Student ID No. 0981029

HtrA1 expression under oxidative stress

酸化ストレス下におけるHtrA1の発現と加齢黄斑変性について

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Professor Narutoku Sato	(Member)

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SUMMARY

Age-related macular degeneration (AMD) is a retinal disease that carries a high risk of causing blindness in the elderly. AMD is the most common cause of blindness in western countries. AMD is a disorder of the central retina, i.e. the macula region, which contains a dense layer of photoreceptors. Below the photoreceptors lies the retinal pigment epithelial (RPE) cell layer which has several important functions, such as phagocytosis of outer segments of photoreceptors, nutrient transport, and maintenance of ion balance. It also forms a part of the blood-ocular barrier. Posterior to the RPE layer is the Bruch membrane and the choroid with blood capillaries. The hallmarks of early phase of AMD are the robust accumulation of lipofuscin in RPE cells and drusen deposits between the RPE and the Bruch membrane. Accumulation of drusen results in the development of two clinical forms of AMD, 1) atrophic, the dry form of AMD and 2) exudative, the wet form of AMD. Neovascularization through the Bruch membrane and the RPE layer is a diagnostic marker for wet AMD. Those detrimental changes cause secondary harmful effects on rod and cone photoreceptors and these cause loss of vision. Oxidative stress and genetic predisposition have been proposed as major contributing factors for the onset of AMD. Recently, it is reported that SNPs in the promoter region of the HtrA1 gene on chromosome 10q26 are a major genetic risk factor for AMD. The HtrA1 gene encodes a secretory serine protease, which is activated by binding to collagen and degrades extracellular matrix proteins such as proteoglycans. The high-risk SNPs increase the expression of HtrA1, and the

HtrA1 protein is found to accumulate in drusen. HtrA1 is known to participate in the oxidative stress response induced by estrogen in hamster kidney cells. Based on these findings, we hypothesize that oxidative stress, in combination with the genetic change in the promoter region, induces persistent and excessive HtrA1 expression which results in the degradation of elastic laminae of the Bruch membrane and choroid which leads to the formation of vascular abnormalities.

We established conditions of applying oxidative stress to mouse embryonic fibroblast (MEF) and a non-transformed human RPE cell line (ARPE19) to measure the induction of HtrA1 expression and to assay oxidative stress-induced premature senescence. We chose H_2O_2 as the stressor and concentrations which are low enough so that H_2O_2 does not affect the cell viability after treatment (0.075 mM or 0.15mM).

We isolated MEF cells from wild type (HtrA1(+/+)), heterozygous (HtrA1(+/-)) and HtrA1 knockout (HtrA1(-/-)) mice. When HtrA1(+/+) MEF cells were treated with 0.15 mM H_2O_2, both the mRNA and protein levels of HtrA1 were increased after treatment. The H_2O_2 treatment induced p21 expression, a marker for cell senescence, in HtrA1(+/+) MEF cells. The induction of p21 after H_2O_2 treatment (0.075 mM) was higher in HtrA1(+/-) MEF cells than in HtrA1(-/-) MEF cells (2-fold at day 12). We also confirmed enhanced senescence in HtrA1(+/-) MEF cells as compared with HtrA1(-/-) MEF cells by SA-β-galactosidase staining. Viability assay showed that HtrA1(+/-) MEF cells were more viable than HtrA1(-/-) MEF cells after treatment with various concentrations of H_2O_2. To confirm these data, we overexpressed protease-inactive mutant HtrA1 or wild type HtrA1 in HtrA1(-/-) MEF cells and then

treated the cells with 0.075 mM H_2O_2. We found that p21 and SA-β-galactosidase activity was induced more in the MEF cells expressing wild type HtrA1 than in cells expressing inactive HtrA1. The primary pathological site of AMD is the RPE layer. We, therefore, analyzed induction of HtrA1 upon oxidative stress in ARPE19. When ARPE19 was treated with 0.15 mM H_2O_2, the expression (mRNA and protein) of HtrA1 was increased after 24 hours. The highest expression was obtained 3 days after treatment. Under these stress conditions, p21 expression and SA-β-galactosidase activity were also increased, suggesting the increase in HtrA1 expression was associated with induction of senescence. We overexpressed wild type HtrA1 or protease-inactive HtrA1 in the ARPE19 cells and then treated the cells with 0.15 mM H_2O_2. We found that p21 and SA-β-galactosidase activity was induced more in the ARPE19 cells expressing wild type HtrA1 than in the cells expressing inactive HtrA1. From these data, it was suggested that oxidative stress induces HtrA1 expression and that HtrA1 prevents cells from cell death but enhances oxidative stress-induced premature senescence.

We next examined the p38 signaling cascade which is known to induce premature senescence and a survival mechanism under oxidative stress. We found that HtrA1(+/-) MEF cells showed higher p38 phosphorylation after H_2O_2 treatment (0.075mM) than HtrA1(-/-) MEF cells. We then treated MEF cells with a high concentration (0.6 mM) of H_2O_2 and assayed for short term activation of p38. p38 was phosphorylated more quickly in HtrA1(+/-) MEF cells than in HtrA1(-/-) MEF cells. These data suggest that HtrA1 enhances p38 activation under oxidative stress. To

confirm these data, we overexpressed wild type HtrA1 or the protease-inactive HtrA1 in HtrA1(-/-) MEF cells and then treated the cells with 0.075 mM and 0.6 mM H_2O_2. We found that p38 phosphorylation was induced more quickly in the MEF cells expressing the wild type HtrA1 than in cells expressing the inactive HtrA1. From these data, we conclude that increased HtrA1 expression enhances oxidative stress-induced premature senescence and prevents cell death through activation of p38.

I. INTRODUCTION

The HtrA (high temperature requirement A) family of serine proteases is well conserved among different species ranging from bacteria, plants, to humans (Fig. 1A). The defining feature of hundreds of family members is the combination of a conserved trypsin-like protease domain with one or more C-terminal PDZ domains (Clausen et al., 2002). Each family member has characteristic functional domains and could have unique functions. The major common function of HtrA1 family members is protein quality control under various stress conditions.

1.1 Bacterial HtrA

E. coli has three HtrA proteins, DegP, DegQ, and DegS, which are localized in the periplasmic space (Swamy et al., 1983). HtrA/DegP was initially identified in *E. coli* by two phenotypes of null mutants. Mutants either showed an increased sensitivity to elevated temperatures (HtrA) or failed to digest denatured proteins in the periplasm (DegP) (Strauch and Beckwith, 1988; Lipinska et al., 1989).

DegP degrades heat-denatured or misfolded proteins in the periplasm, thus protecting cells from the toxic effects of aggregated proteins (Lipinska et al., 1990; Spiess et al., 1999). DegP is a hexamer of staggered association of two trimers with PDZ domains protruding from the hexameric structure. The PDZ domain recognizes and binds hydrophobic patches of denatured polypeptides, and presents the substrates to the catalytic cavity inside the hexamer for digestion (Krojer et al., 2002; Merdanovic et al., 2011). The periplasmic β-amylase MalS protein is a natural substrate of DegP. DegP not only degrades misfolded MalS but also functions as a chaperone that enhances

the folding of nascent MalS into the correct structure. The switch between these two functions is facilitated by the temperature. Chaperone activity occurs at temperatures below 28^{o}C. Proteolytic activity increases dramatically at temperatures above 28^{o}C (Swamy et al., 1983; Lipinska et al., 1990; Spiess et al., 1999; Kim et al., 1999).

DegP has a regulatory function, too. It contributes to the activation of the CpxR/A two-component regulatory system in *E. coli* (Fig. 1B). The CpxR/A system senses perturbations in the bacterial cell envelope and responds by activating the transcription factor CpxR, which results in the enhanced expression of numerous protein folding and degrading activities (Isaac et al., 2005; Ruiz and Silhavy, 2005). CpxA is a cytoplasmic membrane-spanning protein and functions as a sensor kinase. In normal conditions, an inhibitory protein, CpxP, binds the periplasmic domain of the CpxA and inactivates its kinase activity. Under protein-folding stress, misfolded proteins bind CpxP and release CpxP from CpxA, resulting in the activation of CpxA. Activated CpxA phosphorylates and activates CpxR. DegP digests the misfolded protein-CpxP complex and keeps CpxA in the activated state (Ruiz and Silhavy, 2005; Isaac et al., 2005; Clausen et al., 2011).

DegQ has the similar substrate specificity as DegP (Kolmar et al., 1996). Overexpression of DegQ in DegP null mutants allows the cells to recover from sensitivity to elevated temperature. DegQ has, therefore, a complementary function to DegP (Waller et al., 1996).

In contrast to DegP and DegQ, DegS has solely a regulatory function (Ades et al., 1999; Alba et al., 2002; Clausen et al., 2011). DegS regulates a stress response σ factor (Fig. 1B), σ^{E}, which is responsible for the transcription of various stress response genes (Ades et al., 1999; Alba et al., 2001). In normal conditions, σ^{E} is inhibited by RseA, a

membrane-spanning protein that binds σ^E in the cytoplasm and prevents it from binding to RNA polymerase (De La Penas et al., 1997; Missiakas et al., 1997; Campbell et al., 2003). Stress conditions, such as heat shock, denature the outer membrane porins (OMPs). Denatured OMPs binds to the PDZ domain of DegS and activates its protease activity. Activated DegS cleaves the periplasmic domain of RseA, resulting in the release of σ^E from the RseA-σ^E complex (Mecsas et al., 1993; Alba et al., 2002; Ades et al., 1999; Ades et al., 2003).

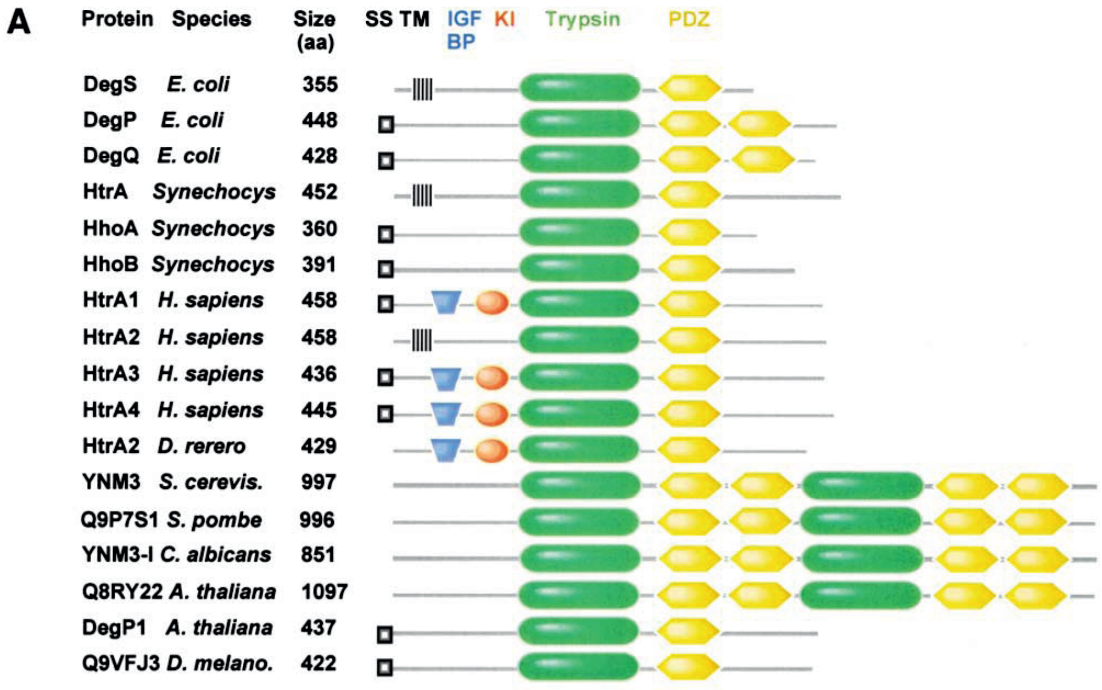
Expression of DegP is induced in response to heat shock, ethanol addition, and oxidative stress (Loosmore et al., 1998; Skórko-Glonek et al., 1999; Rauter et al., 2003). The expression of HtrA proteins in other bacteria, such as *H. influenza*, *L. lactis*, and *L. helveticus* are also upregulated by heat shock, ethanol addition, puromycin treatment, osmotic stress, and hydrogen peroxide (Loosmore et al., 1998; Smed et al., 1998; Foucaud-Scheunemann et al., 2003). An increased expression of HtrA is required for the survival of *Y. enterocolica, S. mutants,* and *L. monocytogenes* under heat shock or oxidative stress (Yamamoto et al., 1996; Diaz-Torres et al., 2001; Wonderling et al., 2004).

HtrA of Gram-positive and Gram-negative pathogenic bacteria is essential for virulence. This outcome is based on the characteristics of HtrA as an oxidative stress protein. The HtrA proteins of these pathogens degrade damaged proteins that are produced by reactive oxygen species released from the host defense system (Jones et al., 2001).

1.2 Plant HtrA

Plants have many HtrA genes; *Thaliana* has 16 HtrA homologues. Five of

them (Deg1, Deg2, Deg5, Deg7, and Deg8) are located in the chloroplast (Itzhaki et al., 1998; Huesgen et al., 2009). Like bacterial DegP, plant HtrA/Deg1 has two functions (Fig. 1C). As a chaperone, Deg1 interact with a photosystem reaction center protein D2 and enhance its assembly with another subunit, D1. As a protease, Deg1 participates with the FTSH (Filamentation temperature-sensitive H) protease in the degradation of photodamaged D1. Deg1 degrades the thylakoid-luminal loops of D1. Then FTSH extracts and degrades the remaining transmembrane helices of the D1 protein from the thylakoid membrane (Kapri-Pardes et al., 2007; Sun et al., 2010; Clausen et al., 2011).



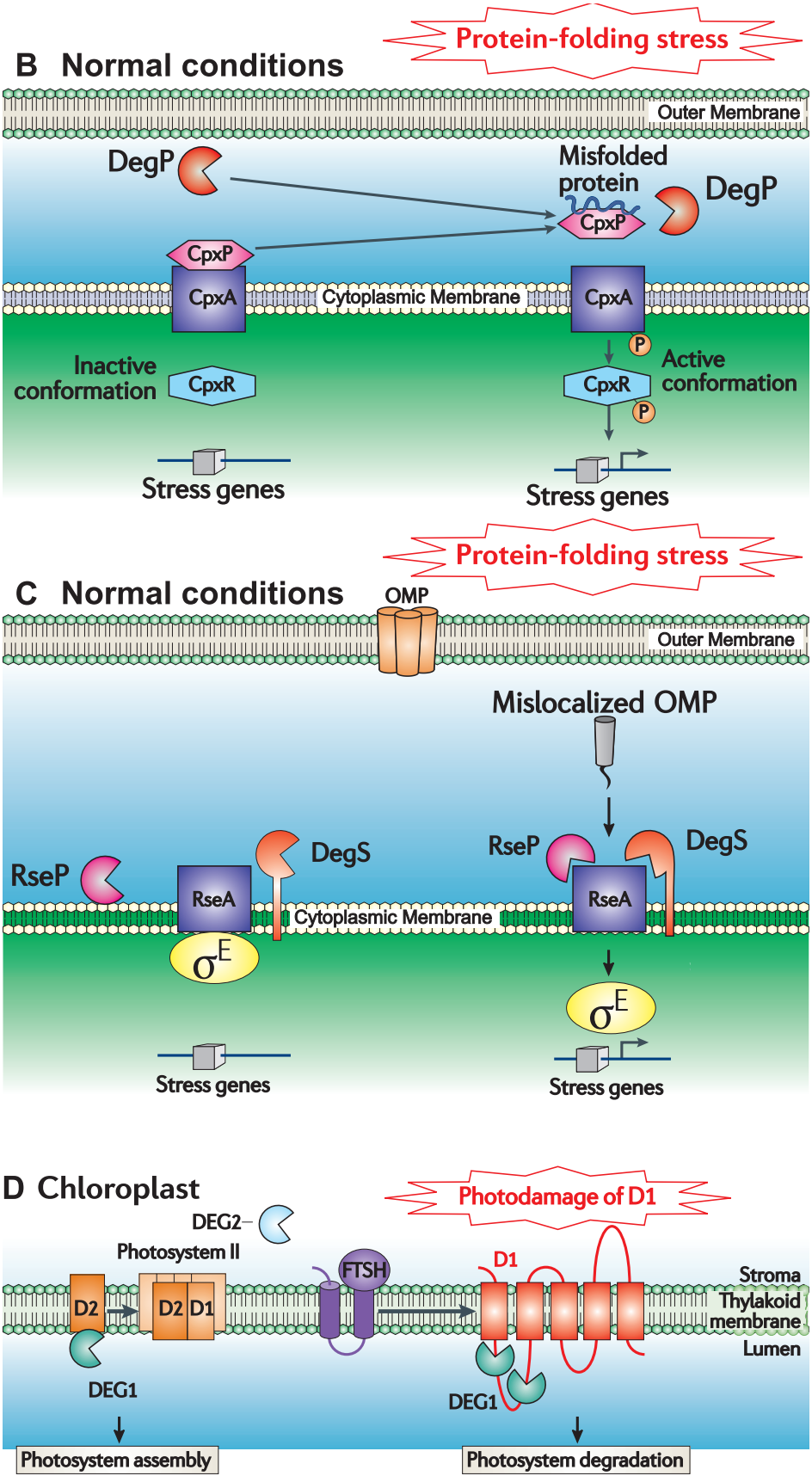

B Normal conditions
Protein-folding stress
Outer Membrane
DegP
Misfolded protein
DegP
CpxP
CpxP
CpxA
Cytoplasmic Membrane
CpxA
P
Inactive conformation
CpxR
CpxR
Active conformation
P
Stress genes
Stress genes
C Normal conditions
Protein-folding stress
OMP
Outer Membrane
Mislocalized OMP
RseP
DegS
RseP
DegS
RseA
Cytoplasmic Membrane
RseA
σE
σE
Stress genes
Stress genes
D Chloroplast
Photodamage of D1
DEG2
Photosystem II
FTSH
D1
Stroma
Thylakoid membrane
Lumen
D2
D2
D1
DEG1
DEG1
Photosystem assembly
Photosystem degradation

Figure 1.A. Domain organization of HtrA family members from bacteria to humans. SS, signal peptide; TM, transmembrane segment; IGFBP, insulin growth factor binding domain; KI, Kazal protease inhibitor domain; Trypsin, serine protease domain; PDZ, PDZ domain (Clausen et al., 2002). **B & C *Escherichia coli* HtrA proteases regulate protein quality control. B.** In normal conditions, CpxP binds the periplasmic domain of the CpxA and inactivates its kinase activity. Under protein-folding stress, misfolded proteins bind CpxP and release CpxP from CpxA, resulting in the activation of CpxA. DegP degrades the substrate-CpxP complex. Activated CpxA activates CpxR. Activated CpxR upregulates stress genes. **C.** In normal conditions, the cytoplasmic membrane protein RseA binds and keeps σ^E in an inactive conformation. Under protein-folding stress, mislocalized outer-membrane proteins (OMPs) bind to PDZ domain of DegS and activates its protease activity. Activated DegS cleaves the periplasmic domain of RseA, resulting in the release of σ^E. σ^E upregulates stress genes. **D. Plant HtrA proteases regulate photosystem assembly and degradation.** Five *Arabidopsis thaliana* HtrAs (Deg1, Deg2, Deg5, Deg7, and Deg8) are located in the chloroplast. As a chaperone, Deg1 interact with a photosystem reaction center protein D2 and assists in the assembly of photosystem II. As a protease, Deg1 participates with FTSH (Filamentation temperature-sensitive H) protease in the degradation of photodamaged D1. Deg1 degrades the thylakoid-luminal loops of D1. Figure 1B,C,D modified from figures in Clausen et al., 2011.

1.3 Mammalian HtrA

Mammalian homologues of HtrA are HtrA1, HtrA2, HtrA3, and HtrA4 (Clausen, T et al., 2002). HtrA1 was originally identified as a gene that was downregulated in SV40-transformed human fibroblasts (Zumbrunn and Trueb, 1996). Mammalian HtrA1 has a secretory signal sequence, an Insulin-like growth factor binding protein (IGFBP)-like domain, and a Kazal type protease inhibitor (KI) domain in the N-terminal side of the protease domain, and a PDZ domain in the C-terminal side (Truebestein et al., 2011). HtrA3 and HtrA4 share the same domain structure and high homology with HtrA1. HtrA1 is secreted out of cells and degrades various extracellular matrix proteins. The PDZ domain of HtrA1 is required to stimulate the protease

activity through interaction with hydrophobic amino acid sequences in the C-terminal ends of various proteins (Murwantoko et al., 2004).

During embryo development, mouse HtrA1 is expressed in specific areas where signaling by TGF-β family growth factors plays important regulatory roles. Actually, HtrA1 can inhibit TGF-β signaling. The protease activity of HtrA1 is essential for this inhibition (Oka et al., 2004). HtrA3 also inhibits the signaling of TGF-β. Since HtrA1 and HtrA3 expression overlaps with each other in various tissues, they might have complementary functions (Tocharus et al., 2004). The expression of HtrA4 is low and HtrA4 has not been well characterized.

1.3.1 HtrA2 and human disease

In contrast to HtrA1, -3 and -4, HtrA2 is a mitochondrial membrane protein with a transmembrane domain in the N-terminal region (Challa et al., 2007). Its catalytic domain and the PDZ domain localize in the intermembrane space. HtrA2 is cleaved in the N-terminal region and the C-terminal fragment containing the catalytic and PDZ domains is released and leaked into the cytoplasm when the cell receives apoptotic stimuli. Cytoplasmic HtrA2 induces apoptosis directly through its protease activity or by binding to XIAP (Vande Walle et al., 2008).

HtrA2 knockout mice do not show signs of decreased apoptosis, but rather exhibit striatal neurodegeneration and die prematurely. The neurological symptoms of HtrA2 KO mice are characteristic of the parkinsonian syndrome in humans (Martins et al., 2004). HtrA2 is probably involved in the protein-folding stress response in mitochondria and its absence causes mitochondrial malfunctions that lead to neural degeneration and Parkinson's disease. Actually, mutations in the *HTRA2* gene are

reported in patients of familiar Parkinson's disease in Belgium and Germany. These mutations resulted in defective activation of the protease activity of HtrA2 (Strauss et al., 2005; Bogaerts et al., 2008).

1.3.2 HtrA1 and human diseases

High HtrA1 expression has been reported in rheumatoid arthritis (Tsuchiya et al., 2005; Grau et al., 2006), osteoarthritis (Hu et al., 1998; Chamberland et al., 2009), preeclampsia (Ajayi et al., 2008), Duchenne muscular dystrophy (Bakay et al., 2002), and Alzheimer's disease (Grau et al., 2005). In arthritic cartilage, HtrA1 seems to degrade extracellular matrix proteins and aggravates arthritis. Recombinant HtrA1 is known to digest cartilage matrix components, such as aggrecan, biglican, decorin, fibromodulin, Type II collagen, and fibronectin (Tsuchiya et al., 2005; Grau et al., 2006; Chamberland et al., 2009).

HtrA1 degrades aggregated and fibrillar forms of tau, a protein involved in various neurological diseases (Tennstaedt et al., 2012). The expression level of HtrA1 in the brain increases as the aggregated tau increases (Tennstaedt et al., 2012). HtrA1 also degrades the amyloid precursor protein fragment Aβ that is accumulated in the blood vessel walls of Alzheimer's disease patients (Grau et al., 2006).

Loss-of-function mutations of human HtrA1 gene cause CARASIL (cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy). This hereditary disease is characterized by ischemic, non-hypertensive, cerebral infarction which accompanies alopecia and spondylosis in early adulthood. Cerebral small arteries of CARASIL patients show a distinct type of arteriosclerosis: arteries show intimal thickening, dense collagen fiber deposition, hyaline degeneration of the tunica

media, and loss of vascular smooth-muscle cells. Probably, the loss of HtrA1 activity causes accumulation of extracellular matrix components in the cerebral small vessel wall. TGF-β signaling is constantly stimulated in the CARASIL vessel wall, due to the loss of TGF-β-inhibitory HtrA1. TGF-β enhances extracellular matrix production and regulates cell differentiation. Increased TGF-β signaling, therefore, may contribute to the excessive production of the extracellular matrix and abnormal differentiation of vascular smooth muscle cells (Hara et al., 2009).

The expression of HtrA1 is downregulated in ovarian cancer (Shridhar et al., 2002), malignant melanoma (Baldi et al., 2002), Burkitt's lymphoma (Nie, et al., 2003), mesothelioma (Baldi, et al., 2008), primary brain tumors, and several other cancer cell lines (Chien et al., 2004). Overexpression of HtrA1 in highly invasive melanomas suppresses proliferation and migration of the tumor cells (Baldi et al., 2002). Moreover, cell death of ovarian cancer cells can be induced by HtrA1 via its protease domain. HtrA1-induced cell death is not attenuated by the broad-spectrum caspase inhibitor zVAD(OMe)fmk (Chien et al., 2004). The protease activity of HtrA1 itself, therefore, can induce cell death. HtrA1 enhances cytotoxicity of widely used tumor chemotherapeutic agents, paclitaxel, and cisplatine. Ovarian or gastric tumor patients who have a higher expression of HtrA1 show a better response to cisplatin-based combination chemotherapies than patients who have lower levels of HtrA1 (Chien et al., 2006). All these findings show that HtrA1 has tumor suppressor activity.

1.4 Age-related macular degeneration (AMD)

SNPs in the promoter region of the HtrA1 gene are a major genetic risk factor for age-related macular degeneration (AMD) (DeWan, et al., 2006; Yang et al., 2006).

AMD is a retinal degenerative disease and is the leading cause of irreversible visual impairment in individuals over the age of 55 years all over the world (Coleman et al., 2008; Ambati et al., 2003). The prevalence of AMD in Asian populations aged 40-79 years was 7.36% (Kawasaki et al., 2010).

