J Mol Hist (2017) 48:225–234
DOI 10.1007/s10735-017-9716-5

ORIGINAL PAPER

Decreased interaction between ZO-1 and occludin is involved in alteration of tight junctions in transplanted epiphora submandibular glands

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Received: 2 February 2017 / Accepted: 9 March 2017 / Published online: 22 March 2017

Abstract Tight junctions (TJs) in salivary epithelium play an important role in regulating saliva secretion. Autologous transplantation of submandibular glands (SMGs) is an effective method to treat severe dry eye syndrome. However, epiphora occurs in some patients 6 months after transplantation. We previously found that the acinar TJs are enlarged in rabbit SMGs after long-term transplantation, but the exact TJ components involved in the epiphora are still unknown. Here, we found that the mRNA and protein expression of ZO-1 and occludin were increased in the transplanted SMGs obtained from epiphora patients, while other TJs were unchanged. The intensity of ZO-1 and occludin at the apicolateral membranes as well as occludin in the cytoplasm were increased in epiphora SMGs, but the interaction between ZO-1 and occludin was decreased as evidenced by both co-immunoprecipitation assay and co-immunofluorescence staining. Mechanically, the expression of casein kinase 2α (CK2α) and CK2β, which was reported to affect occludin modification and the interaction of occludin with ZO-1 in previous literatures, were increased in epiphora glands. Moreover, activation of muscarinic acetylcholine receptor (mAChR) by carbachol directly decreased the interaction between ZO-1 and occludin and increased the acinar TJ width in the freshly isolated human SMGs, whereas these effects were abolished by pretreatment with CK2 inhibitor. Taken together, our findings suggest that decreased interaction between ZO-1 and occludin might contribute to the epiphora occurred in the transplanted SMGs, and mAChR together with the intracellular molecule CK2 might be responsible for the alteration of TJs in epiphora glands.

Keywords Epiphora · Submandibular gland · Transplantation · Tight junction · Casein kinase 2

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Introduction

Dry eye syndrome is a common ophthalmologic disorder characterized by reduced or lack of tear production with serious complications, including visual disturbance, corneal surface damage, and even loss of sight. Autologous transplantation of submandibular gland (SMG) with insertion of Wharton's duct into the upper conjunctival fornix, which provides permanent substitution of tears, is an effective way to treat severe dry eye syndrome (Geerling et al. 1998; Sieg et al. 2000). However, it is notable that the secretion pattern changes after transplantation since the blood supply of the transplanted SMG is reconstructed by microsurgery, while the gland is denervated without nerve anastomosis during the operation. Secretion of the

transplanted SMG is decreased several months and then followed by excessive secretion, or epiphora, which appears in more than 40% of patients 6 months after surgery. Severe epiphora may lead to mild discomfort at rest and at room temperature, while worsened symptoms occur upon exercise or under high temperature (Su et al. 2015; Yu et al. 2004). These patients have to take secondary gland reduction surgery to reduce the secretion from the transplanted SMG. Therefore, understanding the secretory mechanism involved in the transplanted gland is of great importance for finding an effective therapy to ameliorate epiphora and avoid more surgeries.

Under physiological conditions, fluid and electrolyte secretion in salivary glands is accomplished through both aquaporin 5 (AQP5)-mediated transcellular pathway and tight junction (TJ)-based paracellular pathway (Gonzalez-Mariscal et al. 2003; Kawedia et al. 2007; Tsukita et al. 2001; Zhang et al. 2013; Abe et al. 2016). We previously found that the content of AQP5 is reduced in lipid microdomains while increased in non-lipid microdomains in transplanted SMGs from epiphora patients, suggesting that AQP5 trafficking might partly involve in the epiphora (Ding et al. 2014). Recently, increasing evidence demonstrates the importance of TJs in regulating salivary secretion, and the aberrance in TJ expression and/or disruption in TJ structure are closely related with secretory disorders (Cong et al. 2012; Ewert et al. 2010; Zhang et al. 2013; Abe et al. 2016). In a rabbit model of SMG autotransplantation, acinar TJ width is increased on postoperative days 90 and 180 in hypersecretory SMGs (Yang et al. 2017). However, the exact TJ molecules that contributed to epiphora and the related signaling pathway are still unknown.

Therefore, the present study was designed to explore the alteration of TJ expression and structure in the transplanted SMGs obtained from epiphora patients, and further investigated the mechanism involved in the regulation of salivary epithelial TJs. This may allow us to understand the molecular mechanism of the transplanted glands, and help to explore potential targets to treat epiphora in SMG transplanted patients.

Materials and methods

Human participants and ethics

Transplanted SMG samples were collected from six individuals (ages 24–45 years; three males) who underwent partial gland reduction for epiphora within 6 to 12 months (mean: 8.95 months) after transplantation. SMGs from six individuals (ages 52–59 years; four males) who underwent functional neck dissection for primary oral squamous cell carcinoma without irradiation or chemotherapy were used as controls. All control samples were confirmed to be histologically normal. The research protocol was approved by the Institutional Review Board of Peking University Hospital of Stomatology, and all participants had signed an informed consent document prior to tissue collection.

