Université de Montréal

The Implication of Cell-derived Microvesicles in Retinal Pigment Epithelium Degeneration

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Ce mémoire intitulé :

The Implication of Cell-derived Microvesicles in Retinal Pigment Epithelium Degeneration

L'implication des microvésicules d'origine cellulaire dans la dégénérescence de l'épithélium pigmentaire de la rétine

Présenté par

Saeideh Shani

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Abstract

In industrialized countries, degeneration of the macula is the most common cause of irreversible vision loss after 65 years of age and is termed age-related macular degeneration (AMD). This multifactorial disease comprises two types according to the stage of progress: dry AMD (70% of AMD cases) and wet AMD (30% of AMD cases). Dry AMD causes alterations in the retinal pigment epithelium (RPE) whose role in retina homeostasis is critical. These functional and structural alterations are manifested by accumulation of metabolic and cellular waste products in the form of drusen, which appear as lipid-enriched deposits between the RPE and Bruch's membrane. Oxidative stress is the main contributing factor, which can derive RPE cell blebbing activity and senescence. Despite extensive research; there is still no cure for dry AMD.

Our laboratory has long been focusing on the effects of microparticles originating from different cells, such as RPE-derived microparticles (RMPs). Microparticles (MPs) are membrane fragments that are released into environment upon cell activation or apoptosis and are responsible for various biological functions. microRNAs are selectively enriched in microparticles. According to our RNA sequencing profile analysis, let-7f is one of the most abundant microRNA (miRNA) in RMPs, and it may mediate oxidative stress-induced retinal cell dysfunction.

The principal objective of this work was to explore the molecular events responsible for RMP-induced RPE cell dysfunction, emphasizing the disruptive signal of let-7f. Our findings strongly indicate that RMPs released by RPE cells under oxidative stress accelerate RPE degeneration and cause subretinal deposits. Given the results regarding let-7f function, this miRNA seems to be the main suspect exerting the detrimental effect of microparticles in RPE cells.

In conclusion, verification of the role of RMPs and downstream players such as let-7f in retinal degeneration should inspire the development of therapeutic targets against AMD.

Keywords: RPE-derived microparticles (RMPs), oxidative stress, RPE cell dysfunction, Senescence, phagocytosis, miRNA let-7f, dry-AMD.

Résumé

La dégénérescence maculaire liée à l'âge (DMLA), est la cause la plus fréquente de perte de vision irréversible dans les pays industrialisés, chez les personnes de plus de 65 ans.

La DMLA est une maladie multifactorielle qui existe sous deux formes: la forme sèche et la forme humide. La forme humide qui représente 30% des cas, se caractérise par une néovascularisation choroïdienne pathologique. Des traitements efficaces existent et consiste à bloquer l'action du VEGF. La forme sèche affecte la grande majorité (70%) des patients, demeure actuellement sans traitement. Elle se caractérise par une altération de l'épithélium pigmentaire rétinien (EPR) qui joue un rôle majeur dans l'homéostasie de la rétine. Ces altérations fonctionnelles et structurales se manifestent par des accumulations dans la membrane de Bruch de déchets métaboliques et cellulaires, les drusens. Il a été démontré que le stress oxydatif est le principal facteur qui conduit à la formation de blebs et à la sénescence des cellules de l'EPR.

Notre laboratoire s'intéresse aux effets des microparticules provenant de différents types cellulaires telles que les microparticules dérivées de l'EPR (RMPs). Les microparticules (MPs) sont des fragments membranaires libérés dans l'environnement lors de l'activation cellulaire ou de l'apoptose. Ils sont responsables de diverses fonctions biologiques. Fondé sur notre profil de séquençage de l'ARN, nous avons constaté que les microparticules sont enrichies en microARNs (miARNs). Le let-7f est l'un des miARNs les plus abondants dans les RMPs, et est impliqué dans le dysfonctionnement des cellules rétiniennes induit par le stress oxydatif.

L'objectif principal de ce travail est d'étudier les mécanismes d'action des RMPs responsables du dysfonctionnement des cellules de l'EPR. Nos résultats indiquent clairement que dans des conditions de stress oxydatif les RMPs peuvent accélérer la dégénérescence de l'EPR et provoquer des dépôts sous-rétiniens. Selon nos résultats, le let-7f semble jouer un rôle majeur dans l'effet néfaste des RMPs sur les cellules de l'EPR.

En conclusion, l'étude du rôle des RMPs dans la dégénérescence rétinienne met en évidence l'intérêt de stratégie visant les miRNA tel que le Let-7f comme cibles thérapeutiques pour traiter la DMLA.

Mots-clés: Microparticules dérivées de l'EPR (RMPs), stress oxydatif, dysfonctionnement des cellules EPR, sénescence, phagocytose, miARN let-7f, DMLA sèche.

Table of content

List of tables

List of figures

List of acronymes

~ ..... Approximately

% ..... percent

e.g. ..... for example

i.e. ..... for example

µL ..... microliter

µg ..... microgram

µM ..... micromolar

°C ..... degrees Celsius

List of abreviations

AMD..... Age-related macular degeneration

BrM ..... Bruch's membrane

β-Gal..... Beta-galactosidase

CDKI ..... Cyclin-dependent kinase inhibitors

ClO^- ..... Hypochlorite

cm..... Centimeter

CO_2 ..... Carbon dioxide

CTL ..... Control

DDR ..... DNA damage response

DNA ..... Deoxyribonucleic acid

EVs..... Extra cellular vesicles

FBS ..... Fetal bovine serum

FGF ..... Fibroblast growth factor

FITC ..... Fluorescein isothiocyanate

G protein..... Guanine nucleotide-binding proteins

GA ..... Geographic Atrophy

H_2O_2 ..... Hydrogen peroxide

HQ..... Hydroquinone

JMD..... Juvenile macular degeneration

LMP..... Lymphocyte-derived microparticles

mCRP ..... Membrane complement regulatory protein

MFI ..... Mean fluorescent intensity

miRNA ..... MicroRNA

MMP 2..... Matrix metalloproteinase-2

MP ..... Microparticle

MPO ..... Myeloperoxidase

mRNA ..... Messenger RNA

mtDNA ..... Mitochondrial DNA

μm ..... Micrometer

NF-κB ..... Nuclear factor kappa-light-chain-enhancer of activated B cells

NIH..... National Institute for Health

nm..... Nanometer

NO ..... Nitric oxide

NO_3^- ..... Peroxynitrite radical

NTA..... Nanoparticle tracking analysis

O^-_2 ..... Superoxide anion

OH ..... Hydroxyl radical

PBS..... Phosphate buffered saline

PEDF ..... Pigment epithelium-derived factor

POS ..... Photoreceptor outer segments

PRs ..... Photoreceptors

PSer ..... Phosphatidylserine

RhoA ..... Ras homolog family member A

RMPs..... RPE- derived microparticles

RNA ..... Ribonucleic acid

ROCK I..... Receptor-operated channel I

ROCKII ..... Receptor-operated channel II

ROI..... Reactive oxygen intermediate

ROS ..... Reactive oxygen species

RP..... Retinitis pigmentosa

RPE ..... Retinal pigment epithelium

SA-β-gal ..... Senescence-associated β-galactosidase

SOD..... Superoxide dismutase

TCA..... Trichloroacetic acid

TNF ..... Tumor necrosis factor

TRAIL ..... TNF-related apoptosis-inducing ligand

TRAILR2..... TRAIL receptor 2

UV ..... Ultraviolet

VEGF ..... Vascular endothelial growth factor

To my love and life Hamed, Nasrin and Saeid

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This work was carried out at the CHU Sainte-Justine, associated with the Université de Montréal. First, I would like to thank my research director Dr. Hardy, whose throughout this work his office was always open whenever I encountered any trouble, for the support, the humanity and the wise advice that he gave me. I would also like to thank the people and the organizations that helped me and without whom this work could not have been done:

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Introduction

Before discussing the main subject, oxidative stress linked to degeneration of the retinal pigment epithelium (RPE) and the potential implications of the miRNA let-7f, we will briefly describe the human eye ultrastructure to facilitate understanding of the results. We will pay particular attention to the pathological effects of RPE-derived microparticles (RMPs). This part provides a general overview of the anatomy and physiology of the eye and ophthalmologic disease pathology.

1. Eye anatomy

The mammalian eye is a sense organ in which photoreceptors react to light, thus enabling visual perception. As in a camera, light reflected from an object passes through the eye, chemical signals arise, and sensitive cells within the eye are exposed to these chemicals. This stimulus, in turn, is electrically transmitted to the visual cortex of the brain and results in the perception of a processed image.

The eye globe is an approximately 2.5 cm in diameter and composed of two segments: the anterior and posterior (Figure 1).

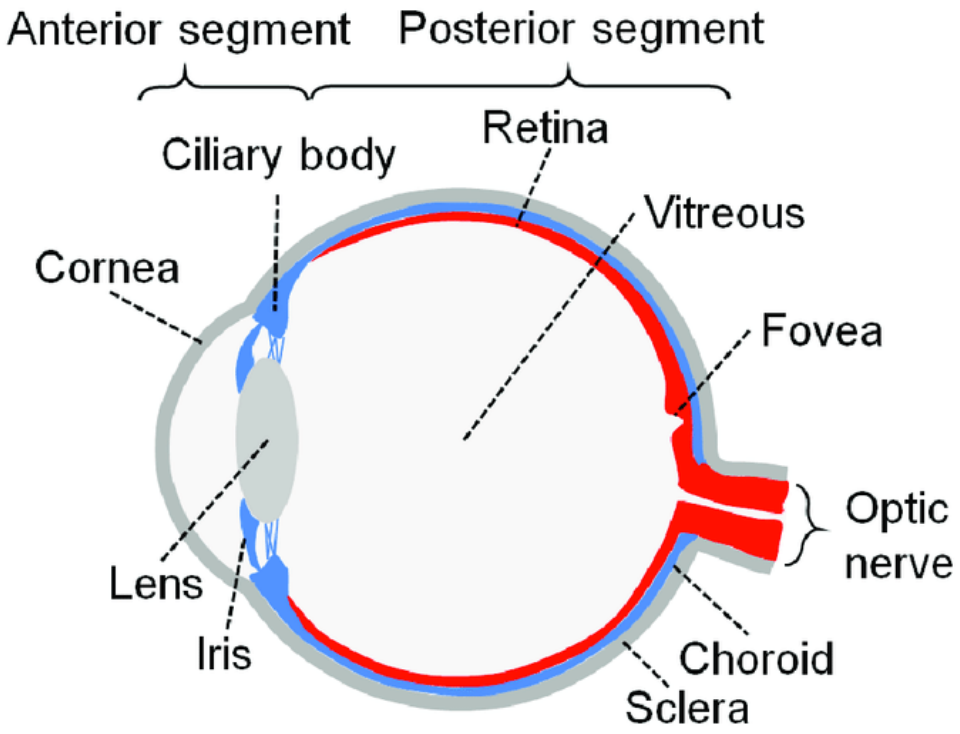

Figure 1: Schematic structure of human eye

Human eye consist of two segments: anterior segment contains the cornea, lens, iris and ciliary body; Posterior segment contains retina, choroid, sclera and vitreous (Adapted from Chuang et al [1]).

1.1. Anterior segment

The anterior segment usually comprises the front one third of the eye, but age and sex parameters may affect on its depth and volume. The anterior chamber in aged males diminishes more than that in females [2].

The anterior segment includes four portions, from front to back: the cornea, iris, ciliary body, and lens (Figure 2).

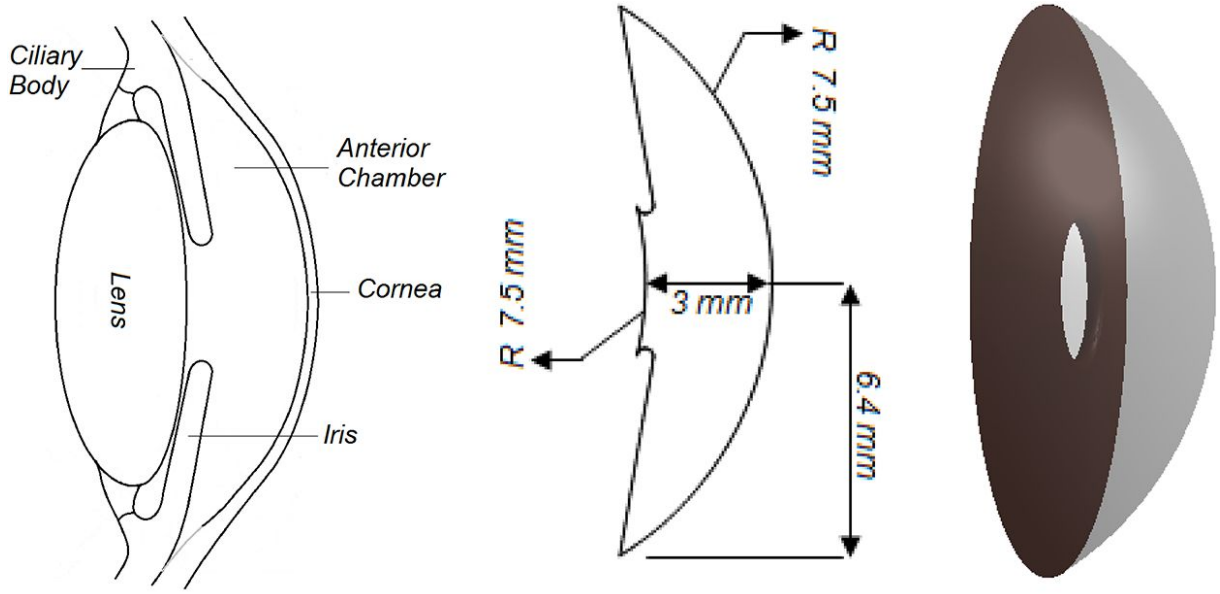

Figure 2: Anterior segment detailed anatomy

The anterior segment of eye includes four constituent including the cornea, iris, ciliary body, and lens (adapted from Modarreszadeh et al [3] with a little modification).

1.1.1. Cornea

The cornea is the outermost layer of the eye globe. Despite being transparent, it protects the globe from direct contact and separates the inside structure from the outside, similarly to a window. It has two main functions: first capturing and concentrating light and second protecting the eye structure [4].

1.1.2. Iris

The iris is a flat, colored, ring-shaped membrane behind the cornea, with an adjustable circular opening (pupil) in the center. In fact, what is called eye color is defined by that of the iris. Iris contractility can adjust the pupil diameter to allow more or less light to reach the retina [4].

1.1.3. Ciliary body

The ciliary body is a backward continuation of the iris and is composed of muscles and processes; it is a ring-shaped thickened area that separates the posterior chamber and the vitreous body. The ciliary processes secrete a transparent gelatinous fluid in the space behind the lens called the vitreous humor, which makes up almost four-fifth of the eyeball volume. The ciliary muscle enables the shape of the lens to be changed to adjust the focus on near or far objects (a process called accommodation) [5].

1.1.4. Lens

The lens is a transparent biconvex structure beside the cornea; it helps to concentrate light to the retina, while changing the convex angle under ciliary body contractility [5].

1.2. Posterior segment

The posterior segment makes up the back two-thirds of the eyeball and includes the retina, choroid, sclera, and vitreous. In general, a posterior segment begins immediately after the lens and includes all optical structures behind it. These constituents are further explained below (Figure 3).

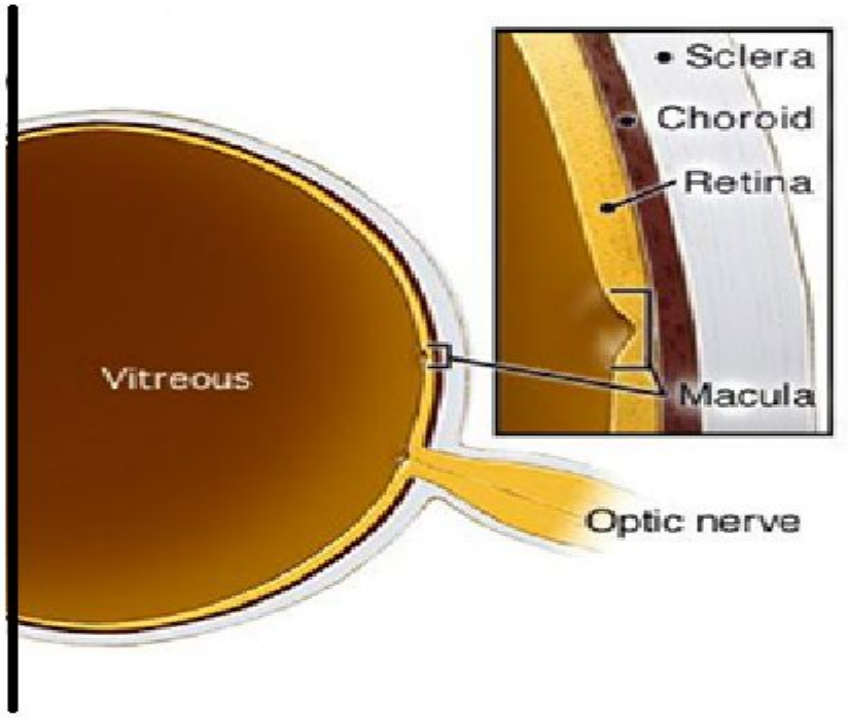

Figure 3: Posterior segment anatomy

Posterior segment contains retina, choroid, sclera and vitreous (adapted from: http://www.precisionfamilyeyecare.com/eye-encyclopedia/the-anatomy-of-the-eye-posterior/).

