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DISSERTATION

Characterization of temperature-sensitive transient receptor potential channel melastatin 8 (TRPM8) in cultivated human ocular surface cells

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Dedicated to Annette, Ida and Bruno

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Abstract

Background and aim: The dry eye syndrome (DES) is one of the most common ophthalmologic disorders worldwide. DES is often associated with *Pterygium conjunctivae*, a hyperproliferative and vascularized, benign conjunctival tumor. Hyperosmolarity of the tear film and increased activation of transient receptor potential channel vanilloid subtype 1 (TRPV1) are crucial pathogenetic factors in the development of inflammation on the ocular surface. TRPV1 is involved in thermal sensation, osmosensitivity and mediating inflammation. So far, treatment options only provide symptomatic relief by artificial tear substitution and topical anti-inflammatory drugs. Previous data indicate an inhibitory effect on TRPV1 channel mediated calcium-signaling via activation of another TRP channel (melastatin 8, TRPM8). Therefore, this thesis aims to further characterize these TRPs and the effects of known and novel modulators in connection with DES and pterygium. **Methods:** SV40-transfected immortalized human corneal epithelial cells (HCEC), human conjunctival epithelial cells (HCjEC) and primary cultivated human pterygium epithelial cells (hPtEC) were used as *in vitro* cell models. As heterologous TRPM8 transfection model, an osteosarcoma cell line (U2osTRPM8, B2) was used for transfected cells (TRPM8) and non-transfected controls (wild type). Changes in the intracellular calcium concentration ($[Ca^{2+}]_i$) were investigated with fluorescence calcium imaging. The planar patch-clamp technique was employed to measure underlying whole-cell currents. **Results:** Physical cold stimulation (< 10 °C), application of menthol (500 µM), icilin (15 – 60 µM) as well as the novel endogenous thyroid hormone metabolite $3T_1AM$ (thyronamine; 1 µM) increased $[Ca^{2+}]_i$ and whole-cell current density in HCEC. This effect could be suppressed by the TRP channel blockers BCTC (10 – 20 µM) and lanthanum-III-chloride (La^{3+}; 500 µM), respectively. $3T_1AM$ and menthol also elicited calcium responses in TRPM8-transfected cells whereas non-transfected controls did not show any significant effect. Capsaicin (CAP; 20 µM) induced increases in whole-cell currents and calcium transients in HCEC. Notably, these responses were inhibited by pre-incubation with either icilin (15 µM) or $3T_1AM$ (1 µM). In HCjEC, icilin (60 µM) induced reversible calcium transients. The calcium response to icilin (60 µM) in hPtEC differed significantly from HCjEC. **Conclusions:** This study demonstrates the functional expression of TRPM8 channels in HCEC, HCjEC and hPtEC for the first time. $3T_1AM$ mediates its effects through TRPM8 channel activation. Furthermore, TRPM8 activation by the artificial cooling compound icilin or by a thyroid hormone derivative ($3T_1AM$) inhibited TRPV1-linked calcium signaling pathways. Therefore, $3T_1AM$ may be a possible new target in connection with the TRPs to develop an alternative treatment of DES.

Abstrakt

Hintergrund und Zielsetzung: Das Syndrom des trockenen Auges (DES) ist eine der häufigsten ophthalmologischen Erkrankungen weltweit. DES ist oftmals mit *Pterygium conjunctivae* assoziiert, einer benignen, vaskularisierten Bindehautwucherung. Ein hyperosmolarer Tränenfilm und damit verbundene erhöhte Aktivierung des transient receptor potential channels vom Vanilloidsubtyp 1 (TRPV1) sind entscheidende Pathogenesefaktoren in der Entwicklung der Entzündung der Augenoberfläche. TRPV1 ist beteiligt an der Temperaturwahrnehmung, Osmosensitivität und Entzündungen. Die einzig verfügbare Therapie ist symptomatisch mit künstlichen Augentropfen und topischen anti-inflammatorischen Medikamenten. Vorangehende Studien zeigten einen inhibitorischen Effekt auf TRPV1-induzierte Kalziumströme durch die Aktivierung eines anderen TRP-Kanals (TRP melastatin 8 (TRPM8)). Diese Arbeit charakterisiert diese TRP-Kanäle sowie Effekte von bekannten und neuartigen TRP-Modulatoren in Verbindung mit DES und Pterygium. **Methoden:** SV40-transfizierte immortalisierte humane Korneaepithelzellen (HCEC), humane Konjunktivaepithelzellen (HCjEC) und primär kultivierte humane Epithelzellen von Patienten mit *Pterygium conjunctivae* (hPtEC) wurden als *in vitro* Zellmodelle verwendet. Weiterhin wurde eine TRPM8-transfizierte Osteosarkomzelllinie (U2osTRPM8, B2) und deren Wildtyp als heterologes Transfektionskontrollmodell verwendet. Veränderungen der intrazellulären Kalziumkonzentration ($[Ca^{2+}]_i$) wurde mit Fluoreszenz-Ca^{2+}-Imaging untersucht. Planares Patch-Clamping wurde verwendet, um Ganzzellströme zu messen. **Ergebnisse:** Physikalische Kühlung (< 10 °C), Menthol (500 µM), Icilin (15 – 60 µM) sowie der neuartige endogene Schilddrüsenhormonmetabolit $3T_1AM$ (Thyronamine; 1 µM) erhöhten die $[Ca^{2+}]_i$ und die Ganzzellstromdichte in HCEC. Dieser Effekt konnte durch die TRP-Kanalblocker BCTC (10 – 20 µM) und Lanthanum-III-Chlorid (La^{3+}; 500 µM) unterdrückt werden. $3T_1AM$ und Menthol führten ebenfalls in TRPM8-transfizierten Zellen zu messbaren Kalziumeinströmen, wohingegen in nicht-transfizierten Zellen kein Effekt messbar war. Auch Capsaicin (CAP; TRPV1 Agonist; 20 µM) induzierte Ganzzellströme und Kalziumeinströme in HCEC. Diese konnten durch Präinkubation mit entweder Icilin (15 µM) oder $3T_1AM$ (1 µM) geblockt werden. In HCjEC induzierte Icilin (60 µM) reversible Kalziumeinströme. Diese unterschieden sich signifikant von denen, die Icilin (60 µM) in hPtEC auslöste. **Zusammenfassung:** Die vorliegende Studie zeigt erstmals die funktionale Expression von TRPM8 in HCEC, HCjEC sowie hPtEC. $3T_1AM$ aktiviert TRPM8. TRPM8-Aktivierung durch Icilin oder $3T_1AM$ inhibiert die TRPV1-vermittelte Kalziumsignalkaskade. Deshalb stellt $3T_1AM$ in Verbindung mit TRP-Kanälen eine mögliche neue Therapieoption bei DES dar.