Reagents and antibodies

Carbachol and tetrabromocinnamic acid (TBCA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against ZO-1 and occludin were purchased from Life Technologies (Cat. 402200 and 331500, Carlsbad, CA, USA). Antibodies against casein kinase 2 (CK2) α and β were purchased from Santa Cruz Biotechnology (sc-6479 and sc-12739, Carlsbad, CA, USA). Antibody against GAPDH was purchased from Abmart (M20006, Shanghai, China). Other chemicals and reagents were of analytical grade.

Reverse transcription-polymerase chain reaction (PCR)

Total RNA was isolated with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was prepared from 2 μg of total RNA with RevertAid First Strand cDNA Synthesis Kit (Promega, Madison, WI, USA). The primer sequences were listed in Table 1. Real-time PCR was performed with DyNAmo Color Flash SYBR Green qPCR Kit according to the manufacturer's instructions under Thermo PikoReal Real-Time PCR Systems (Thermo Fisher Scientific, Waltham, MA, USA).

Western blot analysis

Human SMG tissues were homogenized with lysis buffer (containing 50 mmol/L Tris–HCl, pH 7.2, 150 mmol/L NaCl, 1 mmol/L ethylenediamine tetraacetic acid, 1 mmol/L phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.1% SDS, and 0.1% sodium deoxycholate) using a polytron homogenizer as described previously (Ding et al. 2014). The homogenates were centrifuged at 12,000×*g* for 10 min at 4 °C. The supernatants were collected, and the protein concentration was measured by the Bradford method. Equal amounts of proteins (40 μg) were separated on 9% SDS–PAGE, transferred to polyvinylidene fluoride membranes, and probed with primary antibodies. Blots were then probed with horseradish peroxidase-conjugated secondary antibodies (ZSGBBIO, Beijing, China), and the target proteins were detected by an enhanced chemiluminescence reagent (Pierce Biotechnology, Rockford, IL, USA). GAPDH was used as a loading control.

Table 1 Primers for human tight junction components mRNA

Gene	Upper primer (5′-3′)	Lower primer (5′-3′)	Genebank
ZO-1	CCTTCAGCTGTGGAAGAGGATG	AGCTCCACAGGCTTCAGGAAC	NM_003257
ZO-2	CCAGGAAGCACAGAATGCAAG	GCGCTTGGAGTGTTCTGACAG	NM_004817
ZO-3	GCGTTAACAGCGACTACGAGA	CATAGGTTCGCATGCTGTCCT	NM_014428
Occludin	GCCTTCACCCCCATCTGACTA	CCAGTCCTCCTCCAGCTCATC	NM_002538
Claudin-1	GCAGAAGATGAGGATGGCTGT	CCTTGGTGTTGGGTAAGAGGT	NM_021101
Claudin-2	GCCATGATGGTGACATCCAGT	TCAGGCACCAGTGGTGAGTAG	NM_020384
Claudin-3	GGACTTCTACAACCCCGTGGT	AGACGTAGTCCTTGCGGTCGT	NM_001306
Claudin-4	CAAGGCCAAGACCATGATCGT	GCGGAGTAAGGCTTGTCTGTG	NM_001305
Claudin-5	GGCACATGCAGTGCAAAGTGT	ATGTTGGCGAACCAGCAGAGT	NM_003277
Claudin-7	CTCGAGCCCTAATGGTGGTCT	CCCAGGACAGGAACAGGAGAG	NM_001307
Claudin-11	CTGATGATTGCTGCCTCGGT	ACCAATCCAGCCTGCATACAG	NM_005602
Claudin-16	CGTGAAATTCCTCCCTGATGA	CAGCAGGTGAGAACAGCTCCA	NM_006580
GAPDH	CTTTGGCATTGTGGAAGGGCTC	GCAGGGATGATGTTCTGGGCAG	NM_002046

Immunofluorescence

Frozen specimens (7 μm) from human SMGs tissues were fixed in cold acetone, and incubated with antibodies against ZO-1 and/or occludin at 4 °C overnight and then incubated with Alexa Fluor 488 and/or 594-conjugated secondary antibody at 37 °C for 2 h. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Fluorescence images were captured under confocal microscope (Leica TCS SP8, Wetzlar, Germany). The average fluorescence intensity in acini and the ratio of apicolateral to total intensity from ten randomly chosen acini from each section was measured by ImageJ Software (National Institutes of Health, Bethesda, MD, USA).

Transmission electron microscopy

SMGs were fixed in 2% paraformaldehyde-1.25% glutaraldehyde. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (H-7000 electron microscope, HITACHI, Tokyo, Japan). For morphometric analysis, the distance between neighboring acinar TJs (shown as TJ width) from four sections of ten randomly selected fields in each section were measured and averaged by the use of ImageJ software blindly by two examiners as described previously (Nighot and Blikslager 2010).