1.2.1. Sclera

The sclera is the outer membrane of the eye; it is composed primarily of collagen and fewer elastic fibers, and it does not posses any vessels. This structure resembles that of connective tissue. It covers 80% of the eye and continues forward, thus forming the cornea [4].

1.2.2. Choroid

A layer between the retina and sclera, called the choroid, is approximately 200 μm thick at birth and steadily diminishes with age [6]. The choroid contains blood vessels, melanocytes, fibroblasts, resident immunocompetent cells, and collagenous and elastic matrix. Given that the choroid is the vascular layer of the eye, it is considered one of the most highly vascularized tissues of the body. Its function has long been known to supply oxygen and nutrients to the retina. It contains five layers, which begin immediately behind the retina and include respectively, Bruch's membrane, the choroiocapillaris, Haller's and Sattler's layers (the two vascular layers), and the suprachoroidea [7].

1.2.3. Retina

The retina has several layers. The layer containing nerve fiber is the innermost one, whereas the outermost layer is made of photoreceptors including cones and rods. The photoreceptors are specialized cells that convert light into nervous signals.

The photoreceptors lie on a cell layer called the retinal pigment epitelium (RPE), which itself receives light and performs chemical emmision, thus producing neural impulses that connect to Bruch’s membrane from the choroid. The RPE layer is further discussed below (Figure 4).

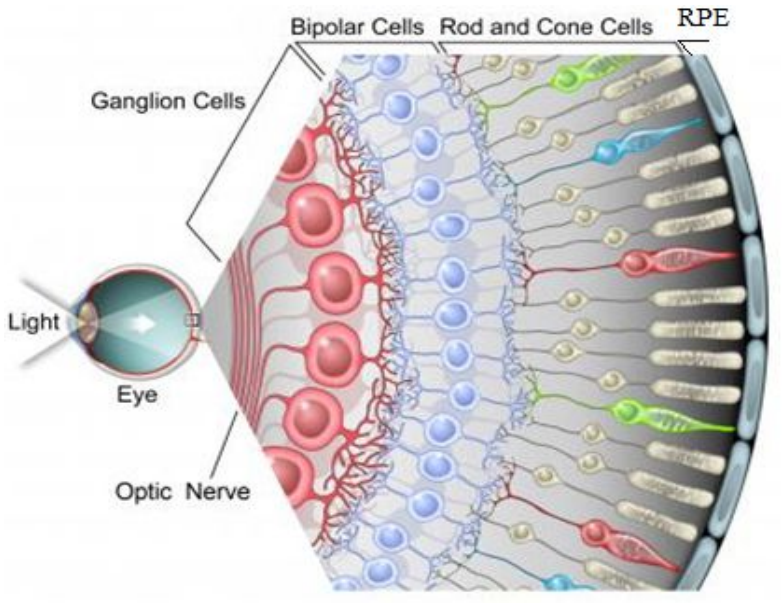

Figure 4: Retina's schematic structure

Figure shows the arrangement of different layers: respectively, from inner to outer side: nerve fiber layer (ganglion cells), bipolar cells, photoreceptors (rods and cones) and RPE layer (adapted from: https://news.feinberg.northwestern.edu/2012/04/retina_research/) (®image courtesy of Jamie Simon, Salk Institute)

In addition, in the direction of a suppositional straight line between an object, the center of the lens, and the retina is a small area around the optical nerve of approximately 0.5 cm with a high concentration of photoreceptors, which are specialized to provide vision accuracy and detail. It is responsible for central vision, whereas the other parts of the retina process only peripheral perception. Any damage to this spot can ruin central vision.

1.2.3.1. Retinal pigment epithelium (RPE)

This pigmented layer consists of a hexagonal cell monolayer behind the photoreceptors. Retinal pigment epithelium (RPE) cells are originated from the optic

neuroepithelium. Apical side of these cells possesses multiple villi projecting forwardly to contact with the outer segments of the photoreceptor cells. Morever cells are kept contacted by tight, adherens and gap junctions in lateral sides. Eventually basal side contacts the underlying basal membrane (Bruch's membrane) [8].

The RPE serves many supportive functions in the retina, such as cycling visual molecules, photoreceptor feeding, and removal of debris from the outer segments of the photoreceptors (Figure 5).

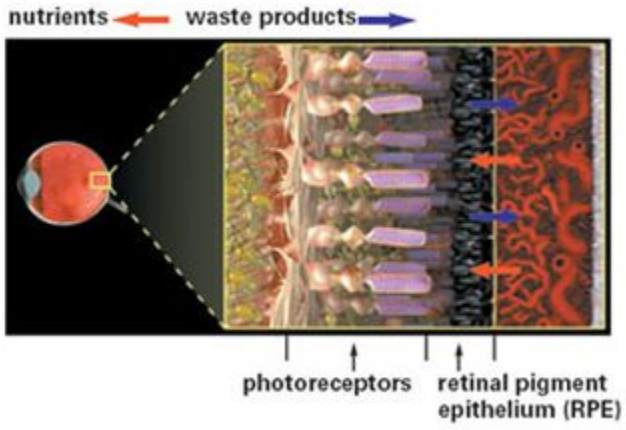

Figure 5: nourishment and waste management in photoreceptors

Retinal pigment epithelium (RPE) supplies the photoreceptors with nutrients and throws out the waste products from outer segment of photoreceptors (adapted from: https://www.eyecenteroftexas.com/services/eye-diseases/retina/macular-degeneration/).

Moreover, the RPE organizes a barrier between the retina and the choroid, through tight junctions (zonula occludens) in constituent cells. This arrangement results in an external blood-retinal barrier that physiologicaly prevents choroidal blood vessel penetration to the retina. The RPE has a photoprotective role, owing to the presence

of pigmented granules, including melanin and lipofuscin, which provide a protective shield while absorbing light [9].

The RPE is directly involved in the maintenance of the visual system integrity via the enzyme RPE65, which transforms all-trans-retinyl ester to 11-cis-retinol, a product necessary for rhodopsin photopigment in the outer segments of photoreceptors (Figure 6) [10].

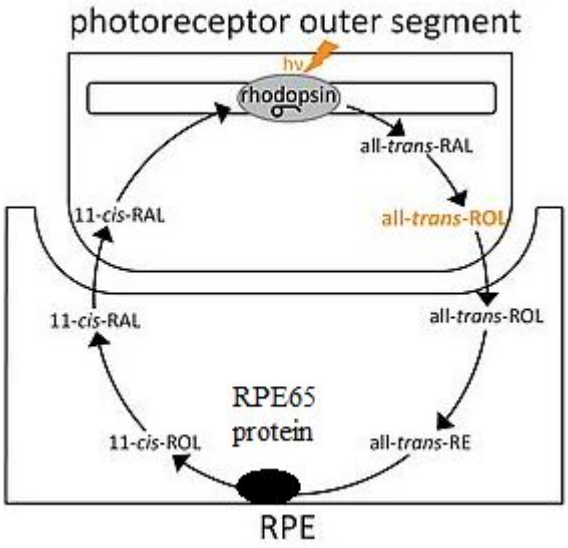

Figure 6: The role of RPE65 in visual cycle

RPE65 is critical for vitamin A metabolism in visual cycle that is responsible for converting photon to electrical pulses (adapted from: http://www.vision-research.eu/index.php?id=1180 by modification).

It also contributes to the daily renewal of the outer segments of photoreceptors [11]. Furthermore, the RPE regulates the transport of nutrients and oxygen to photoreceptors and the removal of metabolic waste from the choroid [12]. In addition,

the RPE expresses several key factors implicated in retinal development, such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF) [13-15].

1.2.4. Vitreous

The vitreous is a clear gelatinous fluid that fills the eyeball from the lens to the retina. Its responsibility is to equilibrate the pH and to nourish the eye layers. In addition, this fluid also facilitates ocular movement [16].

2. Age-related macular degeneration (AMD)

AMD is a multifactorial, neurodegenerative disease of the retina affecting people above 65 years of age [17]. It is the leading cause of severe, irreversible vision loss in the developed world. This global disease annually affects over 8 million older people.

AMD is distinguished by a loss of central vision and resolution due to dysfunction and the death of photoreceptors (PRs) in the central part of the retina called the macula (Figure 7). AMD also detrimentally affects the retinal pigment epithelium (RPE) and Bruch's membrane.

As the older population grows, the number of patients with currently non-treatable AMD becomes large, and AMD becomes an important health problem. According to the statistic data, 17,100 new cases of neovascular (wet) AMD and 180,000 new cases of geographic-atrophy (dry) AMD are recorded annually in Canada [18].

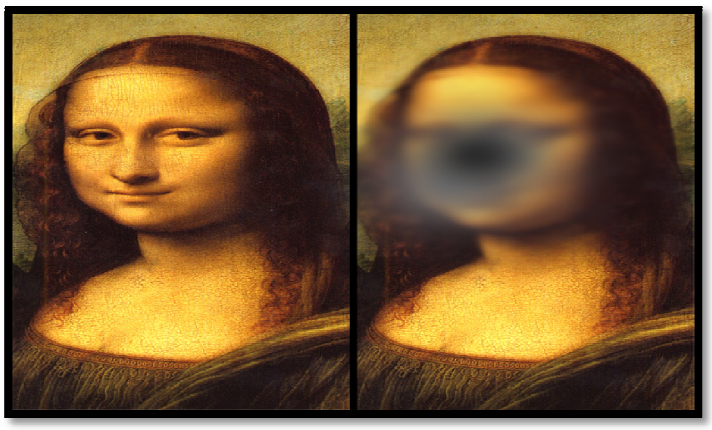

Figure 7: AMD interrupts detail visual recognition and central vision

The difference between a normal vision and that of AMD patient, as seen, AMD patients have problem in face recognition (adapted from Seiple et al [19]).

2.1. Pathophysiology of AMD

AMD is categorized as depending on neovascularization changes or its progression stage [20]. Here, a well-known categorization was used, in which there are two general types of AMD: atrophy (dry type) AMDand neovascular exudative (wet type) AMD. It is possible for a person to have both dry and wet AMD at the same time in two different eyes. Moreover in the same globe it is not unusual for the severity to progress from the dry to the exudative type.

Because the onset and development of both forms of age-related macular degeneration do not follow any specific template, AMD diagnosis is too complicated to make in the earliest stages (Table I).

AMD Assortment	Specific hallmarks
Normal condition	No drusen, No pigmented abnormality
Normal aging condition	Small drusen less than 63 μm
Early AMD (dry)	Medium drusen (63-125 μm), No pigmentary disorder
Geographic Atrophy (GA)	Large drusen (125-250 μm), AMD pigmentary abnormality
Late AMD (wet)	Neovascularization and/or GA

Table I: Clinical assortment of AMD [21].

2.1.1. Atrophy age-related macular degeneration (dry type)

This is the first stage of AMD and the most prevalent form. Published information indicates that approximately 85% of AMD cases are of this type. In the early phase, this type is not easily recognizable by unexperienced clinicians, owing to the presence of non-tangible vision difficulties.

Nevertheless, the clinical hallmark of dry AMD is drusen, which are small, yellow deposits accumulating between the RPE and Bruch's membrane (Figure 8). In fact, drusen are the compound of lipid and protein with the minor presence of trace elements. The origin of these components is not clear but according to the literatures it is likely produced by contribution of RPE and choroid. At least 40% of drusen content is lipid mostly including esterified cholesterol and phosphatidylcholine, the rest is composed of minimum 129 different proteins such as apolipoproteins [22-25].

These lipid accumulations slowly break down the macula and steadily demolish the retina tissue [20, 26, 27]. Although drusen are not always directly associated with AMD, they increase the risk of AMD onset. Drusen deposits vary in diameter; but only those sized between 63 and 250 μm can be characteristics of AMD [28].

These yellow accumulations can gradually develop, and in the late stage of dry AMD may progress to geographic atrophy (GA). In advanced dry AMD or GA, the region of atrophy extends, and this is followed by the disappearance of photoreceptors over time, thus resulting in visual loss (Figure 9) [29].

Vitamin supplementation may decrease the probability of dry AMD in some cases [30]

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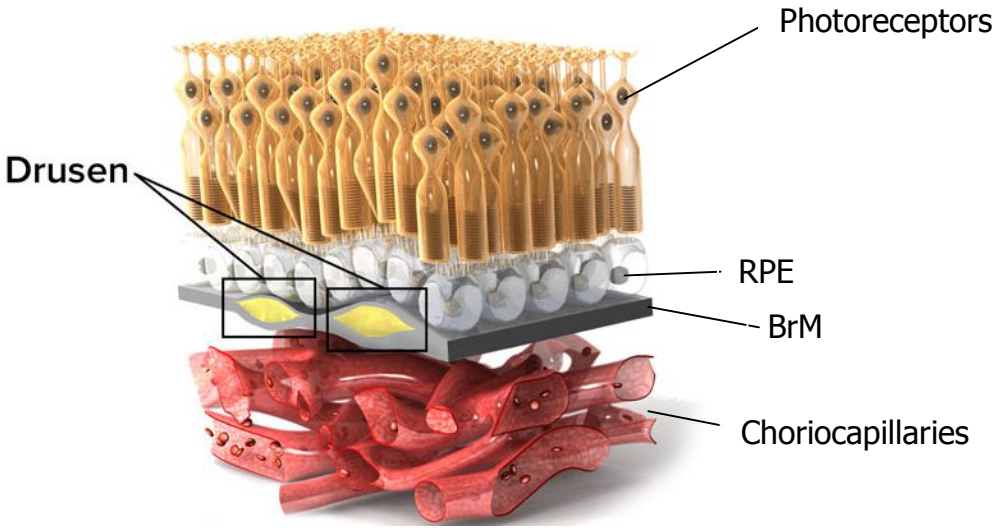

Figure 8: Dry AMD is recognized with drusen hallmark

Lipid spot accumulation between RPE and BrM named drusen which probably interfere with signal transfer from photoreceptors to the downstream counterparties (adapted from: http://www.scienceofamd.org/learn/)

2.1.2. Neovascular (exudative) age-related macular degeneration (wet type)

Patients with Atrophy age-related macular degeneration (dry AMD) are mostly susceptible to wet type (Figure 9). Uncommon blood vessels spread into the retina and over time penetrate the retina layer and may leak into the retina and induce macular swelling. This fibrovascular complex can perturb the normal RPE structure. This kind of damage is severe and can lead to legal blindness; individuals in this stage may have GA at the same time.

