

学校编码: 10384

密级 _____

学号: 24520130154315

厦 门 大 学

博 士 学 位 论 文

睑板腺功能障碍引起的眼表病变及其分子
机制研究

The pathophysiology of meibomian gland dysfunction
related ocular surface diseases and the molecular
mechanism

李 三 明

指导教师姓名: 李 炜 教授

专 业 名 称: 生理学

论文提交日期: 2016 年 4 月

论文答辩日期: 2016 年 5 月

2016 年 4 月

厦门大学学位论文原创性声明

本人呈交的学位论文是本人在导师指导下,独立完成的研究成果。本人在论文写作中参考其他个人或集体已经发表的研究成果,均在文中以适当方式明确标明,并符合法律规范和《厦门大学研究生学术活动规范(试行)》。

另外,该学位论文为()课题(组)的研究成果,获得()课题(组)经费或实验室的资助,在()实验室完成。(请在以上括号内填写课题或课题组负责人或实验室名称,未有此项声明内容的,可以不作特别声明。)

声明人(签名):

年 月 日

厦门大学学位论文著作权使用声明

本人同意厦门大学根据《中华人民共和国学位条例暂行实施办法》等规定保留和使用此学位论文，并向主管部门或其指定机构送交学位论文（包括纸质版和电子版），允许学位论文进入厦门大学图书馆及其数据库被查阅、借阅。本人同意厦门大学将学位论文加入全国博士、硕士学位论文共建单位数据库进行检索，将学位论文的标题和摘要汇编出版，采用影印、缩印或者其它方式合理复制学位论文。

本学位论文属于：

（ ） 1.经厦门大学保密委员会审查核定的保密学位论文，
于 年 月 日解密，解密后适用上述授权。

（ ） 2.不保密，适用上述授权。

（请在以上相应括号内打“√”或填上相应内容。保密学位论文应是已经厦门大学保密委员会审定过的学位论文，未经厦门大学保密委员会审定的学位论文均为公开学位论文。此声明栏不填写的，默认为公开学位论文，均适用上述授权。）

声明人（签名）：

年 月 日

摘要

第一部分：睑板腺功能障碍诱导的小鼠干眼动物模型的建立

目的：睑板腺功能障碍(MGD)是引起蒸发过强型干眼最常见的病因，但是MGD引起的眼表面病变的病理生理过程目前还知之甚少。本研究的主要目的是建立蒸发过强型干眼的动物模型，并观察该类型干眼的眼表病理变化过程。

方法：用裂隙灯显微镜观察外异蛋白基因(Ectodysplasin A, EDA)突变鼠(Tabby鼠)的眼表病变过程，用荧光素钠染色方法检测Tabby鼠的角膜上皮缺损状况，用酚红棉线检测小鼠的水性泪液分泌，对角膜和眼睑进行H&E染色和油红染色。用扫描电镜观察眼表面上皮细胞的微绒毛变化。用蛋白质电泳(Western blot)和/或实时定量PCR(qRT-PCR)方法检测眼表上皮K12、K10、SPRR1B、MUC5AC、MUC1、MUC4、MUC13和MUC15等基因或蛋白的表达情况。

结果：8周龄至16周龄Tabby鼠的眼表相继出现角膜上皮缺损，角膜基质混浊，角膜新生血管长入以及角膜血管翳形成等病变。Tabby鼠的水性泪液分泌没有减少，但体外泪液蒸发时间显著变短。Tabby鼠角膜上皮细胞表面微绒毛明显减少、缩短。Tabby鼠的结膜杯状细胞分布密度没有变化，但其MUC5AC和MUC5B表达显著增加。Tabby鼠角膜上皮K10和SPRR1B的表达于出生后第4周开始上升，并随时间逐渐增强，这说明Tabby鼠角膜出现鳞状上皮化生。

结论：Tabby鼠的眼表病变与蒸发过强型干眼的眼表病变相似，可以作为睑板腺功能障碍诱导的干眼小鼠动物模型。对Tabby鼠眼表病变的研究将有助于我们更深入地了解睑板腺在维持眼表健康中的作用，为今后开发筛选新药物治疗这类疾病提供帮助。

