [26]. Taken together, it was suspected that Fn causes alveolar TJ barrier function breakdown (particularly in CLDN1 and JAM-A) which in-turn results to neutrophil accumulation leading to an increase in alveolar space and paracellular permeability, thereby, resulting in respiratory distress.

Discussion

Fn is a commensal bacteria that could become pathogenic leading to the development of oral diseases and systemic diseases which include respiratory diseases [2, 30]. In an Fn-related disease scenario, Fn is capable of demolishing the oral epithelial barrier, thereby, making Fn a pathogen [31]. This would suggest that Fn may likewise affect other epithelial barriers. Throughout this study, there was an attempted to show the effects of Fn presence in the alveolar epithelium.

Alveoli are found in the terminal airspaces of the lungs and are responsible for gas exchange between the lung airspaces and the circulatory system [32]. Alveolar epithelium is heterogenous with squamous epithelium covering 90% of the alveolar surface area [33] and the alveolar intercellular junction comprised of mostly TJs which in-turn are critical for epithelial barrier function [34]. Respiratory diseases remain a leading cause of mortality and morbidity worldwide, thereby, is considered a serious threat to the general population and public health [35, 36]. Moreover, respiratory viruses have been reported to promote bacterial adhesion to respiratory epithelial cells which in-turn may promote bacterial colonization along the respiratory tract, thereby, contribute to the development of respiratory disease [36]. This would mean that any disruption in the alveolar TJ may result to bacterial adhesion along the alveolar surface which eventually may lead to a respiratory disease state. Considering the results demonstrate that Fn is capable of disrupting TJ gene expression, it is proposed the following sequence of events in a Fn-related oral disease scenario that may putatively lead to a respiratory disease situation: (1) high Fn concentration potentially disrupts alveolar TJ resulting in the loss of alveolar epithelial barrier function; (2) Fn-related alveolar TJ disruption leads to an accumulation of neutrophils which in-turn increases alveolar space; (3) Fn-related increase in alveolar

space results to an increase in paracellular permeability; and (4) viral and/or bacterial pathogens enter the alveoli leading to a respiratory disease state.

In summary, the results postulate that Fn may directly affect TJs proteins that are directly exposed to the alveolar surface. Moreover, Fn can putatively cause neutrophil accumulation leading to an increase in alveolar space. Furthermore, Fn has the potential to cause an increase in paracellular permeability in the alveoli. Taken together, the results would speculate that Fn may putatively aid in the development of a respiratory disease situation.

References

[1] K. Imai, T. Iinuma, and S. Sato, Relationship between the oral cavity and respiratory diseases: Aspiration of oral bacteria possibly contributes to the progression of lower airway inflammation. Jpn Dent Sci Rev 57 (2021) 224-230.

[2] C.A. Brennan, and W.S. Garrett, *Fusobacterium nucleatum* - symbiont, opportunist and oncobacterium. Nat Rev Microbiol 17 (2019) 156-166.

[3] J.A. Bastos, C.G. Diniz, M.G. Bastos, E.M. Vilela, V.L. Silva, A. Chaoubah, D.C. Souza-Costa, and L.C. Andrade, Identification of periodontal pathogens and severity of periodontitis in patients with and without chronic kidney disease. Arch Oral Biol 56 (2011) 804-811.

[4] C.W. Kaplan, X. Ma, A. Paranjpe, A. Jewett, R. Lux, S. Kinder-Haake, and W. Shi, *Fusobacterium nucleatum* outer membrane proteins Fap2 and RadD induce cell death in human lymphocytes. Infect Immun 78 (2010) 4773-4778.

[5] R. Suzuki, N. Kamio, T. Kaneko, Y. Yonehara, and K. Imai, *Fusobacterium nucleatum* exacerbates chronic obstructive pulmonary disease in elastase-induced emphysematous mice. FEBS Open Bio 12 (2022) 638-648.

[6] O.H. Wittekindt, Tight junctions in pulmonary epithelia during lung inflammation. Pflugers Arch 469 (2017) 135-147.

[7] F. Rezaee, and S.N. Georas, Breaking barriers. New insights into airway epithelial barrier function in health and disease. Am J Respir Cell Mol Biol 50 (2014) 857-869.