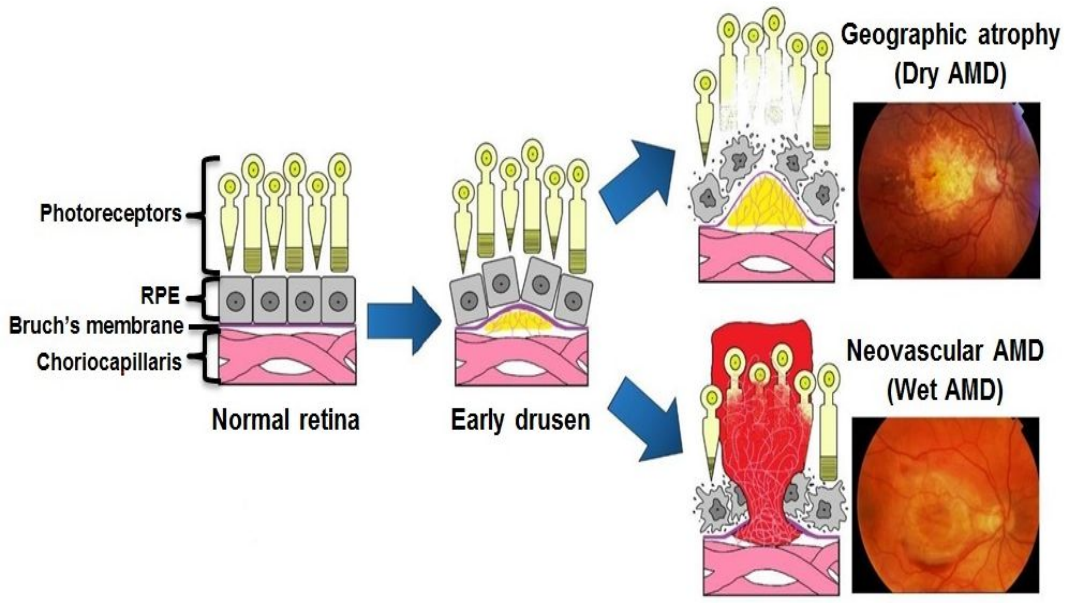

Figure 9: Pathogenesis milestones in AMD progression

Early stage can lead to GA or wet type as the figure shows in dry type drusen is a hallmark and in wet type, abnormal neovascular spreading into to the retina is a characteristic (adapted from: http://retina-amd.org/menu/eye-disease-amd/)

2.2. Ethiology of AMD

AMD is a multifactorial disease that may have different risk factors, with different proportions of hereditary and environmental factors [31]. As its name indicates, age is the greatest risk factor and is inevitable. However, age is not the only cause of AMD. Some of the risk factors cannot be controlled, such as age or genetics, but several others are controllable. Below, the AMD risk factors are briefly explained:

a) **Age:** The risk increases after the age of 65 years old [17].

b) **Sex:** Females are more prone to AMD than males [32].

c) **Family history:** The risk of AMD is mostly associated with a family history of AMD [33].

d) **Obesity:** Being overweight is weakly linked to the progression of AMD, especially in late stage and advanced AMD [34].

e) **Fatty diets:** Studies have indicated that people who regularly consume saturated fats are predisposed to AMD, whereas consuming seafood enriched in unsaturated fats can reduce this risk [35, 36].

f) **Light exposure:** Studies have demonstrated that exposure to visible light and blue light are implicated in AMD development because of their detrimental effects on photoreceptors. Most ophthalmologists recommend wearing sunglasses in the presence of ultraviolet (UV) or sun light exposure [37].

g) **Smoking cigarette:** The literature has confirmed an association between smoking and AMD. Moreover the British Medical Journal has reported that public health experts from the University of Manchester believe that smoking is the most important risk factor for AMD, so smokers have a four times greater

risk of AMD than non-smokers [38]. Research has demonstrated that benzene-1,4-diol (hydroquinone), the most abundant pro-oxidant compound in cigarette smoke, can cause structural perturbations such as changes in matrix metalloproteinase-2 (MMP 2) levels and collagen IV as well as sub-RPE blebbing, all of which are related to AMD pathogenesis [39, 40].

Beyond these explanations, oxidative injury or oxidative stress is the most important underlying factor associated with triggering AMD pathogenesis through different risk factors [25]. Oxidative stress is a process of cellular damage caused by reactive oxygen intermediates (ROIs), which generally play an essential role in age-related disorders such as cancer, Alzheimer's disease and AMD [25, 41, 42].

3. Oxidative stress

Epidemiological, clinical and experimental evidence together indicate that chronic oxidative stress is a primary contributing factor to AMD. In fact, oxidative stress is a predominance of free radicals versus antioxidant protection; it constitutes a biological attack on organisms [43] (Figure 10).

Understanding the free radical concept requires a primary knowledge of chemistry. Free radicals are defined as one or more atoms with an unpaired electron, which makes them unstable and highly reactive. To obtain stability, they must donate an electron to or gain an electron from another molecule [44, 45]. Their principal danger emerges when they attack other structural bio-macromolecules in the body. These

types of reactions are called oxidative, and over time they can lead to dysfunction or cell death.

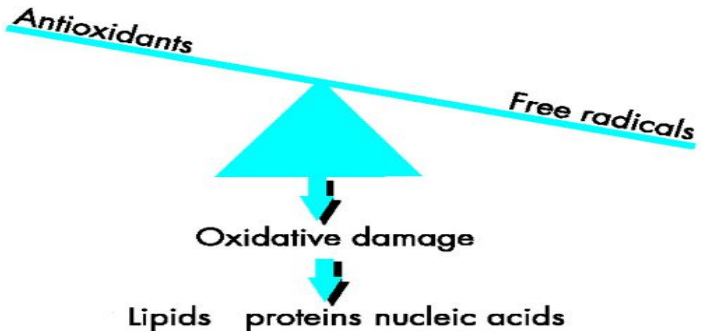

Figure 10: Interdependence between oxidative stress and cellular damage

An imbalance between antioxidants and free radicals can threaten all biological molecules including nucleic acid, proteins and lipids (adapted from Kelly [46]).

Free radicals are reactive oxygen species (ROS), including hydroxyl radical (OH), superoxide anion (O^{-}_{2}), hypochlorite (ClO^{-}), hydrogen peroxide (H_2O_2), peroxynitrite radical (NO_3^{-}), and nitric oxide (NO).

The body's defense mechanisms against ROS can be external or internal. Antioxidants are primarily external chemicals that can attenuate the deleterious effects of ROS; such chemicals may be human made, such as tocopherols, ascorbic acid, and glutathione, or can be absorbed from natural sources such as fruits. The other protection scheme includes enzymatic ROS scavengers such as catalase, peroxidase, and superoxide dismutase (SOD). These enzymes detoxify ROS through catalyzing chemical changes in their structure or reacting with lone electron pairs to

extinguish their reactivity, so that they cannot damage biological molecules [45] (Figure 11).

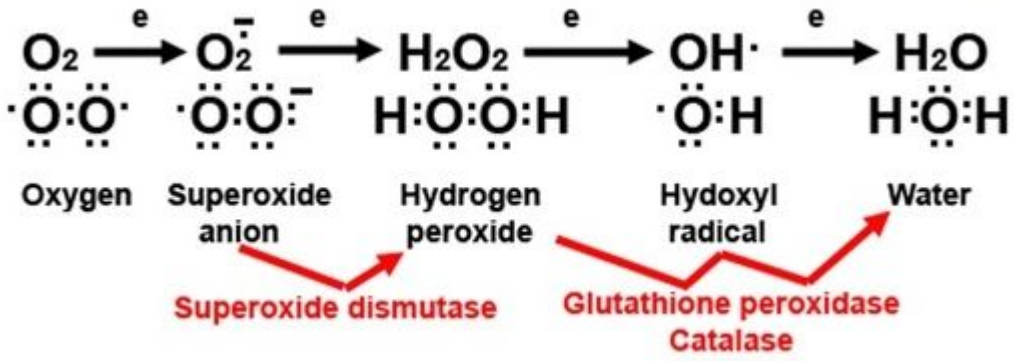

Figure 11 : Enzymatic antioxidant systems

Superoxide dismutase is the major constituent of enzymatic protection residing in mitochondrial matrix, this enzyme can catalyze the dismutation of the superoxide radical to oxygen or hydrogen peroxide. Glutathione peroxidase and catalase are specialized to detoxify of hydrogen peroxide (adapted from Fukai et al [47]).

The best-known role of mitochondria is to produce energy through respiration. through which the electron transport chain occurs in the inner mitochondrial membrane. This transport is mainly responsible for the production of ROS in the course of oxidative phosphorylation and has the potential for electron leakage [43]. Given the crucial function of mitochondria and their delicate structure, the presence of oxidative stress including lipid peroxidation, protein damage, and mitochondrial DNA mutation (mtDNA) is highly detrimental. Furthermore the mitochondria play a major role in apoptosis [48].

ROS are produced largely as by-products of mitochondrial respiration and subsequently can damage macromolecules. In turn, cells compromised by oxidative

stress trigger signals mediating senescence and age-related diseases [49]. Because early stages of senescence and membrane blebbing are thought to be present in the RPE, researchers have sought to explore whether these two conditions might share oxidative stress as a main cause. If so, oxidative stress may provoke RPE blebbing followed by secretion of RPE-derived microparticles, whose subretinal deposition is a sign of senescence and blebbing as a diagnostic feature of AMD [50].

3.1. Cellular senescence and aging

One of the major effects of oxidative stress is the DNA damage response (DDR), which is followed by cellular senescence [51-58]. Senescence refers to the induction of an irreversible halting of mitosis. Recent studies have indicated that its role is a double-edged sword: on the one hand, it has a physiological function in tissue repair and development, whereas on the other hand, it is the major cause of aging and age-related disease [59].

Several lines of evidence suggest that RPE cell senescence plays a key role in the pathology of AMD. First, senescent RPE cells have been observed in primate retinas and in human cultured RPE cells; they exhibit altered morphology and reach confluency at a lower cell density [60-62]. These changes reduce their ability to form an effective barrier between the retina and the choroid [62]. Second, oxidative cell damage accelerates senescence, after which immune dysfunction can arise [63, 64]. Premature senescence has been implicated as a potentially important

pathophysiologic mediator of RPE atrophy and ultimately cell death through changing the size of the telomeres [65].

The hallmarks of senescence can be divided into three stages: primary, which is a gradual and unavoidable process throughout life and is the body's fate; antagonistic, which involves confronting and responding to the damage; and integrative, which is the result of a cellular response and aging phenotype [66].

3.1.1. Causes and characterization of senescence

As mentioned above, senescence is a sign of aging that can be specified by multiple cellular changes, such as decreasing telomere length, increased DNA oxidation, senescence-associated β-galactosidase (SA-β-gal) activity, increased cyclin-dependent kinase inhibitors such as p16/p15/p21, and epigenetic causes.

a) Telomere length: Telomeres, at the ends of chromosomes, are protective structures aiding in genome stability. These repetitive sequences become shorter in every DNA replication and finally lead to a constant cell cycle arrest (in G1 phase) known as replicative senescence [67]. Recent studies have reported a correlation between telomere size and age-related disease: the average telomere length in senescent cells is less than that in normal cells [68-71], in addition, telomere length is subject to change in response to stress and lifestyle [6, 69, 72, 73]. Dow and Harley (2016) reported that telomerase activator-65 significantly can add telomeric DNA in RPE chromosomal ends so it is thought to be a candidate treatment for AMD [74].

b) DNA oxidation: The oxidation of proteins and lipids may cause lipofuscin and drusen, whereas DNA oxidation may provoke cell cycle arrest [75]. Because the DNA damage repair system becomes exhausted with age, genomic inconsistencies accumulate and in turn expedite aging [76].

c) β-galactosidase activity: β-galactosidase is a lysosomal enzyme that catalyzes the hydrolysis of β-D-galactoside into basic units of sugars in the presence of water. According to Gray et al., the senescence phenotype is characterized by an increase in total β-galactosidase activity [77]. In other words, senescence-associated β-galactosidase (SA-β-Gal) is a lysosomal β-galactosidase that is a specific marker for senescent cells in acidic pH ($\simeq$ 6.0) [78]; it is a reliable proxy to detect senescence in vitro.

d) Cyclin-dependent kinase inhibitors (CDKI): CDKIs are a group of proteins inhibiting cyclin-dependent kinase. They have several activities in tumor suppressor networks, cell growth arrest at G1 phase, and eventually aging. These inhibitors include P16, P15, P21, and P57 [79].

e) Epigenetics: Epigenetics also plays an important role in the beginning of aging. It can act as an engine switch that accelerates or delays age-related disease. Among different epigenetic incidents, the major effects of miRNAs are widely known in cellular senescence. miRNAs, a group of small non-coding RNAs with a length of 21–25 nucleotides, primarily interfere in gene silencing and/or post-transcriptional regulation [80]. The multistep maturation of miRNA is orchestrated by a variety of regulatory factors; consequently, imbalance and/or dysregulation of these factors can lead to a disruption in miRNA expression and result in the

progression of various diseases [81]. In addition, they can protect against oxidative stress, through a fast and reversible response ensuring proper gene expression or regulation depending on the type of stress [82-84]. ROS-induced RPE cell damage has a significant function in the pathogenesis of AMD. Several studies have verified that miRNAs are implicated in the regulation of RPE cell survival, by their ability to make mRNAs methylated following oxidative stress [85, 86]. Taken together, we can postulate that miRNA are likely involved somehow in AMD pathogenesis.

3.2. RPE blebbing and microparticles (RMPs)

3.2.1. Microparticle definition

In cell biology, a bleb is an irregular bulge in the plasma membrane of a cell, which is caused by localized decoupling of the cytoskeleton from the plasma membrane. After cell activation either by agonists or by physical or chemical stress, bleb formation results. Virtually all cell types can release membrane fragments called microparticles (MPs), generated from blebs, which represent specific characteristics of activated cells [87, 88].

MPs arise from phospholipid rich, submicron blebs (0.1 μm–1 μm) released from the plasma membranes of various types of cells in a normal state (basal level) or after apoptosis or cell activation by biological and oxidative stress (elevated level) [88-93].

They contain a variety of bioactive molecules, including proteins, biolipids, and nucleic acids such as DNAs, RNAs, and miRNAs derived from the original cells.

The protein and lipid profiles of MPs may be considered a phenotypic snapshot of the cell from which they originated. Recent studies have demonstrated that miRNAs are also carried in circulating extracellular vesicles (EVs) and are probably transferred to other cells, there by altering the functions of those target cells [94, 95]. In addition, miRNAs in EVs, through the lipid bilayer, are protected from environmental stresses that may degrade miRNA. In this context, the miRNA content of vesicles can be applied to predicting disease onset and prognosis [96].

MPs are potent biological agents capable of interfering with biological signals, interacting with target cells, and eliciting both beneficial and detrimental responses. These effects are because of their ability to regulate vascular function and gene expression during inflammation and oxidative stress (Figure 12) [87, 97-102].

MPs have some similarities with apoptotic bodies and exosomes. Apoptotic cell death is a regulative process that maintains the homeostasis of a multicellular organism. Apoptosis causes cell fragmentation, after which apoptotic bodies form. These contain nuclear material surrounded by a membrane rich in phosphatidylserine (PSer) and are larger than the MPs [103] (greater than 1000 nm) (Figure 12).

Exosomes are extruded into the extracellular matrix after fusion of multivesicular endosomes with the plasma membrane. They are smaller than the MPs in diameter, varying between 50 and 100 nm. Their membranes are enriched in tetraspanines and are poor in PSer (Figure 12).

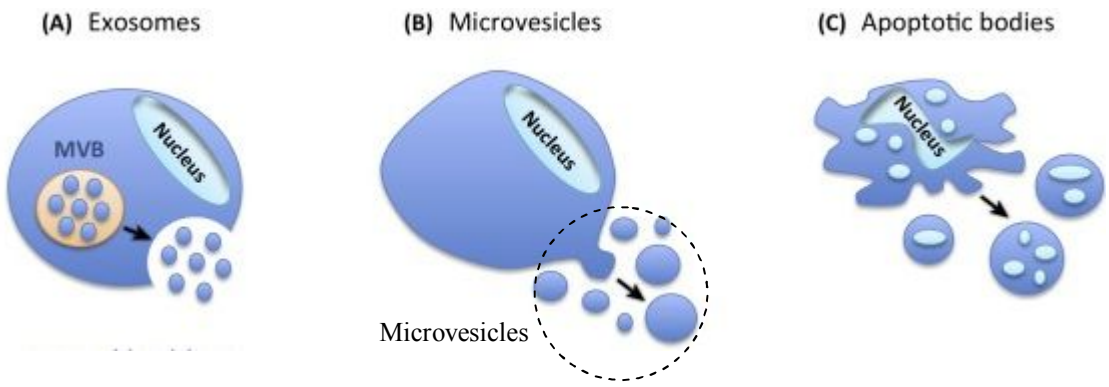

Figure 12: Different types of extra cellular vesicles (EVs)

Till now three types of EVs have been distinguished, A) exosomes are carried and secreted by intracellular vesicles; B) microvesicles (also called MPs or ectosomes) which are budding out and; C) apoptotic bodies or cell fragmentation from late stage of apoptosis [104].

3.2.2. Microparticle formation

MPs generated by affected cells after stress cause the rearrangement of membrane phospholipids. An early event involves the reorganization of plasma membrane lipid asymmetry, alongside externalization of phosphatidylserine. Lipid rafts and their accompanying cholesterol and proteins are concentrated where the membrane buds [105], thus suggesting that MPs arise from lipid rafts or from regions with high raft content [106].

Under physiological conditions, the phospholipids are distributed asymmetrically within the lipid bilayer. Phosphatidylcholine and sphingomyelin are found on the outer layer, and phosphatidylserines and phosphatidylethanolamines are located on the inner layer of the membrane.

Any stimulus induces a calcium influx into the cytosol, thereby resulting in a reorganization of the cytoskeleton (proteolytic activity of calpain) and modulating the activity of transmembrane transporters of phospholipids including flippase, floppase, and scramblase. In the resting cell, these transporters retain the lipid asymmetry of the membrane, with early externalization of phosphatidylserine. Flippase maintains the distribution of resting phospholipids, whereas floppase directly disrupts activated cell asymmetry, and eventually scramblase externalizes phosphatidylserine during apoptosis or cell activation [87]. Other signaling proteins are also involved in MP release. The small G proteins of the RhoA family (ROCK-I and ROCK-II) are activated during budding by caspases. Caspase-activated ROCK-I promotes rearrangement of the cytoskeleton while regulating the interactions between actin and myosin. ROCK-II, via caspase 2, is also involved in the release of MPs [107, 108].

