Overexpression of HTRA1 Leads to Ultrastructural Changes in the Elastic Layer of Bruch’s Membrane via Cleavage of Extracellular Matrix Components

Sarah Vierkotten, Philipp S. Muether, Sascha Fauser*
Center of Ophthalmology, University of Cologne, Cologne, Germany

Abstract
Variants in the chromosomal region 10q26 are strongly associated with an increased risk for age-related macular degeneration (AMD). Two potential AMD genes are located in this region: ARMS2 and HTRA1 (high-temperature requirement A1). Previous studies have suggested that polymorphisms in the promotor region of HTRA1 result in overexpression of HTRA1 protein. This study investigated the role of HTRA1 overexpression in the pathogenesis of AMD. Transgenic Htra1 mice overexpressing the murine protein in the retinal pigment epithelium (RPE) layer of the retina were generated and characterized by transmission electron microscopy, immunofluorescence staining and Western Blot analysis. The elastic layer of Bruch’s membrane (BM) in the Htra1 transgenic mice was fragmented and less continuous than in wild type (WT) controls. Recombinant HTRA1 lacking the N-terminal domain cleaved various extracellular matrix (ECM) proteins. Subsequent Western Blot analysis revealed an overexpression of fibronectin fragments and a reduction of fibulin 5 and tropoelastin in the RPE/choroid layer in transgenic mice compared to WT. Fibulin 5 is essential for elastogenesis by promoting elastic fiber assembly and maturation. Taken together, our data implicate that HTRA1 overexpression leads to an altered elastogenesis in BM through fibulin 5 cleavage. It highlights the importance of ECM related proteins in the development of AMD and links HTRA1 to other AMD risk genes such as fibulin 5, fibulin 6, ARMS2 and TIMP3.

Introduction
Age-related macular degeneration (AMD) is the most common cause of central blindness in the elderly population. Both genetic and environmental factors play important roles in the development of AMD. Smoking and dietary intake have been identified as the major environmental risk factors for developing AMD besides age [1,2]. To identify genetic risk factors of AMD, genome-wide linkage studies had been conducted by several groups [3,4,5]. Two major susceptibility loci were identified: at chromosome 1q31, variants in the complement factor H (CFH), and variants at chromosome 10q26. These two loci account for more than 50% of the disease risk [6].

Two potential AMD genes reside in this region: ARMS2 (age-related maculopathy susceptibility 2) and HTRA1 (high-temperature requirement factor A1). Ever since, there is considerable controversy on which gene plays a causal role in AMD [7,8,9,10]. Strong linkage disequilibrium across the region probably makes genetic studies unsuitable to solve this question. Recently, Tong et al. (2010) [11] suggested that polymorphisms in both genes were genetic risk factors of AMD. Polymorphisms in the promotor region were reported to increase expression levels of HTRA1 [12,13], although others could not confirm these findings [10,14].

HTRA1 is a member of a family of serine proteases characterized by a highly conserved trypsin-like protease domain and a C-terminal PDZ domain. A 22 amino acid signal peptide at the N-terminus marks the HTRA1 protein for secretion. It is involved in degradation of extracellular matrix (ECM) proteins like fibronectin [15] and aggrecan [16]. Elevated HTRA1 levels have been associated with arthritic disease [15,17,18]. Therefore, it seems to be an important protein of ECM homeostasis and turnover. Reduced HTRA1 activity did not repress signaling by the TGF-ß family and resulted in familial ischemic cerebral small-vessel disease [19,20,21]. The involvement of the ECM in the pathogenesis of AMD is further supported by additional AMD risk genes such as TIMP3 (tissue inhibitor of metalloproteinases-3), which inhibits MMPs (matrix metalloproteinases) and is involved in degradation of the ECM [22], and ARMS2, which interacts with several ECM proteins [23].

To date, there is no physiological evidence of HTRA1 involvement in AMD pathogenesis. The aim of this study was the functional analysis of HTRA1 overexpression in the retinal pigment epithelium (RPE) of the mouse eye. Here, we show for the first time that an elevated expression of HTRA1 in RPE cells leads to a change of Bruch’s Membrane (BM) composition in vivo.