[8] E.E. Schneeberger, and R.D. Lynch, The tight junction: a multifunctional complex. Am J Physiol Cell Physiol 286 (2004) C1213-C1228.

[9] L. Gonzalez-Mariscal, A. Betanzos, P. Nava, and B.E. Jaramillo, Tight junction proteins. Prog Biophys Mol Biol 81 (2003) 1-44.

[10] P. Shannon, A. Markiel, O. Ozier, N.S. Baliga, J.T. Wang, D. Ramage, N. Amin, B. Schwikowski, and T. Ideker, Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 13 (2003) 2498-2504.

[11] D. Koschutzki, and F. Schreiber, Centrality analysis methods for biological networks and their application to gene regulatory networks. Gene Regul Syst Bio 2 (2008) 193-201.

[12] Y. Gon, K. Matsumoto, M. Terakado, A. Sekiyama, S. Maruoka, I. Takeshita, Y. Kozu, Y. Okayama, C. Ra, and S. Hashimoto, Heregulin activation of ErbB2/ErbB3 signaling potentiates the integrity of airway epithelial barrier. Exp Cell Res 317 (2011) 1947-1953.

[13] Y. Shintani, S. Maruoka, Y. Gon, D. Koyama, A. Yoshida, Y. Kozu, K. Kuroda, I. Takeshita, E. Tsuboi, K. Soda, and S. Hashimoto, Nuclear factor erythroid 2-related factor 2 (Nrf2) regulates airway epithelial barrier integrity. Allergol Int (Suppl) 64 (2015) S54-S63.

[14] B.R. Stevenson, J.D. Siliciano, M.S. Mooseker, and D.A. Goodenough, Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. J Cell Biol 103 (1986) 755-766.

[15]Y. Soini, Claudins in lung diseases. Respir Res 12 (2011) 70.

[16] J.M. Anderson, and C.M. Van Itallie, Physiology and function of the tight junction. Cold Spring Harb Perspect Biol 1 (2009) a002584.

[17] R. Kaarteenaho, H. Merikallio, S. Lehtonen, T. Harju, and Y. Soini, Divergent expression of claudin -1, -3, -4, -5 and -7 in developing human lung. Respir Res 11 (2010) 59.

[18] K. Umeda, J. Ikenouchi, S. Katahira-Tayama, K. Furuse, H. Sasaki, M. Nakayama, T. Matsui, S. Tsukita, and M. Furuse, ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. Cell 126 (2006) 741-754.

[19] W.T. Kuo, L. Zuo, M.A. Odenwald, S. Madha, G. Singh, C.B. Gurniak, C. Abraham, and J.R. Turner, The tight junction protein ZO-1 is dispensable for barrier function but critical for effective mucosal repair. Gastroenterology 161 (2021) 1924-1939.

[20] A. Raya-Sandino, A. Castillo-Kauil, A. Dominguez-Calderon, L. Alarcon, D. Flores-Benitez, F. Cuellar-Perez, B. Lopez-Bayghen, B. Chavez-Munguia, J. Vazquez-Prado, and L. Gonzalez-Mariscal, Zonula occludens-2 regulates Rho proteins activity and the development of epithelial cytoarchitecture and barrier function. Biochim Biophys Acta Mol Cell Res 1864 (2017) 1714-1733.

[21] D.M. Comer, J.S. Elborn, and M. Ennis, Comparison of nasal and bronchial epithelial cells obtained from patients with COPD. PLoS One 7 (2012) e32924.

[22] W. Feng, J. Guo, H. Huang, B. Xia, H. Liu, J. Li, S. Lin, T. Li, J. Liu, and H. Li, Human normal bronchial epithelial cells: a novel *in vitro* cell model for toxicity evaluation. PLoS One 10 (2015) e0123520.

[23] J.F. Holter, J.E. Weiland, E.R. Pacht, J.E. Gadek, and W.B. Davis, Protein permeability in the adult respiratory distress syndrome. Loss of size selectivity of the alveolar epithelium. J Clin Invest 78 (1986) 1513-1522.

[24] C.B. Coyne, T.M. Gambling, R.C. Boucher, J.L. Carson, and L.G. Johnson, Role of claudin interactions in airway tight junctional permeability. Am J Physiol Lung Cell Mol Physiol 285 (2003) L1166-L1178.