1 Introduction

The dry eye syndrome (DES) is one of the most common ocular health problems worldwide [1]. In general, due to a hyperosmolar tear film, patients experience foreign body sensation, pain and inflammation of the ocular surface [2]. As a result, this leads to increases in epithelial shedding and loss of tight junctional barrier function [3], thereby causing an increased vulnerability to infection by infiltrating pathogens in affected patients [4]. DES can also be a side effect of neoplastic formations such as *Pterygium conjunctivae* [5,6], a benign ocular tumor that starts at the limbus and spreads across the cornea.

Currently, there are no therapeutic options that selectively target the pathogenesis of DES. The majority of the available treatment options is merely symptomatic and ranges from the substitution of artificial tear solution to (invasive) punctual plug/occlusion [2]. There is only one therapy regimen so far that directly targets the inflammatory cascade (topical Cyclosporine A or steroid application), but these agents have severe side effects such as bacterial or fungous (super) infection, cataract formation and intraocular pressure spikes [7,8]. Therefore, further research is required in order to develop alternative treatment options in DES therapy.

Previous research showed that a hyperosmolar tear film results from declines in either tear fluid formation and/or Meibomian glandular lipid secretory activity [4]. Such dysfunction induces epithelial cell volume shrinkage [9] and can lead to innate immune cell activation and inflammation [10]. Osmosensitivity and mediating inflammation are two key properties of a membrane-bound calcium channel superfamily – the transient receptor potential channels (TRPs) [11]. There is emerging evidence that drug targeting of TRP channels is a viable approach for treating this disease [12-14].

1.1 The transient receptor potential channel superfamily

Ion channels of the TRP channel superfamily contribute to changes in intracellular calcium in neurons and non-excitable cells [15]. Since their first discovery in *Drosophila melanogaster*, seven subfamilies have been described, six of them mammalian [16]. Every TRP channel contains six transmembrane subunits (S1 - S6), with a pore-forming loop between S5 and S6 and intracellular located NH_2- and COOH-termini [16]. Chemically related TRP subtypes are thought to form homo- and heteromultimers when they are co-expressed in the same cell [16]. Such combination of TRP channel proteins can form a huge diversity of channels with numerous interactions, biological functions and physical properties.

The TRPM (melastatin) subgroup contains eight members (TRPM1-8) [11]. TRPM8, also known as menthol receptor, is commonly referred to as endogenous *"cold receptor"*, because it is

activated by moderate cooling from ~23 - 28 °C [16]. So far, the expression of functional TRPM8 channels in ocular surface cells was only shown in human corneal endothelial cells [17]. Further studies should aim to identify functional TRPM8 expression in other ocular surface cells due to suggestive evidence that TRPM8 might interact with another subtype of the TRP superfamily – the TRPV1 (vanilloid 1) [17-19]. The TRPV (vanilloid) subfamily consists of six mammalian members. The most investigated TRPV channel is the TRPV1 (*"capsaicin receptor", "pain receptor", "heat receptor"*). In addition, this is also the best-characterized member of the TRPV subfamily in terms of expression pattern, characteristics and clinical translation of its manipulation [20]. This channel integrates both thermal and chemical stimuli, leading to the sensation of noxious heat at innocuous temperatures [11].

1.2 TRPs in ocular surface cells and relevance in dry eye syndrome

For ocular surface cells, TRPV1 expression has been shown in human corneal epithelial cells (HCEC) [21], human corneal endothelial cells [22] as well as in human conjunctival epithelial cells (HCjEC) [23] and corneal fibroblasts [24] [reviewed in 25,26]. The TRPV1 receptor is involved in the corneal wound healing process [27,28]. Since corneal scratching and wounding through a lack in lubrication is a common result in dry eye disease, this receptor might also play a crucial role in the aggravation of DES. According to Okada et al., loss of TRPV1 (TRPV1 $^{-/-}$) in a mice corneal wound healing model affected declines in inflammation and fibrosis/scarring during the healing process, thus improving the outcome [28]. On the other hand, Sumioka et al. recently reported that TRPV1 signaling is necessary in order for corneal wounds to heal adequately [27]. Also using a TRPV1 knock-out mouse model, they were able to show, that in TRPV1-deficient mice, re-epithelialization was impaired by the suppression of both cell migration and proliferation [27]. The absence of TRPV1 attenuated the expression of interleukin 6 (IL-6), a pro-inflammatory agent, in response to epithelial debridement [27]. Therefore, TRPV1 activation is needed to achieve satisfactory epithelial wound healing, but might also have negative effects on the visual outcome.

Like TRPV1, TRPM8 is another thermo-sensitive TRP channel that is expressed in the human corneal endothelium [17], ocular tumor cells such as uveal melanoma cells [19] and corneal nerve fibers [29]. In the latter, upon activation it increases lacrimation and blinking rate [29]. Therefore, TRPM8 activation *in vivo* could dampen DES symptomatology. In addition, TRPM8 activation suppressed the cellular calcium response usually following TRPV1 activation in HCjEC [14]. A similar observation was recorded in eye tumor cells [18]. Furthermore, in a mouse model of colitis, TRPM8 activation in gastro-epithelial cells inhibited inflammatory

responses following TRPV1 activation [30], thus rendering TRPM8/TRPV1 interaction a novel and promising target to inhibit inflammatory responses due to a hyperosmolar stimulant in DES. A recently discovered endogenous thyroid hormone metabolite, 3-monoiodo-L-thyronamine, ($3T_1AM$), has been detected to activate TRPM8 in HCjEC [14]. Chemically, this metabolite is closely related to the thyroid hormones T_3 and T_4 [31]. Under physiological conditions, it is present in the human serum [32,33]. In addition, $3T_1AM$ elicits a number of systemic effects including reductions in cardiac drive [34] and the respiratory quotient along with hyperglycemia [35,36] as well as rapid hypothermia (in rodents) [31,37]. $3T_1AM$ generally interacts with the human trace amino acid receptor 1 (hTAAR1) in several brain regions as well as with mitochondrial targets [38]. $3T_1AM$ interactions with alpha2-adrenergic receptors in HCjEC were shown recently [39]. Owing to its TRPM8 activating property, $3T_1AM$ could be an interesting new agent in mediating TRPV1 activity inhibition, thus avoiding inflammation caused by DES.