Results
Generation of transgenic mice and evaluation of HTRA1 expression
Founder mice were generated by microinjection of the transgene into the male pronucleus of one-cell embryos, which
were then transplanted into pseudopregnant females that gave birth to founder mice. The expression of the transgenic construct was assessed by PCR on genomic DNA. Transgene-specific primers amplified a transgene cDNA-specific 417-bp sequence in transgenic, but not in the WT mice (Fig. 1A). Sequencing of the transgene showed the correct sequence of the complete Rpe65/Htra1 construct.

To determine the Htra1 mRNA level of transgenic mice compared to WT, we performed relative quantification by real-time PCR (Fig. 1B). As a control for experimental variability we used beta-actin (Actb) as normalizer gene. The fold change in Htra1 gene expression was calculated with the Pfaffl method and revealed the highest Htra1 gene expression in transgenic line no. 2 with a 2.79 fold increase compared to WT mice. This is consistent with findings by Yang et al. [13] who demonstrated a 2.7 fold mRNA increase of HTRA1 in the RPE of patients genotyped for the risk variant. Therefore, our Htra1 transgenic mice may be regarded as a physiological model of HTRA1 overexpression and may reflect the situation in AMD patients carrying the risk variant. Offspring from line no. 2 mice were then generated by mating a transgenic parent with a C57BL/6N mouse and expanded by at least six back-crosses. Animals were kept heterozygous for the transgene.

Since the mRNA levels do not necessarily correlate with protein levels, we performed Western Blot analysis with RPE/Choroid lysates of transgenic and WT mice to confirm HTRA1 overexpression on protein level. Here, we found moderate expression of HTRA1 protein in C57BL/6N mice, while the Htra1 transgenic mice showed an increase in expression (Fig. 1C). When we conducted densitometric analysis of our Western Blot experiments, we detected a 2.68-fold overexpression of HTRA1 protein in the transgenic mice compared to WT mice (Fig. S1). Thus, mRNA and protein levels of HTRA1 did correlate in our transgenic mice and both demonstrated a physiological overexpression as seen in AMD patients.

To monitor the secretion of HTRA1, we cultured primary RPE cells from WT and Htra1 transgenic mice and performed Western Blot analysis of cell culture supernatants (Fig. 1D). Here, we detected HTRA1 in the supernatant of WT as well as transgenic mice. This finding clearly demonstrates the secretion of HTRA1 from primary cultured RPE cells into the medium. Note that the secretion of HTRA1 was increased in Htra1 transgenic mice.

Furthermore, we evaluated HTRA1 expression in other tissues to demonstrate that the overexpression was restricted to the RPE due to the Rpe65 Promoter, and that HTRA1 overexpression in the RPE was not due to any strain variances. Figure 1E illustrates that no overexpression of HTRA1 protein could be found in brain, liver, spleen or kidney of transgenic mice when compared to WT mice.

Morphological evaluation of Htra1 transgenic mice

Morphological screening of WT C57BL/6N and Htra1 transgenic mice up to one year of age via hematoxylin-eosin (HE) staining on paraffin cross sections revealed no differences in choroid, BM and RPE (Fig. 2A). In addition, we observed no differences in vasculature by immunofluorescence for the endothelial cell marker Type-IV collagen (ColIV) (Fig. 2B).

Ultrastructural changes in the elastic layer of Bruch’s membrane in Htra1 transgenic mice

To further investigate the structural differences, we performed transmission electron microscopy (TEM). Here, Htra1 transgenic mice revealed ultrastructural changes in the elastic layer of BM up to one year of age (Fig. 3 B, D, F and H). The elastic layer of Htra1