[25] L.A. Mitchell, C. Ward, M. Kwon, P.O. Mitchell, D.A. Quintero, A. Nusrat, C.A. Parkos, and M. Koval, Junctional adhesion molecule A promotes epithelial tight junction assembly to augment lung barrier function. Am J Pathol 185 (2015) 372-386.

[26] G. Balamayooran, S. Batra, M.B. Fessler, K.I. Happel, and S. Jeyaseelan, Mechanisms of neutrophil accumulation in the lungs against bacteria. Am J Respir Cell Mol Biol 43 (2009) 5-16.

[27] J.C. Horvat, M.R. Starkey, R.Y. Kim, S. Phipps, P.G. Gibson, K.W. Beagley, P.S. Foster, and P.M. Hansbro, Early-life chlamydial lung infection enhances allergic airways disease through age-dependent differences in immunopathology. J Allergy Clin Immunol 125 (2010) 617-625, 625 e1-625 e6.

[28] A.L. Humlicek, L.J. Manzel, C.L. Chin, L. Shi, K.J. Excoffon, M.C. Winter, D.M. Shasby, and D.C. Look, Paracellular permeability restricts airway epithelial responses to selectively allow activation by mediators at the basolateral surface. J Immunol 178 (2007) 6395-6403.

[29] S.P. Kantrow, Z. Shen, T. Jagneaux, P. Zhang, and S. Nelson, Neutrophil-mediated lung permeability and host defense proteins. Am J Physiol Lung Cell Mol Physiol 297 (2009) L738-L745.

[30]Y.W. Han, *Fusobacterium nucleatum*: a commensal-turned pathogen. Curr Opin Microbiol 23 (2015) 141-147.

[31] Y. Chen, T. Shi, Y. Li, L. Huang, and D. Yin, *Fusobacterium nucleatum*: the opportunistic pathogen of periodontal and peri-implant diseases. Front Microbiol 13 (2022) 860149.

[32] C.E. Overgaard, L.A. Mitchell, and M. Koval, Roles for claudins in alveolar epithelial barrier function. Ann N Y Acad Sci 1257 (2012) 167-174.

[33] J.D. Crapo, B.E. Barry, P. Gehr, M. Bachofen, and E.R. Weibel, Cell number and cell characteristics of the normal human lung. Am Rev Respir Dis 126 (1982) 332-337.

[34] A.I. Ivanov, C.A. Parkos, and A. Nusrat, Cytoskeletal regulation of epithelial barrier function during inflammation. Am J Pathol 177 (2010) 512-524.

[35] G. Huang, and F. Guo, Loss of life expectancy due to respiratory infectious diseases: findings from the global burden of disease study in 195 countries and territories 1990-2017. J Popul Res 39 (2022) 1-43.

[36]V. Avadhanula, C.A. Rodriguez, J.P. Devincenzo, Y. Wang, R.J. Webby, G.C. Ulett, and E.E. Adderson, Respiratory viruses augment the adhesion of bacterial pathogens to respiratory epithelium in a viral species- and cell type-dependent manner. J Virol 80 (2006) 1629-1636.