1.3 Aim of study

Based on the aforementioned findings, the following postulations were suggested:

#1: The human corneal epithelium, human conjunctival epithelium and pterygial tissue express functionally active, cold-sensitive TRPM8 channels that have similar characteristics as TRPM8 channels in the human corneal endothelium. *In vitro* cell models and fluorescence calcium imaging as well as patch-clamping will be used to validate TRPM8 functional expression.

#2: It is likely that there is an interaction between TRPM8 and TRPV1 and that TRPM8 activation even inhibits the TRPV1 channel.

#3: $3T_1AM$ activates TRPM8 and is therefore an interesting alternative option to suppress TRPV1-induced calcium influx.

2 Methods

2.1 Cell cultivation

Simian vacuolating virus 40 (SV40)-transfected immortalized human cornea epithelial cells (HCEC) were kindly provided by Paulsen et al. and Reichl et al. They are an established cell model for the human corneal epithelium [40]. The cells were cultured as previously described [12]. Briefly, the cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal calf serum (FCS), 20 mg/ml insulin and 5 % antibiotics (penicilin and streptomycin). 1 – 3 days prior to the calcium imaging measurements, the cells were seeded on dishes with sterile glass cover slips and grown in a humidified atmosphere with 5 % carbon dioxide (CO_2) and 95 % ambient air. Cell subcultivation by cell passage was performed with

accutase. Accutase activity was quenched with serum-supplemented growth medium. Washing with phosphate buffered saline (PBS) and change of the growth medium was performed three times a week.

Freshly isolated conjunctiva tissue was donated by a patient with pterygium. The excision was performed at the Department of Ophthalmology, Campus Virchow-clinic, Charité - University of Medicine Berlin. Tissue samples were treated enzymatically (collagenase IV) and then placed on glass coverslips in six multi-wells filled with the aforementioned nutrient medium. After a week, the cells were gathered and put onto six smaller cover slips in two wells. After 1 – 3 days, cells were used for calcium imaging measurements.

2.2 Chemicals and solutions

Capsazepine and icilin were purchased from Cayman Chemical Company (Ann Arbor, Michigan, U.S.A.). BCTC and fura-2/AM were purchased from TOCRIS Bioscience (Bristol, United Kingdom). $3T_1AM$ was commercially synthesized by Dr. R. Smits (ABX advanced biochemical compounds, Radeberg, Germany) and purified by Dr. R. Thoma (Formula GmbH Pharmaceutical and Chemical Development Company, Berlin, Germany). It was kindly provided by Daniel Ratmann from the group of Josef Köhrle (Institute of Experimental Endocrinology, Charité University Berlin). Medium and supplements for cell culture were purchased from Life Technologies Invitrogen (Karlsruhe, Germany) or Biochrom AG (Berlin, Germany). Accutase was purchased from PAA Laboratories (Pasching, Austria). All other reagents were purchased from Sigma (Deisenhofen, Germany). For calcium imaging recordings, Ringer-like (control) bathing solution containing 150 mM NaCl, 6 mM CsCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES-acid and 1 mM glucose at pH 7.4 was used. Planar patch-clamp recordings were performed in an internal solution containing 50 mM CsCl, 10 mM NaCl, 60 mM CsF, 20 mM EGTA, and 10 mM HEPES-acid at pH 7.2 and 298 mOsm (provided by Port-a-Patch©, Nanion, Munich, Germany). External solution (Nanion, Munich, Germany) contained 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM D-glucose monohydrate and 10 mM HEPES-acid at pH 7.4 and 298 mOsm according to Fertig et al. [40].

2.3 Intracellular calcium imaging

The fluorescent dye fura-2/AM (am = acetoxymethylester) is widely used to detect very small changes in free cytosolic calcium (range: nM). This dual wavelength dye responds to stimulation (light) by definite wavelengths (340 nm to 380 nm – absorption maximum) with the emission of a longer wavelength (510 nm – emission maximum). It is possible to measure very small

changes of intracellular [Ca^{2+}] even in a single cell, if the measured area is narrowed down to a certain degree. The cells were pre-incubated with fura-2/AM (2 µM) for 15 – 45 minutes. During this time, the dye passively diffused into the cell. Within the cell, the acetoxymethylester is cut off by enzymes, making the dye non-diffusible and keeping it inside the cell. Part of the fura-2/AM is binding to free calcium ions and responds to 340 nm. The non-calcium binding part responds to 380 nm. While at 340 nm the dye responds to an increase of calcium with an increase of fluorescence, at 380 nm it decreases. These wavelengths are the specific excitation wavelengths for fura-2/AM, because a clear difference of fluorescence can be expected when intracellular Ca^{2+} concentration is changing. After pre-incubation, the cells were washed several times in a Ringer-like (control) bathing in order to remove any cell debris and superfluous dye. A photomultiplier was used to detect the fluorescence light. The ratio of the two wavelength-traces was calculated, which correlates directly to the changes in free cytosolic calcium as described by Grynkiewicz et al. in 1985 [42]. TIDA software for Windows (v5.0 by HEKA, Lamprecht, Germany) was used to track the fluorescence signals. The ratio of the two recorded traces (f_{340nm}/f_{380nm}) eliminates potential confounders such as cell thickness and different concentrations of fura-2/AM inside the cells. Primary calcium data were normalized according to the calcium baseline level. All experiments were performed at approximately 21 °C room temperature.