**Figure 1. Genotyping and protein analysis of Htra1 transgenic mice.** (A) PCR of genomic DNA from transgenic and WT mice. Transgenic mice show amplification with a visible band at 417 bp (tg) as can be seen in the positive control (+). WT mice (wt) as well as the negative control (−) show no amplification. (B) Quantitative real-time PCR determination of Htra1 mRNA levels from total RNA isolated from posterior eyes of C57BL/6N and transgenic lines no. 1 to 4 mice. An increase of Htra1 mRNA in Htra1 transgenic lines compared to WT was observed. Transgenic line no 2 shows the highest rate with a 2.79 fold increase (3mice/C57BL/6N and 12mice/transgenic line: 3 independent experiments, 3mice/group). Data are expressed as means ±SD. Relative mRNA levels were normalized to Actb and expressed as fold change relative to control. (C) Western Blot analysis of RPE/Choroid lysates from 3 month old WT (wt) and transgenic (tg) mice. Transgenic mice show a higher expression of HTRA1 protein compared to WT (1mice/group, 6 independent experiments). Expression of ACTB serves as a loading control. Image is representative. (D) Western Blot analysis of primary RPE cells supernatant. Cells were cultured till confluent and medium was removed 24 hours after exchange of fetal bovine serum-free medium. The experiment clearly demonstrates secretion of HTRA1 from RPE cells (1mice/group, 6 independent experiments). Note that the expression level of HTRA1 is higher in transgenic cell culture supernatants. Image is representative. (E) Western Blot analysis of tissue extracts from 3 month old WT (wt) and transgenic mice (tg). There are no differences in HTRA1 expression found in brain, liver, spleen and kidney of transgenic mice compared to WT (1mice/group, 6 independent experiments). Expression of ACTB serves as a loading control. Image is representative. doi:10.1371/journal.pone.0022959.g001
transgenic mice appeared fragmented with spots of electron dense material (asterisk Fig. 3 D and H), while the elastic layer of control mice appeared thick and continuous (Fig. 3 A, C, E, G). To ensure that the discontinuities are not merely localized irregularities, the TEM images were made at the central retina as well as the periphery and graded through blinded and randomized examination by several independent graders. Fig. S2 indicates the widely spread morphological changes in the Htra1 transgenic mice compared to WT.

**Expression of ECM proteins in RPE/choroid lysates of WT and Htra1 transgenic mice**

Due to the changes observed in BM of Htra1 transgenic mice in TEM, we conducted Western Blot analysis of RPE/choroid lysates to investigate the expression of major components of the ECM as well as the expression of ECM proteins that are involved in elastogenesis in Htra1 transgenic compared to WT mice. Figure 4A clearly demonstrates that the expression of nidogen 1, elastin microfibril interface-located protein (EMILIN1), fibulin 4 and lysyl oxidase-like 1 (LOXL1) was comparable in WT and Htra1 transgenic mice. Interestingly, expression levels of fibulin 5, which has elastogenic activity and tropoelastin were reduced in RPE/choroid lysates of Htra1 transgenic mice compared to WT. Densitometric analysis of our Western Blot findings confirmed the reduction of fibulin 5 and tropoelastin levels (Fig. S3). Western Blot analysis also revealed higher expression of fibronectin fragments in transgenic mice (4B). These findings support the notion of an altered ECM homeostasis in Htra1 transgenic mice.

**Distribution of nidogen 1 and nidogen 2 in BM**

We performed immunofluorescence staining on paraffin sections of Htra1 transgenic and WT mice to evaluate the presence of nidogen 1 and nidogen 2 in BM. Even though we detected degradation of nidogen 1 and nidogen 2 in our in vitro digestion assay, we observed no differences in the distribution of nidogen 1 and nidogen 2 in BM of WT and transgenic mice (Fig. 5).

**Proteolysis of ECM proteins by HTRA1**

To analyze the effects of HTRA1 proteolytic activity on ECM components of BM, we purified recombinant human His-tagged...
HTRA1 lacking the N-terminal IGFBP/MAC25 domain (Δmac25HTRA1 plasmid kindly provided by Prof. Dr. Ehrmann, Duisburg-Essen, Germany). To ensure that the bands seen on Coomassie-stained SDS-Gel for the HTRA1 recombinant protein were indeed HTRA1 protein, we excised the two bands and processed them for mass spectrometry. The data showed that the two bands corresponded to human HTRA1 (Fig. S4). Proteolytic activity of recombinant HTRA1 was confirmed by β-casein digestion (Fig. S5). An Inhibitor of HTRA1 (NVP-LEB748 kindly provided by Novartis) was used as a control to ensure that degradation of proteins was specifically due to HTRA1 proteolysis and not due to any possibly co-purified bacterial proteases. It was demonstrated to inhibit proteolytic activity by a dose-dependent manner with an IC₅₀ of 0.21 μm as determined by HTRA1-dependent digestion of resorufin-labelled casein in an earlier study by Grau et al. [15].

Figure 3. Transmission electron micrographs of BM. TEM images showing the elastic lamina of BM in WT (A, C, E, G) and Htra1 transgenic (B, D, F, H) mice (A–D at the age of 3 month; E–H at the age of 12 month). A, B and E, F at original 3000 × magnification and C, D and G, H at original 20000 × magnification. Note the fragmentation of the elastic lamina in Htra1 transgenic mice seen as discontinuities in contrast to the continuous elastic lamina of the WT mice. These discontinuities were observed in all transgenic mice that were analyzed (1 mice/group, 6 independent experiments). Image is representative. BM, Bruch’s membrane; Lv, Lumen vasculare (Lumen of a vessel); Mi, Mitochondria; Pc, Processus cellularis (cell process); Spi, Spatium intermembranosum (intermembranous cleft).