In contrast, the TNF-related apoptosis-inducing ligand (TRAIL) and respective receptor TRAIL-R2 are involved in the secretion of endothelial-derived MPs through NF-κB signaling [109]. All these complex events cause budding of the membrane and spreading of vesicles out from the origin membrane (Figure 13).

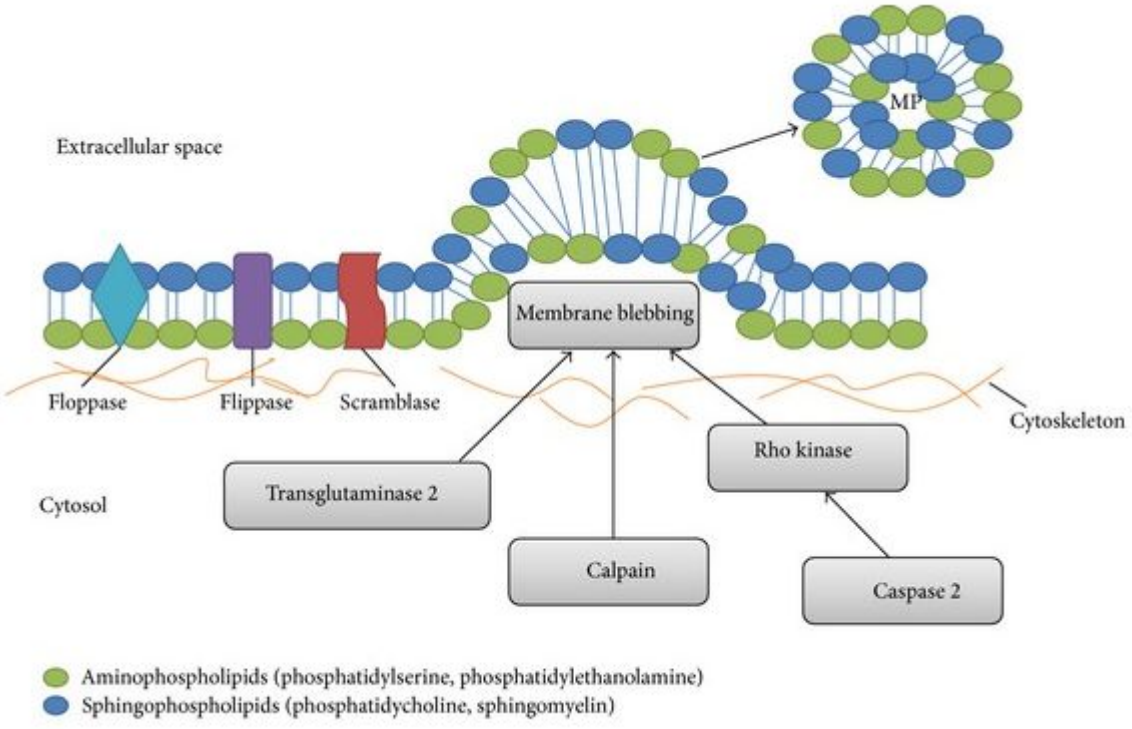

Figure 13 : Cell membrane changes in line with microvesicles formation [110].

3.2.3. Retinal pigment epithelium derived microparticles

As demonstrated, oxidative stress induces the formation of blebs in human RPE while triggering a rearrangement response in the actin cytoskeleton [40, 111-117]. Subsequently, these membrane-derived blebs accumulate and then result in subretinal deposit formation [40, 118-121]. The excessive accumulation of any lipid-rich extracellular deposit between the RPE and Bruch's membrane is a prominent histopathologic feature that gives rise to the earliest clinical hallmark of AMD [122, 123]. Although the proteomic profile of RPE blebs has revealed a total protein profile comprising proteins (n=314) predominantly involved in oxidative phosphorylation, cell junctions, cytoskeleton regulation, and immunogenic processes [117]. Because such

deposits have the original cell's characteristics, they can be characterized by RPE markers such as RPE65 [124].

Our laboratory has provided the first demonstration that RPE cells take up MPs originally derived from the same cells under oxidative stress. RMP uptake is a receptor-mediated process that consequently prompts host RPE cell senescence and eventually cell death. These findings strongly suggest that RMPs function as mediators exacerbating the oxidative damage to RPE cells, and indicate a pathological role of RMPs in AMD [125]. To the best of our knowledge, RMPs contain nucleic acids, and RNA sequencing of RMPs has revealed that miRNAs are selectively enriched in RMPs.

At the National Institutes of Health (NIH), the Biomarkers and Surrogate Endpoint Working Group has formulated criteria for biomarkers of diseases as follows. Biomarkers should be: (a) an objectively measurable characteristic representing a physiologic processes, pathogenic processes or pharmacological response to a therapeutic intervention; (b) a characteristic or variable reflecting how a patient feels, functions or survives; and (c) intended to substitute for a clinical endpoint to predict either clinical benefit or harm of medicinal strategies, on the basis of epidemiological, therapeutic, pathophysiological, or other scientific evidence [126]. According to these criteria, miRNA can be considered as a biomarker.

One of the highly conserved miRNAs is the let-7 family, whose members have a key role in gene regulation and cell senescence. Zhou et al. showed that let-7 family members are expressed in retinal and choroidal endothelial cells [127] beside that AMD patients revealed 2.6 times more expression of miRNA let-7 based on serum

concentration [128]. Among members of this family, let-7f miRNA is one of the most abundant in RMPs. According to the literature, let-7f affects cell function in many other cell types [129-132]. We hypothesized that let-7f may mediate the expected effects of RMPs on RPE cell functions.

4. Thesis Objectives

Given the literature and previous laboratory findings, the roles of microvesicles such as lymphocyte-derived microparticles (LMPs) in AMD have been demonstrated [133, 134]. In this context, we sought to investigate whether RMPs contribute to RPE cell dysfunction either specifically due to miRNA let-7f or otherwise.

Our work was organized around the following objectives:

1) To investigate the role of oxidative stress in aging and RMP production.
2) To consider whether RMPs contribute to RPE cell dysfunction in vitro.
3) To assess in vivo RMP production and to examine the role of RMPs in the development of RPE cell dysfunction.
4) To explore the molecular events responsible for RMP-induced RPE cell dysfunction, emphasizing the potential let-7f signal.

Verification of the role of RMPs on the retinal degeneration and understanding of the associated mechanisms should support the development of therapies for AMD.

Results

This thesis contains two articles:

A) **Extracellular microparticles accelerate oxidative damages to retinal pigment epithelial cells**

B) **The implication of miRNA let-7f in the degeneration of retinal pigment epithelium in vitro**

Article 1

Extracellular microparticles accelerate oxidative damages to retinal pigment epithelial cells

Extracellular microparticles accelerate oxidative damages to retinal pigment epithelial cells

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Running Title: RMPs induce RPE cell dysfunction

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Keywords: RPE-derived microparticles (RMPs), oxidative stress, RPE cell dysfunction, senescence, phagocytosis, dry-AMD

Abstract

Purpose: Oxidative stress is a primary contributing factor to early dry age-related macular degeneration (AMD). Oxidative injury to the retina may promote extracellular microparticles (RMPs) released from retinal pigmented epithelium (RPE). This study was designed to investigate whether RMPs cause RPE cell dysfunction.

Methods: RMPs were isolated from human ARPE-19 cells under oxidative stress. The oxidative stress-induced RMPs *in vitro* and *in vivo* were characterized using Nanosight and FACS analysis. Fluorescent DiI-labelled RMPs were used to assess uptake into RPE cells. MTT assay and [^{3}H]-thymidine incorporation assay were used to determine cell viability and cell proliferation respectively. Expression of cell cycle genes was analyzed by quantitative RT-PCR and Western blot. The effects of RMPs on RPE cell senescence and phagocytosis were assessed.

Results: Oxidative stress induced extracellular vesicles (EVs) released from RPE cells; RMPs constitute over 95% of the EVs. Significantly, more RMPs were released from aged RPE cells. RMPs were taken up by RPE cells in a time-dependent manner; however blockage of CD36 attenuated the uptake process. The decrease of RPE cell viability and cell proliferation by exposing with RMPs was associated with an increased expression of cyclin-dependent kinase inhibitors p15 and p21. Moreover, RMPs enhanced senescent and interrupted phagocytic activity in RPE cells.

Conclusion: This is the first study to demonstrate that oxidative-induced RMPs produce a strong effect of inducing RPE cell degeneration. This finding leads to the postulate that RMPs exacerbate oxidative stress damage to RPE cells, which may uncover a potentially relevant process in the genesis of dry AMD.

Introduction

Age-related macular degeneration (AMD) represents the leading cause of vision loss in the elderly, which has a devastating impact on quality of life.[1, 2] The most common form of AMD is dry AMD, with which 85-90 percent of AMD patients are diagnosed.[3] The key features of dry AMD are the degeneration of the retinal pigment epithelium (RPE), and drusen formation.[4, 5] Clinical and experimental evidence points to oxidative stress as a primary contributing factor to AMD by promoting RPE cell dysfunction and ultimately cell death.[6-8]

RPE is a single layer of pigmented epithelial cells with highly organized structure and tight junctions. The structural and functional integrity of RPE is critical for maintaining photoreceptor functions and survival.[9] One of the major effects of oxidative stress is the induction of cellular senescence and apoptosis.[10-18] Senescent RPE cells exhibit altered morphology and change in growth rate;[19, 20] these changes could reduce their ability to form an effective barrier between the retina and the choroid.[19]

RPE cells are among the most actively phagocytic cells in nature. RPE cells ingest and metabolize lipid-rich photoreceptor outer segments (POS) for visual factor recycling and to prevent debris buildup.[20] Impairment of RPE phagocytosis would lead to accumulation of material within the RPE and deposition of abnormal material within Bruch's membrane.[21] Drusen are extracellular lipid- and protein-containing deposits that accumulate mainly between the RPE and Bruch's membrane, they are a hallmark of aging and early AMD.[22] There is evidence suggesting that drusen are formed from extracellular microvesicles (EVs) derived from RPE cells.[18, 23]

In general, EVs include microparticles (MPs), exosomes, and apoptotic bodies. MPs are a heterogeneous population of shed microvesicles of 100 to 1000nm in size, formed from activated or stressed cells; exosomes are released from exocytosis of multivesicular bodies with 50 to 100 nm in diameter.[24] Several studies reported the role of RPE-derived exosomes in AMD,[23, 25-27] and there have been very few reports of RPE-derived MPs (RMPs) contributing to sub-RPE deposits.[18, 28, 29] However, the effect of RMPs on RPE cell functions has not yet been established. To gain a better understanding of the pathological roles of RMPs, we isolated and characterized the RMPs from cultured RPE cells exposed to oxidative stress conditions. Most interestingly, we found that RMPs reduced RPE cell viability and impeded RPE cell functions. These findings may reveal a novel pathogenic mechanism of oxidative stress in the development of dry AMD.

Methodology

Cell culture

ARPE-19, a spontaneously immortalized cell line of human retinal pigment epithelium, was purchased from American Type Culture Collection (ATCC, CRL-2302), and maintained according to standard procedures. Primary mouse RPE cells were isolated from different ages of C57BL/6 or $Sod^{-/-}$ knockout mice of different ages and cultured as we described previously.[30] For cell purity confirmation, confluent primary cells were fixed with 4% paraformaldehyde followed by immunohistochemistry staining with Alexa Flour 488 conjugated anti-RPE65 antibody (Novus; NB100-355AF488) (See Supplementary **Fig. S1**). All animal experiments were performed

according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care Committee of CHU Sainte-Justine (Montreal, QC, Canada).

Isolation and characterization of RMPs derived from ARPE-19 cells

Induction of RMPs was performed as described with modification.[29] Briefly, confluent ARPE-19 cells were cultured with medium containing sequential FBS reduction for 6 days. On day 7, cells (or primary mouse RPE cells at passage 2) were exposed to 10 microunits myeloperoxidase (MPO) for 90 minutes followed by exposure to hydrogen peroxide (H_2O_2) at 100 μM for 24 hours. At the end of the exposure time, isolation of RMPs was performed as described previously.[31] RMPs were then characterized with annexin V staining[32] **(**See Supplementary **Fig. S2)**. To produce fluorescent lipophilic membrane dye (DiI) labelled RMPs (DiI-RMPs), ARPE-19 cells were exposed to DiI for 12 hours before MPO exposure.

Extracellular vesicles (EVs) size analysis and quantification

To analyse EVs secreted from ARPE-19 cells with or without oxidative stress, cells were plated at 10^6 cells/5 cm^2 and cultured without 100μM of H_2O_2 for 24 hours. Differential centrifugations (i.e. 2000×g, and 12,000×g) at 4°C were performed to purify RMPs from culture supernatants. EVs profiling was analyzed by NanoSight particle-tracking system (NanoSight Ltd.) as described [33] or FACS analysis by measuring the amount of Annexin V^+ MPs.

RMPs uptake assay

a. Uptake of DiI-RMPs by RPE cells *ex vivo*: RPE/choroid explants were prepared as we described previously.[30] RPE/choroidal explants were incubated with DiI-RMPs and fluorescent images were taken at different incubation time points.

b. Uptake of DiI-RMPs by ARPE-19 cells *in vitro*: ARPE-19 cells were incubated with 10μg/ml of DiI-RMPs. At different time points, cells were collected for spectrofluorometry readings and the mean fluorescent intensity (MFI) was determined as described previously.[34] To investigate whether CD36 is involved in the uptake process of RPE cells, ARPE-19 cells were pre-exposed to 15 μg/mL of CD36 antibodies (ab23680, Abcam) or isotype-matched control antibodies (anti-human IgA, Sigma) for 6 hours, followed by 24 hours DiI-RMPs incubation.

Cell viability assay and proliferation assays

ARPE-19 cells or primary RPE cells were incubated with different doses of RMPs or control vehicle. Cell viability at different time points was determined by MTT assay as described previously.[35] Cell proliferation was evaluated by [^{3}H]-thymidine incorporation assay.[36]

Cell cycle analysis and quantitative RT-PCR analysis

ARPE-19 cells in log phase of growth were exposed to 10μg/mL of RMPs for 24 hours. Cells were harvested for FACS analysis as we described previously.[36] Total RNAs isolation and quantitative analysis of gene expression were performed as we described previously.[36] Gene expression was normalized to 18S. PCR primers

targeting human p15, p21 and 18S were synthesized by Alpha DNA (Montreal, Quebec, Canada) based on the following sequences: p15 forward 5'-CTCTCACCCGACCGGTGCAT-3', reverse 5'-GGTGGGTGGGGGTGGGAAAT-3'; p21 forward 5′-GCAGACCAGCATGACAGATTT-3′, reverse 5′-GGATTAGGGCTTCCTCTTGGA-3′; 18S forward 5'-CGCAGCTAGGAATAATGGAATAGG-3'; reverse 5'-GCCTCAGTTCCGAAAACCAA-3'.

Western blot analysis

ARPE-19 cells were collected after 24 hours exposure to RMPs. Extraction of soluble proteins and fractionation by SDS-PAGE was performed as described previously.[31] The anti-p15 and anti-p21 antibodies (LifeSpan BioSciences, Inc. 1:500) were used to reveal the protein levels of p15, and p21 respectively.

Senescent assay

The confluent RPE primary cells or ARPE-19 cells were exposed to 10μg/mL of RMPs for different times. Senescence-associated β-galactosidase (SABG) staining was performed as described.[37] Images were taken using phase-contrast microscopy and SABG positive cells were counted.

Phagocytic activity assay

Phagocytic activity assay was performed according to a modified method.[38] Briefly, photoreceptor outer segment fragments (POS) isolated from pig eyes were labeled

with 2 mg/mL of fluorescein isothiocyanate (FITC) (Sigma). RPE cells were fed with FITC-POS (10 POS/cell) for different durations. Unbound POS were removed by extensive washing with medium. The fluorescence was recorded at 485/525 nm using Spectramax GeminiXS plate reader. Phagocytosis rate was calculated by fluorescent count for each time point compared to total fluorescent count. In RMPs exposed experiments, RPE cells were pre-exposed to 10μg/mL of RMPs for 12 hours followed by incubation with FITC-POS, the rate of phagocytosis was determined after 4 hours incubation.

Animals

Hydroquinone exposed mice

C57BL/6 female mice (12-month-old) were randomly divided into two groups: mice in the control group (n=8) receiving regular drinking water, and the second group mice (n= 8) receiving drinking water containing 0.8% hydroquinone (HQ) (Fisher Scientific). Mice were kept for 45 days as described[39]. At the end of exposure period, eyes were immediately subjected to dissection of retina and RPE/choroid sheets.

Sod1 knock-out mice

Homozygous 129*Sod1*^{tm1Leb}/j knock-out male mice and age similar 129SF1/J wild-type male mice were purchased from Jackson Laboratory. The RPE/choroid were collected from 12-month-old sod1$^{-/-}$ mice and control mice respectively (n=7 mice for each group), and subjected to MPs characterization analysis.