2.4 Planar patch-clamp recordings

Electrophysiological recordings were obtained through the use of the whole-cell mode of a high throughput semi-automated planar patch-clamp setup. Planar patch-clamp measurements were made in connection with the PatchMaster software (v2.73.1; HEKA, Lamprecht, Germany) and software which controls the pump. On the day of the patch-clamp experiments, the cells were detached from the bottom of the flask by applying trypsin for a short period of time. The reaction was stopped by supplementing DMEM containing FCS. Single cell suspension was prepared in standard extracellular solution. After centrifugation, the medium was exchanged for an external medium. By gently pipetting the medium and the pelleted cells, a single cell solution with a cell density of approximately $1x10^8$ – $5x10^7$/ml was obtained and confirmed with a microscope.

The novel Port-a-Patch© system was used, which is the smallest patch-clamp setup worldwide. Instead of attaching a patchpipette to a single cell, a cell suspension is placed onto a micro-chip with a small aperture. The resistance of the microchip is similar to the resistance of a conventional patch pipette (2.5 – 3 MΩ). A single cell is brought to the aperture by a negative pressure, supplied by a software-controlled pump. Inside the micro-chip, solution similar to intracellular fluid is located. Outside, around the attached cell, extracellular-like solution is

brought onto the chip with a small pipette. In this way, the cell is electrically connected with the electrical system and measurements of very small membrane currents can be performed. These currents were recorded using an EPC 10 patch-clamp amplifier with PatchMaster software for Windows®. Membrane capacitance (C_M) and access resistance (R_S) were calculated by the software. Series resistances, fast and slow capacity transients as well as leak currents (up to ≈ 100 pA) were compensated by the patch-clamp amplifier in connection with the software. All experiments were performed at ≈ 22 °C room temperature, unless stated otherwise. Measurements regarding specific TRPM8 and TRPV1 channel behavior were achieved through the application of voltage step-protocols (10 mV voltage steps at a range of −60 to +130 mV for 400 ms each). Holding potential (HP) was set to 0 mV in order to eliminate any possible contribution of voltage-dependent Ca^{2+}- channel activity. Currents were also recorded by a voltage ramp protocol of −60 to +130 mV range and 500 ms duration 5 s each. Resulting currents were normalized with respect to cell membrane capacitance to obtain current density (pA/pF).

2.5 Statistical analysis

Statistical significance was determined using Student's *t*-test for paired data (p-values: two-tailed) after a passed normality test according to Kolmogorov-Smirnov. In certain cases, non-parametric Wilcoxon matched pairs were used. For non-paired data, Student's *t*-test for unpaired data was used, after a passed normality test. If this was not the case, non parametric Mann-Whitney-U test was performed. Welch's correction was applied if data difference varied too much. Probabilities of $p < 0.05$ were considered to be significant. Statistical tests were performed with GraphPad Prism 5.0, SigmaPlot 12.0 and Microsoft Excel 2010 software. The number of repeats is shown in each case in brackets. All values are means ± standard error of the mean (SEM). This study acknowledges and was performed accordingly to the statutes of the Charité – University of Medicine Berlin for securing good scientific practice [43]. In 2010, the study was approved by the ethics committee of the Charité under file number EA2/076/10 and was recently renewed (July 2015). All patients with pterygium, who provided the primary cells, were required to provide a signed written consent.

3 Results

3.1 Strong cold stimulation, menthol and icilin induce calcium influx in HCEC

Strong physical cold stimulation was achieved by adding cold Ringer-like solution of approximately 8 °C to the measuring chamber. Mean cooling temperature was 8.58 °C ± 0.54 °C

(n = 16). After this (strong) cooling effect, a transient increase of the fluorescence ratio f_{340nm}/f_{380nm} was detectable. Specifically, it went from 1.203 ± 0.002 to 1.227 ± 0.005 ($p = 0.013$, $n = 7$) within thirty seconds. At the end of the experiment, the fluorescence ratio f_{340nm}/f_{380nm} recovered to 1.207 ± 0.002. Furthermore, cells were pre-incubated with the TRPM8 antagonist, 20 µM BCTC, for 20 minutes. After applying cold Ringer-like solution, the fluorescence ratio f_{340nm}/f_{380nm} decreased from 1.2 ± 0.002 to 1.188 ± 0.003 ($p = 0.001$, $n = 9$). There was a recovery detectable as the ratio increased (1.203 ± 0.002) towards the end of the measurements. Notably, the response pattern to strong cold stimulation of the two cell samples differed significantly. As the pre-treated cells presented a decrease in fluorescence, the non pre-treated cells showed an increase (1.227 ± 0.005 and 1.188 ± 0.003, $p < 0.0001$, $n = 7 - 9$). Similar results were obtained by using 500 µM lanthanum-III-chloride (La^{3+}) instead of BCTC.

If the cells were treated with the highly selective TRPM8 channel agonist menthol, HCEC produced a larger Ca^{2+} response. Specifically, 500 µM menthol increased the fluorescence ratio f_{340nm}/f_{380nm} from 1.203 ± 0.004 to 1.279 ± 0.011 after 300 s ($n = 7$, $p = 0.0001$), while in the presence of 10 µM BCTC the ratio merely increased from 1.204 ± 0.009 to 1.236 ± 0.01 in the same time ($n = 6$, $p = 0.1034$, not significant).

Similar results were obtained with 60 µM icilin, which activates TRPM8 but also TRPA1. Application of icilin induced a fast irreversible increase of the fluorescence ratio f_{340nm}/f_{380nm} within 20 seconds from 1.198 ± 0.002 up to 1.372 ± 0.002 ($p = 0.0004$, $n = 5$). There was no recovery effect detectable, as the ratio was still at 1.347 ± 0.061 towards the end of the measurements. The cells that were pre-incubated with 20 µM BCTC showed a slight increase after application of 60 µM icilin from 1.197 ± 0.0008 to 1.218 ± 0.013, which was not considered to be significant ($p > 0.05$, $n = 8$), as was the ratio after 10 minutes (1.206 ± 0.018, $p > 0.05$, $n = 8$). Taken together, these results implicate an involvement of cold-sensitive TRPM8 channels in cellular response to physical cooling and established modifiers of TRPM8 activity.