Immunoprecipitation

SMG lysates were obtained as described previously (Cong et al. 2015). Immunoprecipitation was carried out by incubation with either ZO-1 or occludin antibody and protein A/G beads (Santa Cruz Biotechnology) overnight at 4 °C. The beads were washed three times with lysis buffer and eluted with SDS–PAGE loading buffer. The proteins were separated using 9% SDS–PAGE and used for western blot analysis.

Statistical analysis

Data were presented as means ± SD. Statistical analysis was performed by unpaired Student's *t* test for two groups or by one-way analysis of variance followed by Bonferroni's test among multiple groups using GraphPad software (GraphPad Prism, San Jose, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Expressions of ZO-1 and occludin are higher in the transplanted epiphora SMGs

As shown in Fig. 1a, the mRNA expressions of ZO-1, -2, -3, occludin, claudin-1–5, -7, -11, and -16 were detectable in human SMGs. In the transplanted glands, the mRNA expressions of ZO-1 and occludin were higher than those of control SMGs, while other TJ molecules were not changed (Fig. 1a). The alteration was further confirmed by Real-time PCR detection which showed that the mRNA level of ZO-1 and occludin were significantly increased by 139.7 and 120.2%, respectively (Fig. 1b, c). Western blot analysis identified that the protein expressions of ZO-1 and occludin in epiphora glands were increased by 52.3 and 85.7%, respectively, as compared with those in controls (Fig. 1d, e).

Immunofluorescence images showed that in human control SMGs, ZO-1 and occludin were mainly localized at the apicolateral membranes in acini. However, in epiphora SMGs, the expression of ZO-1 at the apicolateral membranes was obviously enhanced and

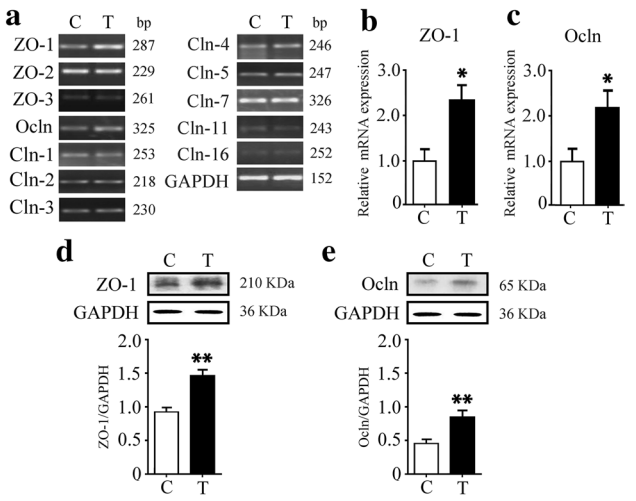

Fig. 1 Expression of tight junction components in human control and transplanted submandibular glands. **a** The mRNA expressions of ZO-1–3, claudin-1~-5, -7, -11, and -16 in control and transplanted glands were detected by reverse transcription-polymerase chain reaction (PCR). **b, c** The mRNA expressions of ZO-1 (**b**) and occludi (**c**) were further confirmed by Real-time PCR. **d, e** The protein expressions of ZO-1 (**d**) and occludin (**e**) in control and transplanted glands were detected by western blot analysis. *C* control, *T* transplanted, *Cln* claudin, *Ocln* occludin. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with controls

semi-quantitative analysis showed that the fluorescence intensity of ZO-1 in acini as well as the ratio of apicolateral to total intensity were significantly increased (Fig. 2a, b). Although the fluorescence intensity of occludin at the apicolateral membranes was enhanced, yet it was mostly dispersed in the cytoplasm in epiphora SMGs, and hence the ratio of apicolateral to total intensity was not significantly changed (Fig. 2c, d).

Ultrastructure of TJs is changed in epiphora SMGs

The alteration in TJ expression can closely affect its barrier function (Zhang et al. 2013). In order to explore whether the TJ barrier was changed in the transplanted epiphora glands, we next observed the morphology of acinar TJs. In human control SMGs, TJs were located at the apical portion between neighboring epithelial cells, and formed a slightly dilated distance with an average TJ width 13.75 ± 0.07 nm. The distance between acinar TJs was enlarged in epiphora glands, and quantitative analysis showed that TJ width was significantly increased by 68.18% (Fig. 3). These results suggested that acinar TJs were "opened" in epiphora glands, which might be involved in hypersecretion after the long-term transplantation.

Interaction between ZO-1 and occludin is decreased in epiphora SMGs

As a TJ cytoplasm protein, ZO-1 directly binds with TJ transmembrane proteins including occludin and claudin family members (Fanning et al. 1998; Tsukita et al. 2001). Previous studies showed that the interaction between ZO-1 and occludin affects the assembly of TJ complex and plays an important role in determining paracellular permeability (Dorfel et al. 2013; Furuse et al. 1994). Accordingly, we found that ZO-1 was co-immunoprecipitated with occludin in human control SMGs. However, the level of ZO-1 co-immunoprecipitated with occludin as well as the level of occludin co-immunoprecipitated with ZO-1 were lower in the transplanted glands (Fig. 4a, b), which suggested that the interaction between ZO-1 and occludin was decreased in epiphora glands.