In vivo RMPs characterization in mice and humans

RPE/choroid tissues were homogenized in annexin buffer followed by two steps centrifugations of 2000 rpm for 15 min and then 10,000 rpm for 20 seconds. The resulting supernatants containing MPs of RPE origin were first labeled with anti-RPE65 antibody conjugated with Alexa Flour 488 (RPE65-AF488, Novus) or FITC mouse IgG2a (BD pharmingen) as isotype control, then incubated with annexin V-Cy5 (BD pharmingen). RMPs were characterized with annexin V^+ and $RPE65^+$ staining by FACS analysis and gated using 1.0-μm beads.[31]

Statistical analysis

Results were presented as means ± SEM from at least three independent experiments performed as duplicates or triplicates. Student's t-test was used to determine the significant differences between two groups. One-way ANOVA followed by post-hoc Bonferroni tests was used for comparison of means from three or more groups. Statistical significance was set at $P < 0.05$.

Results

1. Oxidative stress promoted more extracellular microvesicles (EVs) released from ARPE-19 cells.

Human ARPE-19 cells have structural and functional properties characteristic of RPE cells *in vivo*[40]. ARPE-19 cells exposed to Hydrogen peroxide (H_2O_2) is a commonly used model to test oxidative stress susceptibility or antioxidant efficiency.[41] 100 μM final concentration of H_2O_2 has been proved to be a sub-lethal concentration to

ARPE-19 cells[41-43]. The nanoparticles tracking analysis of extracellular microvesicles (EVs) revealed that EVs released from both normal culture condition and oxidative condition have a similar size distribution pattern, with diameters of 50 to over 600 nm. The average size of these EVs was approximately 200 nm and did not differ between the two groups (mean 207±3.7nm, mode 165.3±18.2nm for normal condition, mean 204.8±3.6nm, mode 150 ±11.0 nm for the oxidative condition, P = 0.94 for mean, P = 0.88 for mode). This result suggested that RPE cell derived EVs contain both exosomes (less than 100nm) and microparticles (size from 100nm~1000nm), but over 95% of EVs are microparticles (**Fig. 1A**). Thus, we designated these RPE derived EVs as RMPs. NanoSight analysis showed significantly more RMPs released from ARPE-19 cells exposed to H_2O_2 compared to control non-stimulated cells (**Fig. 1B,** P<0.01).

2. RPE cells released more MPs under oxidative stress *in vitro* and *in vivo*.

To investigate whether old RPE cells are more sensitive to oxidative stress, we isolated and cultured primary RPE cells from 4-month-old or 24-month-old C57BL/6 mice. We defined age equivalence as 4-month-old C57BL/6 mouse corresponding to human age 25, and a 24-month–old mouse being equivalent to human age 69.[44] After exposure with H_2O_2, RMPs released from RPE cells were determined by FACS analysis measuring the amount of Annexin V^+ MPs released into media. H_2O_2 caused significantly more RMPs to be released from 24-month-old RPE cells compared to 4-month-old RPE cells (**Fig. 2A**). To investigate whether the antioxidant prevented oxidative stress-induced RMPs production, RPE cells were exposed to H_2O_2 with or

without antioxidant (U-83836-E, inhibitor of lipid peroxidation, a derivative of vitamin E). Under oxidative stress, RMPs in the culture medium with U-83836 were significantly less than the group without U-83836. This result suggested that oxidative stress is involved in RMPs production (**Fig. 2B**).

Several studies have shown that exposure to HQ, a potent oxidant, induced nonlethal bleb injury and sub-RPE deposits [29, 39, 45]. To investigate whether oxidants stimulate RMPs release *in vivo*, we analyzed the RMPs in the RPE/choroidal tissue of the mice exposed to HQ. Consistent with other groups finding, HQ exposure induced significantly more RMPs by two folds (**Fig. 2C**).

Retinal dysfunction, sub-RPE deposits, and thickened Bruch's membrane were detected in the $Sod1^{-/-}$ knockout mice deficient in Cu, Zn-superoxide dismutase (Sod1).[46-48] In these 12-month-old $Sod1^{-/-}$ mice, the average amount of RMPs released from the $Sod1^{-/-}$ mouse (n=7) were significantly higher than that from wild type control mice (n=7), Mean ± SEM, 655400 ± 277700 ($Sod1^{-/-}$) vs. 391000 ± 376200 (control) (**Fig. 2D**).

3. RMPs were taken up by RPE cells in a time-dependent manner.

To assess whether RPE cells take up RMPs, we first used the primary RPE cells in RPE/choroid explants. The DiI-RMPs bound or ingested RPE cells were detected by fluorescent microscopy. The presence of DiI-RMPs along the RPE cell membrane and in intracellular compartments was identified. Fluorescence intensity was increased over time (**Fig. 3A**). Similar findings on primary mouse RPE cells were observed *in vitro* on human ARPE-19 cells. Following exposure to DiI-RMPs, ARPE-

19 cells significantly increased DiI staining detectable by spectrofluorometry**,** and increased in a time-dependent fashion (**Fig. 3B**). Nonetheless, when the membrane scavenger receptor CD36 protein on RPE cells was blocked by CD36 specific antibody, the uptake rate was significantly decreased by 47% compared to control vehicle (**Fig. 3C**).

4. RMPs dose-dependently reduced RPE cell viability and suppressed cell proliferation.

To investigate the effects of RMPs on RPE cell growth, we exposed ARPE-19 cells to different concentrations of RMPs at different time points. The cell viability assay showed that RMPs dose-dependently reduced ARPE-19 cell viability (**Fig. 4A)**. The similar time-dependent reduction of cell viability with 20μg/mL RMPs was observed on primary RPE cells (see supplementary **Fig. S3**). Although RMPs at the dose of 10μg/mL did not significantly affect cell viability, they did remarkably suppress both ARPE-19 and primary RPE cells proliferation (**Fig. 4B**). Of special note, the inhibitory effects of RMPs on cell viability of both ARPE-19 and primary mouse RPE were comparable to those of RMPs derived from primary mouse RPE cells (see supplementary **Fig. S4**).

5. RMPs induced RPE cell cycle arrest in G_0/G_1 phase with an increased expression of p15 and p21.

We further evaluated the cell cycle kinetics of RPE cells by FACS. Relative to control, RMPs-exposed cells exhibited an increase in G_0/G_1-phase; conversely, the proportion

of RMPs-exposed cells in S-phase markedly declined (**Fig. 5A)**. We also performed gene expression studies to characterize specific mechanisms of this cell-cycle alteration. We found that mRNA levels of the cyclin-dependent kinase (CDK) inhibitors p15 and p21 were elevated approximately 3.1 and 4.7-fold respectively (**Fig. 5B**). Consistently, RMPs significantly augmented the protein levels of $p15^{INK4b}$ and $p21^{Cip1}$**,** as indicated by a 1.9- and 3.2-fold induction respectively **(Fig. 5C-D).**

6. RMPs induced RPE cell senescence.

Along with the observation that RMPs suppressed RPE cell proliferation, RMPs significantly increased SABG activity in both primary RPE cells (**Fig. 6A-B**) and ARPE-19 cells (**Fig. 6C-D**). The senescent cells are revealed by senescence-associated β-galactosidase (SABG) stained cells in blue (**Fig. 6A and 6C**). The prolonged incubation of RMPs resulted in more significant induction of senescence (**Fig. 6B and 6D**).

7. RMPs reduced phagocytosis activity.

RPE cells are phagocytic cells, and play a vital role in phagocytosis of the tips of the photoreceptor outer segments (POS) for maintaining structural and functional integrity of the retina.[49] To explore the influence of RMPs on RPE cell engulfment of POS, confluent monolayers of RPE cells were co-cultured with FITC-labeled POS for different times. The rate of phagocytosis was increased with incubation period of time (**Fig. 7A**). Nonetheless, RMPs exposure significantly decreased POS phagocytic activity of RPE cells (**Fig. 7B**).

8. Positive correlation between RMPs and lipids deposit in human eyes

RMPs amount in the RPE/choroidal tissue and drusen formation was measured in 35 human cadaver eyes. The finding showed a positive correlation (r= 0.85) between RMPs and drusen formation (**Supplementary Figure S5A**). Moreover in different analysis in age categorized donors (less than 65 and over 65 age), more RMPs (**Supplementary Figure S5C**) and more drusen (**Supplementary Figure S5B**) were found in the older age group (over age 65).

Discussion

Emerging evidence suggests that oxidative injury is a major factor involved in the pathogenesis of early AMD by stimulating RPE cell functional changes and inducing cell apoptosis.[47] Recent research work revealed that oxidative stress also induces RPE to release EVs in such a way as to participate in drusen formation.[18, 29] Non-lethal oxidative injury induced EVs releasing from RPE cells, and these EVs may be trapped between the RPE and its basal lamina as sub-RPE deposits.[29] In addition, Carver et al. also showed that membrane complement regulatory protein microparticles (mCRP-positive MPs) induced by oxidative stress may participate in drusen formation.[18] In line with this finding, we found that ARPE-19 cells released EVs under conditions of non-lethal dose of H_2O_2 stimulation, and that these EVs were MPs mixed with few exosomes. Although oxidative stress induced more EVs release, their size distribution is similar to that of EVs derived from non-oxidative stress condition. Interestingly, oxidative stress caused more RMP release from old primary mouse RPE cells than that from younger RPE cells, which suggested that aged RPE

cells may be more susceptible to oxidative damage than younger cells. The prevention of RMPs release due to oxidative injury by antioxidant U83836 further supports the concept that oxidative stress has primer effects for inducing RMPs release. The *in vitro* observation of oxidative stress stimulating RMPs production were further exhibited in the two mice models of oxidative stress, HQ feeding mice and $Sod^{-/-}$ knock-out mice, which suggested an *in vivo* pathophysiological relevant between oxidative stress and RMPs production.

A plausible role for RMPs in the pathogenesis of dry AMD has been suggested as a likely contributor to build-up of sub-RPE deposits, because of the observation that RMPs can carry components found in drusen, and oxidative stress could trigger or participate in drusen formation by releasing MPs from the RPE.[18, 29, 50] Recently, we have analyzed the RMPs amount in the RPE/choroidal tissue and drusen formation from 35 human cadaver eyes. The resulting data showed a positive correlation between RMPs and drusen formation, and more RMPs and more drusen presented in the old age group (over age 65). However, there is a distinct limitation of the present study of using human cadaver eyes due to the fact that the clinical history, ophthalmologic background, and even the clinical phenotype of AMD could not be defined in these donors.

In this study we addressed another important issue pertaining to oxidant-mediated injury of the RPE that may be accelerated by RMPs. EVs are potent biologic agents implicated in exchanging biological signals, interacting with target cells to induce responses.[51, 52] However, the pathological roles of RMPs cells in AMD are not yet well defined. We have demonstrated previously that microparticles derived

from lymphocytes elicit potent detrimental biological effects through endocytosis of endothelial cells.[31, 35] Here, we showed that RPE cells time-dependently take up RMPs. The membrane scavenger receptor CD36 is expressed in RPE cells. CD36 mediates the uptake of oxidized lipids, which has been implicated in the pathogenesis of AMD.[53] Taking into account the nature of RMPs with an abundance of membrane lipids, we postulated the involvement of CD36 in the RMPs uptake process. This hypothesis was supported by the blockage of CD36 decreasing RMPs uptake.

The taking up of RMPs consequently affected RPE cell fate by: 1) reducing RPE cell viability and cell proliferation in a dose-dependent manner; 2) inducing RPE cell cycle arrest at G0/G1 phase, with increases of the cyclin-dependent kinase inhibitors p15 and p21; 3) inducing RPE cell senescence; and 4) interrupting RPE cell phagocytosis activity. Most importantly, the effects of RMPs on RPE cell senescence and phagocytosis may suggest another implication of RMPs involved in early age-related pathological changes.

The accumulation of senescent cells in tissues is associated with aging and age-related diseases, such as AMD, which have been hypothesized to disrupt tissue structure and function.[54, 55] Although there is no direct clinical evidence of senescence RPE cells related to AMD, a study using of a progeroid mouse model provide evidence to support a causal link between p16Ink4a+ senescent cells and age-related pathologies.[56, 57] Suppression of phagocytosis of RPE cells may also interrupt the physiological process necessary to maintain the health and integrity of the neural retina and choriocapillaris.[9, 58, 59] Thus, these changes in RPE cells induced by RMPs favor accumulation of sub-RPE deposits after oxidant injury.

In conclusion, our findings strongly indicat that RMPs released by RPE cells under oxidative stress may accelerate RPE degeneration (a major feature of AMD). This study supported the RPE oxidant injury hypothesis in which oxidative stress promotes RPE cell release microparticles and accumulation of RMPs leads to RPE cells dysfunction consequently accelerating the subretinal deposits.[6] Importantly, the novel findings provide new information to better understand the pathobiology of dry AMD, which may help to develop preventive and therapeutic strategies for this age-related blinding disease.

Acknowledgments

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Author contributions statements:

S.S designed experiments, prepared figures and wrote manuscript.

C.Y designed experiments, wrote manuscript, edited main manuscript.

H.T helped for figures 1, 2 and 7 preparation.

J.L, S.C edited the manuscript.

P.H designed experiments and edited the manuscript.

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LEGEND

Figure 1. Oxidative stress promoted more extracellular microvesicles (EVs) released from ARPE-19 cells. (**A**). Representative particle size distribution and concentration of EVs released from RPE cells by Nanoparticle Tracking Analysis (NTA). ARPE-19 cells were exposed to 100 μM of H_2O_2 or PBS (control group) for 24 hours. After differential centrifugations, the EVs were collected and subjected to NTA. (**B**) The amount of EVs was expressed as the number of particles/10^6cells. Values were collected from 4 independent assays and expressed as mean ± SEM; $^{**}P<0.01$ vs control.

Figure 2. RPE cells released more MPs under oxidative stress. (**A**) Primary mouse RPE cells (passage 2, isolated from 4-month-old and 24-month-old C57BL/6 mice) were exposed to 100 μM H_2O_2 or PBS (control group) for 24 hours. MPs concentration was determined by FACS through measuring the amount of Annexin V^+ MPs released into media**,** and was expressed as the number of MPs/10^6cells. **P*<0.05, ***P*<0.01 vs control; +*P*<0.05 vs. 4-month-old RPE cells. (**B**) RMPs concentration was determined by FACS analysis after 24 hours exposure to H_2O_2 with or without antioxidant (5μM of U83836)**,** and was expressed as the number of MPs/10^6 cells. ***P*<0.001 vs. control, +*P*<0.05 vs. 24 h exposure to H_2O_2. (**C**) C57BL/6 female mice at 12-month-old received 0.8% HQ in their drinking water or regular drinking water for 45 days. The RMPs production in the RPE/choroidal tissue was detected by FACS analysis, and presented as particles/mg tissue. ***P*<0.001 vs. control. (**D**) The RMPs released from RPE/choroid of 12-month-old $sod1^{-/-}$ mice and control mice were characterized by FACS analysis with Annexin V and antibody against RPE65. The average amount of RMPs were presented as particles/eye (n=7 mice for each group). **P*<0.05 vs control. HQ, hydroquinone; WT, wild type.

Figure 3. RMPs were taken up by RPE cells in a time-dependent manner. (**A**) Representative fluorescent images of DiI-RMPs uptake by RPE cells of mice RPE/choroidal explants. Photos were taken 24, 72 and 96 hours after choroidal explants were incubated with 10 μg/mL of DiI-RMPs respectively (20x magnification). (**B**) Uptake of DiI-RMPs by human ARPE-19 cells was evaluated by spectrofluorometry after RPE cells were incubated with DiI-RMPs for indicated times, and presented as mean fluorescent intensity (MFI). (**C**) ARPE-19 cells were pre-exposed to CD36 antibody or control IgA, followed by 24 hours incubation with DiL-RMPs. The relative uptake was presented as percentage of control group (set as 100%).**P*<0.05 vs. IgA control.

Figure 4. RMPs dose-dependently reduced RPE cell viability and suppressed cell proliferation. (**A**) ARPE-19 cells were incubated with different concentrations of RMPs for indicated times. Cell viability was evaluated by MTT assay, and values were reported as a percent of non-exposed (control) cells. (**B**) ARPE-19 and primary RPE cells were incubated with 10 μg/mL RMPs for 48 hours, cell proliferation was evaluated by [^{3}H]-thymidine incorporation assay. Relative cell proliferation rates were determined and presented as percentage of control. *p<0.05, **p<0.01, ***p<0.001 vs. control.