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Protease assays with purified ECM components were performed to screen for degradation of ECM components. As formerly described [15], fibronectin is a substrate of HTRA1 and was degraded after three hours of incubation with recombinant HTRA1 (Fig. 6A).

Nidogen 1 and nidogen 2, structural constituents of basement membranes, were also degraded by HTRA1 as shown in Fig. 6B and 6C. In contrast, laminin and Type-IV collagen showed no signs of degradation by recombinant HTRA1 in vitro (data not shown). Note that recombinant HTRA1 also degraded recombinant fibrin 5 (Fig. 6D). When using insoluble elastin from human skin as a substrate for HTRA1, we failed to observe any fragmentation bands for elastin (6E). Therefore, HTRA1 seemed to have no elastase activity.

After 18 hours of incubation and in the absence of substrate a loss of HTRA1 could be observed in all Figures. This loss was most likely due to self-degradation of HTRA1 as has been described before for bacterial HtrA [24] and human HTRA1 [25].

TGF-β expression in Htra1 transgenic mice
Since HTRA1 is known to inhibit TGF-β and has an IGF-binding protein (IGFBP) domain, we were interested in the TGF-β and IGF-1 expression in the RPE/choroid layer of Htra1 transgenic mice. We also determined VEGF expression levels because of its pro-angiogenic properties. To investigate this expression, we performed Multiplex assays via the Luminex xMAP Technology (Fig. 7A–C). Transgenic and WT mice showed no significant differences in the concentration of these growth factors at young or old age.

Discussion
Polymorphisms in the chromosomal region 10q26 are highly associated with AMD. Two attractive candidate genes reside in this region: ARMS2 and HTRA1. There is conflicting data concerning the involvement of these genes in the pathogenesis of AMD [9,10,11,12,13]. In this study, we provide the first functional evidence that HTRA1 overexpression in RPE is involved in AMD pathogenesis by fragmentation of the elastic layer in BM.

It has long been postulated that BM is important in AMD development and particularly in formation of choroidal neovascularisation (CNV) in exsudative AMD [26]. Since BM is an acellular layer, it depends on the adjacent RPE and choroidal cells...
Figure 6. Degradation of ECM components by recombinant HTRA1. Purified ECM proteins and purified recombinant HTRA1 were incubated at 37°C in 50 mM Tris-HCl pH7.6, 5 mM CaCl$_2$, 150 mM NaCl over a period of 18 hours (h). (A) Degradation of fibronectin. Fibronectin (FN) shows no sign of degradation when incubated alone. Recombinant HTRA1 degrades fibronectin after three hours of incubation. The inhibitor of HTRA1 completely abolishes degradation of fibronectin by HTRA1. (B) Degradation of nidogen 1. Nidogen 1 (NID1) shows no sign of degradation when incubated alone. Recombinant HTRA1 cleaves nidogen-1 after one hour of incubation. The inhibitor of HTRA1 completely abolishes degradation of nidogen 1 by HTRA1. (C) Degradation of nidogen 2. Nidogen 2 (NID2) shows no sign of degradation when incubated alone. Recombinant HTRA1 cleaves nidogen 2 after one hour of incubation. The inhibitor of HTRA1 completely abolishes degradation of nidogen 2 by HTRA1. (D) Degradation of fibulin 5 by HTRA1. Fibulin 5 (FBLN5) is degraded by HTRA1 already after one hour, but shows no sign of degradation when incubated alone. The
for most of its ECM constituents [27]. With aging a loss of elastic fibers, thickening and calcifications of BM have been observed [28]. In this study, transgenic mice overexpressing HTRA1 showed ultrastructural changes of the elastic layer by TEM. Subsequent Western Blot analysis of major components of the ECM as well as ECM proteins involved in elastogenesis revealed an increase in fibronectin fragments and a reduction of fibulin 5 expression in Htra1 transgenic mice compared to WT in RPE/choroid lysates. In vitro assays showed that HTRA1 degrades fibulin 5 as well as fibronectin. In addition, it also degraded nidogen 1 and nidogen 2, which are important structural constituents of basement membranes. Fibronectin fragments stimulate the release of cytokines and MMPs from RPE cells [29] and therefore can alter the homeostasis of the ECM, which can lead to further degradation of ECM components. Elevated levels of fibronectin fragments are often found in synovial fluids of patients with rheumatoid arthritis [30] and thus are linked to inflammation. Moreover, intact fibronectin is better able to bind collagen and mediate its cell binding, thereby stabilizing the ECM. In contrast, fibronectin fragments have a decreased ability to form and stabilize the ECM.