Activation of mAChR decreases the interaction between ZO-1 and occludin in control SMG

We previously found that muscarinic acetylcholine receptor (mAChR) is hypersensitive in the transplanted SMGs from both epiphora patients and rabbit models (Ding et al. 2014; Yang et al. 2017). To explore whether activation of mAChR affected the interaction between ZO-1 and occludin in SMGs, we treated the freshly isolated human control SMG tissues with carbachol (10 μmol/L) for 10 min. Co-immunoprecipitation assay showed that the content of occludin that interacted with ZO-1 was decreased in carbachol-treated glands as compared with untreated controls. Additionally, we found the consistent results when using occludin antibody to immunoprecipitate ZO-1 (Fig. 4a, b). To further explore the collaboration of ZO-1 and occludin in epiphora SMG tissues, we performed double immunofluorescence staining. The images showed that ZO-1 and occludin were mainly localized at the apicolateral membranes of acini and showed a well co-localization in control SMGs. Although the fluorescence intensity of ZO-1 and occludin at the apicolateral membranes as well as occludin in the cytoplasm were increased in epiphora glands, but their co-localization region became reduced compared with controls (white arrows indicate the co-localization sites in Fig. 4c).

The expression of CK2 is increased in epiphora SMGs

We next explored the possible mechanism that involved in the regulation of TJs in epiphora glands. CK2, consisting of two catalytic subunits (CK2α) and a dimer of regulatory subunits (CK2β), is a Ser/Thr kinase that directly phosphorylates occludin, thereby resulting in an inhibitory effect on the formation of TJ complex (Dorfel et al. 2013). As shown