3.2 Icilin increases whole-cell currents in HCEC

Icilin (15 µM) irreversibly increased inward currents (−60 mV) from −6 ± 1 to −13 ± 2 pA/pF (p ± 0.0005; n = 14 – 17) in HCEC. The non-selective TRP channel blocker lanthanum-III-chloride (La^{3+}, 500 µM) suppressed this rise to −7 ± 3 pA/pF (p ± 0.05; n = 10 – 14). Similar results were obtained regarding the outward currents. Correspondingly, outward currents (+130 mV) increased from +66 ± 13 up to +105 ± 21 pA/pF (p ± 0.01; n = 12 – 15) after application of icilin and they fell after application of La^{3+} to +50 ± 7 pA/pF (p ± 0.05; n = 7). In summary, there is convincing evidence of functional TRPM8 expression in HCEC.

3.3 Icilin increases calcium influx and whole-cell currents in HCjEC

Similar to effects occurring in HCEC, application of icilin (60 µM) induced an irreversible increase of the fluorescence ratio f_{340nm}/f_{380nm} within 2 min from 1.203 ± 0.002 to 1.218 ± 0.01 ($p < 0.01$, n = 7) in cultivated HCjEC. After 600 s, the fluorescence ratio f_{340nm}/f_{380nm} was still at 1.211 ± 0.008 ($p < 0.05$) (recovery) and was also significant. The icilin-induced Ca^{2+} influx was suppressed by the TRP channel blocker La^{3+}. Specifically, La^{3+} reduced the icilin-induced increase of the fluorescence ratio f_{340nm}/f_{380nm} to 1.202 ± 0.003 at 400 s and to 1.183 ± 0.015 at 600 s ($p < 0.01$, n = 5 – 7). Taken together, icilin-induced Ca^{2+} elevation in HCjEC was significantly suppressed in the presence of La^{3+}. Furthermore, icilin (60 µM) also increased the current density in a planar patch-clamp setting from +11.73 ± 12.64 to +65.28 ± 42.09 pA/pF ($p < 0.05$, n = 4). Therefore, these results confirmed functional TRPM8 activity also in HCjEC.

3.4 Icilin induces different calcium response pattern in freshly isolated hPtEC

Application of icilin (60 µM) did not significantly change intracellular Ca^{2+}. However, compared to the HCjEC, the icilin-induced effect was at significantly lower levels ($p < 0.05$, n = 3 – 7), specifically at the end of the measurements (HCjEC: 1.211 ± 0.008, n = 7 and hPtEC: 1.186 ± 0.014, n = 3; $p < 0.01$, n = 3 – 7). This altered response pattern to a chemical agent suggest a modified functional TRPM8 expression in hPtEC compared to the cultivated counterpart (HCjEC).

3.5 $3T_1AM$ increases calcium influx and whole-cell current density through TRPM8 activation in HCEC

HCEC were challenged with 1 µM $3T_1AM$. An immediate increase of the fluorescence ratio f_{340nm}/f_{380nm} from 1.199 ± 0.001 to 1.217 ± 0.003 ($p = 0.001$, n = 6) was evident after 400 seconds. Compared to baseline this was highly significant ($p < 0.0001$, n = 6 – 8). The cells showed no sign of recovery, as the final fluorescence ratio f_{340nm}/f_{380nm} was still at 1.223 ± 0.004 after 600 seconds ($p = 0.002$, n = 6). This elevated level of intracellular calcium differed significantly from the control cells (1.201 ± 0.001 and 1.223 ± 0.004, $p < 0.0001$, n = 6 – 8). Furthermore, investigation of underlying whole-cell currents showed that after application of 1 µM $3T_1AM$ both inward and outward current channel activity increased. Specifically, current density rose from –6.435 pA/pF ± 0.91 to –14.78 pA/pF ± 2.103 ($p = 0.0004$, n = 18 – 26). As La^{3+} was supplemented, current density went back to –5.75 pA/pF ± 1.79 ($p = 0.007$, n = 8 – 18). Similar results were obtained at +130 mV. $3T_1AM$ increased current density from +99.1 pA/pF ± 16.21 to +131 pA/pF ± 31.03 ($p = 0.046$, n = 18). La^{3+} partly suppressed this increase and

brought current density back to +75.88 pA/pF ± 28.85 ($p = 0.042$, $n = 8$). Similar results were obtained with 10 μM BCTC.

In order to validate TRPM8 channel involvement in the cellular response to $3T_1AM$ in HCEC, experiments were conducted using a TRPM8-transfected, heterologous cell line (U2osTRPM8, B2). Menthol, a well known TRPM8-selective agonist, as well as $3T_1AM$ were used to probe for TRPM8 functional expression. The results were compared to the wild type osteosarcoma cell line that does not express TRPM8 channels (U2osWT). 500 μM menthol did not affect $[Ca^{2+}]_i$ in the wild type osteosarcoma cell line. In the transfected counterpart menthol induced a calcium response similar to that recorded in HCEC. 20 μM BCTC suppressed the increase induced by menthol (1.351 ± 0.022 to 1.222 ± 0.005; $n = 3 – 5$; $p < 0.01$). $3T_1AM$ (1 μM) had effects similar to those induced by menthol. The fluorescence ratio f_{340nm}/f_{380nm} increased from 1.206 ± 0.002 to 1.265 ± 0.005 ($n = 5$; $p < 0.05$), which was at lower levels than those induced with menthol (1.351 ± 0.022; $n = 5$; $p < 0.05$). Additionally, the Ca^{2+} response pattern differed significantly suggesting a more complex Ca^{2+} response underlying the cellular response to $3T_1AM$ than menthol. Similar to menthol, 10 μM BCTC blocked the cellular calcium response to $3T_1AM$ (1.265 ± 0.005 to 1.203 ± 0.001; $n = 4 – 5$; $p < 0.01$).

Furthermore, the effective dose of $3T_1AM$ (ED_{50}) was determined. HCEC fluorescence was measured for ten minutes using $3T_1AM$ in varying concentrations. The highest alteration of intracellular $[Ca^{2+}]_i$ in each measurement was used for evaluation. The effect of $3T_1AM$ on free cytosolic calcium varies according to the concentration. $3T_1AM$ induced dose-dependent increases in $[Ca^{2+}]_i$ between 300 nM and 10 μM ($n = 3 – 8$). The calculated ED_{50} value was 1.2 ± 0.0 μM.