Figure 5. RMPs induced RPE cell-cycle arrest in G_0/G_1 phase, accompanied by an increase of p15 and p21. (**A**) Representative graphs depicting cell-cycle progression in ARPE-19 cells exposed to RMPs or without RMPs. The percentage of cells in each cell cycle phase was presented as means ± SEM. *$P < 0.05$, **$P < 0.01$ vs CTL. (**B**) Gene expression in RMPs-exposed ARPE-19 cells was quantified relative to the housekeeping gene and presented as fold induction compared with control (CTL). (**C**) ARPE-19 cells were exposure to 10μg/ml of RMPs for 48 h, and p15 and p21 expression was detected by Western blot. (**D**) The protein levels were normalized to β-actin, and the control condition was set to equal 100%. *P <0.05 vs CTL. p15 and p21 are cyclin-dependent kinase inhibitors $p15^{INK4b}$ and $p21^{Cip1}$ respectively.

Figure 6. RMPs induced RPE cell senescence. Representative images of senescent mouse primary RPE cells (**A**) and senescent ARPE-19 cells (**B**) identified by senescence-associated β-galactosidase (SABG) staining after 48 hours RMPs exposure. Senescent cells were identified by the resulting blue reaction as indicated by arrows. (**C, D**) SABG positive cells were counted using phase-contrast microscopy after RMPs exposure for 24 and 48 hours, and presented as percentage of total cells in a bar graph. **p<0.001 vs. control.

Figure 7. RMPs reduced phagocytosis activity. (**A**) To measure the phagocytosis activity, ARPE-19 cells were incubated with FITC-POS at indicated time points and fluorescence intensity was recorded and presented as percentage of total fluorescence. (**B**) RMPs-pre-exposed RPE cells incubated to FITC-POS for 2 hours. Fluorescence intensity was determined and presented as percentage compared to control group (set as 100%), ***$p<0.001$ vs. Control.

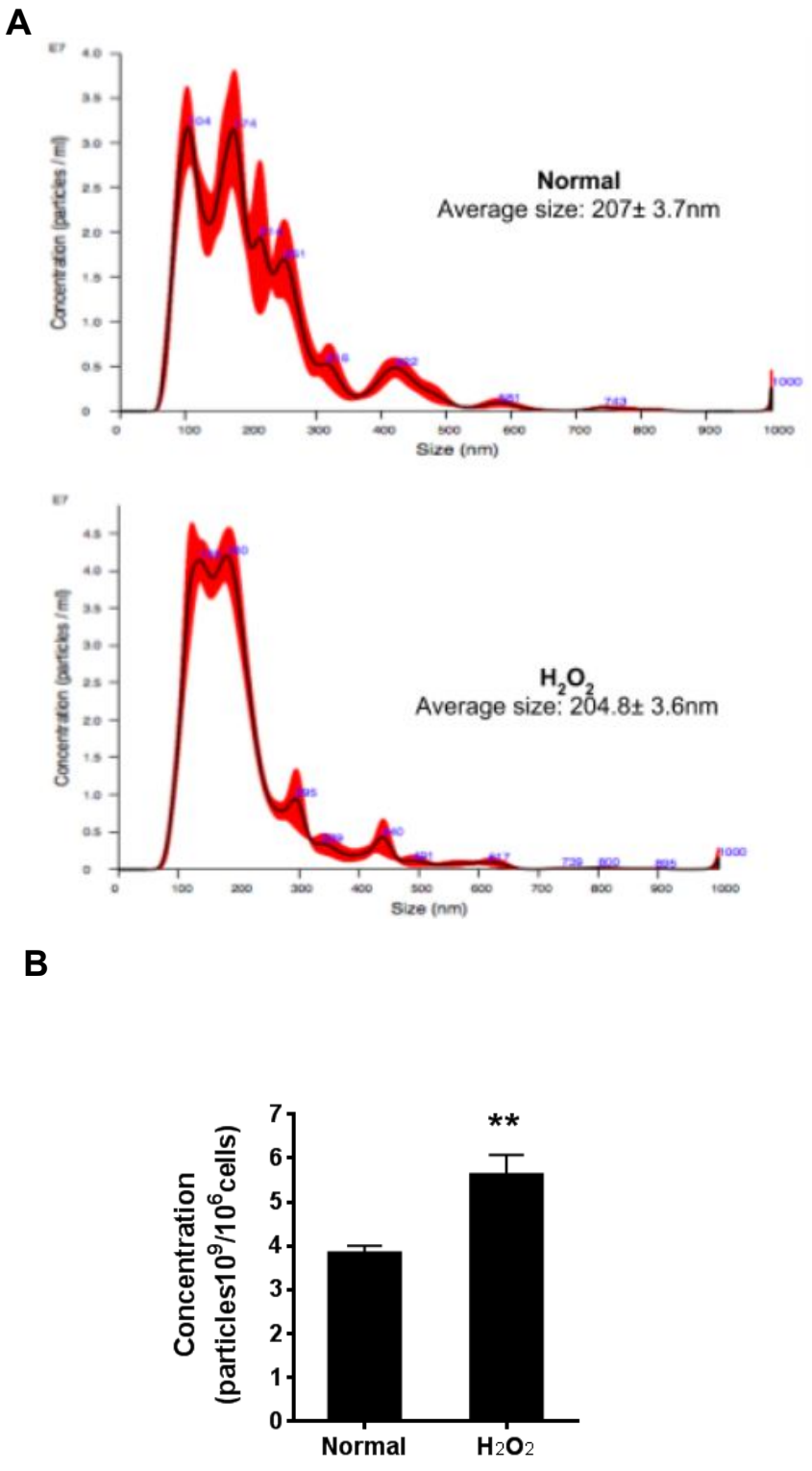


Figure 1. Oxidative stress promoted more extracellular microvesicles (EVs) released from ARPE-19 cells.

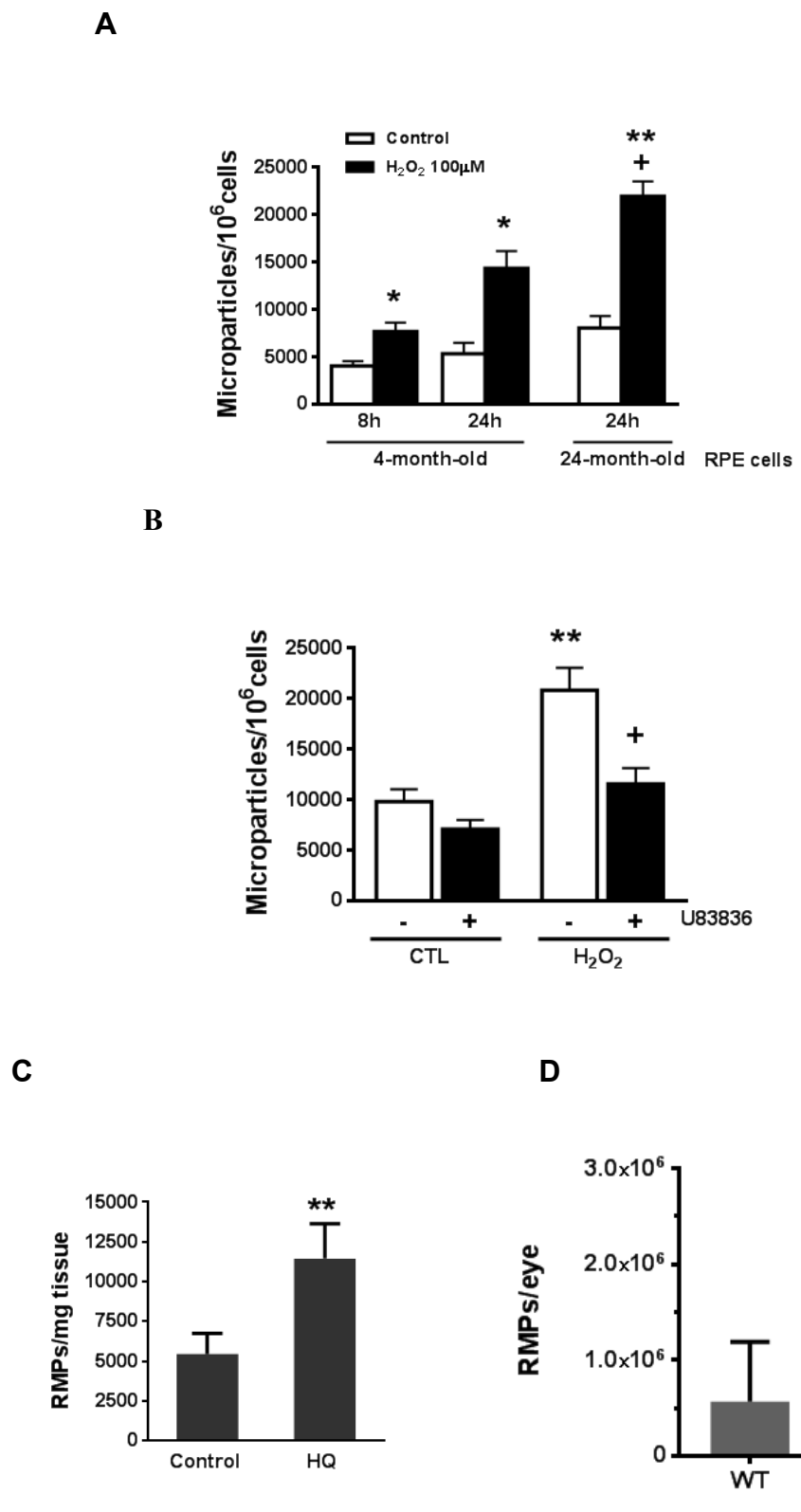
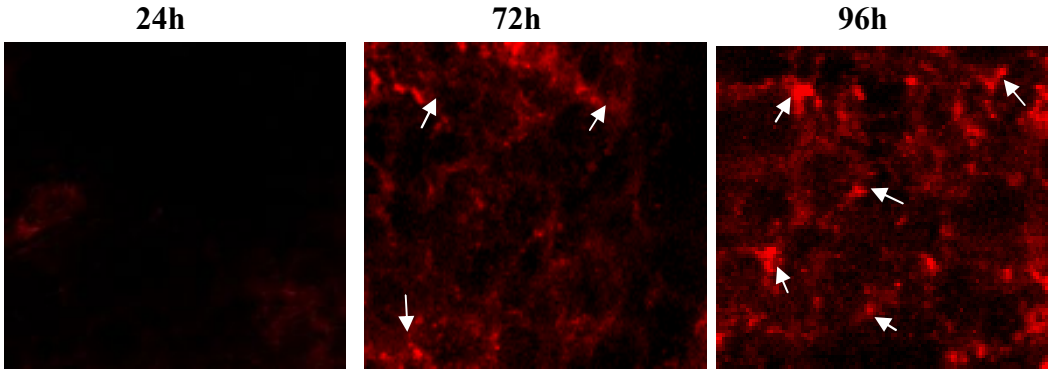


Figure 2. Oxidative stress induced RMPs release form RPE cells in vitro and in vivo.

A



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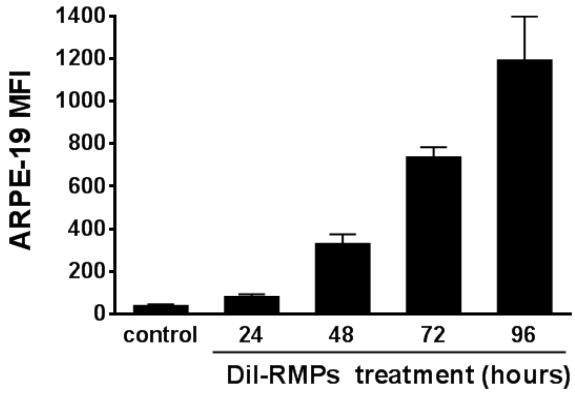

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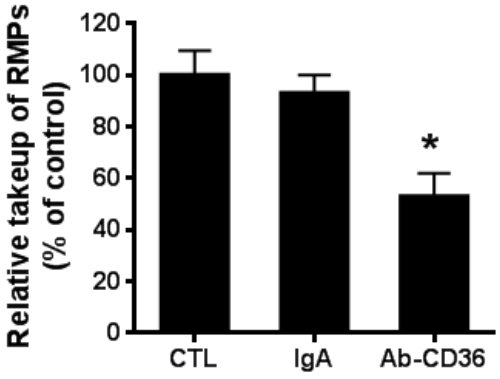
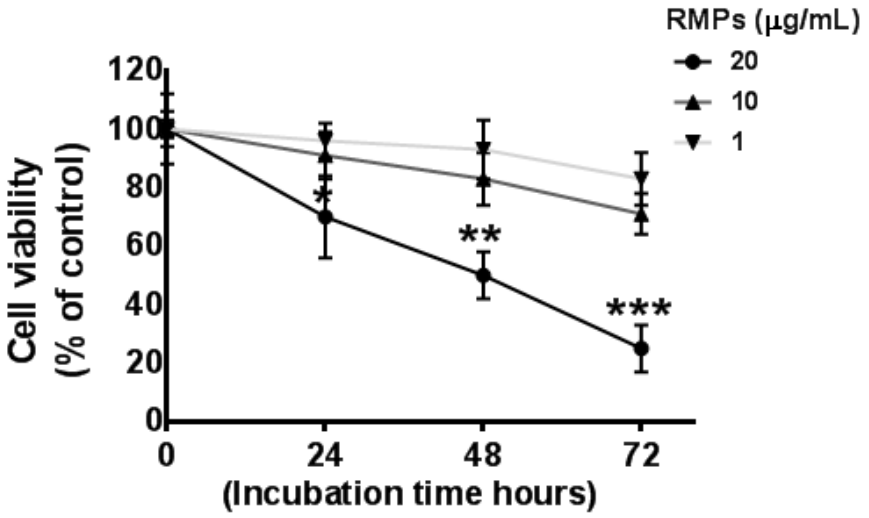


Figure 3. RMPs were taken up by RPE cells in a time-dependent manner.

A



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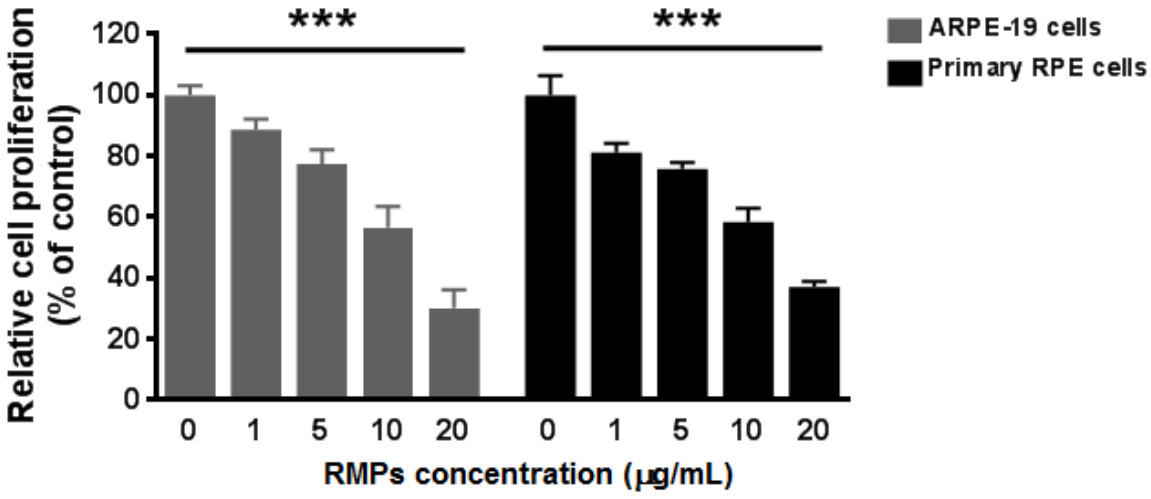


Figure 4. RMPs dose-dependently reduced RPE cell viability and suppressed cell proliferation.