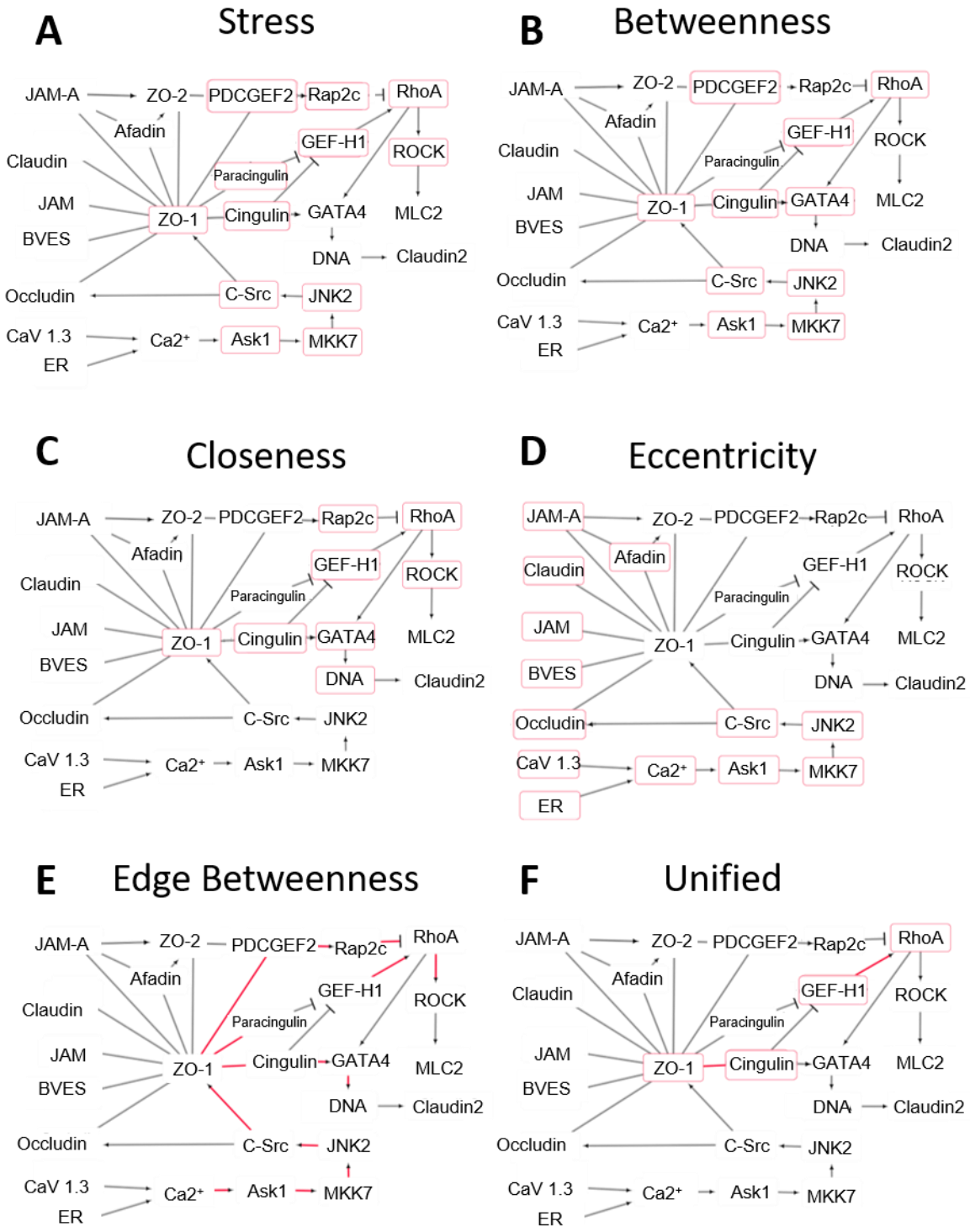

Figure 1. Network design and analysis of tight junction pathways involved in cell polarity and paracellular permeability. Centrality measurements for (A) stress, (B) betweenness, (C) closeness, (D) eccentricity, and (E) edge-betweenness are presented. Nodes found to be significant are indicated in a red rectangular box. Edges found to be significant are indicated as red lines. (F) Unified network combining the significant nodes and edges from all centrality measurements. Nodes found to be significant are indicated in a red rectangular box. Edges found to be significant are indicated as red lines.