While we could observe degradation of nidogen 1 and nidogen 2 in vitro, we failed to observe degradation of nidogen in Htra1 transgenic mice. Our in vitro assays, however, might not reflect physiological conditions due to the fact that we used recombinant HTRA1 without the IGFBP/MAC25 homology domain, which contains an inhibitory element and therefore might have a higher protease activity than endogenous HTRA1.

Human recombinant HTRA1 failed to cleave insoluble elastin from human skin, thereby showing that the fragmentation of the elastic layer in Htra1 transgenic mice is not due to an elastase activity of HtrA1.

Missense mutations of the fibulin 5 gene have been associated with AMD [31] and reduced secretion of fibulin 5 has been found in four AMD mutations [32]. Fibulin 5 is known to be essential for elastogenesis in vivo as observed with fibulin 5−/− mice [33]. Additionally, we were able to demonstrate that transgenic mice also have lower tropoelastin expression in RPE lysates. Fibulin 5 binds to tropoelastin [34,35] and accelerates self-aggregation of tropoelastin, a process called coacervation [36]. Together with the finding of a fragmented elastic lamina in Htra1 transgenic mice, this result indicates an altered elastic fiber formation due to the reduction of fibulin 5. Moreover, our in vitro studies demonstrate that HTRA1 cleaves fibulin 5. In 2007, Hirai et al. [37] reported that the elastogenic organizer activity of fibulin 5 is abrogated by proteolytic cleavage, but failed to identify the responsible serine protease. Here, we report recombinant human HTRA1, a serine protease to cleave fibulin 5 in vitro. Fibulin 5 promotes elastic fiber assembly and maturation by organizing tropoelastin, LTBP-2 and the crosslinking lysyl oxidase-like enzymes LOXL1, 2 and 4 along Fibrillin microfibrils [38,39,40,41]. Besides, Yu et al. [42] described fragmentation of the elastic laminar in mice lacking LOXL1. While we observed a reduction in fibulin 5 and tropoelastin expression, we didn’t observe changes in the expression levels of other proteins that take part in elastogenesis. Therefore, it seems that the reduction of fibulin 5 and neither fibulin 4, nor EMILIN1, nor LOXL1 impairs elastogenesis in Htra1 transgenic mice and is responsible for the fragmentation of the elastic layer in BM as seen in TEM.

This data links HTRA1 into a network of extracellular proteins involved in AMD (Fig. 8). In addition to fibulin 5, other fibulin proteins are implicated in macular dystrophies. Recently, it was shown that ARMS2 interacts with fibulin 6 causing a form of familiar AMD [23]. Elastic fibers in BM seem to be crucial in the pathogenesis in AMD. Both fibulins and EMILIN2 (elastin microfibril interface located protein), another interacting partner of ARMS2 are involved in elastin biogenesis. The importance of elastin is further supported by the fact that the concentration of serum elastin-derived peptides are higher in patients with AMD than in control subjects [43].

Angiogenesis, a prerequisite of CNV in AMD, is promoted by both TGF-ß and VEGF. HTRA1 has been demonstrated to inhibit TGF-ß1 signaling in neuronal cells [44] and is associated with cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL) [19]. To test if HTRA1 inhibition of TGF-ß has any effect on the expression levels of TGF-ß in Htra1 transgenic mice, we measured expression levels in a Laminex assay. There was no significant difference in expression levels for TGF-ß1, 2 and 3 in transgenic compared to WT mice. Thus, we conclude that the inhibition of TGF-ß by HTRA1 is negligible in RPE/choroid lysates of Htra1 transgenic mice.

HTRA1 is also known to have an IGFBP/MAC25 homology domain and is capable of binding IGF-1. Our results exhibit no significant changes in IGF-1 and VEGF expression levels in Htra1 transgenic mice and consequently angiogenesis is not differentially regulated in Htra1 transgenic mice compared to WT mice regarding these factors.