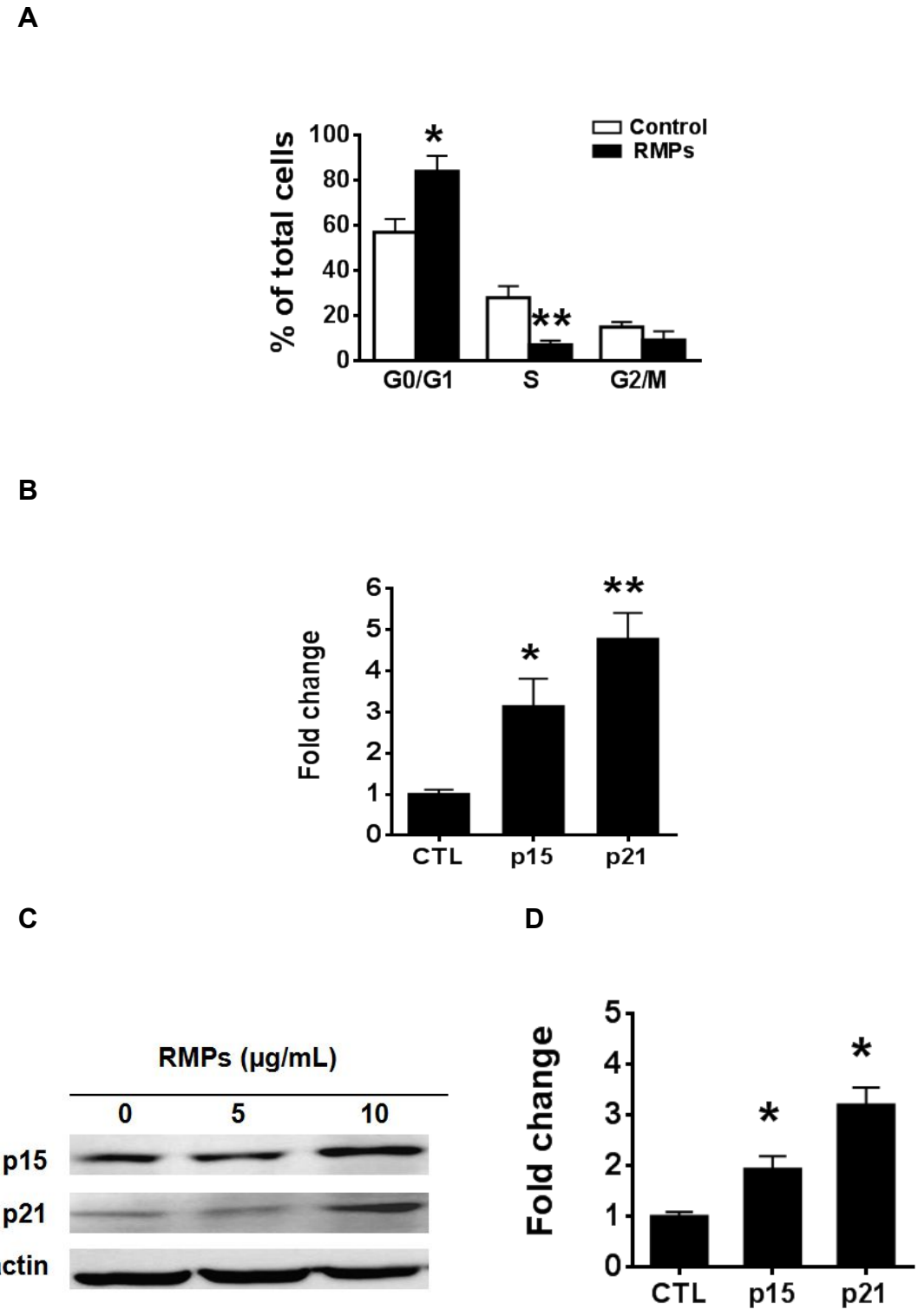


Figure 5. RMPs induce RPE cell-cycle arrest in G_0/G_1 phase which accompanied by an increase of p15 and p21.

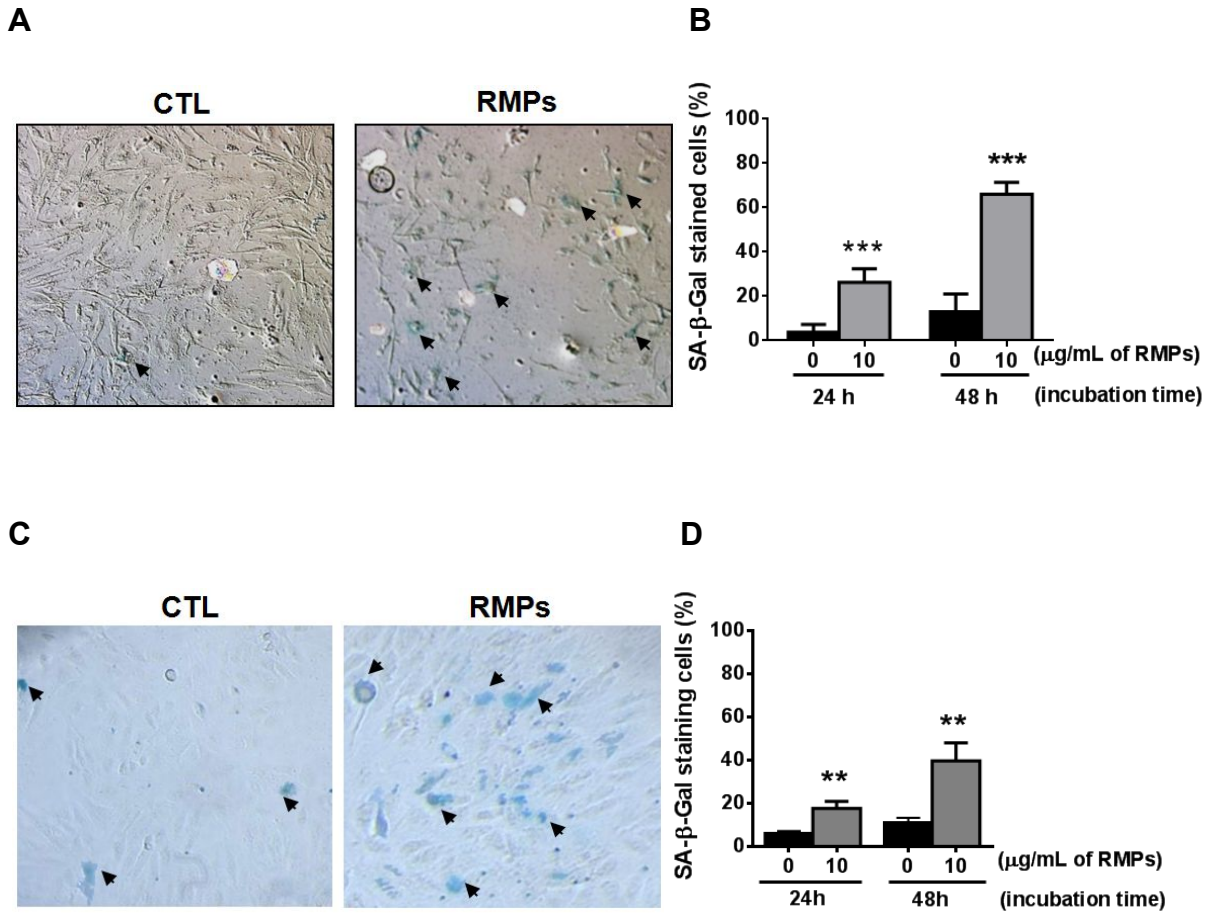
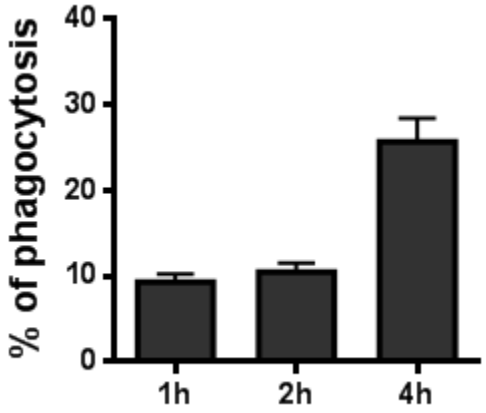


Figure 6. RMPs induced RPE cell senescence *in vitro.*

A



B

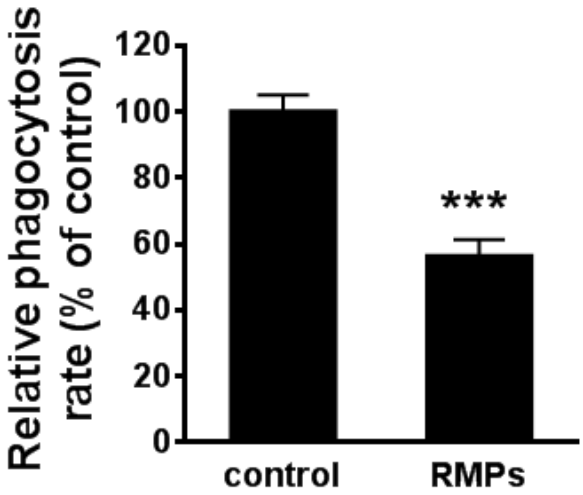


Figure 7. RMPs reduced RPE cell phagocytosis.

Supplementary Data

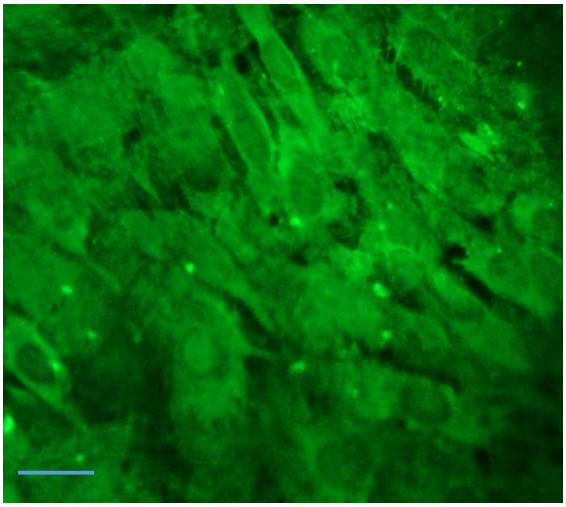
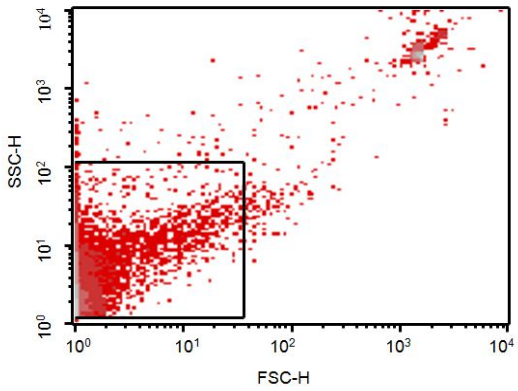


Figure S1. Characterization of primary RPE cells. Primary RPE cells were isolated from mouse pups and verified by RPE cell specific marker RPE65. Confluent primary RPE cells were fixed and stained with anti-RPE65 conjugated with Alexa Fluor 488 in green (Novus; NB100-355AF488). Bar, 50μm.

A



B

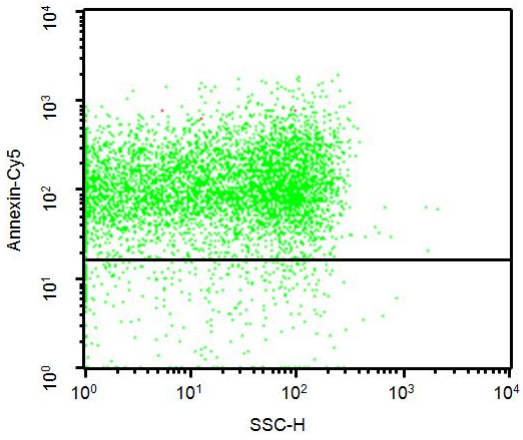


Figure S2. Characterization of RMPs by FACS analysis. (A) Determination of forward (FSC) and side scatter (SSC) characteristics with 1,0-µm beads used to gate RMPs. (**B)** Events at the RMPs gate were further assessed for labeling with annexin V-Cy5 to distinguish true events from electronic noise and thereby increase the specificity of RMPs detection.

RMPs: retinal pigment epithelium cell-derived microparticles.

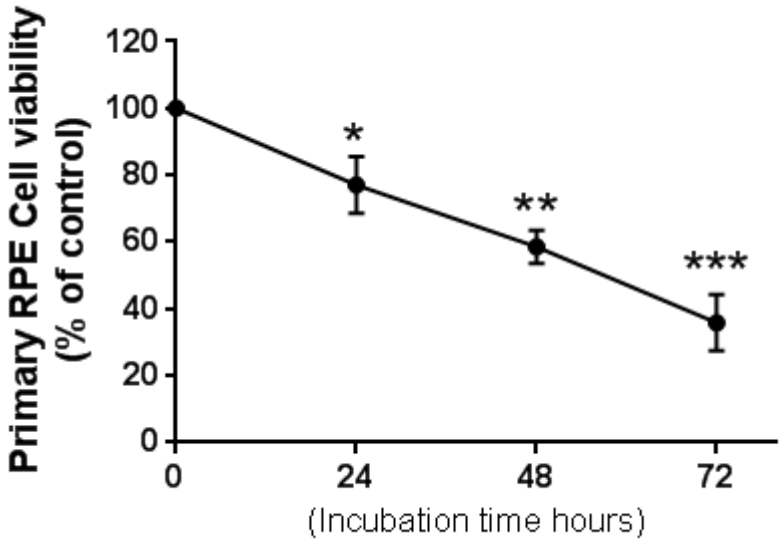


Figure S3. High dose of RMPs (20 μg/mL) reduced RPE cell viability in a time-dependently manner. The cell viability assay was performed after the primary RPE cells exposed to 20 μg/mL of RMPs for indicated hours. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs control.

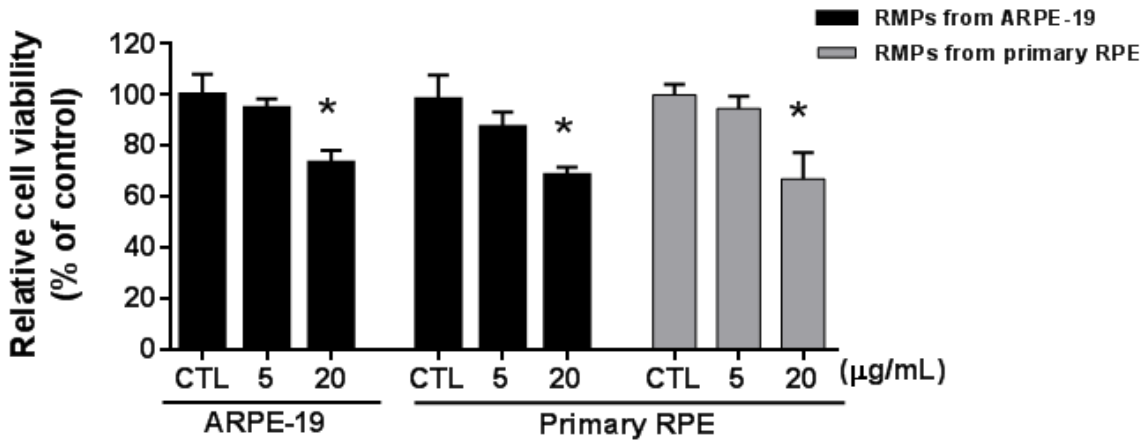


Figure S4. RMPs reduced cell viability of both primary RPE and human ARPE-19 cells. Primary culture mouse RPE cells or ARPE-19 cells were incubated with indicated concentrations of RMPs isolated from either ARPE-19 cells or primary mouse RPE cells under oxidative stress. Cell viability was evaluated after 24 hours incubation. Values were presented as a percent of control group. *$p<0.05$.

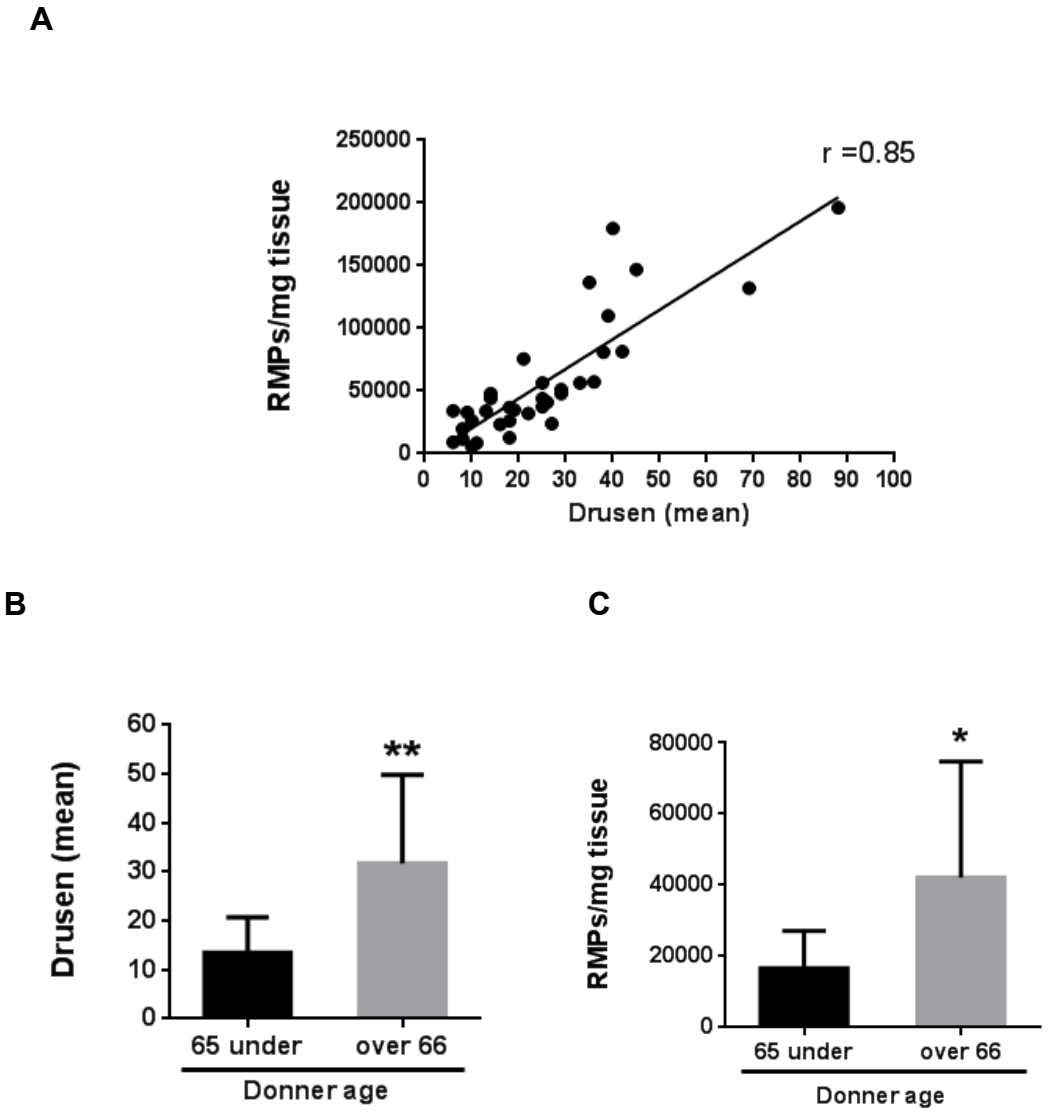


Figure S5. A. Positive correlation between RMPs and lipids deposit in human eyes. Pearson's correlation coefficient r = 0.85, p value <0.0001. **B** and **C. Average drusen counts and RMPs amounts in the two age groups, respectively.**

Methodology

The human cadaver eyes were obtained from eye banks in Québec and conducted according to protocol approved by the ethic Committee of CHU Sainte-Justine

(Montreal, QC, Canada). Research was carried out in accordance with the principles outlined in the declaration of Helsinki. All eyes were stored in a humid chamber after enucleation and transported to the laboratory. Total 35 individual eyes from donners age 65 under (n=12) and age over 65 (n=23) were obtained from the donors aged from 42 to 85 years old.