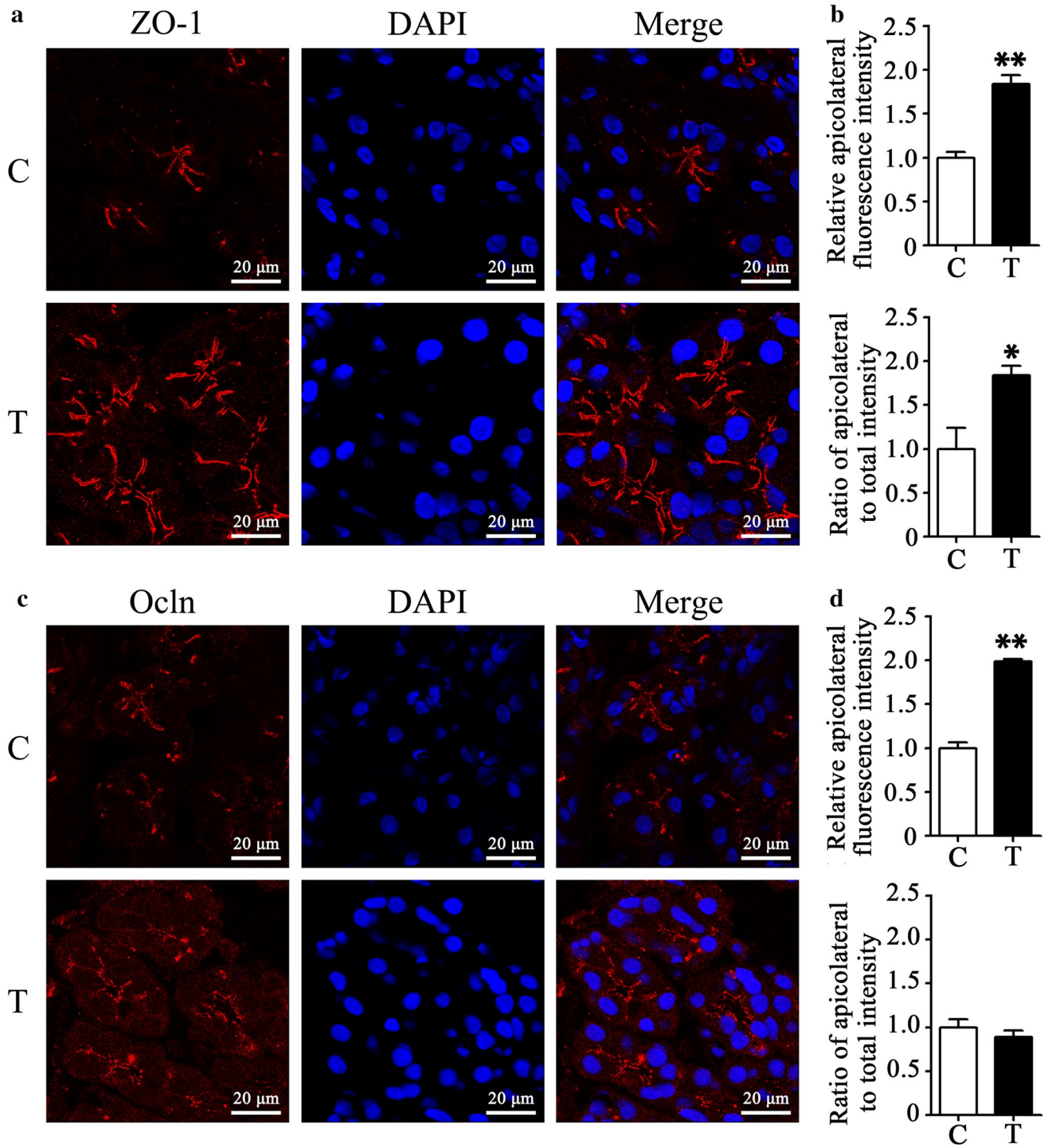

Fig. 2 Distribution of ZO-1 and occludin in human control and transplanted submandibular glands. **a** The distribution of ZO-1 was detected by immunofluorescence staining and observed with a confocal microscope. **b** Quantitative analysis of fluorescence intensity of ZO-1, including apicolateral intensity (*upper*) and ratio of apicolateral to total intensity (*lower*) were performed in ten randomly chosen acini from each section. **c** The distribution of occludin was detected by immunofluorescence staining and observed with a confocal microscope. **d** Quantitative analysis of fluorescence intensity of occludin, including apicolateral intensity (*upper*) and ratio of apicolateral to total intensity (*lower*) were performed in ten randomly chosen acini from each section. *C* control, *T* transplanted, *Ocln* occludin. *Bars*, 20 μm. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with controls

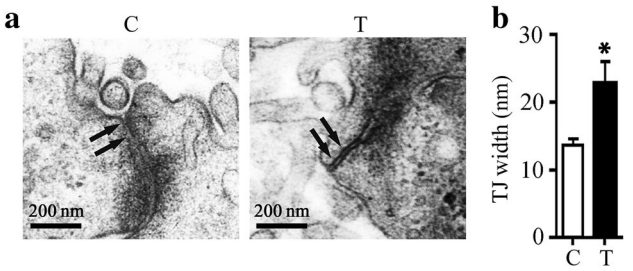

Fig. 3 Ultrastructure of acinar tight junctions (TJs) in human control and transplanted submandibular glands. **a** Representive images of TJ structure under a transmission electron microscope in control and transplanted glands, and black arrows indicated TJ region. **b** The width of acinar TJs was measured from four sections of ten randomly selected fields in each section. *C* control, *T* transplanted. *Bars*, 200 nm. **P* < 0.05 compared with controls

in Fig. 5, the expression of both CK2α and CK2β were significantly higher in epiphora SMGs as compared with those of controls.

CK2 is responsible for carbachol-induced ZO-1/occludin interaction and TJ ultrastructure in cultured human SMG tissues

To investigate the possible role of CK2 on salivary TJs, the freshly isolated human SMG tissues were pretreated with TBCA (1 mmol/L for 30 min), a pharmacological antagonist of CK2. Co-immunoprecipitation assay showed that TBCA pretreatment abolished the carbachol-induced decreased interaction between ZO-1 and occludin, whereas TBCA alone had no influence on their interaction (Fig. 6a, b). These results suggested that carbachol regulated the interaction of ZO-1 and occludin in a CK2-dependent manner in human SMG tissues.