AMD is classified into dry type and wet type (Bird et al., 1995). Dry type AMD is more common than wet type and is related with small, yellow deposits called drusen accumulated between the RPE and Bruch's membrane in the macular region. Wet type AMD causes severe central visual loss and is associated with the vascular abnormalities in the choroid and the subretinal space, specifically beneath the macula (Coleman et al., 2008). Vascular abnormalities of wet type AMD include choroidal neovascularization (CNV) and polyploidal choroidal vasculopathy (PCV). The former is caused by the invasion of new blood vessels from the choroid into the sub-RPE and subretinal spaces. PCV includes the polyploidal vascular dilatation and abnormal branching of the choroid vessels. The abnormal angiogenesis results in excessive leakage of fluid or blood from the newly formed blood vessels. The accumulation of exudate and blood in the macula causes the destruction of photoreceptor cells resulting in blindness.

1.4.1 AMD pathogenesis

The exact pathogenesis of AMD remains elusive. The common risk factors in AMD are age, smoking, hypertension, diet, oxidative stress, and genetic factors. Among environmental factors, age and oxidative stress are the most influential factors (Coleman et al., 2008; Ehrlich et al., 2008; Andreoli et al., 2009; Ding et al., 2009).

The onset of AMD starts in the retinal pigment epithelium (RPE). RPE

functions in essential processes for the maintenance of photoreceptor functions and survival (Glotin et al., 2008). RPE, a single cell layer of pigmented cells, absorbs the light focused by the lens on the retina. RPE provides nutrients to and removes metabolic waste from photoreceptor cells, optimizes ion concentrations in the surrounding tissues, and eliminates fluid from the subretinal space to the blood. RPE phagocytoses and digests the outer segments of photoreceptors, and recycles important substances and returns them to photoreceptors to rebuild light-sensitive outer segments from the bases of the photoreceptors (Strauss, 2005; Gu et al., 2012). Dysfunction of RPE cells will damage the photoreceptors and the surrounding tissues. Oxidative stress and aging which are associated with cumulative oxidative damages are the most common causes of RPE dysfunction.

The retina is subject to constant oxidative damages due to the following reasons: high O_2 tension, excessive exposure to light, high proportion of polyunsaturated fatty acids in the photoreceptor outer segments, and numerous chromophores in the retina. All these processes generate reactive oxygen species (ROS). The phagocytosis by RPE also generates ROS. ROS, such as hydrogen peroxide, singlet oxygen, superoxide anion, and hydroxyl radical causes RPE and choriocapillary injury (Zarbin, 2004). Clinical studies support oxidative damage as a major cause of AMD. Antioxidant vitamin E, ascorbic acid, and β-carotene are effective to delay the progression of AMD. Knockout mice of oxidative stress-associated genes such as super oxide dismutase 1 and 2 showed phenotypes similar to human AMD (Imamura et al., 2006; Justilien et al., 2007; Dong et al., 2009).

During aging, the RPE cells undergo a number of characteristic structural changes, including loss of melanin granules, microvilli atrophy, and the formation of

drusen, and cytoplasmic accumulation of lipofuscin or metabolic debris from incomplete degradation of phagocytosed rods and cones (Ciulla et al., 2001; Gu et al., 2012). These features of aging are observed in the retina of AMD patients, but there are additional pathological changes in AMD. A significant difference was observed in the amount and composition of drusen in retinas with and without AMD (Spraul et al., 1999). These conditions in AMD result in a chronic inflammatory response in Bruch's membrane and in the choroid, and thereby develop atrophy of the retina, PCV and CNV (Moshfeghi and Blumenkranz, 2007).

The senescence-accelerated mouse (SAM P_8) at 12 months of age shows a marked disruption of basal microvilli of RPE and derangement of cellular components. Bruch's membrane thickens uniformly and abnormal deposits of amorphous materials are found in the sub-RPE space. The photoreceptors, RPE, and choriocapillaris of SAM P_8 mice show abnormalities similar to human AMD (Majji et al., 2000). Another senescence-accelerated model (OXYS rats) also shows retinal abnormalities similar to human AMD (Markovets et al., 2011).

1.4.2 Genetic background of AMD

In addition to oxidative stress and aging, genetic background strongly influences the pathogenesis of AMD. Whole genome association studies have shown that chromosome 1q32 is strongly linked with the development of AMD. The initial study of chromosome 1q32 revealed that a variant of complement factor H (*CFH*) gene with a single-nucleoted polymorphism (SNP) at rs1061170 was significantly correlated with AMD. This SNP causes a tyrosine-to-histidine substitution (Y402H) that is located within the binding site for heparin and C-reactive protein (Klein et al., 2005).

Further studies have revealed 20 polymorphisms which show correlation to AMD in and around the *CFH* gene (Li et al., 2006).

A region on chromosome 10q26 is also linked to AMD (Kenealy et al., 2004). SNPs within a region encompassing *PLEKHA1* (pleckstrin homology domain-containing, family A member 1), *ARMS,2* and *HTRA1* are significantly associated with AMD (Jakobsdottir et al., 2005; Fritsche et al., 2008).

It has later been shown that SNPs in the promoter region of *HTRA1* were strongly associated with both dry and wet type AMD (DeWan, et al., 2006; Yang et al., 2006, Cameron et al., 2007; Tuo et al., 2008). *ARMS2* is a putative gene encoding a short open reading frame and located within the promoter region of *HTRA1*. Some high-risk SNPs are located within or near *ARMS2*. An allele of rs2736911 contains a nucleotide deletion and a premature stop codon in *ARMS2*, but this allele is not linked to the onset of AMD. Besides, *ARMS2* encodes a small protein (107 aa) with dubious expression patterns and is conserved only in humans and primates. It seems, therefore, that *ARMS2* is not correlated with the development of AMD (Friedrich et al., 2011).

1.5 HtrA1 and age-related macular degeneration

The high-risk SNPs in the promoter region of *HTRA1* enhance transcription of HtrA1 in homozygous individuals. Lymphocytes and primary RPE cells from AMD patients with homozygous risk alleles express approximately 3-fold higher HtrA1 mRNA than the healthy control (DeWan, et al., 2006; Yang et al., 2006, An et al, 2010).

Several papers have proposed molecular mechanisms through which overexpressed HtrA1 induces damages of choroidal vessels and RPE in AMD. Jones et al. produced transgenic mice expressing human HtrA1 in RPE (Jones et al., 2011). The

transgenic mice show features of PCV, including a branched network of choroidal vessels and polypoidal lesions, severe degeneration of elastic laminae, and tunica media of choroidal vessels. Aged transgenic mice develop occult CNV. Those phenotypes are typical vascular features of wet type AMD. The transgenic mice show degeneration of the elastic lamina of Bruch's membrane, and atrophy of RPE and photoreceptors. Jones et al. also showed that HtrA1 has an elastase activity which may explain the degeneration of elastic laminae of choroidal vessels and Bruch's membrane. Bruch's membrane is the basement membrane of RPE and its damage can injure RPE. Another transgenic mouse line that overexpresses murine HtrA1 in the RPE layer shows essentially the same histological abnormalities (Vierkotten et al., 2011). Vierkotten et al. found overexpression of fibronectin fragments, and reduction of fibulin 5 and tropoelastin in the RPE and choroid layer of the transgenic mouse. Fibulin 5 is essential for the assembly and maturation of elastic fibers. Results of these two transgenic mice indicate that overexpressed HtrA1 is secreted out of RPE and compromises elastogenesis in the elastic laminae of Bruch's membrane and choroidal vessel walls either by degrading elastin or fibulin 5 or both.

Zhang et al. have revealed that HtrA1 knock-out mice exhibit decreased vascular development in the retina and significant upregulation of growth differentiation factor-6 (GDF6) gene in the RPE layer, retina, and brain tissues (Zhang et al., 2012). They suggested that HtrA1 modulates tissue vascularization through GDF6 expression.

Considering that HtrA1 digests various extracellular matrix components and inhibits signaling of TGF-β family growth factors, such as, TGFs, BMPs and GDFs, it is reasonable to assume that the overexpression of HtrA1 induces AMD by changing the extracellular matrix structures and by modifying differentiation and maintenance of RPE

and choroidal vascular cells. It is still unclear how HtrA1 affects the oxidative stress response and the premature senescence of RPE cells.

1.6 Hypothesis

I propose the following mechanisms as etiology of AMD. SNPs in the promoter region of HtrA1 stimulate the HtrA1 expression in the RPE layer. Oxidative stress, a major risk factor of AMD, upregulates HtrA1 expression as well. Genetic variations and oxidative stress increase the HtrA1 expression additively and induce premature cell senescence in the RPE cells. Cell senescence induces degeneration of RPE, triggering the onset of AMD (Fig. 2).

In this study, I try to prove this hypothesis. Recently, in vitro premature senescence models have been established in various kinds of culture cells including ARPE19, a non-transformed human RPE cell line. ARPE19 cells exposed to oxidative stress show authentic senescent phenotypes including hypertrophy of cells, induction of senescence markers such as $p21^{CIP1/WAF1}$ and $p16^{INK4a}$, activation of senescence associated (SA)-β galactosidase, and the characteristic cell cycle arrest (Glotin et al., 2008; Zhu et al., 2009). This model gives a chance for a detailed understanding of the basic mechanisms of AMD development.

We have established the HtrA1 knock-out mouse. By using mouse embryonic fibroblasts (MEFs) derived from the knock-out mouse, we can examine the roles of HtrA1 in cell senescence and oxidative stress with minimum interference from other factors.

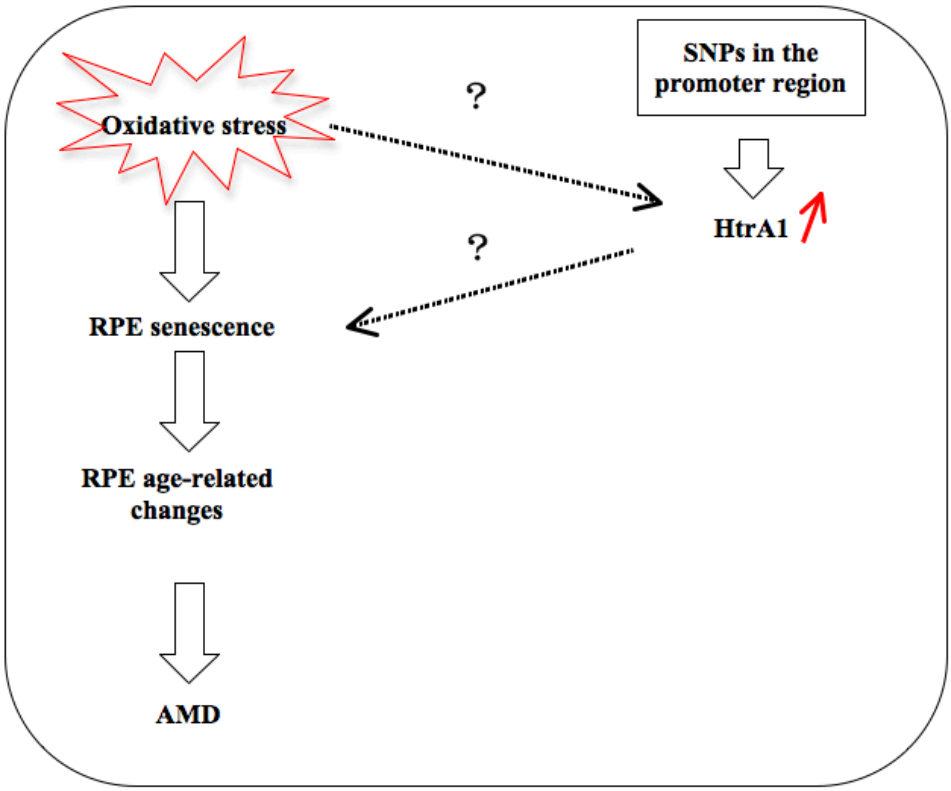


Figure 2. Hypothesis. HtrA1 is upregulated in an individual with high-risk SNPs in the promoter region of the HtrA1 gene. HtrA1 as a stress response protein is further upregulated by persistent oxidative stress in the RPE. The combined effects induce cell senescence and trigger the onset of AMD.

II. MATERIALS AND METHODS

2.1 Cell culture and isolation of primary mouse embryonic fibroblast cells

ARPE-19 cells were cultured in DMEM/F12 (1:1, Invitrogen) containing 2 mM glutamine, 70 μg/ml penicillin, 50 μg/ml streptomycin (Wako) and 10% fetal bovine serum. The cells were subcultured every 3-4 days. Mouse embryonic fibroblasts (MEF) were isolated from HtrA1+/+, HtrA1+/- and HtrA1-/- mouse embryos at 13.5 days of pregnancy (Serrano et al., 1997). Embryos were removed and separated from its placenta and surrounding membranes and were then washed three times with PBS(-) (PBS without Ca^{++} and Mg^{++}). After removal of the head and internal organs, the embryos were washed three times in PBS(-). Each embryo was processed separately. The embryo was minced. Minced tissues were suspended in 1 ml of trypsin-EDTA (0.25% trypsin and 0.02% EDTA in PBS(-)) and then incubated with gentle shaking at 37^{o}C for 15 min. The cell suspension was mixed with 2 volumes of MEF medium (Dulbecco's modified Eagle's medium, Sigma, with 2 mM glutamine, 70 μg/ml penicillin, and 50 μg/ml streptomycin) containing 10% fetal bovine serum (PAA laboratories GmbH). The suspension was filtered through a nylon strainer (mesh: 100/2.54 cm) to remove undigested tissue debris. The filtered suspension was centrifuged at 1000 RPM for 5 minutes. The resultant pellet was resuspended in MEF medium containing 10% FBS and 5 x 10^5 cells from each embryo were plated in a 10 cm culture dish. The remaining cells were used for genotyping by PCR. The culture medium was changed the following day. MEF cells were

maintained at 37°C in a humidified 5% CO_2 chamber. MEF cells were subcultured every 3 days and cells of passage number 3-5 were used for all experiments.

2.2 Treatment procedures

Hydrogen peroxide treatment. MEF cells ($1x10^5$ cells/well) were plated in 6-well plates. After 24 hours, the medium was replaced with a mixture containing 0.075 or 0.15 mM of hydrogen peroxide (H_2O_2, Sigma), and was incubated for 2 hours. Then the medium was changed to the fresh culture medium. Cells were maintained up to 12 days with the medium changed every 3 days. MEF cells were similarly treated with 0.6 mM H_2O_2 , and were harvested directly after various time periods (5, 10, 30, 60, and 120 minutes).

ARPE19 was treated with H_2O_2 according to the published method (Zhu et al., 2009). ARPE19 ($4x10^5$ cells/well) was plated in 6-well plates and cultured for 24 hours. The medium was replaced with a mixture containing 0.15 mM H_2O_2 and was incubated for 2 hours. Cells were then washed with PBS(-), and the culture continued in the normal medium containing 10% FBS for 22 hours. The H_2O_2 treatment was repeated again the next day (day 2). On day 3, the medium was changed to one containing 0.1% FBS and the cells were cultured up to 5 days.

***Tert*-butyl hydroperoxide treatment.** ARPE19 cells ($4x10^5$ cells/well) were plated in 6-well plates and were cultured for 24 hours in the normal medium containing 10% FBS as described above. The culture medium was changed to one containing 0.6 mM *tert*-butyl hydroperoxide (Sigma) and incubated for 30 minutes.

Cells were washed with PBS(-) and then cultured in the medium containing 1% FBS for 1 or 2 days.

Hydroquinone (HQ) treatment. ARPE19 cells ($4x10^5$ cells/well) were plated in 6-well plates in phenol red free medium with 10% FBS and cultured for 48 hours. Then the medium was changed to the phenol red free medium containing 1% FBS and 10 μM HQ (Sigma). The medium with HQ was changed every day and cells were cultured for 3 to 5 days.

UV treatment. ARPE19 cells were plated in the medium containing 10% FBS and were cultured for 24 hours as described for the H_2O_2 treatment. The cells were washed with PBS(-). Cells were exposed to 100 J/m2 of UV light by using a UV cross linker (254-nm, Stratagene). After exposure, the cells were washed with PBS(-) and then cultured in the medium containing 1% FBS for different periods of time (0.5, 12, 24 and 48 hours).

Chloroquine treatment. ARPE19 cells were plated in the medium containing 10% FBS and cultured for 24 hours as described for the H_2O_2 treatment. Then the medium was changed to one containing 1% FBS and chloroquine (Sigma) at various concentrations (0, 20, 40, 60, and 80 μg/μl). After incubation for 24 hours, cells were washed with PBS(-) and were cultured in the medium containing 1% FBS for 24 hours.

2.3 Preparation of mouse and human recombinant HtrA1 proteins

Human or mouse HtrA1 cDNA was subcloned into pCDNA3 plasmid

(Invitrogen). The protease-inactive S328A HtrA1 mutant was constructed by substitution of serine at position 328 with alanine by using PCR mutagenesis. Primers used for PCR mutagenesis are as follows:

Forward 1:5'-CGCAAGCTTCCGTTTTCTA-3'

Reverse 1: 5'-CTAAGGGCCCTCCCGCGTTTCCATAGTT-3'

Forward 2: 5'-TGCAGGGCCCTTAGTAAACCTGGACGG-3'

Reverse 2: 5'-AGCTCATGCCTCGCCTAT-3'.

Recombinant HtrA1 proteins were obtained by a transient transfection of HEK293T cells with a pcDNA3 vector containing human or mouse cDNA of wild type HtrA1 or protease-deficient S328A mutant HtrA1. The empty pcDNA3 was used to prepare the control conditioned medium. Cells cultured in a 10 cm dish were transfected with 10 μg plasmid DNA by the polyethyleneimine (PEI) method (Durocher et al., 2002). After 18 hours, the medium was changed to DMEM with 1% FBS and cells were cultured for another 48 hours. The 48-hour culture medium (CM, conditioned medium) was collected and centrifuged for 1000 rpm for 10 min to remove cell debris. To assay protease activity in the media, CM (6 μl) was incubated with 15 μg β-casein in 50 mM Tris-Cl (pH 7.5) in total volume of 30 μl for 18 hours at 37^{o}C. The digestion products were resolved by SDS-PAGE and the gel was then stained with Coomassie Brilliant Blue (CBB). The amounts of recombinant HtrA1 proteins in the CM were determined by a western blot assay using anti-mouse or anti-human HtrA1 antibody. The amount of human HtrA1 in the CM was determined by comparing band intensities with those of known amounts of purified recombinant human HtrA1 protein

that was produced in *E. coli*.

2.4 Production of human HtrA1 protein in *E. coli*

An N-terminal fragment of human HtrA1 was produced in *E. coli* and purified. The fragment was used for the standard to measure human HtrA1 proteins in CM. A fragment of human HtrA1 cDNA encoding amino acid 1 to 132 was subcloned into pET32a plasmid (Murwantoko et al., 2004). The plasmid was introduced into *E. coli* BL21 (Novagen). Production of the human HtrA1 fragment was induced by incubation in the presence of 1 mM IPTG for 6 hours at 37 ^{o}C. The cell lysate was prepared by sonication and the protein was purified by a Ni-NTA agarose (Qiagen) column. The HtrA1 protein was eluted from the column with 50 mM imidazol and dialyzed against PBS(-). The concentration of purified protein was assayed by the Bradford method.

2.5 Transfection of MEF and ARPE19 cells

MEF ($1X10^5$ cell /well) or ARPE19 ($4X10^5$ cells /well) were plated in a 6-well plate and were then transfected with HtrA1 expression vectors (2 μg DNA/well) by using turbofect (Thermo Scientific) according to the manufacturer's instruction. Cells were used for further experiments 48 hours after transfection.

2.6 Cell viability assay

Cells (1 x 10^4 cells/well) were plated in 96-well microplates and were cultured

for 24 hours. Cells were then treated with various concentrations of H_2O_2 for 2 hours. After treatment, cells were washed and cultured for 18 hours in normal culture medium. Viability of cells was examined using a formazan dye kit (cell count SF solution, Nacalai Tesque) following the manufacturer's instructions. The absorbance at 450 nm (650 nm as background) of each well was measured with a microplate reader (Thermomax, molecular devices).

2.7 Senescence associated-β-galactosidase assay

Senescence associated (SA)-β-galactosidase activity was measured as described previously (Dimri et al. 1995; Severino et al., 2000; Lee and Bar-Sagi et al., 2010). Cells were fixed with 2% paraformaldehyde and 0.2% glutaraldehyde in PBS(-) (pH 7.4) for 5 minutes at room temperature. Cells were then washed twice with PBS(-). Cells were stained at 37^{o}C for 16 hours with the staining solution (comprised of 40mM citric acid/sodium phosphate [pH 6.0], 1% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-gal], 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2mM $MgCl_2$). Cells were washed with PBS(-) twice and then the nuclei were stained with 0.2 μg/ml DAPI for 5 minutes. Cells stained in blue (SA-β-galactosidase positive) and nuclei stained with DAPI (total cell number) were counted under a fluorescence microscope.

2.8 Immunoblotting analysis

ARPE19 cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM

NaCl, 1% Nonidet p-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Nacalai Tesque) and 1 mM PMSF. MEF cells were lysed in the lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Nonidet p-40) containing protease inhibitor cocktail (Nacalai Tesque) and 1 mM PMSF. Supernatant was collected by centrifugation at 10,000 x g for 20 min at 4^{o}C. Protein concentrations were determined by the Bradford method. Cell lysate of equal amounts of total protein (20 μg/well) and 15μl/well of culture medium were resolved on 10% SDS-polyacrylamide gels. The gel was blotted by electrotransfer to PVDF membrane (BioTrace^{TM}, PALL Life Sciences). The membrane was blocked with TBST (50mM Tris/HCl, pH7.5, 100mM NaCl and 0.1% Tween 20) containing 5% nonfat milk for 1 hour at room temperature. The membrane was incubated with a primary antibody diluted in a blocking solution for O/N at 4^{0}C in a sealed plastic bag. Dilutions for primary antibodies were: monoclonal anti-human-HtrA1 (1:2000); polyclonal anti-mouse-HtrA1 (1:2000); anti-p21$^{CIP1/WAF1}$ (1:2000, Santa Cruz); anti-p16^{INK4a} (1:2000, Santa Cruz); anti-p-p38 (1:2000, SAB:11253-1); anti-p-SAPK/JNK (1:1000, cell signaling); anti-p-Erk1/2 (1:2000, cell signaling); anti-p53 (1:5000, Sigma); and anti-pRb (1:5.000, ABM). For the anti-tubulin antibody (1:5000 dilution), the membrane was incubated for 1 hour at room temperature. After incubation with the primary antibody, the membrane was washed with TBST, and then incubated with the appropriate horse radish peroxidase-conjugated secondary antibody. Signals from HRP on the membrane were detected with ECL-prime (Amersham Bioscience) by exposing it on X-ray film (Fuji Photo Film Co.)

2.9 Typing of SNPs in HtrA1 gene

ARPE19 cells in a culture dish were washed with PBS(-) and lysed with a DNA extraction buffer (50 mM Tris-HCl (pH7.6); 25 mM EDTA; 100 mM NaCl; 1% SDS; 0.2 mg/ml proteinase K). The lysate was incubated at 55^{o}C overnight. RNA was digested with 20 μg/ml of RNaseA at 37^{o}C for 1 hour. The lysate was extracted with phenol-CIAA. After centrifugation, the water phase was recovered and DNA was precipitated with an equal volume of 2-propanol. After centrifugation, the DNA pellet was dissolved in TE by incubation at 55^{o}C overnight. SNP typing at rs10490924 (G or C) and rs11200638 (G or A) was performed by the PCR-restriction fragment length polymorphism (RFLP) method. DNA fragments (449 bp for rs10490924 and 569 bp for rs11200638) were PCR amplified using ARPE19 genomic DNA as a template and the following primer sets (Yang et al., 2006; Chowers et al., 2008):

rs10490924 containing fragment:

Forward : 5'-TACCCAGGACCGATGGTAAC-3'

Reverse : 5'-GAGGAAGGCTGAATTGCCTA-3'

rs11200638 containing fragment:

Forward : 5'-CGGATGCACCAAAGATTCTCC-3'

Reverse : 5'-TTCGCGTCCTTCAAACTAATGG-3'.

The PCR cycle was 2 minutes at 95^{o}C, 35 cycles of 30 sec at 95^{o}C, 30 sec at 52^{o}C, and 1 min at 72^{o}C followed by 5 min at 72^{o}C. PCR products were purified by phenol extraction and ethanol precipitation, and digested with 5 units of PvuII (for rs10490924) or EagI (for rs11200638) at 37^{o}C overnight. PvuII and EagI cut G allele

of rs10490924 and rs11200638 polymorphic sites, respectively. DNA fragments were separated on 1.2% agarose gels and visualized by ethidium bromide staining.

2.10 Quantitative RT-PCR

Total RNA was extracted by using Sepasol reagent (Nacalai Tesque) following the manufacturer's instructions. In brief, cells were homogenized in Sepasol followed by incubation at room temperature for 5 minutes. Chloroform was added and incubated at room temperature for 3 minutes. The mixture was centrifuged and the upper phase was recovered. RNA in the upper phase was precipitated with isopropanol, washed with 70% ethanol, and dissolved in DEPC-treated H_2O. Genomic DNA was removed by RNase-free DNase I (Takara Bio Inc.) treatment. Three μg of total RNA was used to synthesize the first strand of cDNA using First-Strand cDNA Synthesis Kit (PrimeScript®II, Takara Bio Inc.) according to the manufacturer's instructions.