In summary, our data suggests that an overexpression of HTRA1 in the RPE leads to an altered BM with fragmentation of the elastic laminar due to a decrease in fibulin 5, which could be shown by Western Blot analysis and degradation by recombinant HTRA1 in vitro. Furthermore, we demonstrate that HTRA1 degrades nidogen 1, nidogen 2 and fibronectin, but not elastin in vitro. Western Blot analysis also confirmed the degradation of fibronectin with a higher expression of active fibronectin fragments in Htra1 transgenic mice. These fragments are known to have proteolytic activity [45] and stimulate the release of cytokines and MMPs, which might further contribute to destabilization of the ECM. This data highlights the importance of ECM related proteins in the development of AMD. It links HTRA1 to other AMD risk genes such as fibulin 5, fibulin 6, ARMS2 and TIMP3.

Materials and Methods

Ethics Statement

This study utilized experiments using mice. All experiments using mice were performed with consent from the Animal Experimentation Committee of the University of Cologne (9.93.2.10.31.07.045). The study did not involve human experiments.

Generation of transgenic mice

A full-length Htra1 cDNA (2030 bp), generated from mouse cDNA by PCR, was cloned directionally after the murine Rpe65 promoter into a pCI plasmid (Promega) (Fig. 9). The Rpe65 promoter confers tissue specific expression as described by
Boulanger et al. [46]. The inclusion of a generic intron is used to further maximize expression. The sequence of the final construct was confirmed (3123 bp) by DNA sequencing. The construct was digested with PagI and Bsu15I followed by purification of the 3.9-kb fragment via agarose gel electrophoresis and millipore membrane filtration. The linearised transgene was used to generate seven founder mice via microinjection (ZMMK Service). The mice were screened for the integrity of the transgene by sequencing through the entire construct. To expand the transgenic lines, the founder mice were crossed into a C57BL/6N background (Charles River Laboratories). All animals investigated were derived from founder no. 2 and were heterozygous for the transgene, as they were generated by mating a transgenic parent with a C57BL/6N mouse.

PCR

Mice were screened for the presence of the transgene by PCR on genomic DNA. Tail pieces were digested overnight at 56°C in lysis buffer (High Pure PCR Template Preparation Kit, Roche), supplemented with 20 μg/ml proteinase K. DNA was isolated and quantified according to the manufacturer’s instructions. For the amplification of the transgene-specific sequence (417 bp) at 56°C, a 5′-primer (5′- GCAGGCTCCTCCAATAAGG -3′) and a 3′-primer (5′- AAGGAAGGAGCGCTAGCAG -3′) were used to produce a 417 bp product. The amplicons were separated by agarose gel electrophoresis and detected by ethidium bromide staining.

Real-time PCR

Total RNA was isolated from RPE/Choroid layers of transgenic and WT mice, at the age of 3 month, using the RNeasy Mini Kit (Qiagen). The cDNA was synthesized with oligo (dT) Primer using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen) followed by a RNase H digestion step. PCR was performed using 12.5 μl of Platinum qPCR Super-Mix-UGD (Invitrogen), 0.25 μl Universal Probe (Roche), labeled at the 5′ end with fluorescein (FAM) and at the 3′ end with a dark quencher dye, PCR Primers and 5 μl cDNA. Real-time PCR was performed using the MP 3000 detection system (Stratagene) and the PCR reaction was carried out at 50°C for 2 minutes and 95°C for 2 min followed by 50 cycles of 95°C for 15 seconds and 60°C for 30 seconds. At the end of the PCR cycles, a melting curve, using a temperature range from 55°C to 95°C with +0.5°C intervals, was generated to test the specificity of the PCR product. A cDNA sample in each experiment was tested in triplicate and each experiment was performed twice. The forward primer for Htra1 was 5′- AGTGGGTCAGGATTCATCGTA -3′ and the reverse primer was 5′- GTGACCACGTGACCATTTGT -3′. As

Figure 7. Luminex Assay of growth factors in RPE/choroid lysates. Concentration of growth factors in RPE/Choroid lysates of transgenic (tg) and WT (wt) mice at 3 month (young) and 12 month (old) of age (4 mice/group, 3 independent experiments). Data are presented as means; bars, ± SD. (A) Concentration of TGF-β isoforms in RPE/choroid lysates. WT and transgenic mice show no significant differences in TGF-β isoform concentration at young or old age. (B) Concentration of IGF-1 in RPE/choroid lysates. WT and transgenic mice show no significant differences in IGF-1 concentration at young or old age. (C) Concentration of VEGF in RPE/choroid lysates. WT and transgenic mice show no significant differences in VEGF concentration at young or old age.