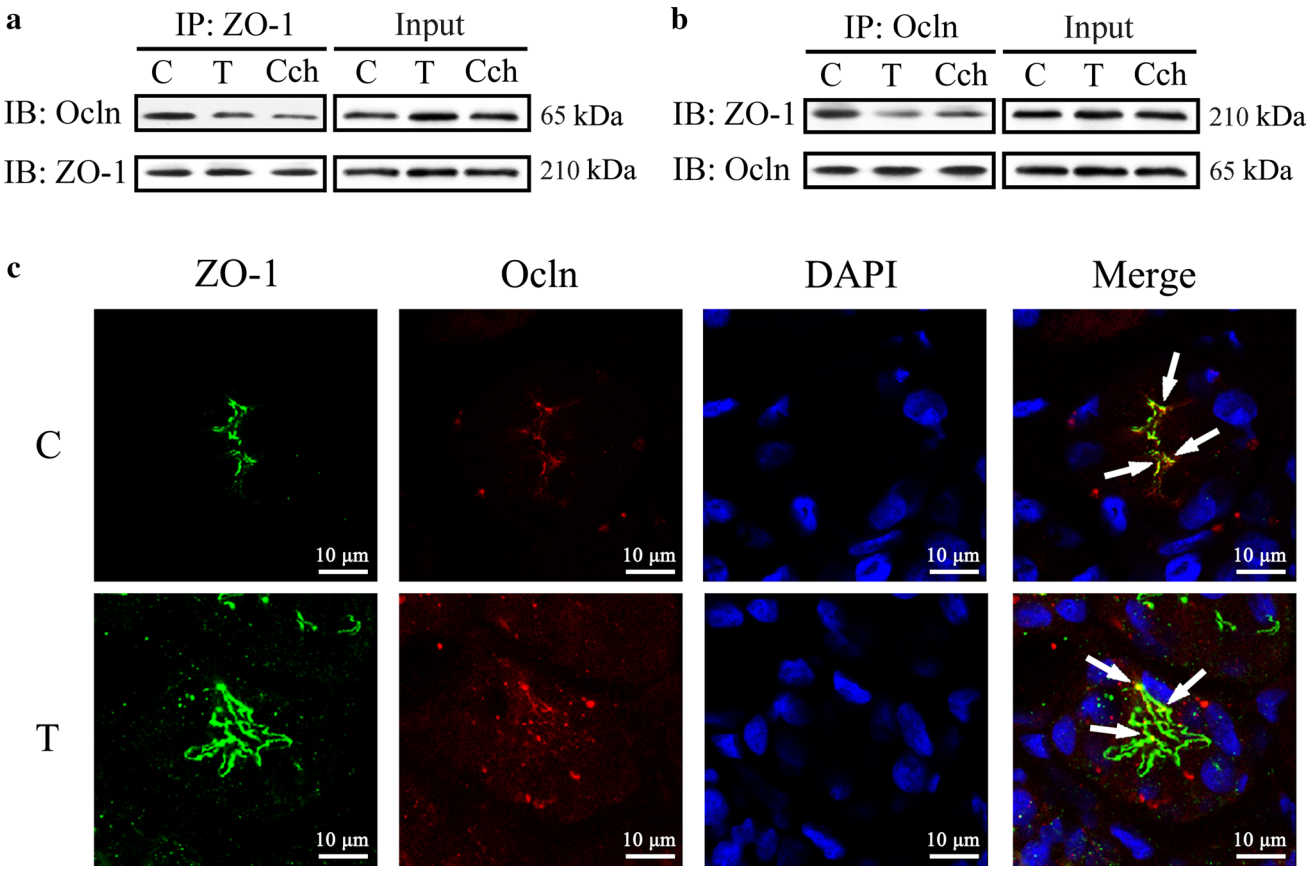

Fig. 4 Interaction between ZO-1 and occludin in human control and transplanted submandibular glands (SMGs), as well as in carbachol-treated human SMG tissues. **a, b** The protein level of occludin that immunoprecipitated with ZO-1 **a** and the level of ZO-1 that immunoprecipitated with occludin were detected by co-immunoprecipitation assay in control and transplanted glans, as well as in the freshly isolated SMG tissues treated with 10 μmol/L carbachol for 10 min. **c** The representative co-immunofluorescence images of ZO-1 and occludin in control and transplanted SMGs. White arrows indicated the co-localization sites of ZO-1 and occludin. *C* control, *T* transplanted, *Ocln* occludin. *Bars*, 10 μm

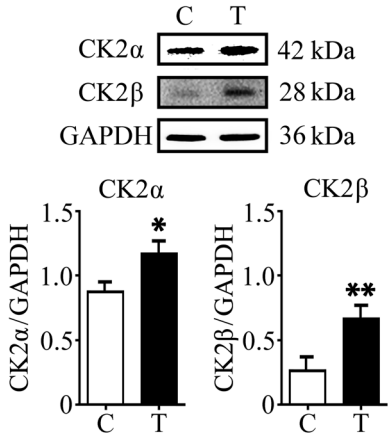

Fig. 5 Expression of casein kinase 2α (CK2α) and CK2β in human control and transplanted submandibular glands. The protein expression of CK2α and CK2β were detected by western blot assay, and semi-quantitative analysis of band intensity were shown below. *C* control, *T* transplanted, *Cch* carbachol. $^{**}P<0.01$ and $^{*}P<0.05$ compared with controls

Furthermore, we observed the effect of CK2 on TJ ultrastructure in control SMG tissues. Carbachol treatment (10 μmol/L, 10 min) enlarged TJ distance between neighboring acinar cells, whereas this effect was not observed in TBCA-pretreated SMG tissues (Fig. 6c). TBCA alone did not affect on acinar TJ ultrastructure. Quantitative analysis showed carbachol significantly increased TJ width by 78.6% compared with that in untreated SMGs. Pretreatment with TBCA suppressed the carbachol-induced increase in TJ width (Fig. 6d). These results suggested that CK2 was responsible for carbachol-modulated the "opening" of TJ structure.

Discussion

In this study, we identified that the interaction of ZO-1 and occludin was decreased though their expression were increased, accompanying with an enlarged TJ distance between neighboring acinar cells in the transplanted SMGs obtained from epiphora patients. Additionally, in the freshly isolated human SMG tissues, carbachol decreased the interaction between ZO-1 and occludin and increased TJ width in a CK2-dependent manner. Moreover, the expression of CK2 was significantly elevated in epiphora SMGs. The results suggest that the alteration in expression and interaction of ZO-1 and occludin might contribute to epiphora after the long-term transplantation, and CK2 is responsible for the mAChR-modulated paracellular permeability and involves in the alteration of TJ structure and function in epiphora SMGs.