3.6 Activation of TRPM8 inhibits TRPV1 activity in HCEC and HCjEC

A possible interaction between TRPV1 channel activity and TRPM8 channel activation in human corneal endothelial cells was previously described by Mergler et al. [17]. Since these channels are co-expressed in HCEC and HCjEC, this series of experiments was initiated to further evaluate an interaction between the TRPV1 channel activity and TRPM8 activation. Therefore, HCEC were pre-incubated with 15 μM icilin for 20 min. The fluorescence ratio f_{340nm}/f_{380nm} rose from 1.205 ± 0.002 to 1.245 ± 0.001 ($p < 0.0001$, $n = 8 – 9$) during these twenty minutes. Then, 20 μM capsaicin was added. In non-pre-incubated cells, the ratio f_{340nm}/f_{380nm} went from 1.196 ± 0.002 to 1.352 ± 0.033 ($p = 0.0005$, $n = 10$). However, the pre-incubated cells also developed a slight increase from 1.245 ± 0.001 to 1.261 ± 0.002 ($p = 0.0003$, $n = 9$), which

was still significant. The compared response pattern to capsaicin of both cell samples showed a statistically relevant difference of 1.352 ± 0.033 and 1.261 ± 0.002 ($p = 0.019$, n = 9 – 10).
Similar results were obtained, when icilin was exchanged for $3T_1AM$ (1 µM) in a comparable setting. The fluorescence ratio f_{340nm}/f_{380nm} increased from 1.199 ± 0.002 to 1.248 ± 0.001 ($p < 0.0001$, n = 6). Then, capsaicin (20 µM) was added. Notably, there was no significant alteration in the fluorescence ratio f_{340nm}/f_{380nm}, as it settled at 1.242 ± 0.005. When exposed to capsaicin, fluorescence in non-pretreated cells increased from 1.196 ± 0.002 to 1.352 ± 0.033 ($p = 0.001$, n = 10) within a few seconds. Furthermore, intracellular [Ca^{2+}] continued at an elevated level of 1.288 ± 0.021 ($p = 0.002$, n = 10) until the end of the experiment.
A patch-clamp recording was conducted to investigate the underlying whole-cell currents in HCEC. $3T_1AM$ was able to increase outward current activity from +78.961 pA/pF up to +128.624 pA/pF corresponding to the previous results in this study using $3T_1AM$. However, this effect was transient. When the current density was back to the original baseline level, 20 µM capsaicin was added. Interestingly, CAP was not able to increase current density like in previous experiments (no additive effect). A similar observation was recorded in HCjEC. At −60 mV, the inward currents in the presence of $3T_1AM$ increased from −10.50 ± 2.33 pA/pF to −23.50 ± 5.16 pA/pF (n = 12 – 14; p ± 0.001). At +130 mV, the outwardly rectifying currents increased from +85.83 ± 21.49 pA/pF to +167.91 ± 19.52 pA/pF (n = 12 – 14; p ± 0.05). Notably, no augmentation of in- and outward currents was detected after replacing $3T_1AM$ with 20 µM CAP.

4 Discussion

The results of this thesis show that HCEC, HCjEC and hPtEC express functional TRPM8 channels. The HCEC cell line is a physiologically relevant and well-established cell model (since 1995) that elicits similar responses compared to those of the freshly isolated counterpart [12,40,44]. Furthermore, an interesting aspect in connection with the recently discovered thyroid hormone metabolite $3T_1AM$ could be found. Specifically, $3T_1AM$ was detected to mediate its cellular effect through activation of the TRPM8 channel in HCEC as well as HCjEC. TRPM8 activation is expected to counter negative effects (inflammation) elicited by TRPV1 activation through exposure to a hyperosmolar tear film in DES.

4.1 Functional expression evidence of TRPM8 in HCEC, HCjEC and hPtEC

This is the first study evidently showing the functional expression of cold-sensitive TRPM8 channels in HCEC, HCjEC as well as in the benign tumor cells of hPtEC. The expression of TRPM8 channels in those tissues is indicated since cellular responses to known putative and well

described TRPM8 agonists were detected. Similar findings were also found in ocular tumor cells [18,19]. This is in accordance with the literature since physical cooling (< 10 °C), icilin as well as menthol all induced calcium responses reflecting this type of TRP channel expression [15,45]. In this thesis, pharmacological blockage of TRPM8 by BCTC suppressed the intracellular calcium increase induced by cooling, menthol and icilin, respectively. Furthermore, icilin induced increases in current density, which were recorded with the planar patch-clamp technique. Outward and inward rectifying current behavior after application of icilin is consistent with functional TRPM8 channel expression in corneal endothelial cells [17] and non-ocular cells [15,45,46]. Another TRP channel blocker (La^{3+}) was also able to suppress icilin-induced increases in current density in the planar patch-clamp recordings. In contrast to the calcium-imaging method, a lower concentration of icilin was used in this setting since the planar patch-clamp technique measures currents across one single cell membrane. As previously shown by Mergler et al., icilin also mediates a recordable effect at this concentration [17].

4.2 Physiological role of TRPM8 in HCEC

Under physiological conditions, the human cornea does not experience temperatures that potentially activate TRPM8 channels. The mean human corneal temperature range varies between 32 °C and 34 °C [47], but TRPM8 channels activate at temperatures from < 23 – 28 °C [16]. However, in DES corneal cooling can occur through excessive evaporation of tear fluid, thus lowering corneal temperature and activating cold receptors in corneal neurons [29] and epithelial cells. Activation of corneal neuronal cold receptors increases blinking rate and tearing rate [29]. The *in vivo* effects of corneal epithelial cold receptor activation are yet to be elucidated. Interestingly, moderately cooled artificial tears provide relief to the eye by reducing corneal and conjunctival sensation [48] and corneal cooling reduced side effects after photorefractive keratectomy [49]. In conclusion, corneal epithelial TRPM8 channels provide a potential advantage in breaking the vicious cycle of DES, because they might sense a loss in tear fluids through evaporation and corneal cooling.