Real time PCR was performed using SYBR green master mix and the lightCycler 480 PCR System (Roche Applied Science). The cDNA samples were run in duplicate for PCR amplification. The cDNA was amplified using the following primers:

Human HtrA1,

Forward : 5'-TGGAATCTCCTTTGCAATCC-3'

Reverse : 5'-TTCTTGGTGATGGCTTTTCC-3'

Human VEGF,

Forward : 5’-CTACCTCCACCATGCCAAGT-3’

Reverse : 5’-GCAGTAGCTGCGCTGATAGA-3’

Human Actin,

Forward : 5’-CCCAGCACAATGAAGATCAA-3’

Reverse : 5’-ACATCTGCTGGAAGGTGGAC-3’

Mouse HtrA1,

Forward : 5’-GCCAAAATCAAGGATGTGGATGA-3’

Reverse : 5’-CAGCAGGACTGGCAGCTTTC-3’

Mouse GAPDH,

Forward : 5’- AACATCATCCCTCATCCAC-3’

Reverse : 5’- CCCTGTTGCTGTAGCCGTAT-3’.

The following conditions were used: 1 cycle of 95°C for 5 minutes; 50 cycles of 95°C for 10 seconds; 55°C for 30 seconds; 72°C for 1 second; followed by the final steps of 95°C for 15 seconds; 60°C for 30 seconds; and 95°C for 15 seconds for dissociation curve analysis. The melting curve for each PCR product was determined according to the manufacturer’s instructions to ensure specific amplification of the target genes. Quantitative values were obtained as the threshold PCR cycle number (Ct) under the conditions where the fluorescent signal of the PCR product increased exponentially. The mRNA level of HtrA1 was normalized to that of human actin or mouse GAPDH.

III. RESULT

3.1 HtrA1 expression was upregulated during cell senescence of RPE

Since RPE is the primary pathological site of AMD, I used ARPE19, a non-transformed human RPE cell line, to examine roles of HtrA1 in oxidative stress. First, I genotyped ARPE19 with respect to the two risk alleles, rs10490924 (LOC387715/ARMS2) and rs11200638 which are strongly linked to the onset of AMD and are responsible for the overexpression of HtrA1 (Yang et al., 2010). ARPE19 cells are heterozygous for high-risk alleles in both of these SNP sites (Fig. 3). ARPE19 cells, therefore, are expected to produce HtrA1 higher than the homozygotes for non-risk alleles, but lower than the homozygotes of high-risk alleles.

The experimental protocol for induction of cell senescence has already been established with ARPE19 (Zhu et al., 2009). In the protocol, ARPE19 cells were treated with 0.15 mM H_2O_2 for 2 hours once each at day 1 and day 2, and the serum concentration of the culture medium was reduce from 10% to 0.1% at the beginning of day 3 to enhance the stress conditions (Fig. 5A). I employed this method in the following experiments. H_2O_2 at 0.15 mM did not cause acute cell death of ARPE19 18 hours after treatment (Fig. 4B).

Exposure of ARPE19 to H_2O_2 under these conditions induced cell senescence of ARPE19. There was more senescence associated (SA)-β-galactosidase positive cells (43.3 %) among ARPE19 cells treated with H_2O_2 at day 5 than among control untreated cells (8.7%) (Fig. 5B). The size of cells became larger after H_2O_2 treatment compared with untreated cells, which is another sign of cell senescence (Fig. 5B, right pictures). Other commonly used senescence markers, $p21^{CIP1/WAF1}$ and $p16^{INK4a}$, were

significantly higher in the H_2O_2 treated cells than in the control cells (Fig. 6A and B). p53 activates transcription of $p21^{CIP1/WAF1}$ (Jackson & Pereira-Smith, 2006), and induction of $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ leads to activation of retinoblastoma (Rb) protein by dephosphorylation (Larsson, 2005; Campisi & Fagagna, 2007). The activated Rb protein inhibits E2F, leading to cell senescence. When ARPE19 was treated with H_2O_2, p53 was increased and the phosphorylated Rb protein (pRb) was decreased compared with untreated cells (Fig. 6A). These data indicated that the two critical senescence pathways, p53-$p21^{CIP1/WAF1}$ -Rb pathway and $p16^{INK4a}$ -Rb pathway were both activated in H_2O_2 treated ARPE19 (Fig. 6).

I examined the HtrA1 expression during this cell senescence process. Since HtrA1 is a secretory protease, HtrA1 in both the cell lysate and the culture medium was assayed. As shown in Figure 6, the amount of HtrA1 in the cell lysate or the culture medium at day 5 was 3.5-fold (Fig. 6A) or 1.6-fold (Fig. 6C) higher, respectively, for ARPE19 treated with H_2O_2 than untreated cells. Quantitative RT-PCR showed that HtrA1 mRNA started to increase at day 2, reached to its maximum at day 3, and then decreased (Fig. 7). HtrA1, therefore, is induced simultaneously with cell senescence of ARPE19. HtrA1 expression in the untreated cells was also increased slightly at day 3 both in the mRNA and the protein levels (Fig. 6 and Fig. 7), probably due to the decrease in serum concentration to 0.1% at day 3.

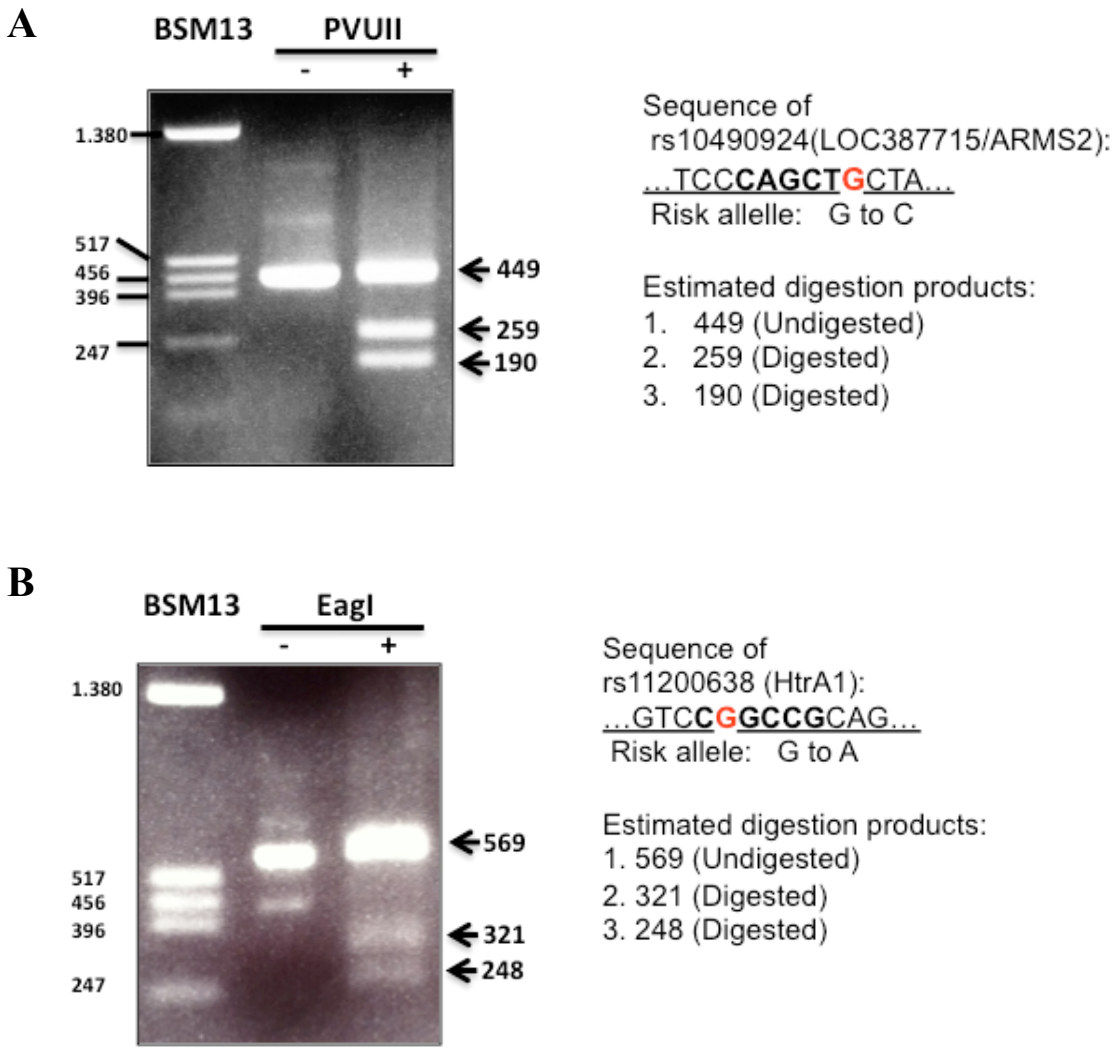


Figure 3. ARPE19 genotyping. Genome DNA of ARPE19 was subjected to PCR to amplify the DNA fragments containing rs10490924 and rs11200638 SNP sites. SNP typing of rs10490924 (**A**) and rs11200638 (**B**) was performed by the PCR-restriction fragment length polymorphism method. Each PCR product was digested with 5 units of PvuII (rs10490924) or EagI (rs11200638) at 37°C overnight. PvuII and EagI both cut G alleles of the SNP sites. Digestion products were separated on 1.2% agarose gels and visualized by ethidium bromide staining. BSM13; size markers, HinfI fragments of the BSM13 plasmid.

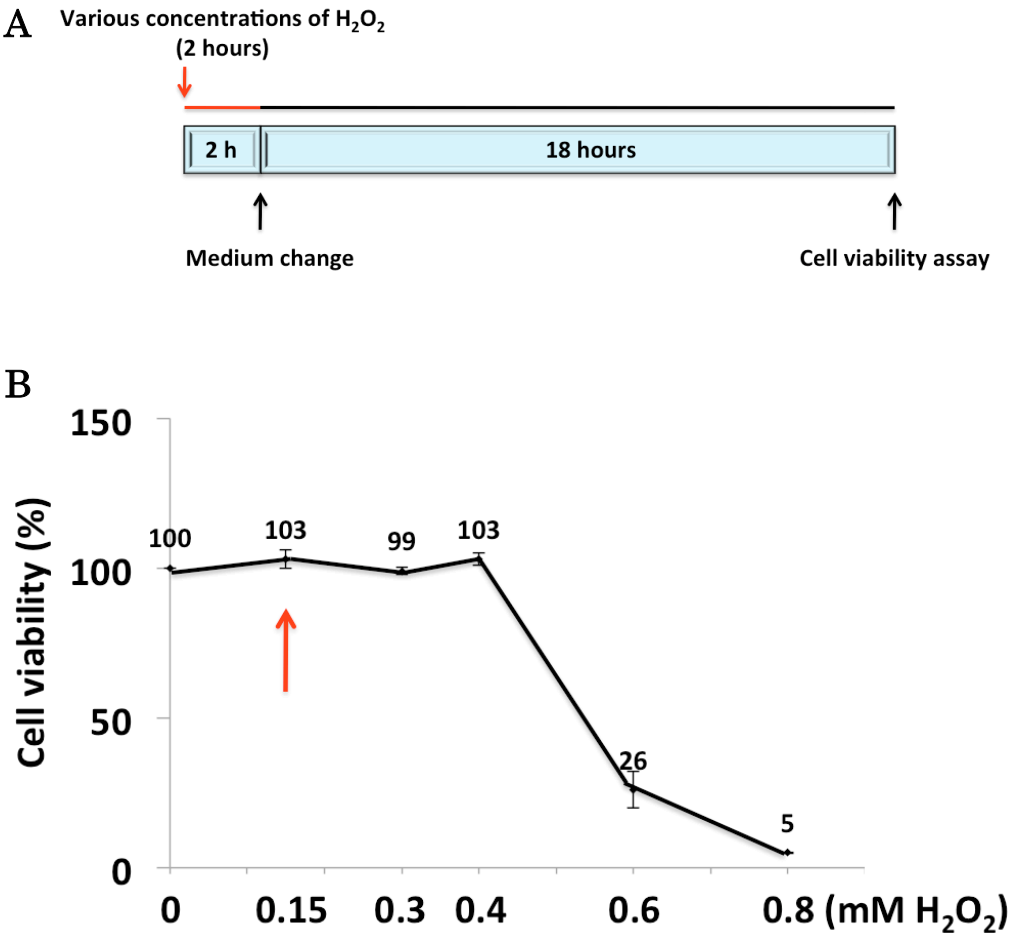


Figure 4. Viability of ARPE19 cells under oxidative stress. **A**. H_2O_2 treatment scheme. ARPE19 cells were treated with various concentrations of H_2O_2 for 2 hours. Cells were washed and cultured in the fresh medium. Cell viability was examined 18 hours after treatment. **B**. Percentage of viable cells. Values represent means ± SD (n=3).

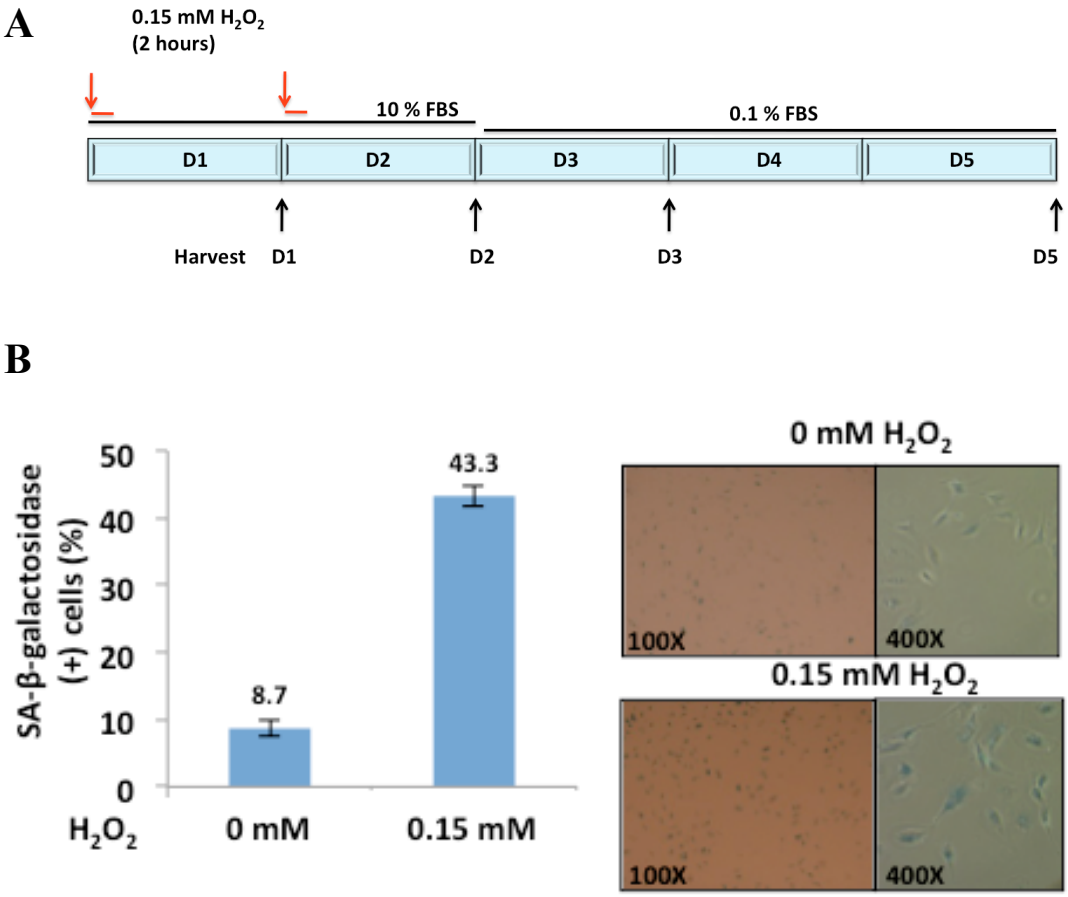


Figure 5. Induction of SA-β-galactosidase activity in ARPE19 cells after H_2O_2 treatment. **A**. H_2O_2 treatment scheme to induce cell senescence in ARPE19 cells. ARPE19 cells were treated with 0.15 mM H_2O_2 for 2 hours. Cells were washed and allowed to recover in the normal medium for 22 hours. Treatment was repeated the next day. At the beginning of day 3, the culture medium was changed to the low serum (0.1% FBS) medium. Cells were harvested at the end of days 1, 2, 3, and 5 and cell lysates and RNA were prepared for further analysis. For SA-β-galactosidase analysis, cells were re-plated at the end of day 5, cultured for another 24 hours, and then stained for SA-β-galactosidase acitvity. **B**. Left graph shows percentage of the SA-β-galactosidase positive cells at day 6. Values represent means ±SD (n=3). Pictures on the left are representative images of the stained cells.

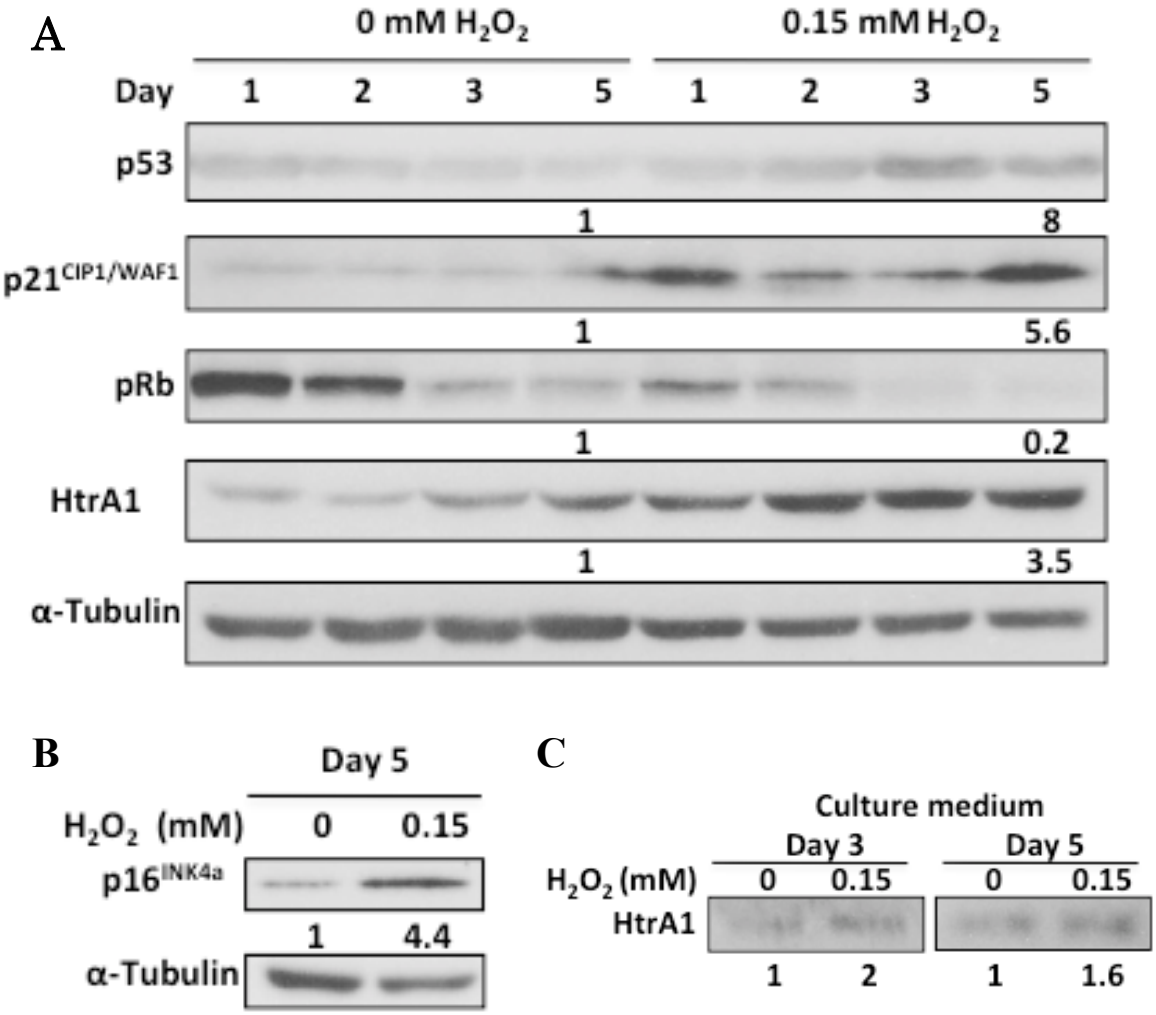


Figure 6. HtrA1 was upregulated during cell senescence of ARPE19. ARPE19 cells were treated with H_2O_2 as described in Figure 5A. Cell lysates (20μg protein/well) and culture media (15μl/well) were subjected to a western blot assay. **A**. Western blot of $p21^{CIP1/WAF1}$, p53, pRb, and HtrA1 in the cell lysate. **B**. Western blot of $p16^{INK4a}$ in the cell lysate at day 5. **C**. Western blot of HtrA1 in the culture media. Western blot with α-tubulin antibody was performed to show equal loading. Expression levels of proteins in the cell lysates were normalized with the amount of tubulin. Fold increase was calculated relative to the value for ARPE19 cells without treatment at day 5. Values below the panel show fold increase.

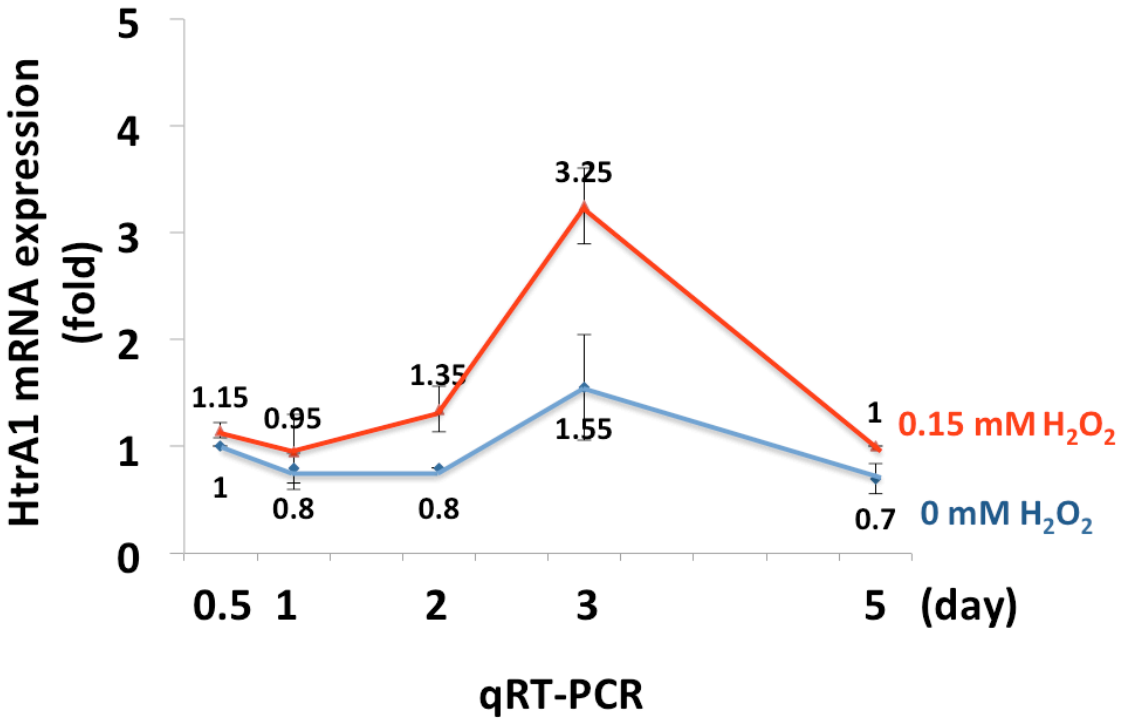


Figure 7. HtrA1 mRNA was induced during cell senescence of ARPE19. ARPE19 cells were treated with H_2O_2 and harvested on days 0.5, 1, 2, 3, and 5 as described in Figure 5. Total RNA was isolated and reverse transcribed to cDNA, and then subjected to quantitative PCR. The expression of HtrA1 was normalized with human actin and relative amounts of HtrA1 mRNA were calculated based on the value for untreated cells at day 0.5. Values represent means ±SD (n=3).

3.2 Induction of HtrA1 expression by other stress inducers

Besides oxidative stress, endoplasmic reticulum (ER) stress is also known to induce HtrA1 expression. Treatment with tunicamycin or dithiothreithol induces HtrA1 mRNA in fetal human RPE cells (Kin-Ng et al., 2011). HtrA1 is also induced in the kidney of the Syrian hamster by oxidative stress caused by estrogen treatment (Zurawa-Janicka et al., 2008). HtrA1, therefore, could be a general responder to stressful conditions. I examined whether HtrA1 was induced in ARPE19 by oxidative stress mediated by inducers other than H_2O_2 or by other types of stress that were linked to the onset of AMD. We used *tert*-butyl hydroperoxide (t-BH), hydroquinone (HQ), and UVC irradiation as other inducers of oxidative stress and chloroquine as a lysozomal stress inducer. As shown in Figure 8, t-BH treatment (0.6 mM for 30 minutes) increased HtrA1 in the cell lysate (2.4-fold) and in the culture medium (6-fold) 2 days after treatment.

Cigarette smoking causes oxidative damage in RPE cells and HQ is the most abundant pro-oxidant compound in cigarette smoke. HQ at 10 μM induced HtrA1 expression in the cell lysate of ARPE19 at day 3 (1.9-fold) and day 5 (2.5-fold). This induction was also evident in the culture medium (Fig. 9).

When ARPE19 cells were treated with 100 J/m^2 UVC, I found the induction of HtrA1 expression in both the cell lysate and the culture medium. As shown in Figure 10, HtrA1 expression in the cell lysate was 2.5-fold higher in the treated cells than in the untreated cells at day 2. Together, all these data suggest that various kinds of oxidative stress inducers could upregulate HtrA1 expression in ARPE19.