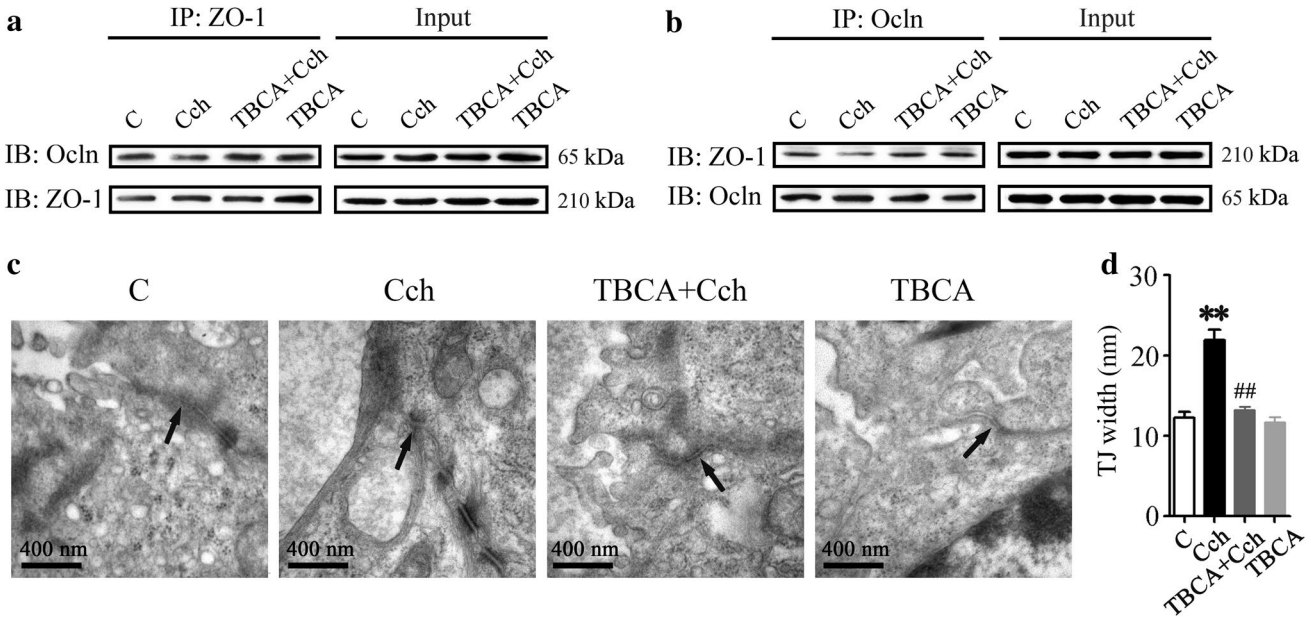

Fig. 6 Role of casein kinase 2 (CK2) in the regulation of ZO-1 and occludin interaction and tight junction (TJ) ultrastructure in human submandibular gland (SMG) tissues. **a, b** The freshly isolated SMG tissues was pretreated with CK2 antagonist tetrabromocinnamic acid (TBCA, 1 mmol/L for 30 min). The interaction between ZO-1 and occludin induced by carbachol (10 mmol/L for 10 min) with or without TBCA pretreatment was detected by co-immunoprecipitation assay. **c** The ultrastructure of acinar TJs induced by carbachol with or without TBCA pretreatment was observed under a transmission electron microscope (**c**), and TJ width was measured from 4 sections of 10 randomly selected fields in each section (**d**). *C* control, *Cch* carbachol. *Bars*, 400 nm. $^{**}P<0.01$ compared with controls. $^{\#}P<0.05$ compared with carbachol-treated SMG tissues

TJs consist of a narrow belt-like structure at the most apical portion of lateral membranes, serving as an indispensable gate for material transport through paracellular pathway (Tsukita et al. 2001). Many studies have revealed the crucial roles of TJs in renal, intestinal, airway, and epidermal epithelium, as well as brain blood endothelium (Coyne et al. 2002; Peerapen and Thongboonkerd 2011; Roxas et al. 2010; Schreibelt et al. 2007). Recently, the role of TJs in salivary epithelium has gained much attention since the TJ-modulated paracellular pathway particularly plays an important role in water, ion, and small particles secretion (Baker 2016). Many secretory stimulators, such as carbachol, capsaicin and adiponectin, are reported to induce salivation by altering TJ properties and increasing paracellular permeability (Cong et al. 2015, 2013; Ding et al. 2013). Moreover, proinflammatory cytokines disrupt TJ barrier function and change TJ content, which might be the mechanism for the hyposecretion occurred in Sjögren's syndrome (Baker et al. 2008; Ewert et al. 2010; Mei et al. 2015; Zhang et al. 2016). These studies suggest that the alteration in TJ expression and structure is closely associated with the secretory function of SMG.