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Figure 8. HTRA1 interacts with ECM proteins. Black arrows indicate cleavage by HTRA1. Proteins encoded by genes known to be related to macular degenerations are circled. Fibulins are delineated by the dashed box. Note that three of five fibulin genes shown have been implicated in maculopathies, and one of five directly interacts with HTRA1. Reference(s) for interactions described earlier are indicated in small boxes. [22,23,31,38,47–53] ARMS2, age-related macular susceptibility 2; ELN, elastin; EMLIN, elastin microfibril interface-located protein; FBLN, fibulin; FN, fibronectin; HTRA1, high-temperature requirement A1; LOXL1, lysyl oxidase-like 1; MMP9, matrix metalloproteinase-9; NID, Nidogen; TIMP3, tissue inhibitor of metalloproteinase 3.

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Universal Probe for HtrA1 PCR we selected Universal Probe No. 83. Actb served as the internal control. The forward primer for Actb was 5’- GCCGCAGGCAGGAGGAGGC-3’ and the reverse primer was 5’- GTGGACTCAGGGCAATGGA-3’. As Universal Probe for Actb PCR we selected Universal Probe No. 101. PCR efficiency represented by a standard curve reached over 95%.

Cell culture
Primary murine RPE cells were isolated from freshly dissected eyes and cultured in medium Dulbecco’s modified Eagle’s medium (high glucose, 5% FCS; Gibco Invitrogen) supplemented with 10% fetal bovine serum (PAA), and antibiotics (100 Units/ml penicillin and 100 µg/ml streptomycin; PAA). Cells were cultured till confluent and medium was exchanged by fetal serum serum-free medium 24 hours prior to Western Blot experiments.

Western blot analysis of murine RPE/choroid Lysates
The expression of HTRA1, fibulin 5, fibulin 4, nidogen 1, EMILIN1, LOXL1 and tropoelastin in the RPE layer of transgenic and WT mice was evaluated by immunoblotting. Briefly, the vitreous and retina were removed and RPE/choroid layers were lysed for 30 min on ice in lysis buffer (RIPA, Bio-Rad Laboratories) supplemented with a mixture of protease inhibitors (Bio-Rad Laboratories). The samples were cleared by a centrifugation step (30 min at 10000 g) and the supernatant was assayed for protein concentration (BCA assay, Thermo Fisher Scientific). Protein (10 µg) was electrophoresed on AnyKD TGX gel (Bio-Rad Laboratories). Proteins were electrophoretically transferred to a PVDF membrane (GE Healthcare) and then blocked in 5% non-fat milk. After washing blots were probed with antibodies to HTRA1 (1:1000, rabbit polyclonal, generated from recombinant HTRA1 lacking the Mac25 homology domain by Pineda), fibulin 5 (1:400, rabbit polyclonal, Santa Cruz Biotechnology), tropoelastin (1:1000, rabbit polyclonal, PR385 Elastin Products Company), fibulin 4 (1:1000, rabbit polyclonal, Santa Cruz Biotechnology), EMILIN1 (1:1000, rabbit polyclonal, Santa Cruz Biotechnology), LOXL1 (1:200, rabbit polyclonal, Santa Cruz Biotechnology), ACTB (1:10000, mouse monoclonal, Abcam). Polyclonal antibodies against nidogen 1 and fibronectin were kindly provided by Ursula Hartmann (Köln, Germany) and insoluble elastin from human skin (Sigma-Aldrich), nidogen 1, nidogen 2 (kindly provided by Manuel Koch, Cologne Systems), nidogen 1, nidogen 2 (provided by Manuel Koch, Cologne). Anti-fibronectin antibody was generated from recombinant fibronectin (Sigma-Aldrich), fibulin 5 (R&D Systems), nidogen 1, nidogen 2 (kindly provided by Manuel Koch, Köln, Germany) and insoluble elastin from human skin (Sigma-Aldrich).