Chloroquine is widely used as anti-malarial and anti-inflammatory drug. Chloroquine inhibits lysosomal functions and causes dysfunction of phagocytosis in

RPE cells (Chen et al., 2011). We treated ARPE19 cells with various concentrations of chloroquine (0, 20, 40, 60, and 80 μg/μl) for 24 hours. Cell death was not significant when ARPE19 was treated with 20 μg/μl of chloroquine, but cytoplasmic changes with many dilated vacuoles were clearly observed, indicating chloroquine was effective as a lysosomal inhibitor at these concentrations (Fig. 11B). Under these conditions, chloroquine increased HtrA1 in the cell lysate. On the contrary, HtrA1 in the culture medium was decreased (Fig. 11C). The total amount of HtrA1 protein, therefore, was not changed considerably with this treatment. Probably, the secretion pathway of ARPE19 cells was disturbed by lysosomal dysfunction caused by chloroquine. I concluded that lysosomal stress does not induce HtrA1 expression.

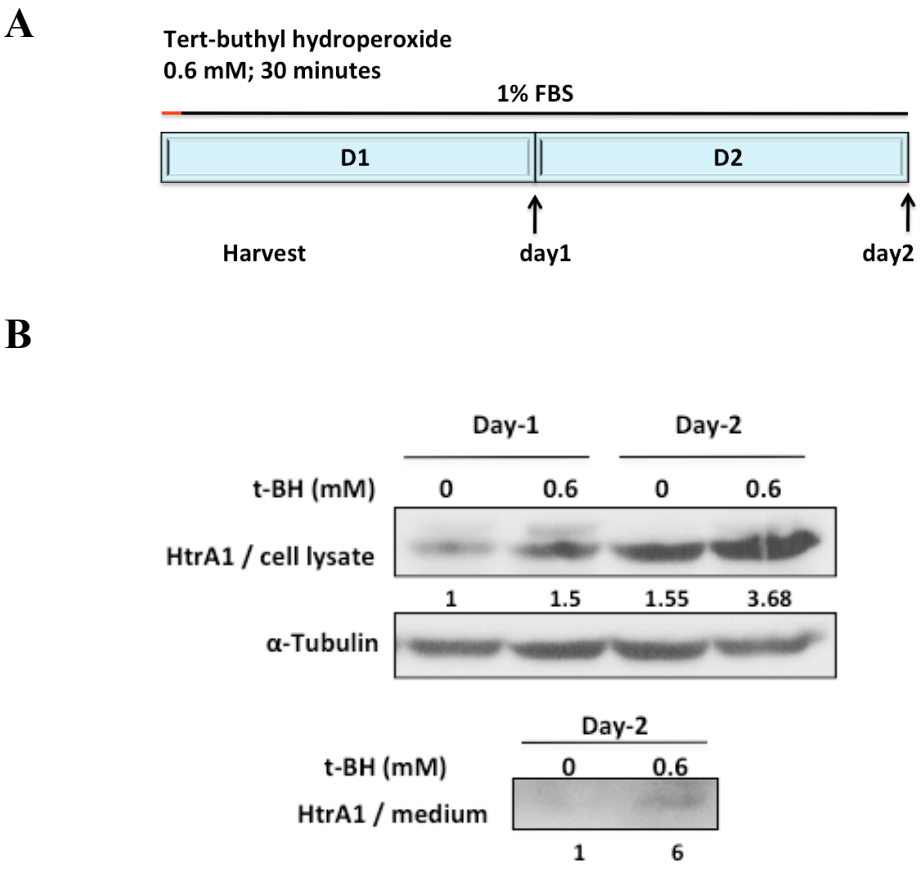


Figure 8. ***tertiary*-butyl hydroperoxide (t-BH) upregulated HtrA1 expression in ARPE19 cells.** **A**. t-BH treatment scheme. ARPE19 cells were treated with or without 0.6 mM t-BH for 30 min, and were washed and cultured for another two days in the medium containing 1% FBS. Cell lysates (20 μg protein/well) and culture media (15 μl/well) were subjected to a western blot assay. **B**. Western blot assay with anti HtrA1 antibody. A Western blot with anti α-tubulin antibody was performed to show equal loading. The expression levels in the cell lysates were normalized with tubulin expression. Fold increase was calculated relative to the value for ARPE19 cells without treatment at day 1 (cell lysates) or day 2 (media) and shown below the panels.

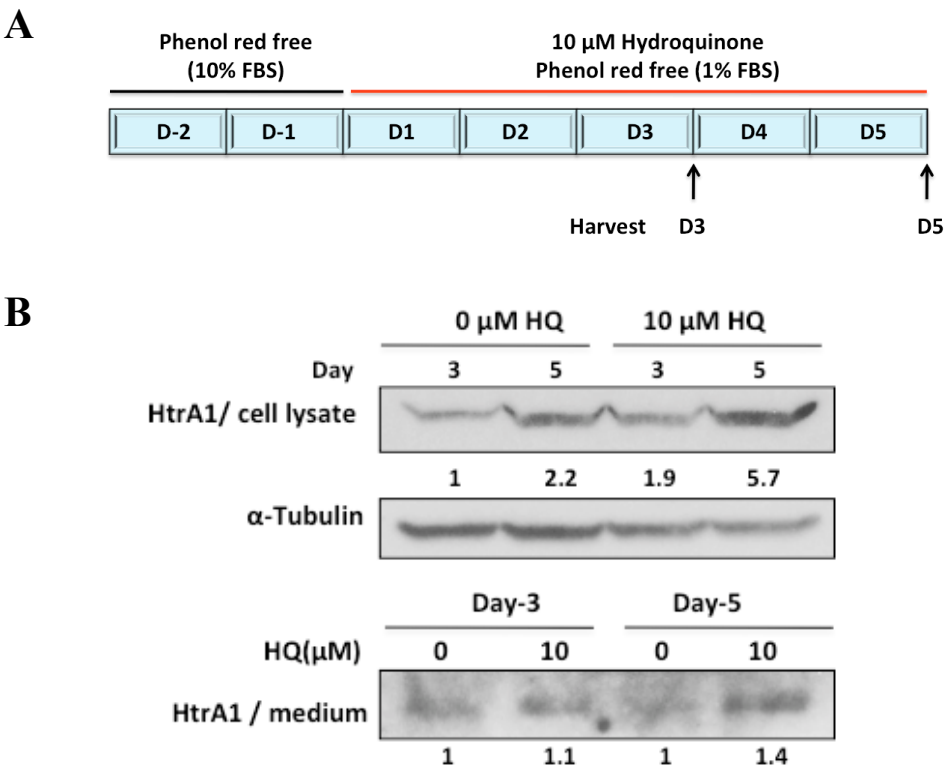


Figure 9. Hydroquinone (HQ) induced HtrA1 expression in ARPE19 cells. **A**. HQ treatment scheme. ARPE 19 cells were cultured in the phenol red free medium with 10 % serum for 2 days and then treated with or without 10 μM HQ in the phenol red free medium with 1 % serum for 5 days. The culture medium was changed every 24 hours. Cell lysates (20 μg protein/well) and culture media (15 μl/well) at day 3 and 5 were subjected to a western blot assay. **B**. Western blot with anti HtrA1 antibody. A western blot with α-tubulin antibody was performed to show equal loading. The expression levels in the cell lysates were normalized with tubulin expression. Fold increase was calculated relative to the value for ARPE19 without treatment at day 3 and shown below the panels.

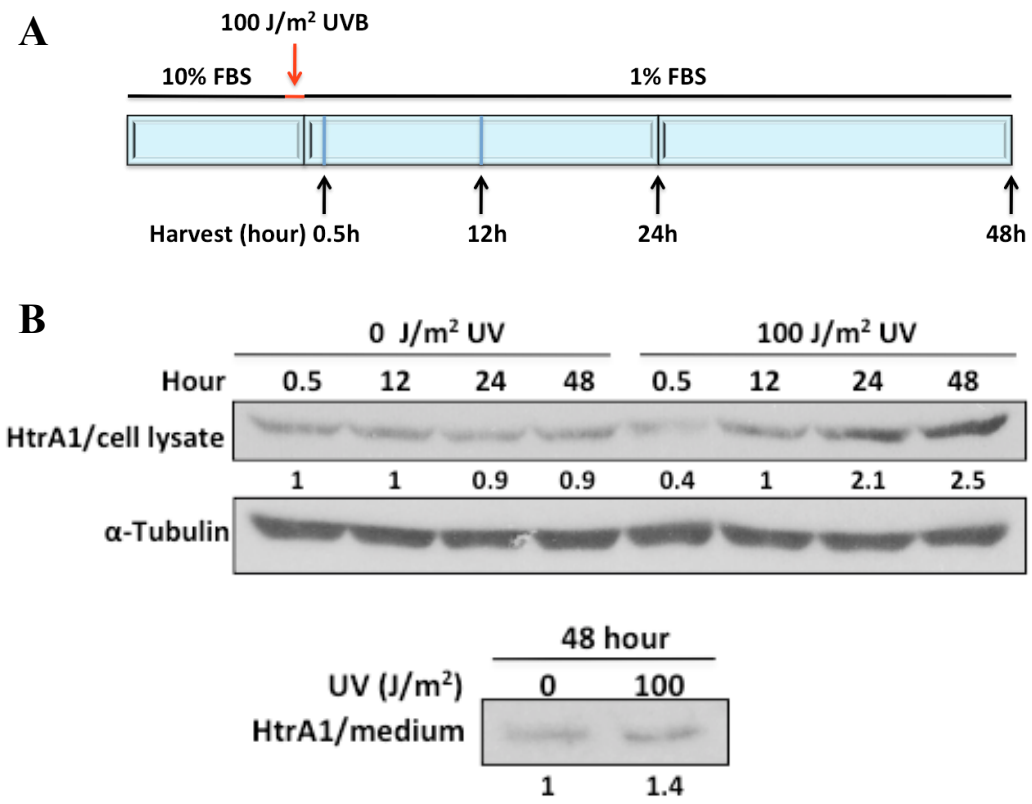


Figure 10. UVC irradiation upregulated HtrA1 expression in ARPE19 cells. **A**. UVC treatment scheme. ARPE19 cells were exposed in PBS(-) to 100 J/m^2 of UVC by using a UV cross linker, and were washed and cultured in the medium containing 1% serum for different periods of time (0.5, 12, 24 and 48 hours). Cell lysates (20 μg protein/well) and the culture media (15 μl/well) were subjected to a western blot assay. **B**. A western blot assay with anti HtrA1 antibody. A western blot with α-tubulin antibody was performed to show equal loading. The expression levels in the cell lysates were normalized with tubulin expression. Fold increase was calculated relative to the value for ARPE19 cells without treatment at day 0.5 and shown below the panels.

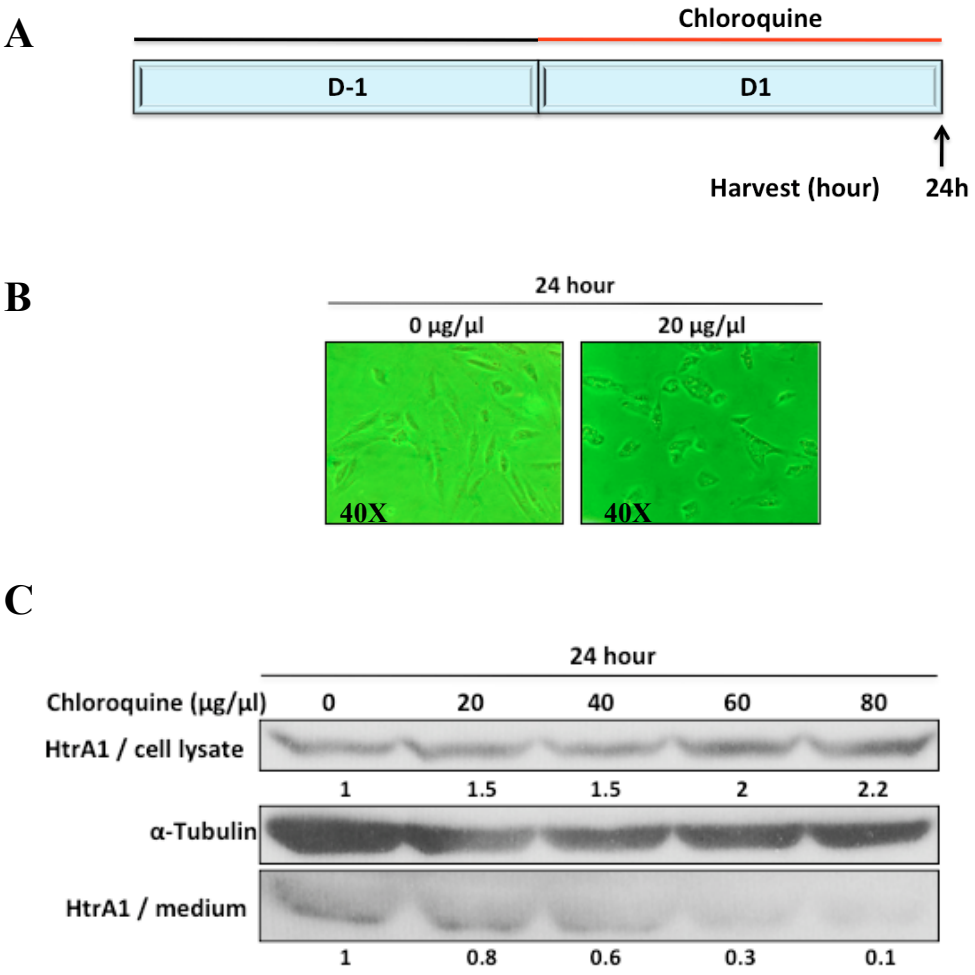


Figure 11. Chloroquine did not induce HtrA1 expression in ARPE19 cells. **A**. Chloroquine treatment scheme. ARPE19 cells were treated with chloroquine at various concentrations (0, 20, 40, 60, and 80 μg/ml) for 24 hours in the medium containing 1% serum. Cell lysates (20 μg protein/well) and culture media (15 μl/well) were subjected to a western blot assay. **B**. Pictures of ARPE19 cells with chloroquine treatment. **C**. A western blot assay with anti HtrA1 antibody. A western blot with α-tubulin antibody was performed to show equal loading. The expression level in the cell lysate was normalized with tubulin expression. Fold increase was calculated relative to the value for ARPE19 cells without treatment and shown below the panels.

3.3 MEF cells induce HtrA1 during cell senescence

To analyze the role of HtrA1 in cell senescence, I next used MEF cells that are most commonly used as primary culture cells for cell senescence studies. We have established the HtrA1 gene knockout mice. I thought that MEF cells isolated from HtrA1 -/-, HtrA1 +/-, and HtrA1 +/+ mice should allow me to study the role of HtrA1 in cell senescence in more detail.

The wild type MEF cells isolated from HtrA1 +/+ mice did not show acute cell death after treatment with H_2O_2 at concentrations of 0.2 mM or lower (Fig. 12B). I chose 0.15 mM H_2O_2 to induce cell senescence in MEF. HtrA1+/+ MEF cells were exposed to 0.15 mM of H_2O_2 for 2 hours and cultured up to day 9 (Fig. 13A). Induction of cell senescence was confirmed by the increase in percentage of SA-β-galactosidase positive cells (60.5%) in H_2O_2 treated cells at day 9 as compared with that (9.7%) for untreated cells (Fig. 13B). Furthermore, $p21^{Cip/WAF1}$ expression was induced at day 1 and up to 3.9-fold at day 9 (Fig. 14A). Significant increase in $p16^{INK4a}$ expression (3.4-fold) was also observed at day 9 (Fig. 14B).

During cell senescence of MEF under these conditions, the HtrA1 protein was increased in the cell lysate (3.6-fold at day 3) and the culture medium (2.7-fold at day 6) (Fig. 14A and 14C). The HtrA1 mRNA level started to increase at day 1, reached the maximum of 3-fold at day 3, and then decreased (Fig. 15). Induction of HtrA1 expression, therefore, is a common response to oxidative stress in both ARPE19 and MEF cells.

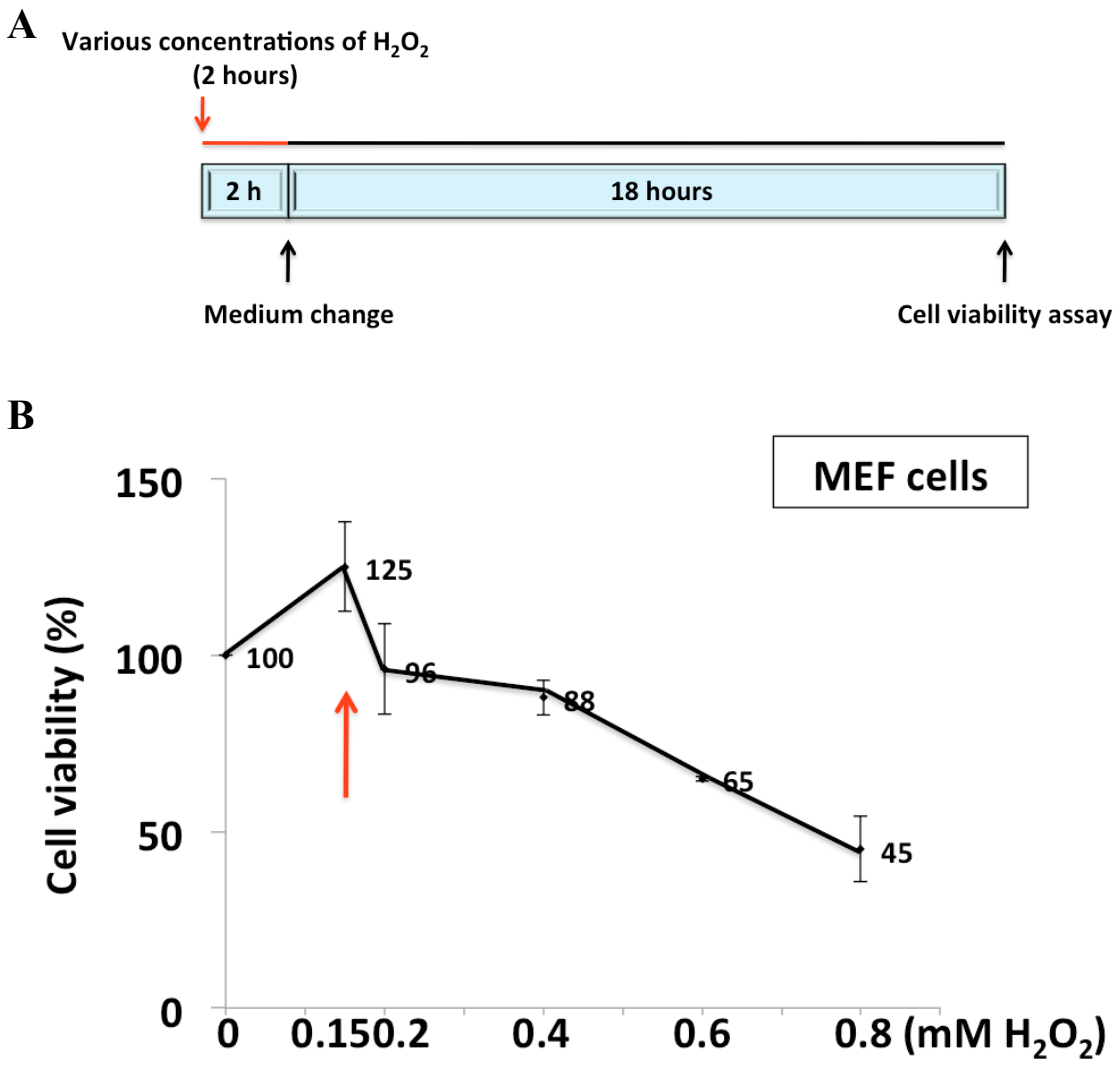


Figure 12. Viability of MEF cells under oxidative stress. **A**. H_2O_2 treatment scheme. MEF cells were treated with various concentrations of H_2O_2 for 2 hours. Cells were washed and cultured in the fresh medium. Cell viability was examined 18 hours after treatment. **B**. Percentage of viable cells. Values represent means ±SD (n=3).

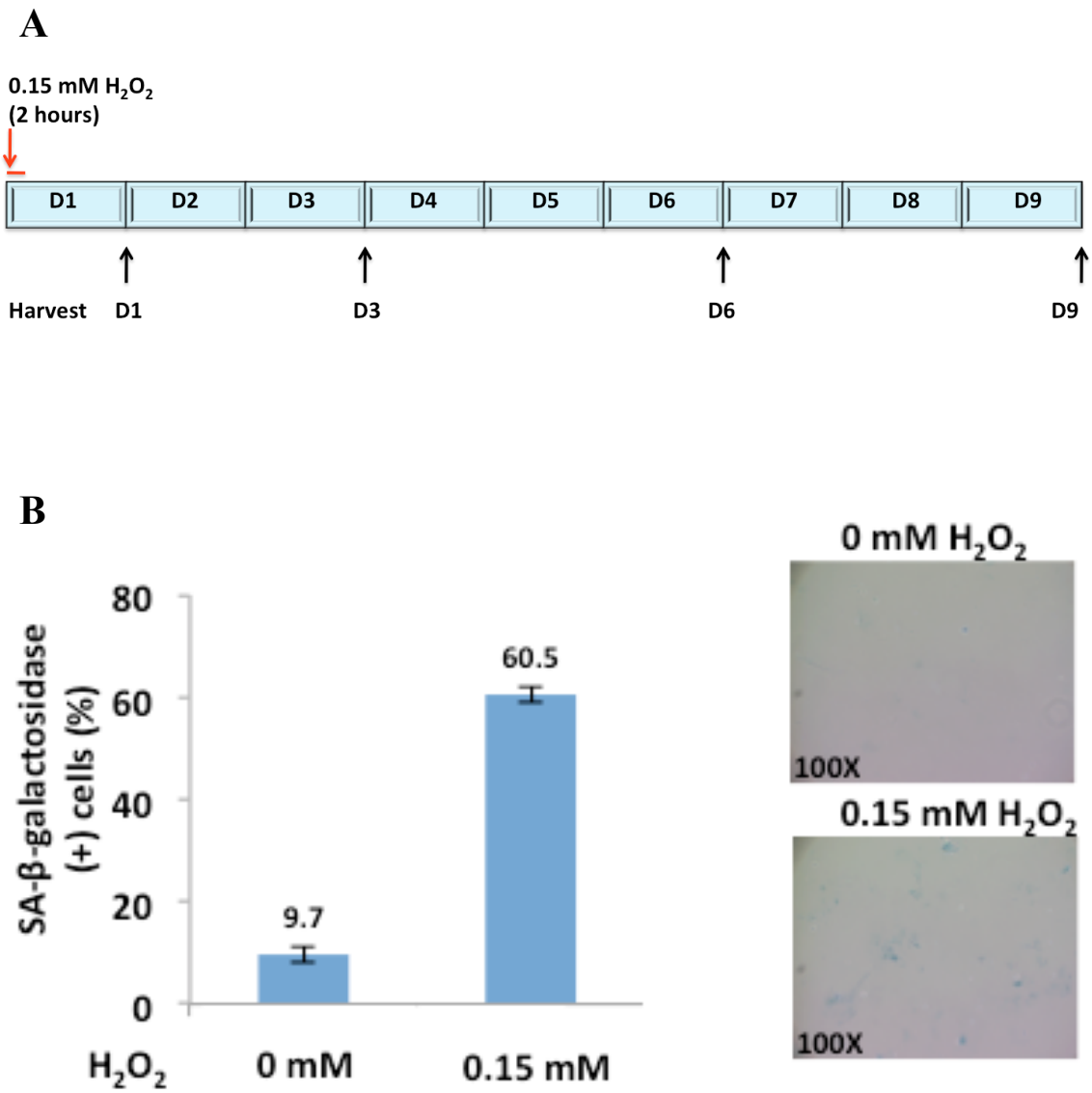


Figure 13. Induction of SA-β-galactosidase activity after H_2O_2 treatment of MEF cells. **A**. H_2O_2 treatment scheme for MEF cells. Cells were treated with 0.15 mM H_2O_2 for 2 hours and were washed and cultured in the normal medium for different periods of time (0, 1, 3, 6, and 9 days). MEF cells not treated with H_2O_2 were used as a control group. **B**. The left graph indicates percentage of the SA-β-galactosidase positive cells at day 9. Values represent means ±SD (n=3). The pictures on the right show representative images of stained cells.