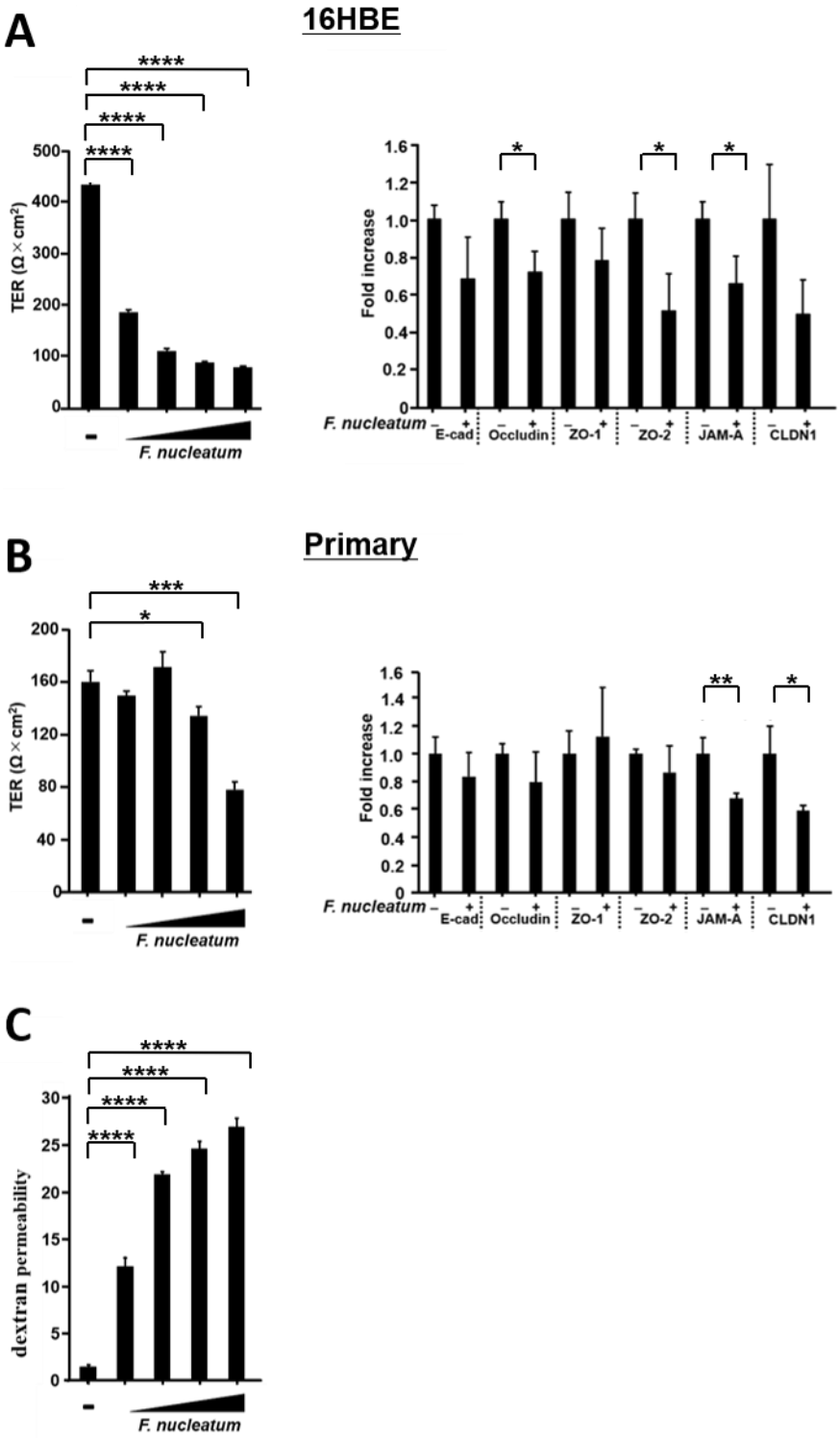

Figure 2. *Fusobacterium nucleatum* lowers transepithelial resistance and decreases tight junction gene expression in immortalized 16HBE and primary human alveolar cells. (A) *F. nucleatum*-treated immortalized 16HBE alveolar cells. (*Left panel*) Transepithelial resistance and (*right panel*) gene expression levels of selected tight junction proteins are presented. (B) *F. nucleatum*-treated primary human alveolar cells. (*Left panel*) Transepithelial resistance and (*right panel*) gene expression levels of selected tight junction proteins are shown. (C) Paracellular permeability measurement of 16HBE alveolar cells with and without *F. nucleatum*-treatment. These experiments were conducted in triplicate and data are presented as the mean ± SD; n = 3 (*, p <0.05; ***, p <0.0005; ****, p <0.0001).