Expression and Purification of HTRA1
The pETAmac25HTRA1 plasmid was kindly provided by Michael Ehrmann (Essen-Duisburg, Germany). E. coli strain DE3 was transformed with the plasmid and grown at 37°C while shaking till it reached an OD600 of 0.5. Protein expression was induced with 0.5 mM IPTG. After 5 hours at 25°C the cells were centrifuged (30 min at 4000 g) and the pellet was resuspended in lysis buffer (500 mM NaH2PO4, 3 M NaCl, pH 7.5) followed by homogenization and centrifugation (30 min at 15000 rpm) Precleared cell lysate was loaded on a Ni2+ NTA Superflow column (QIAGEN) at a flow rate of 3 ml/min. The column was washed with wash buffer I (100 mM Tris, 150 mM NaCl, pH 7.5) and buffer II (100 mM Tris, 150 mM NaCl, pH 7.5, 5 mM β-Mercaptoethanol, 30 mMimidazole) prior to elution in elution buffer (100 mM Tris, 150 mM NaCl, pH 7.5, 5 mM β-Mercaptoethanol, 150 mM imidazole). Fractions of 1 ml were collected and analyzed by SDS-PAGE. The purest fractions were then loaded to a HPS column (Bio-Rad Laboratories) at a flow rate of 3 ml/min. The column was washed with wash buffer I (50 mM NaCl, 50 mM HEPES/NaOH, adjust pH to buffer II) and the protein was eluted with buffer II (50 mM NaCl, 50 mM HEPES/NaOH, 500 mM KH2PO4). Fractions of 4 ml were collected and analyzed by SDS-PAGE. Protein bands were then cut from the gel and analyzed via peptide mass fingerprinting (PMF) by the bioanalytical department of the Center for Molecular Medicine, University of Cologne (CMMC) to confirm that the purified protein is recombinant HTRA1. Purified protein was stored in aliquots in storage buffer (50 mM Tris-HCl, 500 mM NaCl, pH 8.0, 10% glycerol) at -70°C.

Digestion Assays
Degradation of fibronectin (Sigma-Aldrich), fibulin 5 (R&D Systems), nidogen 1, nidogen 2 (kindly provided by Manuel Koch, Köln, Germany) and insoluble elastin from human skin (Sigma-Aldrich) was determined by incubation of recombinant HTRA1 with these proteins in 50 mM Tris-HCl, pH 8.5, 150 mM NaCl for 1, 3 or 18 h at 37°C. ECM proteins and HTRA1 were incubated separately under the same conditions and served as
controls. HTRA1 inhibitor (NVP-LEB748 kindly provided by Novartis) was preincubated with HTRA1 for 20 min at room temperature at a final concentration of 5 µM prior to the addition of ECM substrates. Samples were analyzed on a Silver-stained gel.

Luminex Assays

For all Assays, MILLIPLEX Map Kits from Millipore were used. RPE/Choroid lysates, standards, and quality control samples (25 µl) were added to a pre-wet filter plate in 96-well format followed by addition of premixed microspheres. After an overnight incubation (protected from light; continuous shaking), at 4°C a detection antibodies were added to each well (25 µl) and were incubated for 1 hour at room temperature. The antigen–antibody complex was detected by a phycoerythrin-labeled streptavidin conjugate (25 µl) that was incubated for 30 min at room temperature. Following a wash step, the signal associated with each analyte was measured by analyzing the resulting bead-capture antibody-analyte-detection antibody-Streptavidin-Phycocerythrin conjugate complexes on the Luminex 200. The analyte-specific capture antibodies covalently coupled to spectrally unique microspheric beads provide analytical specificity. The analyte concentration is related to the fluorescence intensity derived from complexed detection conjugates.

Supporting Information

Figure S1 Expression levels of HTRA1 shown by Western Blot and subsequent densitometric analysis. (A) Western Blot analysis of RPE/choroid lysates from 3 month old WT (wt) and transgenic (tg) mice. Transgenic mice show a higher expression of HTRA1 protein compared to WT. Expression of ACTB serves as a loading control. (B) Densitometric analysis of HTRA1 expression. The graph demonstrates the relative signal intensity of WT (wt) and transgenic (tg) mice. Htra1 transgenic mice show a 2.68 fold increase in signal intensity compared to WT mice. (TIF)

Figure S2 Transmission electron micrographs of BM. TEM images showing the elastic lamina of BM in WT (wt) and Htra1 transgenic (tg) mice at the age of 3 months and at the age of 12 months at original 3000× magnification. Note that the discontinuities are no local irregularities. (TIF)

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