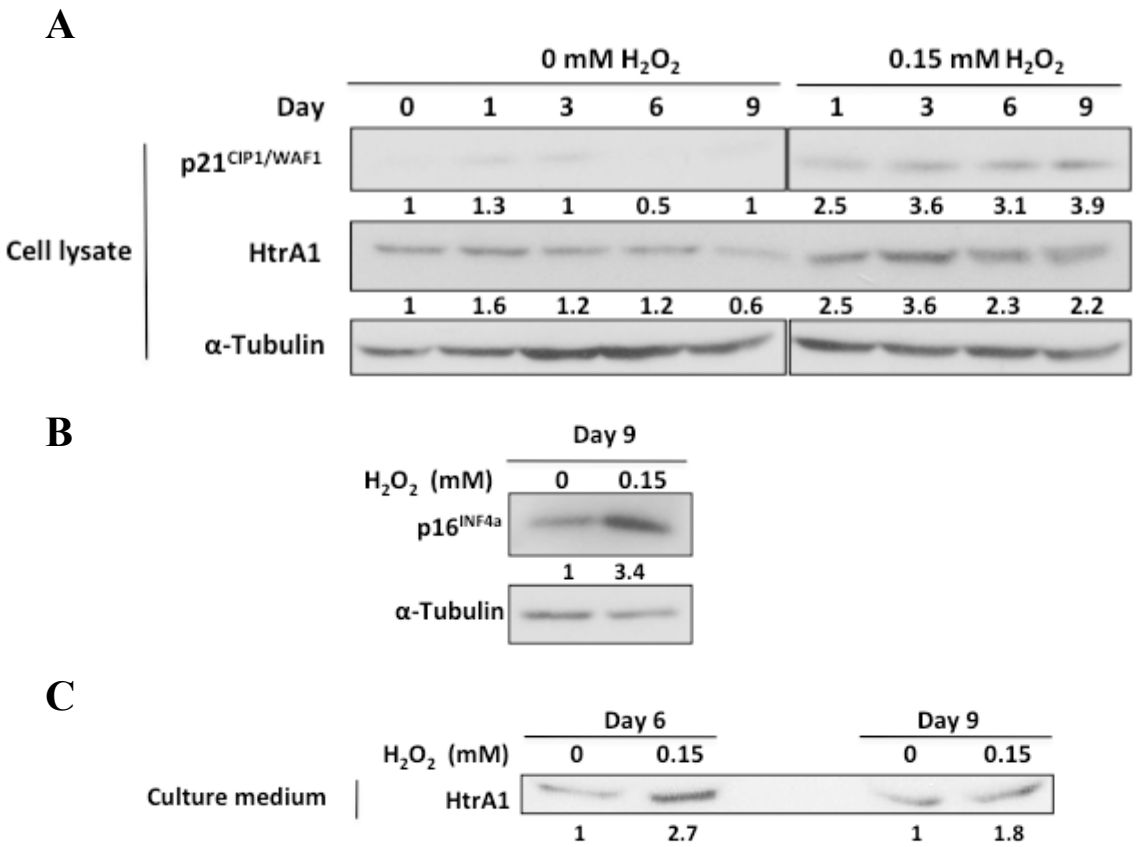


Figure 14. HtrA1 was upregulated during cell senescence of MEF cells. MEF cells were treated with H_2O_2 (Fig. 13). The cell lysates (20 μg protein/well) and the culture media (15 μl/well) were subjected to a western blot analysis. **A**. A western blot of $p21^{CIP1/WAF1}$ and HtrA1 in the cell lysates. **B**. A western blot of $p16^{INK4a}$ at day 9. **C.** A western blot of HtrA1 in the culture media at day 6 and 9. A western blot with anti α-tubulin antibody was performed to show equal loading. The expression level in the cell lysate was normalized with tubulin expression. Fold increase was calculated based on the value for MEF cells without treatment and shown below the panels.

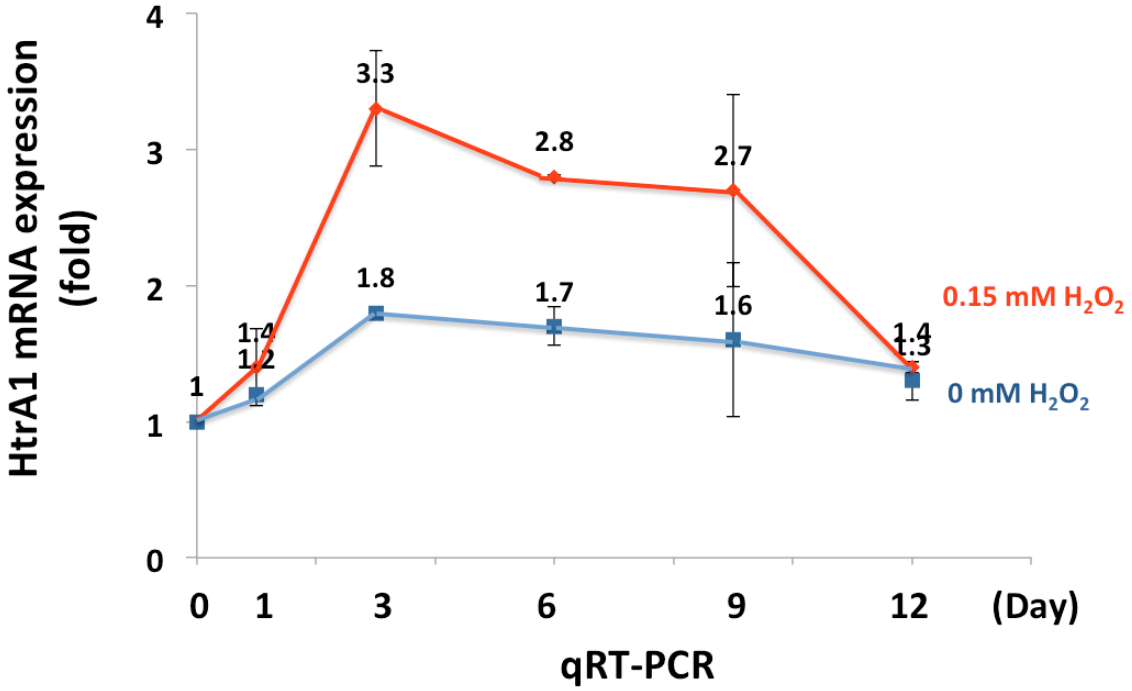


Figure 15. HtrA1 mRNA was upregulated during cell senescence in MEF cells. MEF cells were treated with H_2O_2 and harvested on days 1, 3, 6, 9, and 12. Total RNA was isolated, reverse transcribed to cDNA, and then subjected to quantitative PCR. The expression level of the mouse HtrA1 was normalized with GAPDH. Fold increase was calculated based on the value of HtrA1 mRNA in untreated cells at day 0. Values represent means ±SD (n=3).

3.4 HtrA1 protected cells from cell death but promoted cell senescence induced by oxidative stress

Since bacterial HtrAs have roles in cell survival against heat shock and oxidative stress, I first examined the role of HtrA1 in cell survival under oxidative stress. I analyzed the viability of HtrA1 +/+, HtrA1 +/-, and HtrA1 -/- MEF cells after treatment of H_2O_2. As shown in Figure 16, HtrA1 +/+ and HtrA1 +/- MEF cells were more viable than HtrA1 -/- MEF cells. HtrA1, therefore, protects cells from cell death under oxidative stress. Viability of HtrA1 +/+ and HtrA1 +/- MEF cells was not significantly different at all concentrations of H_2O_2, suggesting that a single copy of the HtrA1 gene is sufficient to give MEF cells full protection against H_2O_2.

Next I investigated the roles of HtrA1 in cell senescence using MEF cells obtained from HtrA1 gene knockout mice. MEF cells undergo spontaneous cell senescence during passage. To obtain a sufficient number of cells of the same passage and the same history, I prepared HtrA1 +/- and HtrA1 -/- MEF cells from embryos obtained from mating of HtrA1 +/- and HtrA1 -/- parents. Since HtrA1 -/- MEF cells were more sensitive to H_2O_2 treatment than HtrA1 +/- MEF cells and have a tendency to die even with the treatment of 0.15 mM H_2O_2 (Fig. 16), I employed 0.075 mM H_2O_2 to induce cell senescence in the following experiments. MEF cells were subjected to H_2O_2 treatment and traced up to 12 days after treatment. As shown in Figure 17, the percentages of SA-β-galactosidase positive cells were higher for HtrA1 +/- MEF cells than for HtrA1 -/- MEF cells. The difference in SA-β-galactosidase positive cells became significant at day 6 and the difference was gradually increased up to day 12. Induction of $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ expressions was higher in the HtrA1+/- MEF cells than in the HtrA1-/- MEF cells (Fig. 18). Therefore, HtrA1 contributes, although

is not essential, to the progression of cell senescence in MEF cells.

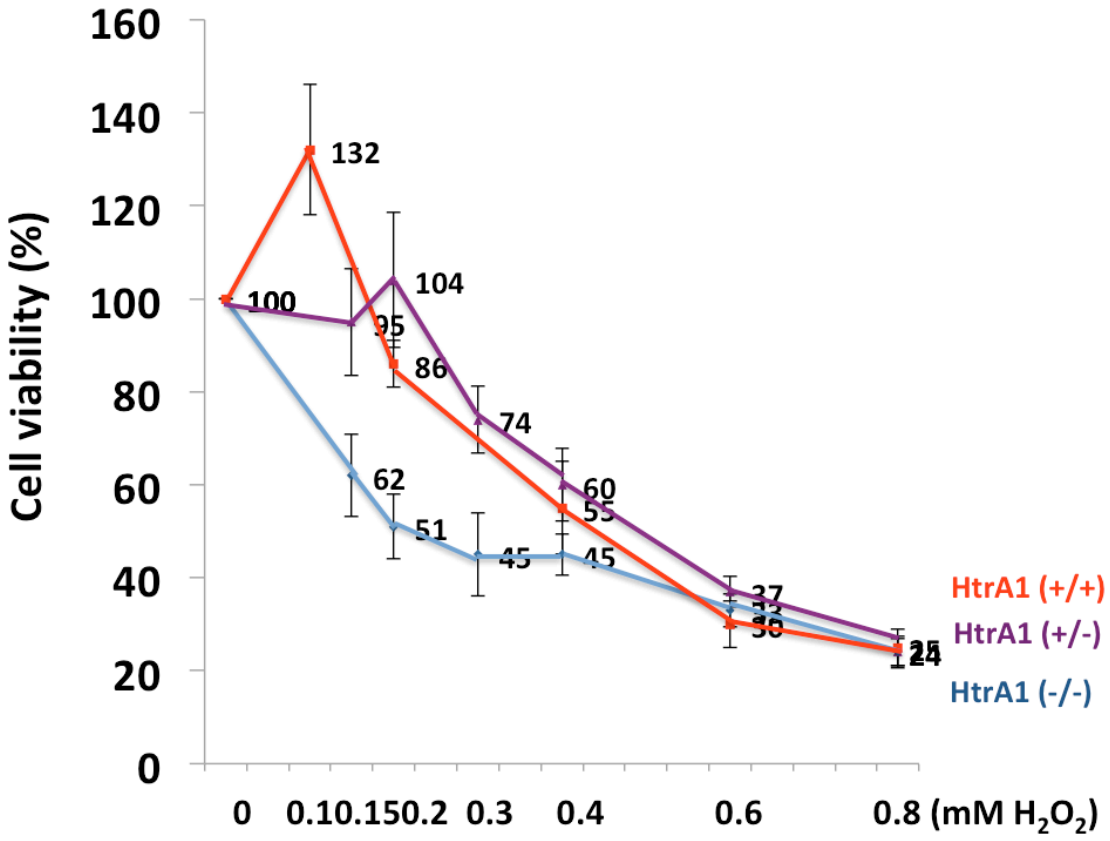


Figure 16. HtrA1 protect cells from cell death under oxidative stress. MEF cells were isolated from HtrA1 +/+, HtrA1 +/-, and HtrA1 -/- mouse embryos and treated with various concentrations of H_2O_2 for 2 hours. Cells were washed and cultured in the normal medium for 18 hours. Cell viability was measured by a colorimetric assay. Values represent means ±SD (n=3).

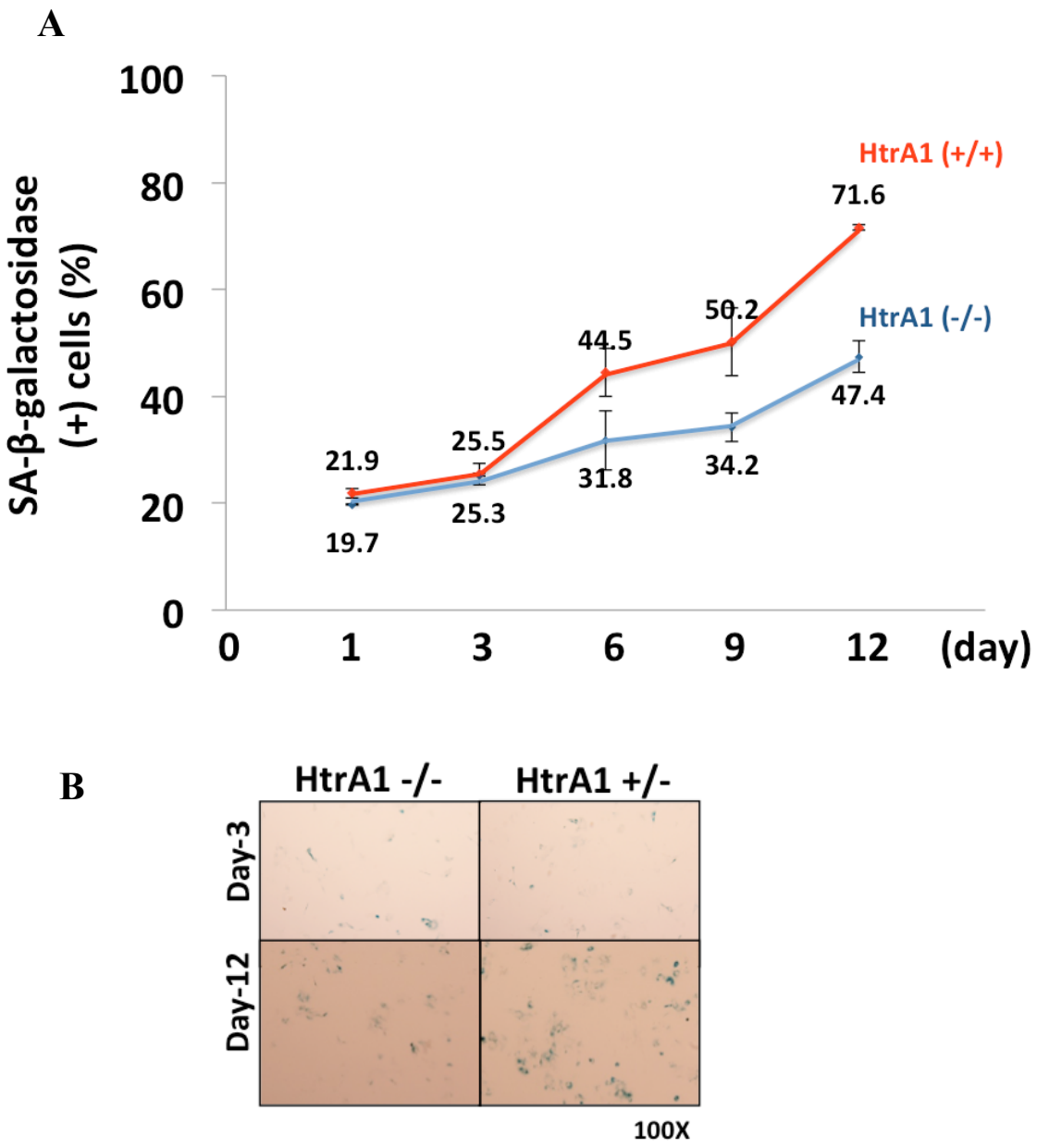


Figure 17. HtrA1 +/- MEF cells increased higher SA-β-galactosidase activity during senescence process than HtrA1-/- MEF cells. **A**. HtrA1 +/- and HtrA1 -/- MEF cells were treated with H_2O_2 (Fig. 13). An SA-β-galactosidase assay was performed on days 1, 3, 6, 9 and 12. Values represent means ±SD (n=3). **B**. Representative images of the SA-β-galactosidase staining at day 3 and day 12.

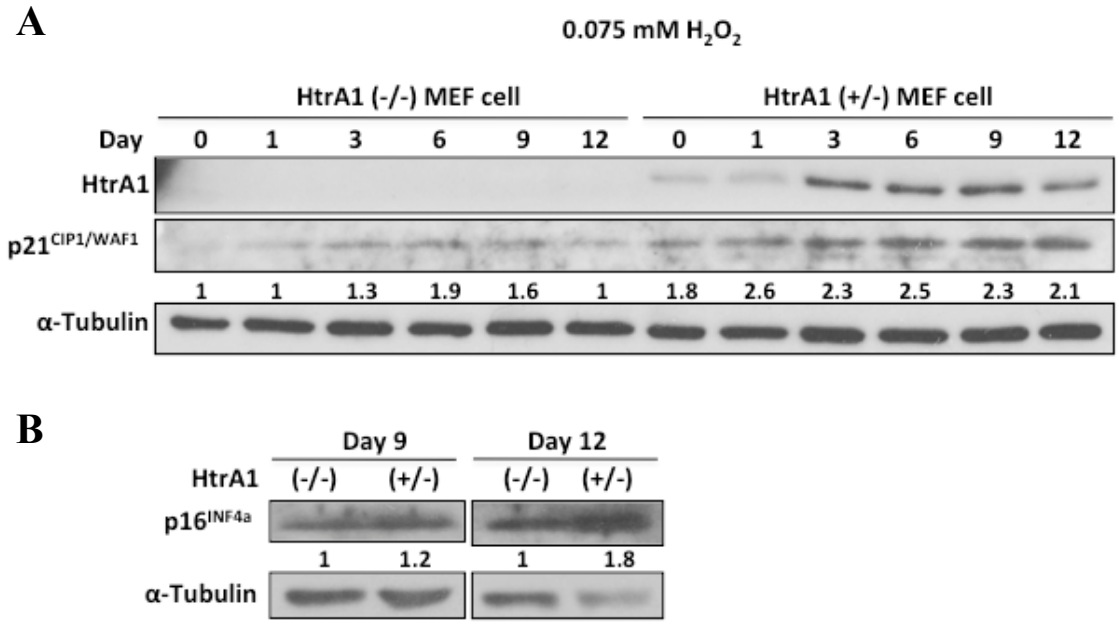


Figure 18. HtrA1 enhanced oxidative stress-induced premature senescence. HtrA1+/- and HtrA1-/- MEF cells were subjected to H_2O_2 treatment as described in Figure 13. Cells were washed and cultured in the normal medium for different periods of time (0, 1, 3, 6, 9, and 12 days). **A**. A western blot assay of HtrA1 and $p21^{CIP1/WAF1}$. **B**. A western blot assay of $p16^{INK4a}$ at day 9 and day 12. A western blot with α-tubulin antibody was performed to show equal loading. The expression of $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ was measured by densitometry of the western blots and normalized with tubulin expression. Fold increase in the expression was calculated based on the value for untreated HtrA1 -/- cells at day 0 (A) or the value for H_2O_2-treated HtrA1 -/- cells at day 9 or day 12 (B). Values below the panel show fold increase.

3.5 Overexpression of HtrA1 in HtrA1 -/- MEF cells promoted cell senescence

To confirm that HtrA1 is responsible for the difference in promotion of cell senescence between HtrA1 -/- and HtrA1 +/- MEF cells, HtrA1 was expressed in HtrA1-/- MEF.

HtrA1 -/- MEF cells were transiently transfected with the mouse HtrA1 expression vector and exposed to 0.075 mM H_2O_2 2 days later. The transfection conditions were adjusted, so that HtrA1 -/- MEF cells produced approximately 10-fold more exogenous HtrA1 proteins than HtrA1 +/- MEF cells. An immunoblotting assay indicated that HtrA1 in the culture medium of transfected HtrA1 -/- MEF cells was 11.8-fold higher at day 2 than the endogenous protein in the HtrA1 +/- MEF culture medium (Fig. 19A). I found that the induction of $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ expressions were 1.6-fold and 2-fold higher, respectively, in the HtrA1-/- MEF cells expressing exogenous HtrA1 than the control cells transfected with the empty expression vector (Fig. 19B). The number of SA-β-galactosidase positive cells was also higher for the HtrA1-/- MEF cells expressing exogenous HtrA1 (45.1%) than the control cells (31.2%). This frequency of SA-β-galactosidase positive cells in MEF cells expressing WT HtrA1 (45.1 %) was comparable with that of HtrA1 +/- MEF cells on day 6 (44.5%) after H_2O_2 treatment (Fig. 17), indicating that the transfection conditions were not extreme, unphysiological conditions.

Interestingly, the protease deficient S328A HtrA1 mutant did not enhance cell senescence (Fig. 19 and Fig. 20). HtrA1, therefore, induces cell senescence in a protease dependent manner.

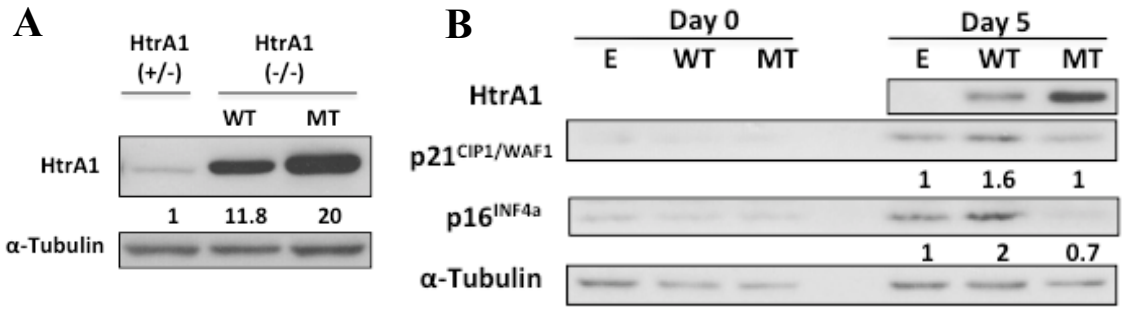


Figure 19. Expression of HtrA1 in HtrA1 -/- MEF cells enhanced induction of $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ after H_2O_2 treatment. HtrA1 -/- MEF cells were transfected with HtrA1 expression vectors containing either no cDNA, mouse HtrA1 (WT) cDNA, or protease deficient mutant HtrA1 (MT) cDNA. Two days later, transfected cells were treated with 0.075 mM H_2O_2 for 2 hours and were washed and cultured for 6 days. **A**. Expression of HtrA1 in transfected HtrA1-/- MEF cells was compared to an endogenous level in HtrA1 +/- MEF cells. **B**. A western blot of $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ at day 6. The expression levels of the proteins were normalized with tubulin expression. Fold increase was calculated based on the values for HtrA1-/- MEF cells transfected with the empty vector and shown below the panels.

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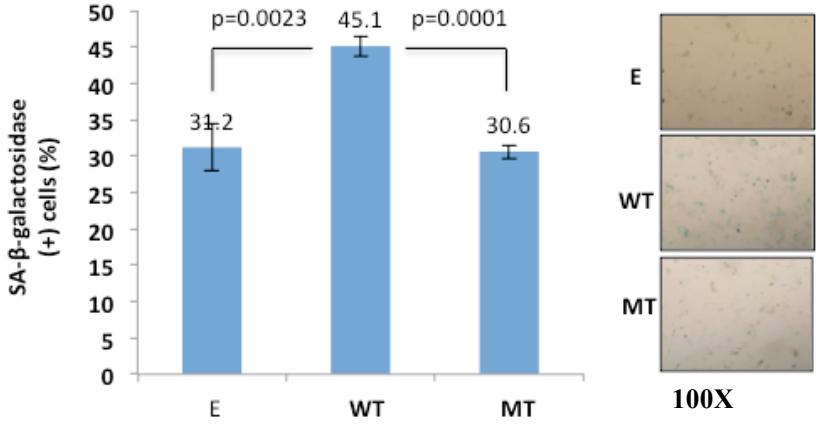


Figure 20. Expression of HtrA1 in HtrA1-/- MEF cells stimulated the induction of SA-β-galactosidase activity after H_2O_2 treatment. HtrA1 -/- MEF cells were transfected with HtrA1 expression vectors and then treated with H_2O_2 as described in Figure 19. Cells were re-plated at day 5 and cultured for another 24 hours. SA-β-galactosidase activity was assayed at day 6. Values represent means ±SD (n=3).

3.6 Recombinant HtrA1 protein enhanced cell survival and cell senescence of HtrA1 -/- MEF

In the previous section, I showed that transient transfection with the HtrA1 expression vector rescued impaired cell senescence of HtrA1 -/- MEF cells. The efficiency of transient transfection of MEF cells was 35-40 % under my experimental conditions. I, therefore, supposed that HtrA1 secreted out of cells should enhance cell senescence of untransfected cells. To examine this possibility, I next investigated whether cell survival and cell senescence were rescued by adding recombinant HtrA1 to the culture of HtrA1 -/- MEF cells. Recombinant wild type mouse or human HtrA1 (WT) and protease-deficient S328A HtrA1 mutant (MT) proteins were produced in HEK293T cells by transient transfection, and culture media (conditioned media, CM) was collected as a source of recombinant proteins. I also prepared CM from HEK293T cells transfected with the control empty expression vector (E). CM of E, WT, and MT were assayed for protease activity using casein as substrate (Fig. 21A). The results confirmed that HEK293T itself did not produce detectable amounts of HtrA1 or any protease activity into the culture medium, and that the exogenous HtrA1 was the only major protease actively in CM of WT-transfected HEK293T cells (Fig. 21A). The amount of mouse recombinant HtrA1 in CM was examined quantitatively by a western blot and compared to that of endogenous HtrA1 produced by HtrA1 +/- MEF cells (Fig. 21B). CM of WT or MT was diluted with CM of E, so that approximately 15 times more recombinant HtrA1 proteins were added to the culture medium when CM was added to 10%.

HtrA1-/- MEF cells were cultured in the presence of 10% CM and exposed to various concentrations of H_2O_2. Viability was assayed after 18 hours. Recombinant HtrA1 rendered HtrA1 -/- MEF cells resistant to cell death, much like the resistance of

HtrA1 +/- MEF (Fig. 22). Protease-deficient HtrA1 (MT) or CM of E did not affect the viability of HtrA1 -/- MEF.

The recombinant HtrA1 protein also induced cell senescence markers, $p21^{CIP1/WAF1}$ and $p16^{INK4a}$, in HtrA1 -/- MEF after H_2O_2 treatment (Fig. 23A). SA-β-galactosidase positive cells were significantly increased in HtrA1 -/- cells cultured with recombinant HtrA1 as compared with the cells cultured with protease-deficient HtrA1 (MT) or with CM of E (Fig. 23B).

All of these data indicate that HtrA1 protects cells from cell death but induces cell senescence upon oxidative stress by functioning from outside the cell in a protease dependent manner.

In the experiments shown in Figure 23, I changed the culture media containing 10% CM every day. One-day old media were checked for the presence of recombinant proteins and remaining protease activity (Fig. 24A and 24B). The data confirmed that recombinant HtrA1 was not degraded and was still fully active after a 24 hour incubation with MEF cells.