关键词：睑板腺功能障碍；干眼；外异蛋白

第二部分：外异蛋白(Eda)促进角膜上皮细胞增殖

目的：EDA基因编码Ectodysplasin A(Eda)蛋白，该基因突变会导致X染色体连锁外胚层发育不良综合征(X-linked anhidrotic-hypohidrotic ectodermal dysplasia, XLHED)。XLHED患者可出现睑板腺发育障碍，但Eda蛋白在眼表的作用并不清楚。

方法:裂隙灯显微镜下观察 Eda 基因突变鼠 (Tabby 鼠) 的眼表病变。采用 ELISA 方法检测人泪液、血浆中的 Eda 浓度。用蛋白质电泳 (Western blot) 和/或免疫荧光 (IF) 以及实时定量 PCR (qRT-PCR) 检测 Eda、Eda 受体 Edar、Ki67、EGFR、磷酸化 EGF (p-EGFR)、磷酸化 ERK1/2 (p-ERK) 的表达。用划痕实验检测 Eda 蛋白对人角膜上皮细胞系 HCE 细胞的迁移作用, 用 CCK8 试剂检测 Eda 蛋白对 HCE 细胞的促增殖作用。

结果: Eda 蛋白高表达于小鼠睑板腺腺泡上, 人泪液中含有 Eda 蛋白, 但血浆中未检测出 Eda 蛋白。在 4-8 周龄 Tabby 鼠, 角膜上皮存在缺损, 角膜上皮厚度较薄。Tabby 鼠角膜上皮的增殖细胞较少, 角膜上皮损伤愈合速度较慢, 在体外培养眼球中发现 Eda 蛋白促进角膜上皮损伤愈合。Tabby 鼠角膜上皮 EGFR、p-EGFR、p-ERK1/2 的表达水平较同龄野生型小鼠低, 体外细胞培养显示 Eda 蛋白促进 HCE 细胞 Ki67、EGFR、p-EGFR 和 p-ERK 1/2 的表达, 且变化趋势与 Eda 蛋白浓度正相关。

结论: 睑板腺分泌 Eda 蛋白进入泪液, 并促进角膜上皮细胞增殖。Eda 重组蛋白有望在临床上用于治疗角膜上皮损伤。

关键词: 外异蛋白; 细胞增殖; 角膜上皮

第三部分: 外异蛋白 (Eda) 通过激活 SHH 信号通路调控上皮屏障

目的: 探讨 Eda 蛋白对角膜和皮肤上皮屏障功能的作用。

方法: 裂隙灯显微镜下用荧光素钠染色观察 Tabby 鼠的眼表病变。用羧基荧光素 (carboxy fluorescein) 做角膜渗透性实验, 用蛋白质电泳 (Western blot) 和/或实时定量 PCR (qRT-PCR), 以及免疫荧光 (IF) 方法检测角膜、皮肤, 以及人角膜上皮细胞系 HCE 细胞的 ZO-1、Claudin-1、Gli-1、Shh 的表达。用 LB 固体培养基进行角膜组织匀浆的细菌培养。

结果: 饲养在普通级环境的 Tabby 鼠的角膜血管翳形成和尾部皮肤溃疡的发病率高于 SPF 级饲养环境。Tabby 鼠的角膜和皮肤上皮紧密连接蛋白 ZO-1 和 Claudin-1 表达水平明显低于同龄野生型小鼠。Eda 蛋白可以显著改善 Tabby 鼠的角膜上皮屏障破坏。体外皮肤培养以及 HCE 细胞培养实验证实 Eda 蛋白可以通过激活 Shh 信号通路来调节 ZO-1 和 Claudin-1 的表达。

结论: Eda 蛋白调控表皮细胞间紧密连接的形成, 对表皮屏障至关重要。我们的发现不仅揭示了 EDA 基因新的功能, 也对 XLHED 相关疾病的发病机制提供新的视角。

关键词: 外异蛋白; 上皮屏障; 紧密连接

厦门大学博硕士论文摘要库

Abstract

Part I. A mouse dry eye model induced by meibomian gland dysfunction

Purpose: Meibomian gland dysfunction (MGD) is the most frequent cause of evaporative dry eye. However, its underlying pathophysiology is largely unknown. Our objective was to gain insight into the ocular surface change induced by MGD.