4.3 $3T_1AM$ activates TRPM8 in HCEC and HCjEC

$3T_1AM$ irreversibly increased calcium influx and whole-cell currents in HCEC as well HCjEC under conditions similar to those *in vivo*. A recovery effect to baseline level could not be observed, suggesting that the cells are not able to reverse the calcium influx caused by $3T_1AM$. $3T_1AM$ also increased $[Ca^{2+}]_i$ in a calcium-free medium in HCjEC [14], suggesting a depletion of cytoplasmatic calcium stores. Additionally, the endogenous metabolite N–Ac–T_1AM of

$3T_1AM$ elicited a calcium response in HCEC, suggesting that this metabolite is still functionally active (unpublished observation by Mergler et al., 2015).

Furthermore, the calcium influx induced by $3T_1AM$ differs in accordance with the applied concentration. The highest alteration in $[Ca^{2+}]_i$ could be detected at 10 µM $3T_1AM$. The calculated effective dose (ED_{50}) of $3T_1AM$ was 1.2 µM. To a lesser extent, $[Ca^{2+}]_i$ increased at 300 nM. At 100 nM, possible alterations in cytosolic calcium were too small to detect. Additionally, TRP channel involvement was confirmed by showing that application of BCTC and La^{3+} inhibited whole-cell currents induced by $3T_1AM$. TRPM8 selectivity was validated by showing that in a heterologous TRPM8 channel expression system the Ca^{2+} transients were comparable to those in HCEC and in both cases inhibited by BCTC. In order to be a viable candidate for future dry eye therapy, the *in vivo* effects of $3T_1AM$ await further evaluation.

4.4 Medical relevance of crosstalk between TRPM8 and TRPV1

The physiological implications of TRPM8 and TRPV1 co-expression, as it occurs in the human corneal endothelium [17] await clarification. This thesis shows a functional co-expression of TRPV1 and TRPM8 in HCEC and HCjEC for the first time.

Notably, TRPM8 activation with icilin or $3T_1AM$ reduced capsaicin-induced calcium transients in HCEC and HCjEC. Furthermore, CAP- and hypertonicity-induced rises of in– and outward current density could be blocked by TRPM8 activation with icilin or $3T_1AM$ [50]. These results strongly suggest a negative feedback effect of TRPM8 activation on TRPV1 channel activity. A similar inhibitory effect has been recorded in HCjEC [14]. Application of CAP to $3T_1AM$-pretreated cells had no additive effect on intracellular Ca^{2+} and whole-cell currents. Instead, capsaicin failed to elicit a cellular response in these cells. Furthermore, results by Khajavi et al. 2015 implicate that TRPM8 activation by $3T_1AM$ also inhibits TRPV1-regulated downstream signaling [14]. Specifically, after application of $3T_1AM$, secretion of interleukin-6 (IL-6), a pro-inflammatory agent, in examined cells significantly decreased [14]. IL-6 is the key mediator of inflammation following TRPV1 activation [51]. In DES, TRPV1 activation is achieved through a hyperosmolar tear film [4]. In brief, activation of TRPV1 induces a calcium influx which activates the transforming growth factor kinase 1 (TAK1) and the mitogen-activated protein kinase (MAPK) - two key steps that ultimately lead to the activation of nuclear factor – kappa b (NF-κB) and increases in IL-6 release [51]. In combination with the crosstalk of TRPM8 and TRPV1 channels in HCEC and HCjEC, inhibition of TRPV1 poses a potential new option to target DES pathophysiology [reviewed in 26]. Also, inhibition of TRPV1 activity in order to avoid inflammatory responses to hypertonicity was recently investigated by Khajavi et al. [13].

The osmoprotectant L-carnitine was identified to directly inhibit TRPV1-related regulatory cell shrinkage following a hypertonic challenge [13]. Similar results in HCEC support these findings (unpublished results by Lucius et al., 2015).
TRPM8 activation by $3T_1AM$ could be an interesting target in order to suppress TRPV1 channel activity, thus avoiding inflammation due to hypertonicity. Several aspects regarding $3T_1AM$ should be clarified and prompt further studies to investigate this thyroid hormone metabolite more thoroughly. Studies have shown that there is in fact a significant concentration of $3T_1AM$ in the human serum [32,33], However, it is still uncertain, whether there is a relevant amount of $3T_1AM$ present in the human tear film under normal conditions and if so, whether it differs in the tear film of patients with DES. Currently, this issue is topic for further investigation at the Charité – Virchow Clinic. The effects of $3T_1AM$ on the cell cycle, cell protein expression and other signaling events under *in vivo* conditions need to be elucidated. In this context, an artificial cornea construct as an alternative to human donor corneas is under development. Using such a temporary cornea construct, permeability studies and measurements of the transepithelial electrical resistance (TER) were already performed [52,53]. This cornea construct may be employed to further evaluate the *in vitro* effect of $3T_1AM$ on inflammatory processes.
Another novelty of this thesis is that functional TRPM8 and TRPV1 expression in (benign) tumor cells such as hPtEC could be demonstrated for the first time. This corresponds with similar findings in other ocular tumor cells like retinoblastoma [18] and uveal melanoma [19]. It is suggested that TRPM8 channel expression might be at lower levels since the calcium response to icilin was significantly weaker than the calcium response pattern in healthy HCjEC (unpublished observation by Lucius 2014). Furthermore, TRPV1 is upregulated in hPtEC, since compared to HCjEC, calcium influx following TRPV1 activation was at significantly higher levels in hPtEC [54]. Since *Pterygium conjunctivae* is often associated with DES [5,6] and TRPM8/TRPV1 interaction could render a novel therapeutic strategy, the implications of alterations in TRPM8 and TRPV1 expression in hPtEC should prompt further investigation.
In conclusion, HCEC, HCjEC and hPtEC express both functional TRPM8 and TRPV1 channels. The data obtained in this thesis indicate that there is an inhibitory effect of TRPM8 activation on TRPV1 activity in HCEC and HCjEC. Such an interaction is expected to counter TRPV1-linked inflammatory signaling pathways (e.g. via the aforementioned NF-κB) as they occur in DES and pterygium as well as in healthy HCEC [10,51,55]. Overall, a novel endogenous TRP channel modulator ($3T_1AM$) has been shown to mediate its cellular effect through TRPM8 activation [14,50] and should therefore be a target for further investigation.