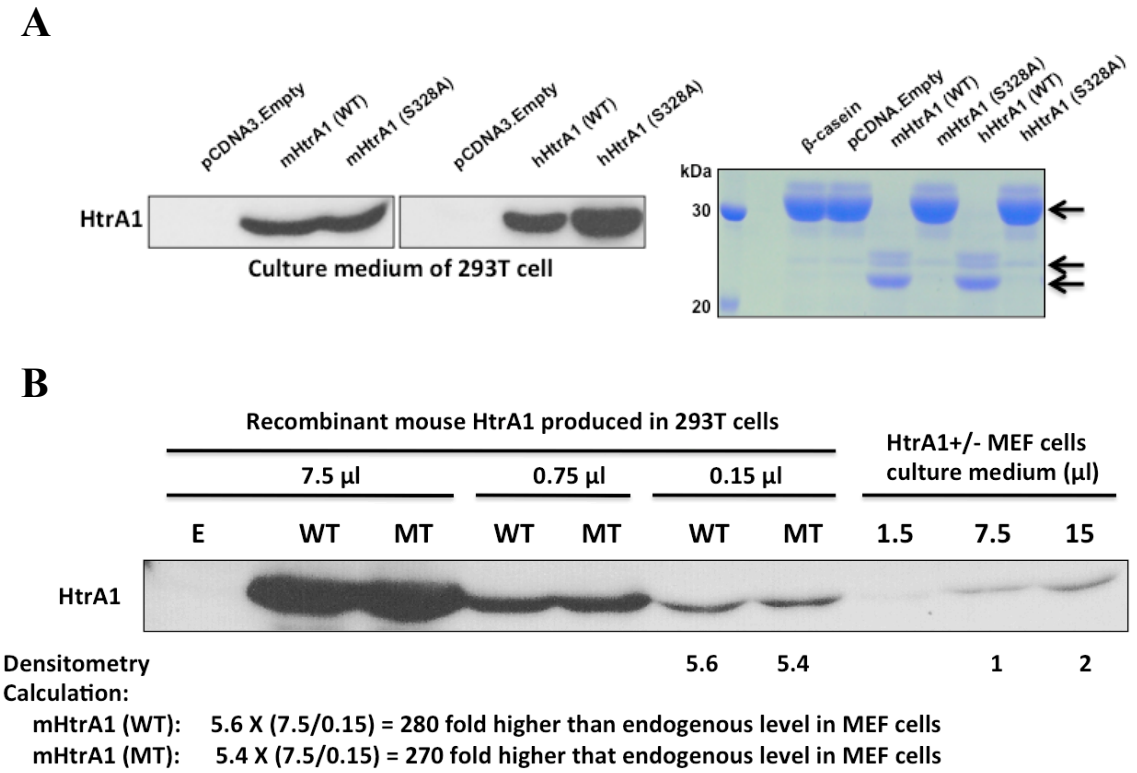


Figure 21. Production of recombinant HtrA1 proteins in HEK293T cells. **A**. A western blot of culture media of HEK293T cells. HEK293T cells were transfected with expression vectors containing either no insert (pcDNA3-Empty), wild type HtrA1 cDNA (mHtrA1 [WT] or hHtrA1 [WT]), or protease-deficient S328A mutant HtrA1 cDNA (mHtrA1 [S328A] or hHtrA1 [MT]) on the left panel. mHtrA1 and hHtrA1 denote mouse and human HtrA1, respectively. HtrA1 protease activity was determined by incubation of the culture media (6 μl) with β-casein (15 μg) at 37°C overnight. Products were separated on a 10% SDS-PAGE and stained with Coomassie Brilliant Blue (CBB) on the right panel. Arrows show β-casein degradation products. **B.** Comparison using a western blot of the amounts of mHtrA1 proteins produced by HEK293T with endogenous HtrA1 produced by HtrA1 +/- MEF cells. Indicated volumes of conditioned media of HEK293T transfected with pcDNA3-empty (E), wild type mHtrA1(WT) or S328A mHtrA1(MT) and the culture medium of HtrA1 +/- MEF were analyzed by a western blot. Values below the panel show relative intensity of the bands.

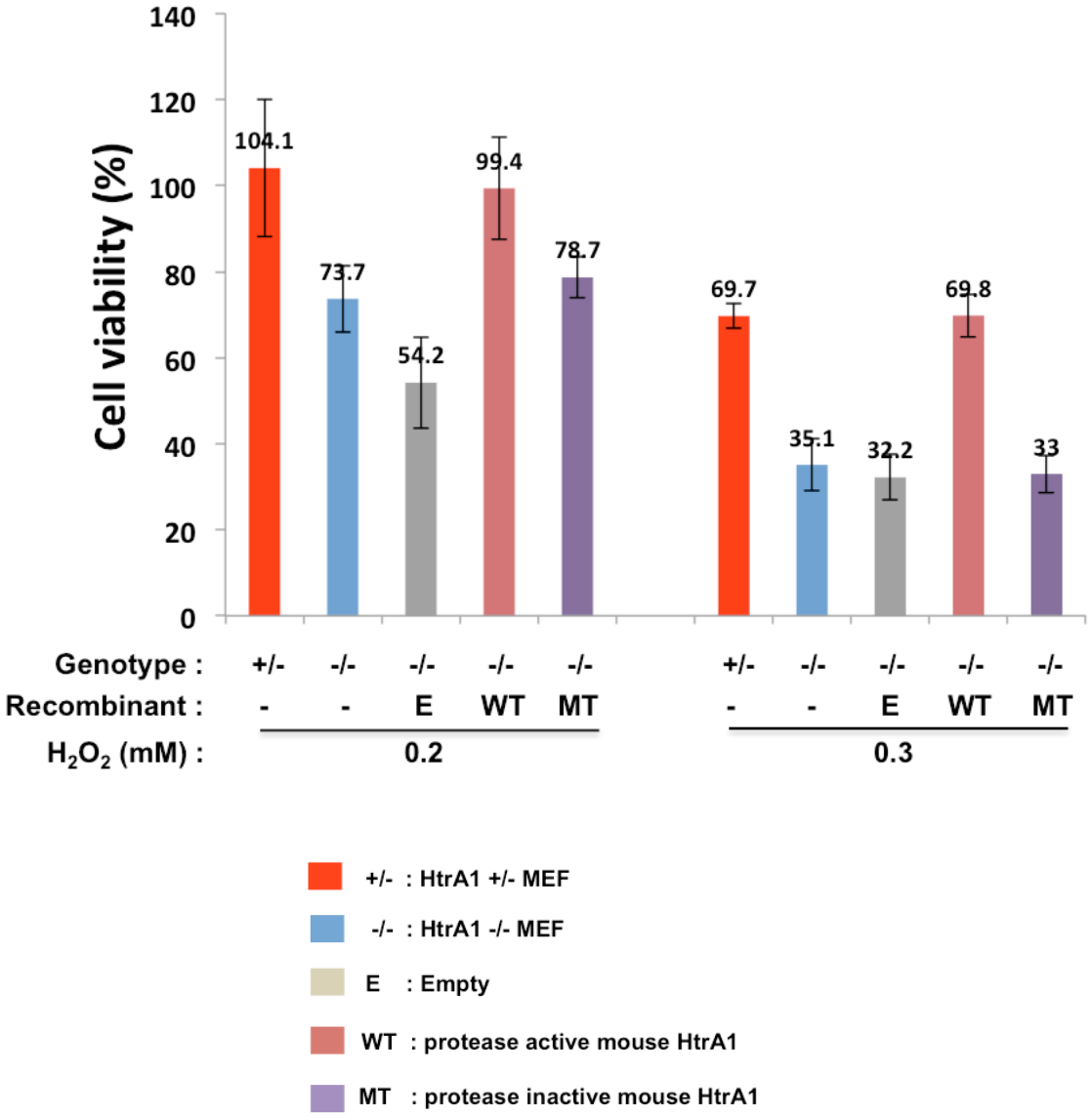


Figure 22. Recombinant HtrA1 in the culture medium protected HtrA1-/- MEF cells from cell death under oxidative stress. HtrA1 +/- MEF cells were cultured in the standard medium. HtrA1 (-/-) MEF cells were cultured in the absence (-) or presence of 10% CM containing either none (E), recombinant HtrA1 (WT), or protease deficient HtrA1 (MT). The cells were treated with 0.2 or 0.3 mM H_2O_2 for two hours. Viability assay was performed 18 hours after treatment. Values represent means ±SD (n=3).

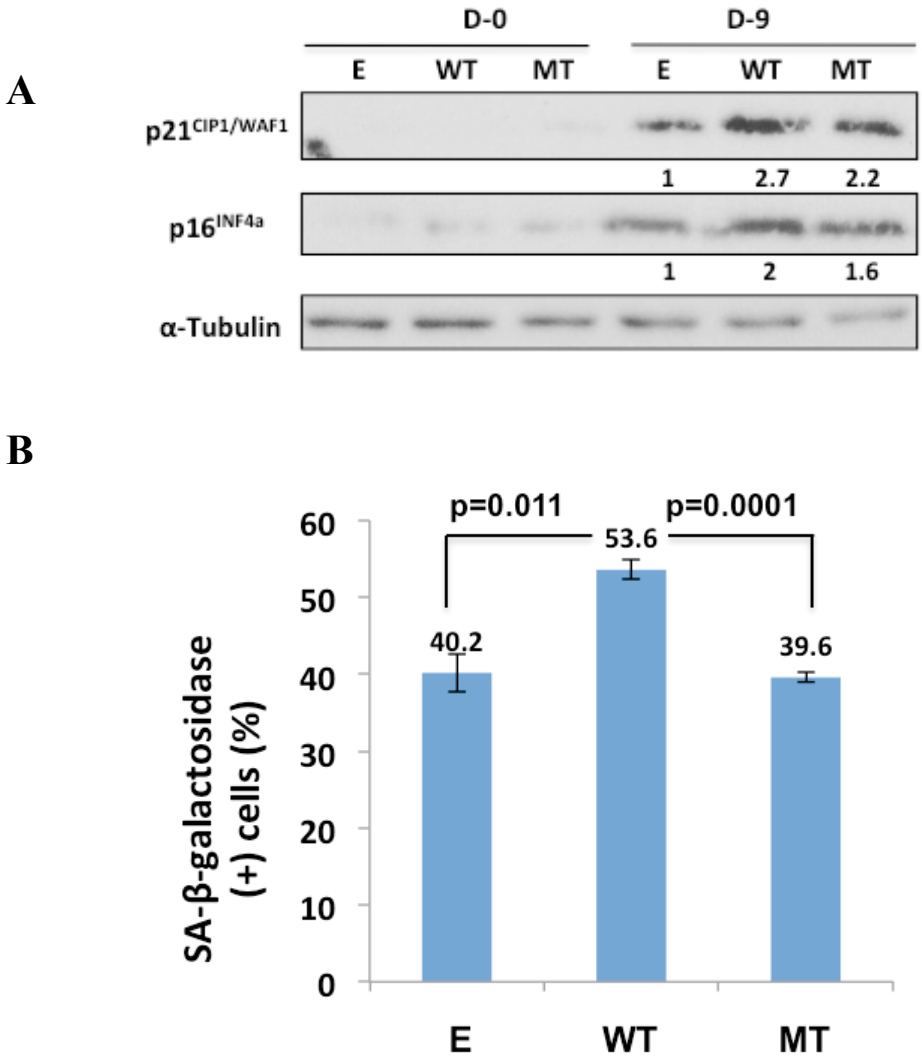


Figure 23. Recombinant HtrA1 induced senescence markers in HtrA1 (-/-) MEF cells after H_2O_2 treatment. **A.** A western blot of $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ at day 9. HtrA1 -/- MEF cells were cultured in the presence of CM and subjected to H_2O_2 treatment. Cell lysates were prepared just before H_2O_2 treatment (day 0) or at day 9 and analyzed by a western blot. Relative expression was determined by the densitometry of bands and normalized with tubulin expression. Fold increase was calculated relative to the value for HtrA1-/- MEF cells cultured with CM of E and shown below the panels. **B.** Percentage of SA-β-galactosidase positive cells. Cells treated as in (A) were re-plated at day 8 and cultured for another 24 hours. SA-β-galactosidase staining was performed at day 9. Values represent means ±SD (n=3).

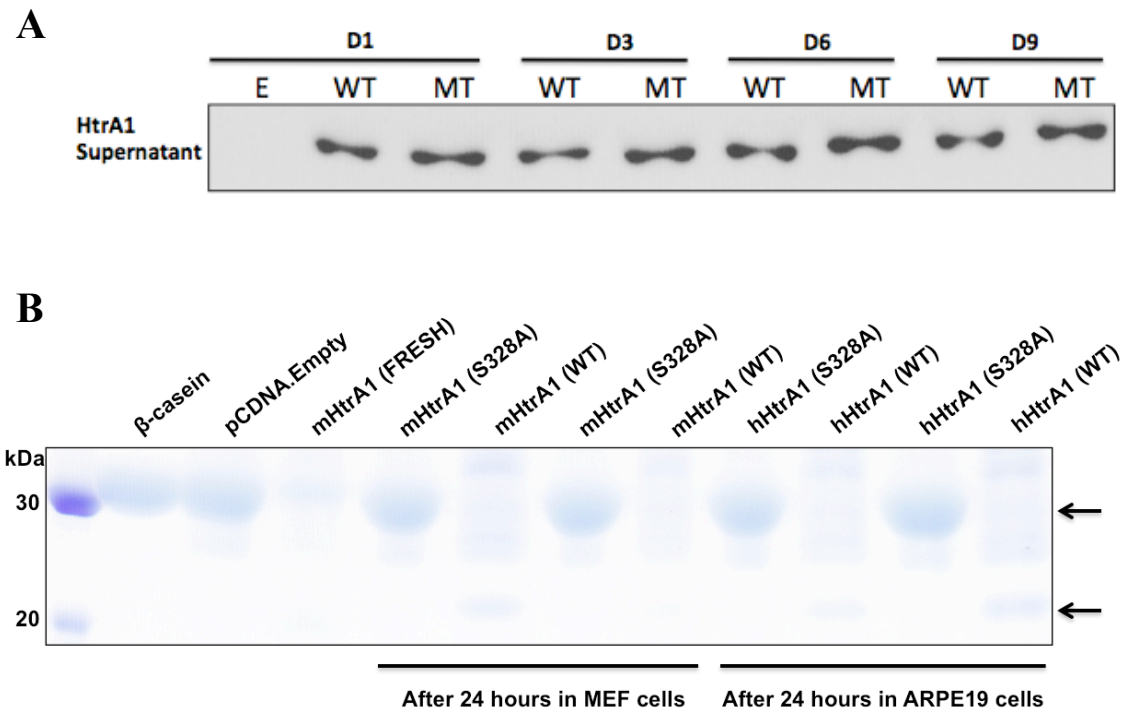


Figure 24. Recombinant HtrA1 proteins and protease activity remaining after a 24 hour culture with MEF. The culture media used in experiments described in Figure 23 were collected and subjected to a western blot and a protease activity assay. **A**. A western blot with anti mouse HtrA1 of the culture media (15 µl/well). **B**. A protease activity assay. The culture medium (20 µl) was incubated with β-casein (15 µg) at 37°C overnight. Products were separated on a 10% SDS-PAGE and stained with CBB.

3.7 HtrA1 promoted cell survival and cell senescence of MEF through the p38 MAPK signaling pathway

MAPK signaling pathways are activated by oxidative stress and modulate the signal pathways for cell survival and/or cell senescence depending on cell types (Iwasa et al., 2003; Sen et al., 2005; Pocrnich et al., 2005; Gutiérrez-Uzquiza et al., 2012). In order to investigate whether HtrA1 affects MAPK signaling cascades, we analyzed the phosphorylation of p38-Mitogen-activated protein kinase (p38 MAPK), c-Jun NH_2-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK1/2) in cells under oxidative stress. First, HtrA1 +/- MEF and HtrA1 -/- MEF cells were treated with high concentration of H_2O_2 (0.6 mM) to detect acute activation of MAPKs. Phosphorylation of p38, JNK, and ERK1/2 MAPK were observed immediately after H_2O_2 treatment and reached their peaks at 120 min (Fig. 25). Phosphorylation of p38, JNK, and ERK 1/2 was quicker and higher in HtrA1 +/- MEF cells than in HtrA1 -/- MEF cells. Next, HtrA1 +/- MEF and HtrA1 -/- MEF cells were treated with a low concentration of H_2O_2 (0.075 mM). As shown in Figure 26, the activation of p38 MAPK was clearly higher in HtrA1+/- than in HtrA1-/- MEF cells at day 1 when the highest p38 MAPK activation was observed under these conditions. Activation of JNK was lower in HtrA1+/- MEF than in HtrA1 -/- MEF. p38 MAPK was the only MAPK that was activated consistently higher in HtrA1+/- MEF than in HtrA1-/- MEF at both low and high concentrations of H_2O_2.

For further confirmation of the involvement of p38 MAPK cascade in HtrA1-enhanced cell senescence, I evaluated the effect of the expression of exogenous HtrA1 in HtrA1-/- MEF cells in the activation of p38 MAPK (Fig. 27). I found that p38 activation was 3.9-fold higher after treatment with 0.6 mM H_2O_2 in the HtrA1-/- MEF cells transfected with the mouse HtrA1 expression vector than cells transfected

with the empty vector (Fig. 27). Expression of protease-deficient mutant HtrA1 had no effect on p38 MAPK activation. Similar results were obtained when transfected cells were treated with a low concentration of H_2O_2. The activation of p38 MAPK was 1.7-fold higher one day after 0.075 mM H_2O_2 treatment in HtrA1-/- MEF cells expressing exogenous HtrA1 than the control-vector transfected cells (Fig. 28, lane E and WT). Again, expression of protease-inactive HtrA1 did not activate p38 MAPK (Fig. 28, lane MT).

The addition of the recombinant HtrA1 protein, but not the mutant HtrA1 protein, to the culture of HtrA1 -/- MEF enhanced p38 MAPK activation 1.4-fold after H_2O_2 treatment (Figure 29). The addition of the HtrA1 protein alone without H_2O_2 treatment did not seem to activate p38 MAPK (Fig. 29, left 3 lanes), suggesting that HtrA1 does not initiate p38 MAPK activation but enhances the response already elicited by oxidative stress.

If p38 MAPK activation is the main pathway for the HtrA1-mediated cell survival and cell senescence, we can expect that inhibitors of p38 MAPK abrogate the effects of HtrA1. To demonstrate effects of p38 MAPK inhibitors, I conducted an experiment similar to that described in Figure 22, but in the presence of SB203580, a specific inhibitor of p38 MAPK. As described in Figure 22, the addition of recombinant HtrA1 enhanced cell survival of HtrA1 -/- MEF to the level of HtrA1 +/- MEF (Fig. 30). However, in the presence of SB203580, the effect of recombinant HtrA1 was completely abolished. SB203580 also abolished induction of $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ (Fig. 31A) and SA-β-galactosidase (Fig. 31B) in HtrA1 -/- MEF by the addition of recombinant HtrA1 to the culture media. As in the case of p38 MAPK activation (Fig. 29), the recombinant HtrA1 protein alone without H_2O_2 treatment did

not induce these senescence markers.

Taken together, all these data indicate that HtrA1 promotes cell survival and cell senescence through p38 MAPK activation.

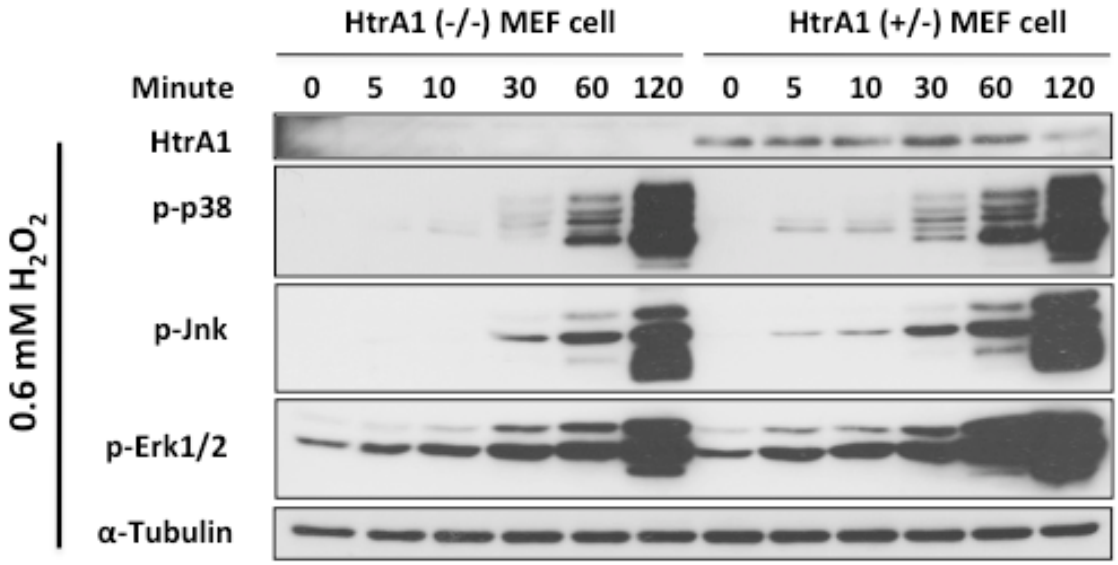


Figure 25. HtrA1 enhanced p38 MAPK activation induced by oxidative stress. HtrA1 +/- and HtrA1 -/- MEF cells were treated with 0.6 mM H_2O_2 for 5, 10, 30, 60, and 120 minutes. Cells lysates were prepared and subjected to a western blot using anti phospho-p38 MAPK (p-p38), phospho-JNK (p-JNK), and phospho-ERK1/2 (p-ERK1/2) antibodies. A western blot with α-tubulin antibody was performed to show equal loading.

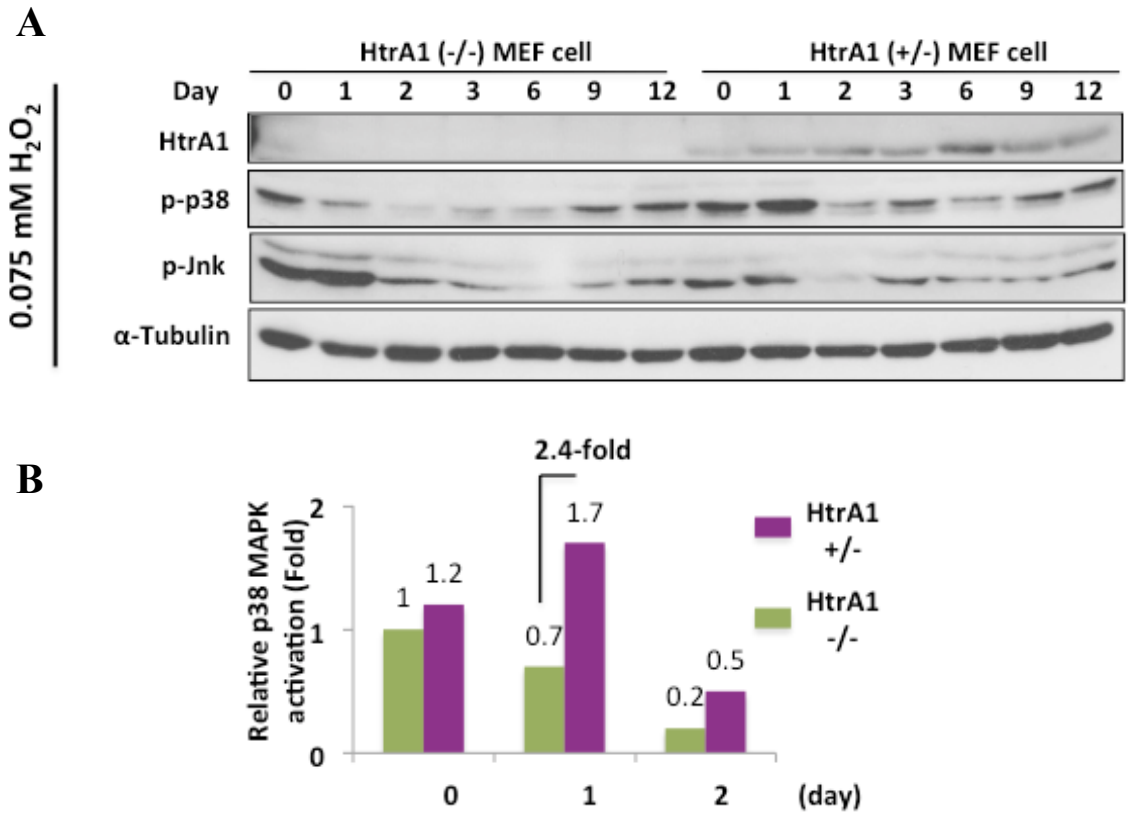


Figure 26. HtrA1 enhanced p38 MAPK activation by oxidative stress. A. HtrA1 +/- and -/- MEF cells were treated with 0.075 mM H_2O_2 to induce cell senescence. Cells were washed and cultured for different periods of time (0, 1, 3, 6, 9, and 12 days). Activation of p38 and JNK and expression of HtrA1 were evaluated by a western blot. **B.** Activation of p38 MAPK by H_2O_2 treatment. Bands of p-p38 on the film shown in A were measured by densitometry and normalized with tubulin expression. Fold activation was calculated relative to the value of p-p38 in HtrA1-/- MEF cells on day 0.