Methods: Ocular surface changes of Ectodysplasin A (EDA) gene mutant Tabby mice were observed under a slit-lamp microscope and corneal epithelia defect was evaluated by sodium fluorescein staining. Corneal sections and eyelids were stained by H&E and oil red. Aqueous tear secretion was measured with phenol red thread tear secretion test. The corneal surface epithelial microvilli change was observed under scanning electron microscope (SEM). K12, K10, SPRR1B, MUC5AC, MUC1, MUC4, MUC13, MUC15 expression were evaluated by immunostaining and/or quantitative real-time PCR (qRT-PCR).

Results: Tabby mice sequentially developed corneal epithelial defects, central corneal stromal edema, neovascularization, and pannus 8 to 16 weeks after birth. Aqueous tear secretion was normal, while ex vivo tear evaporation time were shortened. Corneal epithelial microvilli were less numerous whereas conjunctival goblet cell density was unaffected, but MUC5AC and MUC5B gene expression instead increased. Cytokeratin 10 and 12, SPRR1B gene expression was identified in the corneal epithelium of Tabby mice as early as the 4th week, which is indicative of squamous metaplasia.

Conclusions: Tabby mice are a relevant model of MGD related dry eye. They may lead to better delineate the function of meibomian gland in maintaining ocular surface health, and to identify novel drug options to treat evaporative dry eye disease.

Keywords: meibomian gland dysfunction; dry eye; Ectodysplasin A

Part II. Ectodysplasin A promotes corneal epithelial cell proliferation

Purpose: The EDA gene encodes ectodysplasin A (Eda) which if mutated causes X-linked Hypohidrotic Ectodermal Dysplasia (XLHED) disease in humans. Meibomian gland absent in XLHED patients. Our objective was to identify the effect of Eda protein on the corneal epithelium.

Methods: Ocular surface changes of EDA gene mutant Tabby mice were observed under a slit-lamp microscope. ELISA measured Eda concentration in human serum and tears. Eda, Eda receptor (Edar), Ki67, epidermal growth factor receptor (EGFR), phosphorylated EGFR (p-EGFR), and phosphorylated ERK1/2 (p-ERK) expression were evaluated by immunostaining and/or quantitative real-time PCR (qRT-PCR) and/or Western blot analysis. Eda effects on human corneal epithelial cell line (HCE) proliferation and migration were determined using the cell counting kit-8 (CCK8) assay and scratch wound healing assay, respectively.

Results: Eda was highly expressed in meibomian glands, and it was detected in human tears but not serum. Corneal epithelial integrity was defective and the thickness was reduced in the early postnatal stage of Tabby mice. Corneal epithelial cell proliferation decreased and the epithelial wound healing was delayed in Tabby mice. Eda exposure promoted corneal epithelial wound healing during organ culture. EGFR, p-EGFR and p-ERK were down-regulated in Tabby mice corneal epithelium. Eda treatment up-regulated the expression of Ki67, EGFR, p-EGFR and p-ERK in HCE cells in a dose dependent manner.

Conclusions: Eda protein can be secreted from meibomian glands and promotes corneal epithelial cell proliferation. Recombinant Eda may be used in the treatment of corneal epithelial wounding in a clinical setting.

Keywords: Ectodysplasin A; cell proliferation; corneal epithelium

Part III. Ectodysplasin A regulates epithelial barrier function through activating SHH signaling pathway

Purpose: To investigate the role of Ectodysplasin A (EDA) on the skin and corneal epithelial barrier function.

Methods: The ocular surface of Tabby mice was observed under slit lamp microscope with fluorescein staining. Corneal epithelial barrier function was evaluated with the permeability to carboxy fluorescein. ZO-1, Claudin-1, Gli-1 and Shh expression in the corneal and skin epithelia and HCE cells were performed by

immunostaining and/or Western blot and/or quantitative real-time PCR assay. The cornea bacterial infection was examined by LB solid medium.