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Affidavit

I, Alexander Lucius, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic: **“Characterization of temperature-sensitive transient receptor potential channel melastatin 8 (TRPM8) in cultivated human ocular surface cells”**. I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

____________________ ________________________________

Date Signature

List of selected publications

Alexander Lucius contributed to the publications listed below:

Publication #1: Khajavi N, Reinach PS, Slavi N, Skrzypski M, **Lucius A**, Strauß O, Köhrle J, Mergler S. Thyronamine induces TRPM8 channel activation in human conjunctival epithelial cells. Cell. Signal. 2015;27:315–325.

Impact Factor: 4.32

Contribution: 15 %

Detailed contribution: The author participated in collecting the calcium imaging recordings, analyzing the data and editing the manuscript.

Publication #2: **Lucius A**, Khajavi N, Reinach PS, Köhrle J, Dhandapani P, Huimann P, Ljubojevic N, Grötzinger C, Mergler S. 3-Iodothyronamine increases transient receptor potential melastatin channel 8 (TRPM8) activity in immortalized human corneal epithelial cells. Cell. Signal. 2016;28:136–147.

Impact Factor: 4.32

Contribution: 80 %

Detailed contribution: The author participated in designing the study, writing the manuscript, carrying out a substantial amount of calcium imaging measurements as well as planar patch-clamp recordings, analyzing data and creating diagrams.

Publication #3: Garreis F, Schröder A, Reinach PS, Zoll S, Khajavi N, Dhandapani P, **Lucius A**, Pleyer U, Paulsen F, Mergler S. Upregulation of transient receptor potential vanilloid type 1 channel activity and Ca^{2+} influx dysfunction in human pterygial cells. Invest. Ophthalmol. Vis. Sci. 2016;57(6):2564-77.

Impact Factor: 3.40

Contribution: 10 %

Detailed contribution: The author collected preliminary calcium-imaging data concerning functional TRPM8 channel expression in freshly isolated cells from a patient with pterygium, analyzed the data and participated in editing the manuscript.

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Date, signature and seal of supervising professor doctoral candidate

Selected paper

#1 Thyronamine induces TRPM8 channel activation in human conjunctival epithelial cells

Khajavi N, Reinach PS, Slavi N, Skrzypski M, Lucius A, Strauß O, Köhrle J, Mergler S. Thyronamine induces TRPM8 channel activation in human conjunctival epithelial cells. Cell. Signal. 2015;27:315–325.
http://dx.doi.org/10.1016/j.cellsig.2014.11.015

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#2 3-Iodothyronamine increases transient receptor potential melastatin channel 8 (TRPM8) activity in immortalized human corneal epithelial cells

Lucius A, Khajavi N, Reinach PS, Köhrle J, Dhandapani P, Huimann P, Ljubojevic N, Grötzinger C, Mergler S. 3-Iodothyronamine increases transient receptor potential melastatin channel 8 (TRPM8) activity in immortalized human corneal epithelial cells. Cell Signal 2016;28:136–147.
http://dx.doi.org/10.1016/j.cellsig.2015.12.005

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#3 Upregulation of transient receptor potential vanilloid type 1 channel activity and Ca^{2+} influx dysfunction in human pterygial cells

Garreis F, Schröder A, Reinach PS, Zoll S, Khajavi N, Dhandapani P, Lucius A, Pleyer U, Paulsen F, Mergler S. Upregulation of transient receptor potential vanilloid type 1 channel activity and Ca^{2+} influx dysfunction in human pterygial cells. Invest. Ophthalmol. Vis. Sci. 2016;57(6):2564-77.
http://dx.doi.org/10.1167/iovs.16-19170

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Curriculum vitae

Due to privacy settings, the CV will not be displayed in the online version of this document.

List of publications

The author of this doctoral thesis has been involved in the following work:

1. Mergler S, Zoll S, Straßenburg M, **Lucius A**, Abdelmessih S, Pleyer U. Increased Ca^{2+} influx after activation of heat-sensitive transient receptor potential (TRP) channels in human conjunctival epithelial (HCjE) cells from patients with **Pterygium**. Der Ophthalmologe 2013;110(Suppl.1):111.
2. Garreis F, Mergler S, Gaebel A, Zoll S, **Lucius A**, Straßenburg M, Lude A, Paulsen F. Altered calcium regulation by temperature-sensitive transient receptor potential channels (thermo-TRPs) as a pathophysiologic effect in pterygium. Conference poster at the 7th International Conference on the Tear Film & Ocular Surface: Basic Science and Clinical Relevance in Taormina, 18. - 21.09.2013
3. Garreis F, Mergler S, Schröder A, Zoll S, Khajavi N, **Lucius A**, Straßenburg M, Lude A, Paulsen F. Expression and activity of temperature - sensitive transient receptor potential channels (thermo-TRPs) in human pterygium cells. Conference poster at 109th Annual Meeting of Anatomische Gesellschaft in Salzburg, 24. - 27.09.2014.
4. Khajavi N, Reinach PS, Slavi N, **Lucius A**, Strauß O, Köhrle J, Mergler S. Thyronamine induces TRPM8 channel activation in human conjunctival epithelial cells. Cell Signal 27;315–325, 2015.
5. **Lucius A**, Khajavi N, Reinach PS, Köhrle J, Dhandapani P, Huimann P, Ljubojevic N, Grötzinger C, Mergler S. 3-Iodothyronamine increases transient receptor potential melastatin channel 8 (TRPM8) activity in immortalized human corneal epithelial cells. Cell Signal 28;136–147, 2016.
6. Garreis F, Schröder A, Reinach PS, Zoll S, Khajavi N, Dhandapani P, **Lucius A**, Pleyer U, Paulsen F, Mergler S. Upregulation of transient receptor potential vanilloid type 1 channel activity and Ca^{2+} influx dysfunction in human pterygial cells. Invest Ophthalmol Vis Sci. 2016;57(6):2564-77.

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