By establishing a rabbit SMG transplantation model, we found impaired TJ expression and structure and decreased paracellular permeability in the transplanted hyposecretory SMGs in the early stage after operation, whereas capsaicin improves secretion through modulating expression and function of TJs (Cong et al. 2012), suggesting that TJ might play a crucial role in the transplanted SMGs. However, it is notably that in the long-term stage after transplantation, epiphora is the main complaint since severe epiphora will affect activities of daily life and even cause blurred vision (Geerling et al. 2008; Su et al. 2015; Yu et al. 2004). Recently, we found that TJ width is increased in hypersecretory glands in a long-term rabbit SMG transplantation model (Yang et al. 2017). In the present study, we provided the further evidence that the changes of ZO-1 and occludin expression and function were responsible for alteration of TJ integrity and increase in paracellular permeability in epiphora patients after long-term transplantation of SMGs.

Numerous studies have shown that changes in the content, subcellular distribution and interaction among TJ proteins are often in association with altered TJ structure and barrier function. Occludin played a crucial role in barrier function as evidenced by depletion of occludin in rat SMG-C6 cells which can change the baseline paracellular permeability (Cong et al. 2013). Exposure of intestinal cells to cytokines and bacteria induces endocytosis of occludin and increased paracellular permeability (Yu and Turner 2008; Coyne et al. 2007; Liu et al. 2009). The redistribution of ZO-1 in acute lung injury associates with disruption of barrier integrity in pulmonary epithelium (Jacob and Gaver 2012). The Guk domain of ZO-1 is involved in the recruitment of occludin to be localized at TJ complex, and phosphorylation of occludin prevents its interaction with ZO-1 and destabilizes its assembly at TJs (Elias et al. 2009; Furuse et al. 1994; Schmidt et al. 2001, 2004). Here, we showed that although the contents of ZO-1 and occludin were increased, yet more occludin was translocated from the apicolateral membrane to cytoplasm in epiphora SMGs. More importantly, the interaction between ZO-1 and occludin was significantly decreased in epiphora SMGs. These results indicate that mislocalization and decrease in ZO-1 and occludin interaction might contribute to the disruption in TJ barrier and an opening of paracellular pathway.

The intracellular molecule that mediates the interaction between ZO-1 and occludin was further explored. Occludin is reported to be highly phosphorylated on Ser and Thr residues and herein affects the assembly of TJs (Dorfel and Huber 2012; Raleigh et al. 2011; Rao 2009). CK2 is a ubiquitously expressed Ser/Thr kinase in all eukaryotic organisms, and is able to induce occludin phosphorylation. Treatment with CK2 inhibitors or siRNA results in an enhanced barrier function and reduced paracellular Na^+ flux, whereas knockdown of occludin prevents the CK2-induced change of paracellular permeability in polarized human intestinal epithelial Caco-2 cells, suggesting that CK2-mediated regulation of TJ function is dependent on occludin (Raleigh et al. 2011; Smales et al. 2003). CK2 can also affect the interaction of occludin with ZO-1 and other claudin members in HEK293 cells (Dorfel et al. 2013). However, the role of CK2 in salivary glands remains unknown. mAChR plays an important role in regulation of water and solutes secretion in salivary glands. Activation of mAChR increases paracellular permeability in rat submandibular epithelial cells (Cong et al. 2013). Here, in the freshly isolated human SMG tissues, carbachol directly decreased the interaction between ZO-1 and occludin and pretreatment with CK2 inhibitor abolished the carbachol-induced decreased interaction of ZO-1 and occludin, as well as enlarged TJ width. These results indicate that CK2 was required for the mAChR-modulated paracellular permeability in human SMGs.

Additionally, we have shown that the expression and sensitivity of mAChR were increased in epiphora patients (Ding et al. 2014), and inhibition of mAChR abolishes the hypersecretion in late stage of rabbit SMG transplantation model (Yang et al. 2017). Here, we found that the expression of CK2α and CK2β were elevated in epiphora SMGs, indicating that hypersensitive mAChR in epiphora SMGs might decrease the interaction between ZO-1 and occludin and increase paracellular permeability through the elevated CK2.

Taken together, our data demonstrate that activation of mAChR alters TJ structure and barrier function in human SMGs in a CK2-dependent manner. Decreased ZO-1 and

occludin interaction and elevated CK2 might involve in epiphora after long-term SMG transplantation. Our findings enriched the understanding of the mechanism of mAChR-modulated TJs integrity, clarified the TJ molecules involved in epiphora and provided the possible target for treatment of epiphora after long-term SMG transplantation.

Acknowledgements This study was supported by the National Natural Science Foundation of China 81200799 (CD), 81300893 (XC) and 81470756 (GYY), and Beijing Natural Science Foundation 7173263 (XC).

Author's contributions CD and XC performed the major experiments and wrote the manuscript. XMZ and SLL participated in data interpretation and manuscript improvement. LLW and GYY designed the study, analyzed the data, and wrote the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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