Results: Compromise of tight junction is associated with corneal pannus and tail ulcer in Tabby mice raised in conventional facilities. Tight junction proteins ZO-1 and Claudin-1 were dramatically decreased in skin and corneal epithelia of the Tabby mice. Topical application of recombinant Eda protein could rescue corneal epithelial barrier dysfunction to a large extent in Tabby mice. Moreover, the ex-vivo skin tissue culture and corneal epithelial cell culture indicated that the EDA regulates tight junction formation through activation of sonic hedgehog signaling pathway.

Conclusions: EDA contributes to the barrier function of surface epithelial cells. Our findings revealed new function of EDA signal and shed new light on the pathophysiology and treatment of XLHED related diseases.

Keywords: Ectodysplasin A; epithelial barrier function; tight junction

目 录

摘 要.....	I
Abstract.....	IV
第一章 绪论	1
1.1 睑板腺及其功能	1
1.2 睑板腺分泌的调节	1
1.3 睑板腺功能障碍	2
1.4 睑板腺功能障碍的诊断	3
1.5 睑板腺功能障碍的治疗	3
1.6 睑板腺功能障碍与干眼的关系	4
1.7 研究现状	5
1.8 研究意义	7
第二章 睑板腺功能障碍诱导的小鼠干眼动物模型建立	8
2.1 背景介绍	8
2.2 实验材料	9
2.2.1 主要仪器	9
2.2.2 主要试剂与耗材	10
2.3 实验方法	11
2.3.1 动物饲养和繁殖	11
2.3.2 荧光素钠染色及评分	11
2.3.3 体外泪液蒸发时间测量	11
2.3.4 扫描电子显微镜的角膜样本制备	12
2.3.5 过碘酸雪夫氏 (PAS) 染色	12
2.3.6 H&E 染色	12
2.3.7 油红染色	13
2.3.8 免疫荧光染色	13
2.3.9 RNA 的提取及反转录	13
2.3.10 引物设计	14
2.3.11 Real Time PCR 检测	14
2.3.12 统计方法	15
2.4 实验结果	15
2.4.1 Tabby 鼠角膜病变的发展过程	15
2.4.2 Tabby 鼠睑板腺完全缺失	18
2.4.3 角膜荧光素钠染色结果及其评分	19
2.4.4 泪液分泌的变化及泪腺形态的观察	21
2.4.5 体外泪液蒸发实验	22

2.4.6	Tabby 鼠角膜表层微绒毛的变化.....	24
2.4.7	Tabby 鼠的角膜上皮异常分化.....	24
2.4.8	结膜杯状细胞分布密度及其功能的变化.....	27
2.4.9	Tabby 鼠角膜和结膜的膜结合型黏蛋白表达变化.....	28
2.5	讨论.....	30
第三章	外异蛋白 (Eda) 促进角膜上皮细胞增殖.....	34
3.1	背景介绍.....	34
3.2	实验材料.....	35
3.2.1	主要仪器.....	35
3.2.2	主要试剂和耗材.....	36
3.3	实验方法.....	38
3.3.1	动物饲养和繁殖.....	38
3.3.2	荧光素钠染色.....	38
3.3.3	H&E 染色.....	38
3.3.4	免疫荧光染色.....	38
3.3.5	RNA 的提取及反转录.....	39
3.3.6	蛋白印迹实验 (Western blot).....	40
3.3.7	EDU 标记.....	42
3.3.8	在体角膜上皮损伤修复模型建立.....	43
3.3.9	体外角膜上皮损伤修复模型建立.....	43
3.3.10	人泪液和血浆样本收集.....	43
3.3.11	ELISA.....	43
3.3.12	HCE 细胞培养.....	44
3.3.13	CCK8 实验.....	44
3.3.14	划痕实验.....	44
3.3.15	溶液配制.....	45
3.3.16	引物设计.....	46
3.3.17	统计方法.....	46
3.4	实验结果.....	47
3.4.1	睑板腺可以分泌 Eda 蛋白到泪液中.....	47
3.4.2	Tabby 鼠的角膜上皮厚度变化.....	49
3.4.3	Tabby 鼠角膜上皮在增殖细胞数量减少.....	50
3.4.4	Tabby 鼠的角膜上皮损伤愈合速度异常.....	52
3.4.5	Eda 蛋白加速角膜上皮损伤修复速度.....	54
3.4.6	Eda 蛋白对 HCE 细胞迁移的影响.....	55
3.4.7	Tabby 鼠的角膜上皮 EGF-EGFR 信号通路检测.....	56
3.4.8	Eda 通过激活 EGF-EGFR 信号通路促进 HCE 细胞增殖.....	58
3.5	讨论.....	59
第四章	外异蛋白 (Eda) 通过激活 SHH 信号通路调控上皮屏障..	62
4.1	背景介绍.....	62
4.2	实验材料.....	63
4.2.1	主要仪器.....	63
4.2.2	主要试剂.....	64
4.3	实验方法.....	65