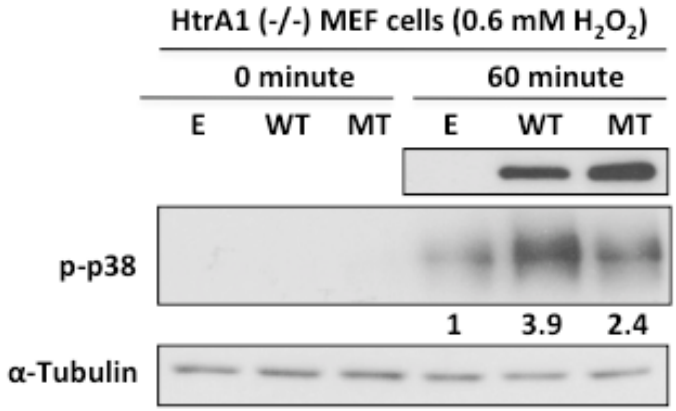


Figure 27. Overexpression of HtrA1 in HtrA1 -/- MEF cells enhanced oxidative stress-induced p38 MAPK activation. HtrA1 -/- MEF cells were transfected with the empty expression vector (E), the expression vector of wild type HtrA1 (WT), and the expression vector of protease-deficient HtrA1 (MT). Two days after transfection, the cells were treated with 0.6 mM H_2O_2. Cell lyates were prepared after 60 minutes and activation of p38 and HtrA1 in the cell lysate were examined by a western blot. Bands of p-p38 MAPK were measured by densitometry and normalized with tubulin expression. Fold activation was calculated based on the value of p-p38 in HtrA1 (-/-) MEF cells transfected with the empty vector and shown below the panel.

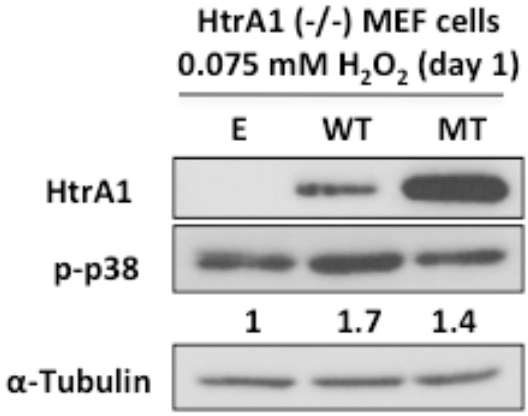


Figure 28. Overexpression of HtrA1 in HtrA1 -/- MEF cells enhanced p38 MAPK activation during cell senescence. HtrA1 -/- MEF cells were transfected with the empty expression vector (E), the expression vector of wild type HtrA1 (WT), and the expression vector of protease-deficient HtrA1 (MT). Two days after transfection, cells were exposed to 0.075 mM H_2O_2. Activation of p38 MAPK and HtrA1 expression were examined 24 hours later by a western blot. The intensity of p-p38 bands was measured by densitometry and normalized with tubulin expression. Fold activation was calculated based on the expression level of p-p38 in HtrA1 -/- MEF cells transfected with the empty vector and shown below the panel.

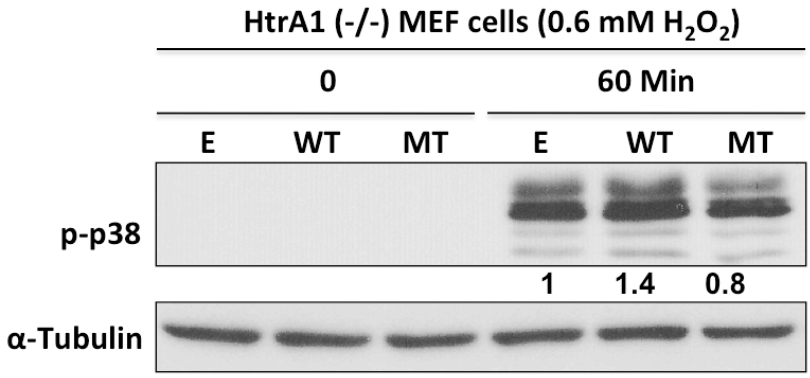


Figure 29. Recombinant HtrA1 enhanced oxidative stress-induced p38 MAPK activation. HtrA1 -/- MEF cells were cultured in the presence of 10% CM of HEK293T transfected either with the empty control vector (E), the HtrA1 expression vector (WT), or the expression vector of protease-deficient mutant HtrA1 (MT) and were treated with 0.6 mM H_2O_2 for 60 min. Cell lysates were prepared and a western blot with anti p-p38 antibody was performed. Intensity of the p38 MAPK band was measured by densitometry and normalized with tubulin expression. Fold activation was calculated based on the value of p-p38 in HtrA1 -/- MEF cells cultured in the presence of CM of E and shown below the panel.

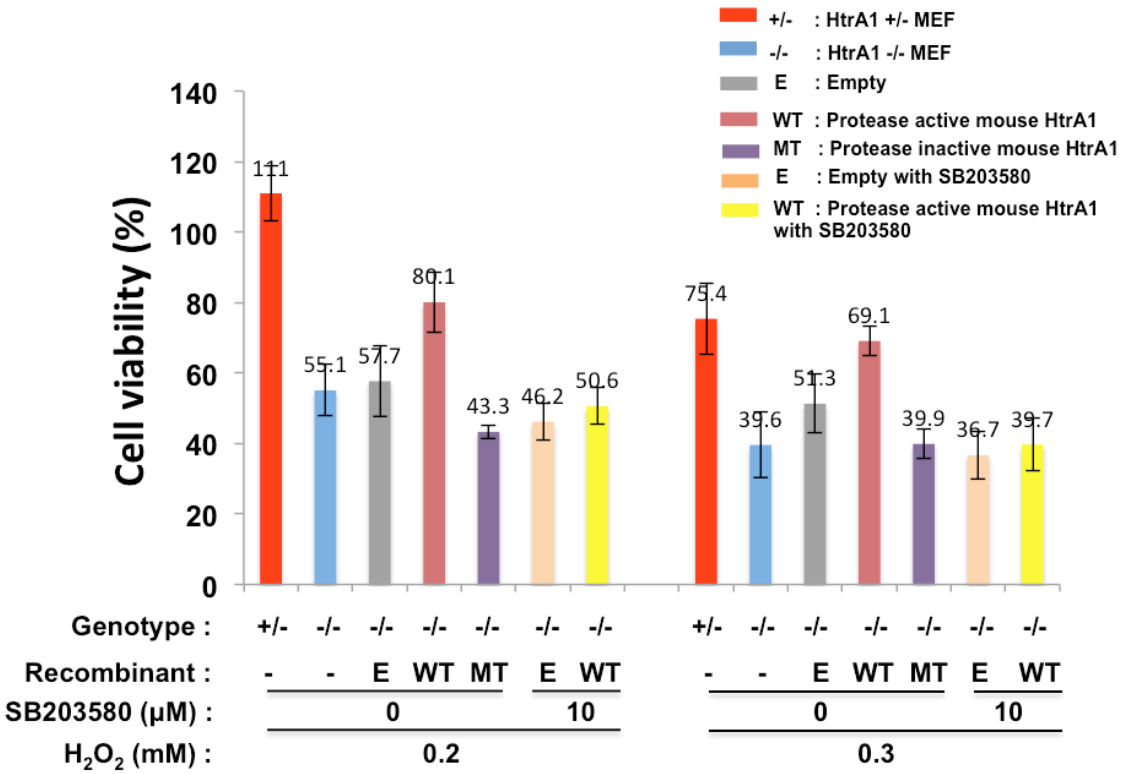


Figure 30. Inhibition of p38 MAPK signal abolished recombinant HtrA1-induced cell survival of HtrA1 -/- MEF cells upon oxidative stress. HtrA1 +/- MEF cells were cultured in the standard culture medium (■). HtrA1 -/- MEF cells were cultured in the standard medium (■), or in the medium containing 10% CM of either E (■), WT (■) or MT (■). HtrA1 -/- MEF cells were also cultured in the presence of 10 μM SB203580 in the medium containing 10% CM of E (■) or WT (■). The cells were treated with 0.2 and 0.3 mM H_2O_2 for 2 hours and cell viability was measured after 18 hours. Values represent means ±SD (n=3).

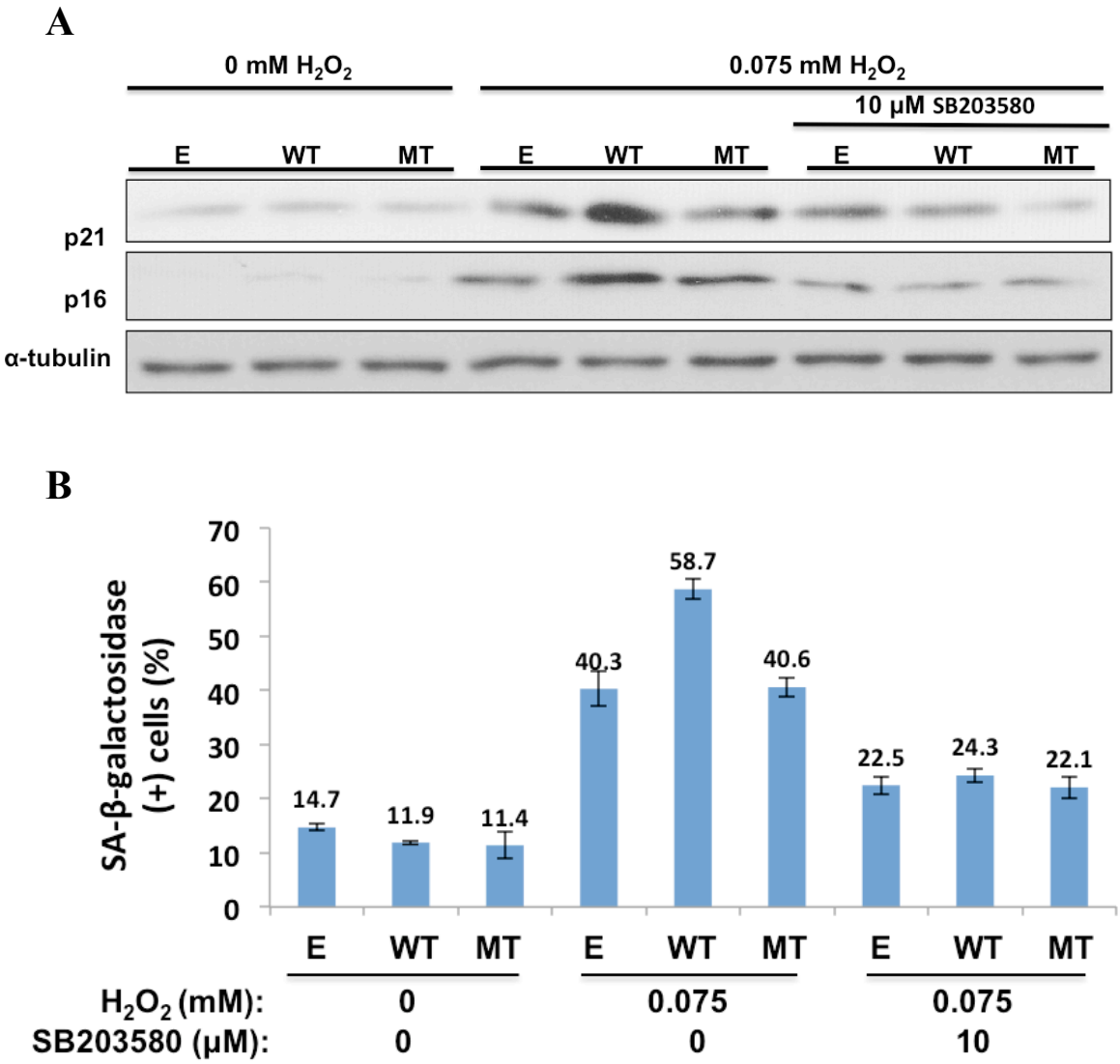


Figure 31. Inhibition of p38 MAPK suppressed HtrA1-enhanced cells senescence in MEF cells. HtrA1 -/- MEF cells were cultured in the medium containing 10% CM of E, WT, or MT in the presence or absence of 10 μM SB203580. Cells were subjected to 0.075 mM H_2O_2 treatment. Cells were harvested at day 9 and cell lyates were prepared. For SA-β-galactosidase analysis, cells were replated at day 8 and cultured for another 24 hours. **A.** A western blot of $p21^{CIP1/WAF1}$ and $P16^{INK4a}$ on day 9. **B.** Percentage of SA-β-galactosidase positive cells at day 9. Values represent means ±SD (n=3).

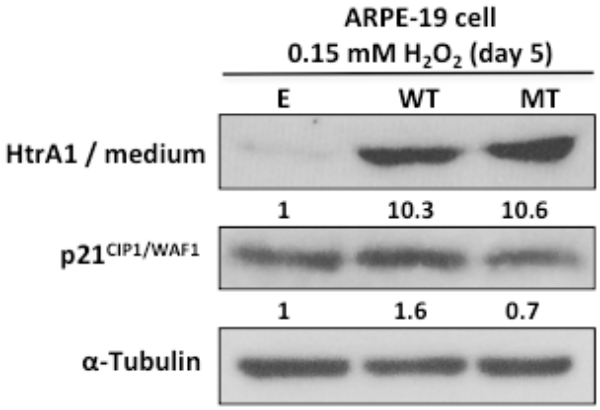
3.8 HtrA1 promoted premature cell senescence in ARPE19 cells

In the preceding sections, I presented data mainly on MEF cells. MEF has been commonly used in cell senescence studies and much information has been accumulated. Furthermore, gene knockout mice with genotypes of HtrA1 -/- and HtrA1 +/- are available in my laboratory, and this makes experiments possible to carry out with HtrA1 -/- MEF. With HtrA1 -/- MEF, I did not need to consider the fluctuation in expression of the endogenous HtrA1 gene. I need, however, to demonstrate if the same conclusions obtained with MEF can be applicable to cells in the RPE, which is the primary etiological site of AMD. Now let us return to the human RPE cell line, ARPE19. I focus in this section whether excessive HtrA1 induces cell senescence in ARPE19.

ARPE19 cells were transfected with the expression vector of human HtrA1 (hHtrA1 WT). As described in Figure 21, transfection conditions were adjusted so that approximately 10 times more HtrA1 was produced in the culture medium of the transfected ARPE19 compared to the endogenous HtrA1 produced by untransfected cells (Fig. 32 A upper panel). The transfected cells were subjected to 0.15 mM H_2O_2 treatment and the expression of senescence markers was examined. As shown in the middle panel of Figure 32A, the induction of $p21^{CIP1/WAF1}$ expression was 1.6-fold higher in the ARPE19 cells overexpressing HtrA1 than in the control cells transfected with the empty vector. Percentage of SA-β-galactosidase positive cells was also higher in the ARPE19 cells overexpressing HtrA1 (55%) than in the control-transfected cells (44.3%) (Fig. 32B). As in the case of MEF (Fig. 19 and 20), the protease-inactive mutant human HtrA1 (hHtrA1 MT) did not show this senescence enhancing activity.

Next I examined the effects of recombinant HtrA1 proteins added to the culture medium of ARPE19. Recombinant human HtrA1 proteins were produced in HEK293T cells as described in Figure 21. Using a western blot, I estimated the concentrations of human wild type HtrA1 (hHtrA1 WT) and mutant HtrA1 (hHtrA1 MT) in the conditioned medium (CM) by comparing them with the amount of recombinant protein produced in BL21 *E. coli* cells (Fig. 33A). I diluted CM of WT and CM of MT with CM of E (CM of HEK293T cells transfected with the empty expression vector) so that 20-times excess (40 ng/ml) of recombinant HtrA proteins over the endogenous HtrA1 (2 ng/ml) were provided when CM was added to 10%. ARPE19 cells cultured in the presence of CM were treated with 0.15 mM H_2O_2 and senescence markers were measured. As shown in the middle two panels of Figure 33B, induction of $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ expressions were higher in ARPE19 cells cultured with recombinant hHtrA1 than in the cells cultured with mutant hHtrA1 or in the presence of CM of E. Expression of p53 was also induced by the addition of recombinant hHtrA1 WT protein (Fig. 33B, top panel). The percentage of SA-β-galactosidase positive cells were significantly increased in the ARPE19 cells cultured with recombinant hHtrA1 WT (60.1%) compared to cells cultured with mutant HtrA1 MT (45.6%) or in the presence of CM of E (43.7%) (Fig. 34). These data with ARPE19 cells are consistent with the data obtained with HtrA1 -/- MEF. Therefore, enhancement of cell senescence by HtrA1 is a common response that is induced by oxidative stress. HtrA1 exerts its activity, at least in part, from outside the cells in a protease activity dependent manner. HtrA1 seems to affect both of the two major senescence pathways, the p53-$p21^{CIP1/WAF1}$ -Rb pathway and the $p16^{INK4a}$ -Rb pathway in ARPE 19 cells.

A



B

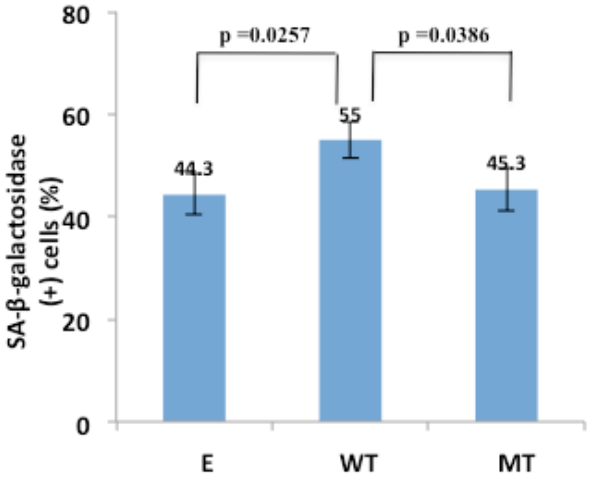


Figure 32. HtrA1 enhanced oxidative stress-induced cell senescence of ARPE19. ARPE19 cells were transfected with the empty vector (E) or the expression vector containing human wild type (WT) HtrA1 or mutant (MT) HtrA1. Two days after transfection, cells were treated with 0.15 mM H_2O_2. Western blot assays of HtrA1 in the culture media and $p21^{CIP1/WAF1}$ in the cell lysates were performed at day 5 after treatment. For SA-β-galactosidase analysis, cells were replated at day 5 and cultured for another 24 hours. **A**. Western blots of $p21^{CIP1/WAF1}$ and HtrA1 on day 5. The expression of HtrA1 and $p21^{CIP1/WAF1}$ was measured by densitometry of the western blot bands. The expression level was normalized with tubulin expression. Fold increase was calculated relative to the value for ARPE19 cells transfected with the empty vector and shown below the panels. **B**. Percentage of SA-β-galactosidase positive cells on day 6. Values represent means ±SD (n=3).

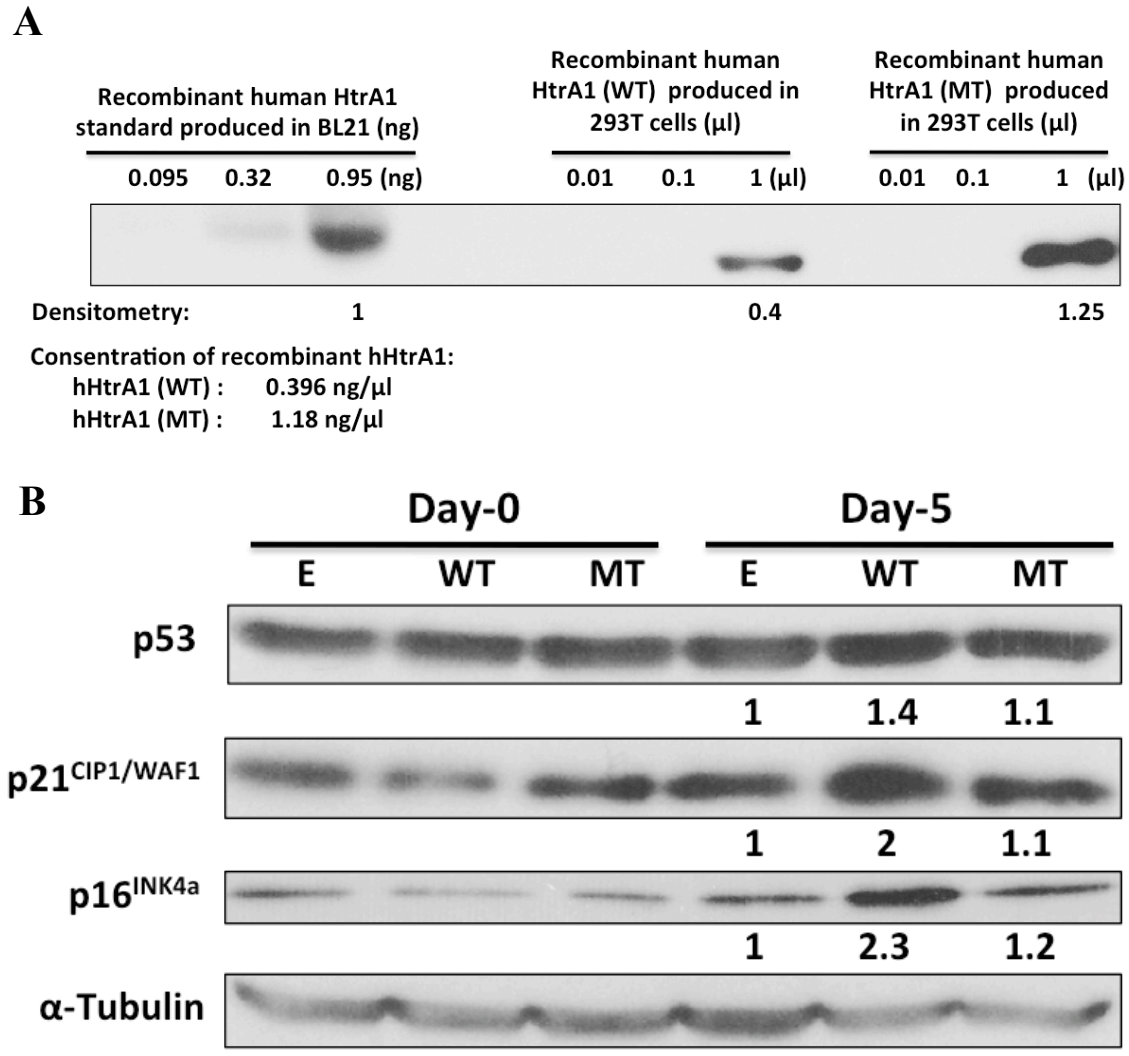


Figure 33. Recombinant HtrA1 induced $p21^{CIP1/WAF1}$ and $p16^{INKa}$ expression in ARPE19 cells. **A.** Recombinant human HtrA1 proteins. The wild type (WT) and protease-deficient mutant (MT) HtrA1 proteins were produced in HEK293T cells as described in Figure 21. The amounts of recombinant proteins were assayed by a western blot. Known amounts (0.095 to 0.95 ng) of a fragment of hHtrA1 protein produced in BL21 *E. coli* was used as standard. **B.** ARPE19 cells were cultured in the presence of 10% CM containing recombinant HtrA1 WT or MT, or in the presence of CM of HEK293T transfected with the control empty vector (E). Recombinant proteins were added 20-fold in excess over the endogenous HtrA1 produced by ARPE19. The cells were treated with 0.15 mM H_2O_2 and the expressions of p53, $p21^{CIP1/WAF1}$, and $p16^{INKa}$ were assayed by western blot at day 5. Values below the panel show fold increase in expression.

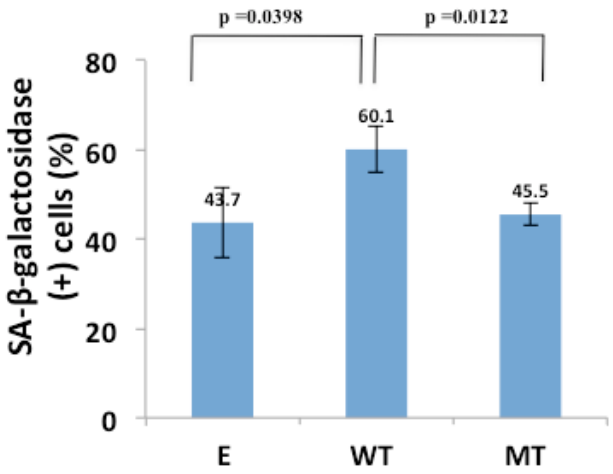


Figure 34. Recombinant HtrA1 induced SA-β-galactosidase activity in ARPE19 cells. ARPE19 cells were treated as described in Figure 33. The cells were re-plated at day 5 and SA-β-galactosidase positive cells were analyzed 24 hours later. Values represent means ±SD (n=3).

3.9 HtrA1 promoted cell senescence of ARPE19 through p38 MAPK pathway

I have shown that in MEF cells, HtrA1 enhances cell survival and cell senescence through the p38 MAPK cascade (Fig. 25 to 31). Here I examined if HtrA1-enhanced cell senescence in ARPE19 was also mediated by the p38 MAPK cascade. As shown in Figures 33 and 34, recombinant HtrA1 enhanced cell senescence of ARPE19 triggered by the treatment with H_2O_2 (also see Fig. 34 A and B, middle 3 lanes). However, in the presence of SB203580, a specific inhibitor of p38 MAPK, inductions of $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ by recombinant HtrA1 was completely inhibited (Fig. 35A, right 3 lanes). Similarly, induction of SA-β-galactosidase activity by recombinant HtrA1 was inhibited by SB203580 (Fig. 35B, right 3 lanes). As in the case of MEF, HtrA1 alone without oxidative stress did not seem to induce cell senescence of ARPE19 (Fig. 35A and B, left 3 lanes). HtrA1 may enhance the cell senescence process already initiated by oxidative stress.

Based on all data in this thesis together, I conclude that the expression of HtrA1 is induced during cell senescence both in MEF and ARPE19 cells exposed to oxidative stress. Induced HtrA1 enhances cell senescence further, but protects cells from cell death. These outcomes depend on the protease activity of HtrA1, which acts in the extracellular space and activates the p38 MAPK cascade inside the cell. The mammalian HtrA1 gene is, therefore, one of the oxidation stress responsive genes, which promote cell survival but induce cell senescence. Elevated expression of HtrA1 caused by genetic polymorphism in the promoter region of HtrA1 together with the induction of HtrA1 by persistent exposure of RPE to oxidative stress may additionally contribute to accelerate cell senescence of RPE cells, leading to the onset of AMD.