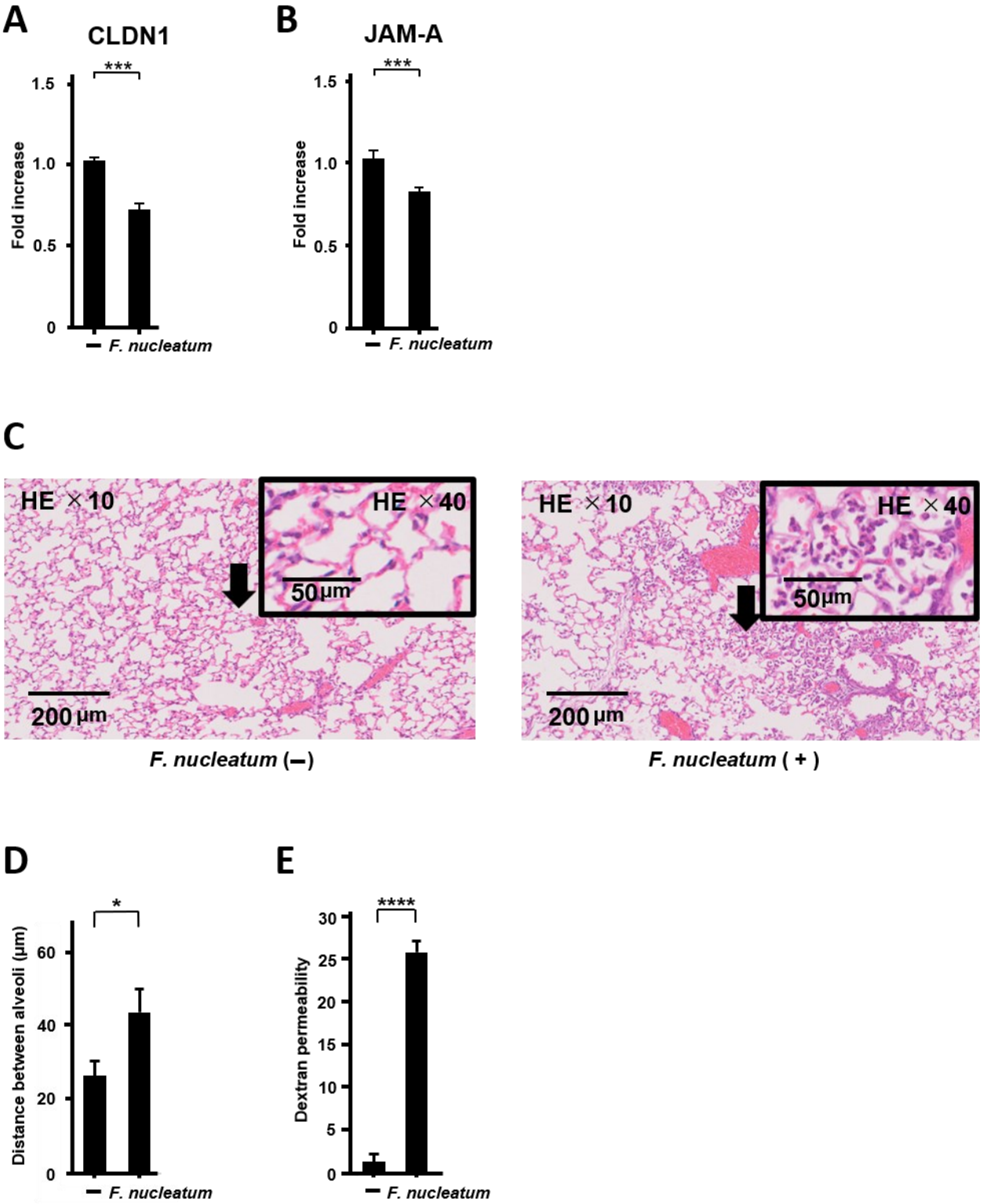

Figure 3. *Fusobacterium nucleatum* affects mice alveolar tissue. (A) CLDN1 and (B) JAM-A gene expression levels of *F. nucleatum*-treated mice alveolar tissue. (C) Histological staining of mice alveolar tissue. Before (*Left panel*) and after (*Right panel*) *F. nucleatum*-treatment of mice alveolar tissue. Reference measurement (μm) is indicated below each panel (*lower left*). Magnification of selected tissue area (*upper right*) is indicated by an arrow. (D) Alveolar distance measurement of mice alveolar tissue with and without *F. nucleatum*-treatment. (E) Paracellular permeability measurement of mice alveolar tissue with and without *F. nucleatum*-treatment. These experiments were conducted in triplicate and data are presented as the mean ± SD; n = 4 (*, p <0.05; ***, p <0.0005; ****, p <0.0001).