4.3.1 动物饲养及繁殖.....	65
4.3.2 Carboxy fluorescein 渗透实验	65
4.3.3 角膜细菌培养.....	66
4.3.4 pcDNA3.1-EDA 表达载体构建及细胞转染.....	66
4.3.5 上皮细胞的分离.....	69
4.3.6 皮肤组织培养.....	70
4.3.7 免疫荧光染色.....	70
4.3.8 蛋白印迹实验 (Western blot)	70
4.3.9 荧光素钠染色及评分.....	70
4.3.10 RNA 收集及 Real time PCR	70
4.3.11 引物设计.....	70
4.3.12 统计方法.....	71
4.4 实验结果.....	71
4.4.1 Tabby 鼠的角膜和皮肤组织长期存在慢性炎症.....	71
4.4.2 不同饲养环境下 Tabby 鼠的角膜血管翳和尾巴溃疡发病率统计	73
4.4.3 Tabby 鼠角膜存在细菌感染.....	75
4.4.4 Tabby 鼠的角膜及皮肤上皮紧密连接蛋白 ZO-1 和 Claudin-1 表达下降.....	76
4.4.5 Eda 重组蛋白能够有效改善 Tabby 鼠的角膜上皮屏障破坏.....	78
4.4.6 Eda 蛋白通过激活 Shh 信号通路促进 ZO-1 和 Claudin-1 蛋白的表达	80
4.5 讨论.....	85
第五章 全文总结	89
参 考 文 献	90
附 录.....	98
致 谢.....	99

Table of Contents

Abstract in Chinese	I
Abstract in English	IV
Chapter I Research Background	1
1.1 Meibomian gland and its function.....	1
1.2 Regulation of Meibomian gland secretion.....	1
1.3 Meibomian gland dysfunction.....	2
1.4 Diagnosis of Meibomian gland dysfunction.....	3
1.5 Treatment of Meibomian gland dysfunction.....	3
1.6 Meibomian gland dysfunction and dry eye.....	4
1.7 Current research situation.....	5
1.8 The significance of this research.....	7
Chapter II A mouse dry eye model induced by meibomian gland dysfunction	8
2.1 Introduction.....	8
2.2 Materials.....	9
2.3.1 Equipments.....	9
2.3.2 Reagents and Supplies.....	10
2.3 Methods.....	11
2.3.1 Animals.....	11
2.3.2 Evaluation of sodium fluorescein staining.....	11
2.3.3 Ex vivo tear evaporation assay.....	11
2.3.4 Ultra-Microstructure of corneal epithelium.....	12
2.3.5 PAS staining.....	12
2.3.6 H&E staining.....	12
2.3.7 Oil red O staining.....	13
2.3.8 Immunofluorescence staining.....	13
2.3.9 RNA isolation and reverse transcription.....	13
2.3.10 Primer design.....	14
2.3.11 Quantitative real-time RT-PCR analysis.....	14
2.3.12 Statistical analysis.....	15