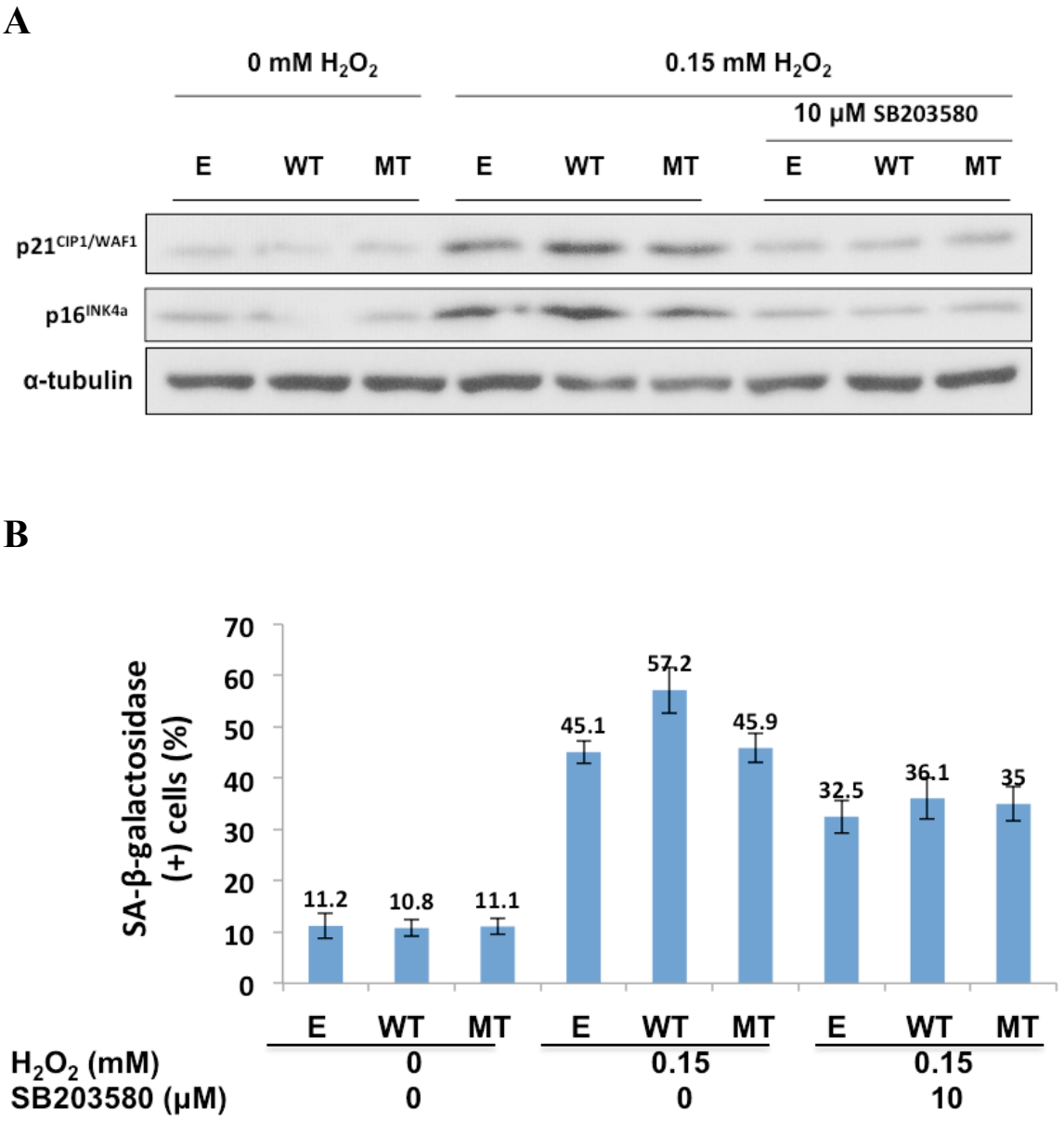


Figure 35. Inhibition of p38 MAPK signal suppressed HtrA1-enhanced cell senescence of ARPE19. ARPE19 cells were cultured in the presence of CM as described in the legend for Figure 34. Cells were treated with 0.15 mM H_2O_2 and were cultured in the presence or absence of SB203580. Cells were harvested at day 5. Cell lysates were analyzed by a western blot. **A.** A western blot assay for $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ expression at day 5. **B.** Percentage of SA-β-galactosidase positive cells at day 6. Cells were re-plated at day 5, cultured for another 24 hours, and stained for SA-β-galactosidase activity. Values represent means ±SD (n=3).

IV. DISCUSSION

4.1 HtrA1 as a genetic risk factor of age-related macular degeneration

Age-related macular degeneration (AMD) has received robust attention from many researchers due to its rising prevalence as a consequence of the increasing number of the elderly in the world. Although the etiology of AMD still remains an enigma, many studies have found that, besides aging, two categories of factors are involved in the onset of AMD: one are genetic factors and the other are environmental factors, such as persistent oxidative stress, smoking, UV irradiation, and excessive fat intake (Cruickshanks et al., 1993; Evans, 2001; Cong et al., 2008; Coleman et al., 2008).

So far, many genes have been reported to show genetic linkages with AMD. Among them, two loci are very strongly and additively linked to the development of AMD. One is the complement factor H (*CFH*) gene on chromosome 1q32 (Klein et al., 2005). The other locus is in the ARMS2/HtrA1 region on chromosome 10q26 (Yang et al., 2006; DeWan et al., 2006). *ARMS2* is a putative gene composed of two exons that encode a hypothetical 107-amino acid protein. *ARMS2* is within 7 kb upstream of the transcription start site of the *HTRA1* gene. SNPs of twelve sites in this 7 kb region and three sites in 5'UT and intron1 of the *HTRA1* gene show linkage to the onset of AMD (Friedrich et al., 2011). High-risk homozygotes at rs11200638 which is in the promoter region of the *HtrA1* gene (625bp upstream of transcription initiation site of the *HTRA1* gene and 3 Kb downstream of the poly (A) site of the *ARMS2* gene) were

reported to show 3- to 4-fold elevated expression of the *HTRA1* gene in lymphocytes or in RPE cells compared to the non-risk homozygotes (Yang et al., 2006; DeWan et al., 2006; An et al., 2010; Yang et al., 2010). There is still controversy about which gene is actually responsible for AMD, because two studies did not detect elevated HtrA1 expression in high-risk homozygous individuals (Chowers et al., 2008; Kanda et al., 2010). A variant of rs2736911 within the *ARMS2* gene results in a premature stop codon at the 38th serine residue, and the mRNA level of this variant is very low (Friedrich et al., 2011). The rs2736911 variant, however, is not risk-associated. *ARMS2*, therefore, are not correlated with the development of AMD. Transgenic mice overexpressing mouse or human HtrA1 in RPE show characteristic choroidal vascular abnormalities of wet type AMD (Jones et al., 2011; Vierkotten et al., 2011). Now, it is widely accepted that the overexpression of HtrA1 in the RPE layer is the primary etiological event of AMD.

The CFH protein, the product of the other high-risk associated gene of AMD, has been recently reported to bind malondialdehyde (Weismann et al., 2011). Malondialdehyde is a decomposition product generated by peroxidation of membrane phospholipids and is a hallmark of oxidative stress. Malondialdehyde makes adducts with proteins and other molecules, generating oxidation-specific epitopes (OSEs). OSEs induce the inflammatory response and innate immune response, thus contributing to inflammatory damages of tissues under oxidative stress conditions. CFH recognizes and binds malondialdehyde epitopes and suppresses inflammatory and immune responses in vitro as well as in vivo (Weismann et al., 2011). An AMD high-risk

polymorphism in the *CFH* gene encodes histidine at the 402 residue (H402) instead of tyrosine (Y402) in the non-risk allele. CFH(H402) hardly binds malondialdehyde epitopes and suppresses inflammation poorly compared to the CFH(Y402) (Weismann et al., 2011). The CFH variant, therefore, should be involved in the onset of AMD by aggravating oxidative stress in RPE. All these data underscore the importance of oxidative stress in AMD.

4.2 HtrA1 as a stress response gene

The stress response genes imply either that the expression of the genes is induced, or that the proteins encoded by them are activated upon stress, or both. In bacteria, the transcription of HtrA genes is induced by various stress conditions such as heat shock, ethanol exposure, osmotic stress, and oxidative stress (Loosmore et al., 1998; Smed et al., 1998; Skórko-Glonek et al., 1999; Foucaud-Scheunemann et al., 2003). Besides, HtrA proteins are activated by binding their PDZ domains to hydrophobic peptide stretches that are exposed on the surface of stress-denatured proteins.

Mammalian HtrA1 has been reported to be upregulated by stress conditions. Endoplasmic reticulum (ER) stress induced by tunicamycin or dithiothreitol treatment increases the HtrA1 mRNA level 3.5-fold or 4.5-fold, respectively, in human fetal RPE cells (Kin-Ng et al., 2011). Oxidative stress mediated by estrogen injection to male Syrian hamster upregulates HtrA1 protein and mRNA 2.5-fold and 1.4-fold, respectively, in the kidney cells (Zurawa-Janicka et al, 2008). In this thesis, I have

shown that HtrA1 is induced by H_2O_2 treatment, typical oxidative stress conditions, in MEF and a human RPE cell line, ARPE19. In accordance with the induction by ER stress and estrogen, the induction by H_2O_2 is 3.5 to 4-fold in the mRNA level, and the HtrA1 protein is also increased accordingly both in the cell lysate and in the culture medium.

PDZ domains recognize hydrophobic stretches of peptides, especially several hydrophobic amino acids at the very C-termini of proteins. Murwantoko et al. have shown that the PDZ domain of HtrA1 recognizes the C-termini of C-propeptides of fibrillar procollagens, including type III collagen (Col3) (Murwantoko et al., 2004). The HtrA1 PDZ does not bind the native trimeric Col3 C-propeptide, because its C-terminus is folded inside the native protein. The HtrA1 PDZ, however, binds the Col3 C-propeptide after denaturation that exposes the C-terminus for binding. Synthetic oligopeptides that mimic the ligands bind the HtrA1 PDZ and activate the proteolytic activity of HtrA1 several fold (Murwantoko et al., 2004). These results indicate that the HtrA1 protease activity can be stimulated under stress conditions. HtrA1, therefore, fulfils the requirements for stress response genes.

I have shown in this thesis that HtrA1 expression is induced by another authentic oxidative stress inducer, *tertiary*-butyl hydroperoxide (t-BH) and by other stress inducers, such as hydroquinone (HQ) and UVC irradiation, which are environmental risk factors of AMD.

HQ is widely used in chemical industries. HQ also occurs in plants as the β-D-glucopyranoside conjugate (arbutin), and free HQ is a major pro-oxidant chemical

in cigarette smoke (Deisinger et al. 1996). People are, therefore, routinely exposed to HQ. Cigarette smoking is a major risk factor for the development of AMD (Kabasawa et al., 2011). I show that HtrA1 expression is induced in ARPE19 by HQ at least in the protein level to the extent comparable to the induction by H_2O_2 or t-BH, authentic oxidative stress inducers (Fig. 9). Exposure to HQ of ARPE19 cells or the primary culture of mouse RPE/choroid cells is reported to dysregulate the expression of pro-angiogenic VEGF and anti-angiogenic pigment epithelial derived factor (PEDF), so that HQ shifts the balance to pro-angiogenesis (Pons and Marin-Castaño, 2011). Neoangiogenesis is a major characteristic of wet-type AMD that causes most visual loss in AMD patients.

Frequent UV irradiation caused by excessive sunlight exposure increases the risk of AMD (Blumenkranz et al., 1986; Cruickshanks et al., 1993). UV irradiation triggers many types of effects that cause cell damage. Oxidative stress is one of the effects which is evident from the increase in ROS, including H_2O_2, from exposure to UV (Peus et al., 1998; Sakurai et al., 2005; Balaiya et al., 2010). UVC irradiation induces HtrA1 protein expression, although to a lesser extent than H_2O_2 does in ARPE19 cells (Fig. 10).

Chloroquine does not seem to induce HtrA1 expression in ARPE19 cells, but it may interfere with the secretion processes of HtrA1 because HtrA1 in cell lysate is increased and HtrA1 in the culture medium is decreased by chloroquine treatment (Fig. 11). Chloroquine is a lysosomotropic agent. Chronic administration of chloroquine induces retinopathy in humans and experimental animals due to lysosomal dysfunction

and the degeneration of RPE and neural retina. In ARPE19 cells, Chloroquine induces lysosomal vacuolation which is a sign of lysosomal dysfunction, accumulation of lipid bodies in the cytoplasm, arrest of phagocytosis, and cell death. Chloroquine induced retinopathy is, therefore, supposed to be a model of AMD (Chen et al., 2011). Phagocytosis and degradation of phagocytosed materials in lysosomes are essential processes of RPE to recycle the outer segment tips of photoreceptors, and dysfunction of these processes may lead to a build-up of protein-lipid waste products as drusen in AMD patients. Oxidative stress induced by H_2O_2 does not induce the vacuolization or lipid accumulation in ARPE19 cells (Chen et al., 2011). Chloroquine, therefore, seems to contribute to degeneration of RPE through a pathway distinct from that for oxidative stress.

As discussed above, HtrA1 is definitely a stress response gene. The expression of HtrA1 is induced by oxidative stress and other types of stress which are considered environmental risk factors for the onset of AMD. It is necessary in the future to test whether the proteolytic activity of the stress-induced HtrA1 protein is activated or not under stress conditions. The N-terminal domains and the C-terminal PDZ domain may regulate the proteolytic activity of HtrA1, because removal of these domains stimulates the proteolytic activity of HtrA1 (Oka et al., 2003). The PDZ domain activates HtrA1 by binding to the ligand peptides (Murwantoko et al., 2005). The N-terminal IGFBP and KI domains are rich in cysteine residues and can function as a sensor for redox conditions of the extracellular space. It is an interesting idea that ROS regulates the protease activity of HtrA1 through the N-terminal domains.

4.3 HtrA1 enhances cell senescence and protects cells from cell death

A normal cell has a finite life span and cannot propagate continuously in a culture because the cell undergoes cell senescence (also called replicative senescence) due to the shortening of telomeres (Hayflick and Moorhead, 1961; Hayflick, 1965). Senescence can be induced in the absence of telomere shortening under various conditions, such as non-telomeric DNA damages, strong mitogenic signals, carcinogenic stress, and oxidative stress. This type of cell senescence is called premature senescence (or stress-induced senescence) because it arises prior to the telomere shortening (Kuilman et al., 2010). Oxidative stress has detrimental effects on a cell even though the cell has its own defense mechanism against oxidative stress. The damaged cell may transform and proliferate infinitely. Cell senescence is a mechanism by which the cell can suppress unregulated growth by arresting cell proliferation. The cell that has become senescent will be more resistant to the programmed cell death, or to other types of cell death (Wang, 1995).

Since HtrA1 was induced by oxidative stress, I expected that HtrA1 should affect cell senescence processes as well as cell survival under oxidative stress. I used MEF cells prepared from HtrA1 (+/+), HtrA1 (+/-), and HtrA1 (-/-) mice with the genetic background of BALB/c to examine functions of the HtrA1 gene on cell survival and cell senescence under oxidative stress conditions induce by H_2O_2 treatment. The usage of HtrA1 knockout MEF cells enables me to obtain unequivocal results on the effects of the presence of one or two copies of the HtrA1 gene. HtrA1 (+/+) MEF and HtrA1 (+/-) MEF cells are more viable than (-/-) MEF cells after treatment of various

concentrations of H_2O_2 (Fig. 16), indicating that the presence of HtrA1 gene renders the cells resistant to cell death upon oxidative stress. On the other hand, HtrA1 (+/-) MEF cells show more advanced senescence, as demonstrated by a higher induction of cell senescence markers than HtrA1 (-/-) MEF cells under oxidative stress (Fig. 17 and 18). Furthermore, overexpression of HtrA1 in HtrA1 (-/-) MEF cells by transient transfection enhances the stress-induced cell senescence (Fig. 19 and 20). The same results have been obtained with ARPE19 cells (Fig. 32). HtrA1, therefore, enhances stress-induced cell senescence and protects cells from cell death.

HtrA1 functions from outside the cell because the recombinant HtrA1 protein added to the cell culture medium enhances cell survival and cell senescence of HtrA1 (-/-) MEF (Fig. 22-24), as well as the cell senescence of ARPE19 (Fig. 33, 34). HtrA1 is a secretory serine protease, and there are many reports showing its protease activity for extracellular matrix proteins (Tsuchiya et al., 2005). There are, however, only a few papers reporting extracellular HtrA1 affecting cell physiology (Oka, et al., 2003; Tiaden et al., 2012). Oka et al. reported that HtrA1 secreted from implanted HEK293T cells changed the cell fate in chick retinal development by inhibiting BMP-4. Tiaden et al. showed that the recombinant HtrA1 protein enhanced osteogenic differentiation of human mesenchymal stem cells. On the contrary, several reports claimed that HtrA1 functioned inside the cells by binding to microtubules or by degrading XIAP, a regulator of apoptosis (Chien et al., 2009; He et al., 2012).

The recombinant HtrA1 protein added to the culture medium restored survival of H_2O_2-treated HtrA1 (-/-) MEF to the level comparable to that of HtrA1 (+/-) MEF

(Fig. 22). In these experiments, the recombinant HtrA1 protein was added at 15 times excess over the level of endogenous HtrA1 produced by untreated HtrA1 (-/-) MEF. We need to consider the following factors to assess the efficacy of the recombinant protein: 1) expression of endogenous HtrA1 is induced in HtrA1 (+/-) MEF but not in HtrA1 (-/-) MEF after H_2O_2 treatment; 2) the recombinant HtrA1 protein may not be fully active because the preparation of the conditioned media includes several steps and the serum contains potent serine protease inhibitors; 3) HtrA1 may act in the immediate vicinity of the cell surface where exogenous proteins penetrate poorly. Considering these factors, I conclude that the major site of action of HtrA1 is the extracellular space, although I cannot completely exclude the possibility that the exogenous HtrA1 protein is internalized by endocytosis and functions inside the cell. The transgenic mice lines overexpressing HtrA1 in RPE show abnormalities which are characteristic to AMD and are attributable to the proteolytic activity of HtrA1 in Bruch's membrane (the basement membrane for RPE) and the blood vessel walls in the choroid layer (Jones et al., 2011; Vierkotten S et al., 2011). These data also support my conclusion that HtrA1 functions in the extracellular milieu.

The recombinant HtrA1 protein enhances not only cell survival but also cell senescence induced by oxidative stress in MEF and ARPE19 (Fig. 23, 33, 34). These activities are completely dependent on the protease activity of HtrA1 because the protease-deficient S328A mutant protein does not show any of these activities. The results with the transgenic mice also support that protease activity of HtrA1 is important to exhibit vascular symptoms of AMD (Jones et al., 2011; Vierkotten S et al., 2011).

The N-terminal region of HtrA1 shows homology to IGFBP-rP1/mac25 which is a potent tumor suppressor gene and an inducer of cell senescence or apoptosis in cancer cell lines in vitro (Sato et al., 2007). My data, however, indicate that the N-terminal region does not have activities in enhancing cell senescence and survival.

4.4 HtrA1 acts through p38 MAPK to enhance cell senescence and survival

Oxidative stress activates all three major MAPK signal cascades in MEF cells (Zhou et al., 2006). Activation of ERK1/2 usually leads cells to growth and survival, while activation of p38 MAPK and JNK, which are canonical stress signaling kinases, are associated with diverged cell fates such as cell growth, differentiation, cell senescence, apoptosis, and cell survival, depending on types and severity of stress, cell types, and cell conditions (Finkel and Holbrook, 2000; Matsuzawa and Ichijo 2000; Martindale and Holbrook, 2002). Among the three MAPK cascades, the p38 MAPK signaling cascade plays pivotal roles in the induction of cell survival and cell senescence in response to oxidative stress (Iwasa et al., 2003; Pocrnich et al., 2009; Cai et al., 2011; Gutiérrez-Uzquiza et al., 2012). In ARPE19 cells, inhibition or knockdown of p38 MAPK by chemical inhibitors or siRNA induces cell death under oxidative stress, while activation of p38 MAPK enhances cell survival (Pocrnich et al., 2009). Similarly, in MEF cells, activation of p38 MAPK enhances cells survival under oxidative stress (Gutiérrez-Uzquiza et al., 2010).

In my experimental conditions with sub-lethal doses of H_2O_2 (0.075mM to 0.15 mM), oxidative stress with H_2O_2 activates p38 MAPK higher in HtrA1 (+/-) MEF than

HtrA1 (-/-) MEF, suggesting that HtrA1 enhances activation of p38 MAPK (Fig. 26). I have confirmed this result by transient expression of HtrA1 in HtrA1 -/- MEF (Fig. 27 and 28) and by the additions of recombinant HtrA1 proteins to the culture medium (Fig. 29). Furthermore, SB203580, a specific inhibitor of p38 MAPK, completely abolished the enhancing activity of recombinant HtrA1 protein on both cell survival and cell senescence upon oxidative stress (Fig. 30 and 31). I have obtained almost the same results with ARPE19 cells (Fig. 35). All these data indicate that HtrA1 enhances cell senescence and cell survival mainly, if not exclusively, through p38 MAPK. The activation of p38 MAPK requires the protease activity of HtrA1, because the protease-inactive S328A mutant HtrA1 protein does not enhance the p38 activation both in MEF and ARPE19 cells.

The mechanism through which the extracellular HtrA1 protease activates the p38 MAPK cascade in a protease dependent manner remains unclear. There are, however, several reports which suggest possible mechanisms. Polur et al. examined the induction of HtrA1 expression in the join cartilage of mouse osteoarthritis models and found that the expression of HtrA1 was associated with the expression of discoidin domain receptor 2 (Ddr2). They proposed that the secreted HtrA1 digests extracellular matrix molecules, remaining type II collagen fibrils which are a poor substrate for HtrA1 intact in the pericellular space. Type II collagen fibrils free of associated proteins bind Ddr2 more efficiently and stimulate intracellular signaling pathways, one of which is the p38 MAPK cascade (Xu et al., 2010; Polur et al., 2010; Lin et al., 2010). In another possible mechanism, fragments of fibronectin, vitronectin, or other

extracellular matrix proteins are suggested to activate cell surface receptor molecules which are upstream of p38 MAPK. Fibronectin and vitronectin are good substrates of HtrA1. These proteins contain RGD motifs which bind integrin and stimulate p38 MAPK (Xiong et al., 2002; Xiao et al., 2004; Humphries, et al., 2006). Fragmentation of these proteins may release RGD containing peptides which bind integrin more efficiently than the intact proteins. In fact, the addition of fibronectin fragments to the culture medium activates p38 MAPK in bovine aortic endothelial cells (Orr et al., 2005) and induces IL-6, TNF-α and MCP-1 (an intracellular signal for inflammation) in RPE cells (Austin et al., 2009).

HtrA1 alone does not activate the p38 MAPK cascade in MEF and ARPE19 cells (Fig. 31 and 35), just as the aforementioned HtrA1 alone does not enhance cell senescence and cell survival. HtrA1 enhances the p38 MAPK activation already triggered by oxidative stress. Whatever the molecular mechanism is, it should explain this phenomenon.

I have not examined the signaling pathway which leads the p38 MAPK activation to cell senescence in MEF or ARPE19. p53-$p21^{CIP/WAF}$ and $p16^{INK4a}$-Rb signaling cascades are the main pathway for premature senescence in mouse and human cells (Hara et al., 1996; Gil and Peters, 2006). p38 MAPK may send activation signals to these two pathways. It is known that p38 MAPK upregulates $p21^{CIP/WAF}$ expression indirectly through p53 activation or activates $p21^{CIP/WAF}$ directly by phosphorylation and stabilization (Kim et al., 2002). p38 MAPK is also known to upregulate $p16^{INK4a}$ expression. Detailed analysis of these signaling pathways may be a promising project

in the future.

4.5 HtrA1 in the onset of AMD

Here in this thesis, I have accumulated evidence that support my hypothesis (Fig. 2). Genetic variations of the HtrA1 gene and oxidative stress additively enhance the HtrA1 expression. The increase in HtrA1 expression is not very strong in my in vitro system. Since AMD is a chronic disease of the elderly, persistent exposure to oxidative stress and constantly elevated expression of HtrA1 over a long period of time, although at a low level, may be essential to invoke damages in the RPE. The oxidative stress may be aggravated by the variant of CFH which fails to block oxidation-specific epitopes (OSEs) to induce inflammatory response and innate immune response. Subsequent processes that lead to the full symptoms of AMD may be as follows. Elevated HtrA1 induces premature senescence in RPE cells through activation of p38 MAPK. Senescent RPE cells lose phagocytic/lysosomal activities that are essential to process waste products of the photoreceptor outer segments, leading to the accumulation of drusen. At the same time, excessive HtrA1 diffuses to Bruch's membrane and moves into the choroid layer where it degrades proteins of the extracellular matrix, such as elastin, fibulin5, and proteoglycans, leading to dilapidation of Bruch's membrane and the choroidal blood vessel walls. The damages of Bruch's membrane, the basement membrane for RPE, may accelerate degeneration of RPE. The damages in choroid vessel walls result in polyploidal choroidal vasculopathy, and in the later stages, to choroidal neovascularization. The latter step may require

induction of VEGF. I have preliminary data suggesting that HtrA1 induces VEGF expression in ARPE19 cells and probably in other cells, too. Excessive HtrA1, therefore, may explain not only the senescence of RPE but also the neoangiogenesis, two essential etiological features of AMD.

Anti-oxidants such as vitamin E are commonly used medicines for AMD patients. Inhibition of VEGF by monoclonal antibodies is now the most hopeful therapy for AMD. My data suggest inhibition of HtrA1 or p38 MAPK can also be a promising remedy against AMD.

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VI. REFFERENCES

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