2.4 Results	15
2.4.1 Ocular surface manifestations in Tabby mice.....	15
2.4.2 Absence of Meibomian gland in Tabby mice.....	18
2.4.3 Evaluation of sodium fluorescein staining.....	19
2.4.4 Tear secretion and histology of lacrimal gland.....	21
2.4.5 Ex vivo tear evaporation test.....	22
2.4.6 Microrvilli change on the ocular surface.....	24
2.4.7 Aberrant corneal epithelial cell differentiation.....	25
2.4.8 Goblet cell distribution and functional changes.....	27
2.4.9 Epithelial mucin expression profile changes.....	29
2.5 Discussion	30
Chapter III Ectodysplasin A promotes corneal epithelial cell proliferation	34
3.1 Introduction	34
3.2 Materials	35
3.3.1 Equipments.....	35
3.3.2 Reagents and Supplies.....	36
3.3 Methods	38
3.3.1 Animals.....	19
3.3.2 Sodium fluorescein staining.....	38
3.3.3 H&E staining.....	38
3.3.4 Immunofluorescence staining.....	38
3.3.5 RNA isolation and reverse transcription.....	39
3.3.6 Western blot.....	40
3.3.7 EDU labling.....	42
3.3.8 Corneal epithelial wound healing in vivo.....	43
3.3.9 Corneal epithelial wound healing ex vivo.....	43
3.3.10 Human tear and serum collection.....	43
3.3.11 ELISA.....	43
3.3.12 HCE cell culture.....	44
3.3.13 CCK8 assay.....	44
3.3.14 Scratch wound healing assay.....	44
3.3.15 The preparation of regents.....	45
3.3.16 Primer design.....	46

3.3.17 Statistical analysis.....	46
3.4 Results.....	47
3.3.1 Meibomian gland secretes Eda protein.....	47
3.3.2 The thinner corneal epithelium in Tabby mice.....	49
3.3.3 Corneal epithelial cell proliferation decreased in Tabby mice.....	50
3.3.4 Corneal epithelial wound healing delayed in Tabby mice.....	52
3.3.5 Eda promotes epithelial cell proliferation.....	54
3.3.6 Effect of Eda on HCE cell migration.....	55
3.3.7 EGF-EGFR signaling in Tabby mice corneal epithelium.....	56
3.3.8 Eda activated EGF-EGFR signaling pathway in HCE cells.....	58
3.5 Discussion.....	59
Chapter IV Ectodysplasin A regulates epithelial barrier function though activating SHH signaling pathway.....	62
4.1 Introduction.....	62
4.2 Materials.....	63
4.3.1 Equipments.....	63
4.3.2 Reagents and Supplies.....	64
4.3 Methods.....	65
4.3.1 Animals.....	65
4.3.2 Measurement of epithelial permeability to carboxy fluorescein.....	65
4.3.3 Corneal bacterial culture.....	65
4.3.4 pcDNA-EDA plasmid transfection.....	66
4.3.5 The separation of epithelial cell.....	69
4.3.6 Skin tissue explants culture.....	70
4.3.7 Immunofluorescence staining.....	70
4.3.8 Western blotting.....	70
4.3.9 Evaluation of sodium fluorescein staining.....	70
4.3.10 RNA isolation and quantitative real-time RT-PCR analysis.....	70
4.3.11 Primer design.....	70
4.3.12 Statistical analysis.....	71
4.4 Results.....	71
4.4.1 Inflammation in cornea and skin of Tabby mice.....	71
4.4.2 The incidence of corneal pannus and tail ulcer in different bleed environments.....	73
4.4.3 Corneal bacterial infection in Tabby mice.....	75

4.4.4 Declines in epithelial tight junctional protein ZO-1 and Claudin-1 expression.....	76
4.4.5 Recombinant Eda protein topical application rescues corneal epithelial barrier dysfunction.....	78
4.4.6 Eda regulates ZO-1 and Claudin-1 expression through the sonic hedgehog signaling pathway.....	80
2.5 Discussion	85
Chapter V Summary	89
References	90
Appendices	98
Acknowledgement	99

Degree papers are in the “[Xiamen University Electronic Theses and Dissertations Database](#)”.

Fulltexts are available in the following ways:

1. If your library is a CALIS member libraries, please log on <http://etd.calis.edu.cn/> and submit requests online, or consult the interlibrary loan department in your library.
2. For users of non-CALIS member libraries, please mail to etd@xmu.edu.cn for delivery details.