Briefly, the anterior segment of each eye was removed, and eye globe was flatted by four incisions crossing mid-macula. One quarter of the ocular containing macula was used to analyze RMPs, one quarter of macula lining flap tissue was sectioned and stained by Oil Red O (Sigme, O0625-25G) for labeling neutral triglycerides and lipids. DAPI staining was used to determine cell nuclei.

Note: There is a distinct limitation of the present study of using human cadaver eyes due to the fact that the clinical history, ophthalmologic background, and even the clinical phenotype of AMD could not be defined in these donors.

Article 2

The implication of miRNA let-7f in the degeneration of retinal pigment epithelium in vitro

The implication of miRNA let-7f in degeneration of retinal pigment epithelium in vitro

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Running Title: Pathophysiological role of let-7f in the RPE dysfunction

Word count: 2,425

Keywords: RPE-derived microparticles (RMPs), miRNA let-7f, oxidative stress, RPE cell dysfunction, senescence, dry-AMD

Abstract

Background: Age-related macular degeneration (AMD) is the leading cause of vision loss in the elderly. MicroRNAs play a major role in post transcriptional regulation while interfering in signalling pathways. In several vision diseases, miRNA dysregulation has been demonstrated; therefore the expression of the let-7 family of miRNA precursors in retina disorders is of interest. According to our previous study, the function of retinal pigment epithelium cells (RPE) is strongly affected by RPE-derived microparticles (RMPs). However, the active components of RMPs have not yet been identified. Through RNA sequencing, we found that the microRNA let-7f is one of the most abundant microRNAs in RMPs. This study was aimed at investigating whether let-7f is responsible for the development of RPE dysfunction and downstream consequences threatening vision health.

Methods: Cell viability assay and proliferation assay were performed to assess the effects of let-7f in RPE cells and to choose the pathophysiological dose of let-7f in RPE cells. Let-7f-transfected RPE cells were subjected to cell cycle analysis and senescence-associated β-galactosidase (SA-β-Gal) assays. Western blotting was used to evaluate protein expression. To investigate the inhibitory effect of let-7f inhibitor on RMPs, we subjected RMP-exposed RPE cells to viability assays and western blotting in the presence or absence of let-7f inhibitor.

Result: Our results indicated that let-7f (50 nM) adversely affected RPE cell growth and decreased the proliferation of RPE cells. This miRNA caused cell cycle arrest in G0/G1 and consequently resulted in a considerable increase in senescence-associated β-galactosidase activity and the expression of the senescence proteins

p15 and p21. In addition, the results of RMP and let-7f inhibitor exposure supported our data.

In conclusion, these findings suggest a potential role of let-7f as a mediator exacerbating RPE cell damage, which is likely to be followed by AMD.

Introduction:

Vision-related cell function is detrimentally compromised when aging results in an irreversible state aggravated by degenerative diseases such as age-related macular degeneration (AMD) [1].

Macular degeneration in the elderly is one of the most severe causes of blindness in developed countries [2]. To date, several therapies have been developed for advanced age-related macular degeneration (wet AMD), primarily neovascularisation inhibitors [3]. As expected, these treatments can be rationally applied in late stages in which vascularisation crosses the retina-blood barrier. Such therapeutics are generally expensive; therefore, early diagnosis at the time of onset (before neovascularisation) together with an ideal preventive strategy would be valuable to halt the progression to the wet type. Actually, there is neither an absolute cure nor a method to inhibit degeneration during the dry stage. The number of current therapeutics is expected to maintain life expectancy satisfactorily until more effective preventive treatments emerge [4].

Retinal epithelium malfunction can be epigenetically driven by non-coding RNAs affecting gene expression patterns, and/or gene translation [1]. MicroRNAs (miRNAs) are conserved short strands of 17–22 nucleotides that regulate cellular function post-

transcriptionally, in either proliferative or suppressive mode [5]. One of the highly conserved miRNAs is the let-7 family, whose members have a key role in gene regulation and cell senescence [6].

We provided the first demonstration that retinal pigment epithelium (RPE) cells take up microparticles, which are initially expelled under oxidative stress (unpublished data). The uptake of RPE-derived microparticles (RMPs) is a receptor-mediated process that consequently provokes host cell senescence or death. This finding strongly suggests that RMPs may exacerbate the oxidative damage to RPE cells, thus addressing the pathological role of RMPs in AMD [7]. To the best of our knowledge, RMPs contain nucleic acids whose sequencing profiles indicate miRNA enrichment. Moreover, among these microRNAs, let-7f is one of the most abundant. According to the literature, let-7f affects cell function in many cells other than those involved in vision [8-11]. Although the holistic effect of RMPs on macular degeneration is mainly accepted, we further postulate that let-7f may mediate the influence of RMPs on RPE cell functions and AMD pathogenesis.

Methodology:

ARPE19 cells were purchased from the ATCC (CRL-2302™). At passage two, the cells were seeded in a T75 flask and cultured until confluent at 37°C under 5% CO_2. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% fetal bovine serum (FBS) from Gibco.

For isolating RPE primary cells, newborn C57BL/6 mice were purchased from Charles River Laboratories (St. Constant, QC, Canada). Procedures were performed

in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The Animal Care Committee of CHU Sainte-Justine (Montreal, QC, Canada) approved the protocol. The miRNA mimic let-7f (has-let-7f-5p) was obtained from Ambion (USA, TX) in the amount of 5 nmol. The miRNA inhibitor of let-7f (has-let-7f-5p) was purchased from Ambion (USA, TX) in the amount of 5 nmol.

To confirm the results, most of the experiments were verified in primary RPE cells in addition to the ARPE-19 cells.

Primary RPE isolation and culture

Primary RPE cultures were obtained as described previously from 9-day-old mouse pups [12, 13]. The animals were euthanized, and their eyes were enucleated and kept overnight at room temperature in DMEM (Gibco, CA) in the dark, then incubated for 45 minutes with 2 mg/mL trypsin/collagenase I at 37°C in a water bath. After neutralization with DMEM containing 10% FBS, RPE cells were isolated. RPE cells were plated in six-well LabTek plates (Nunc; Thermo Fisher Scientific, Inc., New York, NY) with RPE from one eye per well in DMEM containing 10% FBS and antibiotics (100 units/ml penicillin G and 100 mg/ml streptomycin sulfate, Gibco) [14]. To confirm the purity, cells were stained at 100% confluence with anti-RPE65 conjugated with Alexa Fluor 488 (Novus; NB100-355AF488) after fixation by paraformaldehyde (PFA) 4% in phosphate buffered saline (PBS).

RMP isolation and characterization

Induction of RMPs was performed as previously described with modifications [15]. Briefly, ARPE-19 cells were plated on plates coated with collagen IV/laminin and grown to confluence. Cell growth was reduced through medium containing sequentially decreasing FBS for 6 days. On day 7, the cells were exposed to 10 microunits of myeloperoxidase (MPO) for 90 minutes, then exposed to hydrogen peroxide (H_2O_2) at 100 μM for 24 h. At the end, RMPs were isolated as described previously [16]. The RMPs were then characterized by annexin V staining [17]. Protein concentration was measured with the Bradford method [18].

Viability

RPE cells (0.2×10^6) in the second passage were cultured in a 1:1 (vol/vol) mixture of DMEM and Ham's F12 medium (Gibco, CA) containing 3 mM L-glutamine, 10% FBS (Gibco, CA), and antibiotics (100 units/ml penicillin G and 100 mg/ml streptomycin sulfate, Gibco, CA). Cells were grown to 70% confluence in 24 well plates (Nunc; Thermo Fisher Scientific, Inc., New York, NY) and then were transfected with different doses of the miRNA mimic let-7f (25 nM and 50 nM) via a TransIT-X2 dynamic delivery system (Mirus CO, USA).

In the other experiment, cells were transfected with miRNA let-7f (50nM) with a TransIT-X2 dynamic delivery system (Mirus CO, USA); or RMPs (10 μl/ml) isolated in our lab as described previously; or first expsosed to RMPs (10 μl/ml) and then transfected with let-7f inhibitor (50 nM).

After 48 h, cell viability assay was assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Among chromogenic viability assays, the MTT assay is the most commonly used method [19]. Then 0.5 mg/mL MTT was added to each well, and the cells were incubated at 37°C for 2 h.

Formazan extraction was performed with isopropanol, and the amount of formazan was determined colorimetrically with a spectrophotometer (CLARIOstar, BMG Labtech) at 600 nm for each group; three separate samples were collected and measured [20].

Proliferation determined by [3]-thymidine incorporation assays

ARPE-19 cells (4×10^4) were seeded onto 24-well plates. After 24 h, the cells were transfected with miRNA let-7f (25 nM, 50 nM), exposed to [^{3}H]-thymidine (1 µCi/ml) and incubated. After 48 h of incubation, we aspirated the medium, washed the tray with cold trichloroacetic acid (TCA 5%), and lysed the cells. Finally, thymidine incorporation was determined through scintillation counting (PerkinElmer, Waltham, MA, USA) [21].

Cell cycle analysis

ARPE-19 cells were cultured in 35-mm tissue-culture dishes (0.25×10^6 cells/dish). The culture medium was replaced with new medium without FBS to synchronize them after 24 h. On the third day, cells were transfected with the miRNA mimic let-7f (50 nM) through TranseIT-X2 dynamic delivery in DMEM/F12 with 10% FBS for 48 h. After exposure, the cells were collected, washed with PBS, fixed in 70% ethanol and

maintained on ice for at least 1 h after a minimum of 1 h at -20°C. After two more washes with PBS, the cell pellets were stained with propidium iodide (PI) solution containing PBS, 50 µg/mL PI, and 10 µg/ml DNase-free RNase. The DNA fluorescence of PI-stained cells was measured by excitation at 488 nm and monitored through a 630/22-nm band pass filter with flow cytometry. A minimum of 10,000 cells was analyzed per group, and the DNA histograms were gated and further analyzed with software to estimate the percentages of cells in different phases of the cell cycle [22].

Senescence-associated β-galactosidase assays

Senescence-associated β-galactosidase is a hydrolase enzyme that catalyzes the hydrolysis of β-galactosides into monosaccharides only in senescent cells.

Cells were trypsinized at passage 3 and seeded into 24 well plates on sterile coverslips, which had been incubated with 200 µL attachment factor for 1 h. Cells were seeded at 60,000 cells per well in 0.5 ml DMEM and 10% FBS. After 24 h of incubation at 37°C under 5% CO_2, cells were transfected with serum-free medium (OptiMEM) and 50 nM let-7f miRNA (10 µM stock) with the TransIT-X2 dynamic delivery system. Then, 48 h later, according to the protocol described by Eccles, M et al [23], ARPE-19 cells were stained for SA-β-galactosidase to investigate the effect of the miRNA let-7f on RPE cells. Stained cells were observed under an inverted microscope for the development of blue-green color. For quantification, images were taken with phase-contrast microscopy, and positive cells were counted.

Western blot analysis

ARPE-19 cells were collected after 48 h exposure to RMPs with or without let-7f inhibitor or with let-7f alone. Extraction of soluble proteins and protein expression by SDS-PAGE was performed according to Yang et al. [16]. Briefly, samples were loaded (50 μg/mL) on 18% separating gel, and after being blocked with 5% milk, the membrane was exposed to primary antibodies including anti-p15 INK4b unconjugated rabbit polyclonal antibody (LifeSpan BioSciences, Inc. 1:500) and anti-p21 rabbit polyclonal antibody (Elabscience, USA; 1:1000), or anti-β-actin monoclonal unconjugated antibody (Novus, CA; 1:2500) as a loading control. Subsequently, goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, CA; 1:5000) was used to detect the protein levels of p15 and p21. Proteins were detected with an ECL western blotting detection system (PerkinElmer, Inc.) via Image Quant LAS 500, and densitometry values were quantified in Image J software.

Statistical analysis

Results are shown as means ± SEM from at least three independent experiments performed in duplicate or triplicate. The t-test was used to define the significant differences between two groups. One-way ANOVA was used for comparison of the means from three or more groups. Statistical significance was set at $p < 0.05$.

Results:

Let-7fs' ffect on RPE cell viability To investigate the effect of miRNA let-7f on RPE cells, we exposed cells with different doses of let-7f and found that 48 h incubation with 50 nM let-7f significantly decreased RPE cell growth (Figure 1A).

Because let-7f is one of the most abundant miRNAs in RMPs, we examined the inhibitory effect of let-7f via let-7f inhibitor exposure in RPE cells exposed to RMPs. After 48 h incubation, cell viability assays showed that the inhibition of let-7f partly prevented RPE decreased viability induced by RMPs (Figure 1B).

Let-7f suppressed RPE cells proliferation.

Along with the observation of the effect of let-7f on RPE cell viability, the miRNA let-7f at 50 nM concentration significantly suppressed proliferation (Figure 2).

Let-7f caused cell cycle arrest at G0/G1.

To explore the anti-proliferative effect of let-7f on RPE cells, we observed its effect on cell cycle circulation via flow cytometry analysis. The cell cycle showed no significant change in the percentage of cells in G2/M after exposure to 50 nM let-7f for 48 h, whereas the percentage in G0/G1 phase increased significantly in the 50 nM groups. There was also a significant decrease in S phase in the 50 nM groups compared with the control group (Figure 3).

Let-7f induced senescence in RPE cells.

After understanding the effect of let-7f on cell cycle arrest, we considered very likely that RPE cells undergo senescence and aging. Therefore, to detect senescence, we analyzed senescence-associated β-galactosidase. Compared with the control group, the transfected group showed more aged cells; thus, let-7f caused senescence in RPE cells (Figure 4A, 4B).

Age-related protein expression increased in RPE cells exposed to let-7f.

When the cell cycle arrested at G0/G1 and subsequent senescence increased, we reasoned that the expression of senescence proteins would also increase. We found that let-7f induced the expression of the cyclin dependent kinase inhibitors p15 and p21 (Figure 5A). In contrast, let-7f inhibitor exposure suppressed the effect of RMPs and decreased the expression of the cyclin dependent kinase inhibitors p15 and p21 (Figure 5B).

Discussion:

Given our previous results explaining the effects of RMPs on RPE cell dysfunction, we performed RNA sequencing of RMPs and then determined let-7f abundance in RMPs. Our present results showed that let-7f represses cellular growth and proliferation in a dose dependent manner, and this effect is followed by cell cycle arrest in G0/G1 phase and an increase in the cyclin-dependent kinase inhibitors p15 and p21. Together, these changes may finally result in senescence as a deleterious consequence. We reasoned that exposure to the let-7f inhibitor might potentially attenuate RMPs' interference with cell cycle signals. As expected, inhibitor exposure

decreased the expression of p15 and p21 proteins. These proteins and their relatives are implicated in cell senescence, the potential fate of tumor cells that fail to proliferate as well as some other cells; consequently, they may serve as biomarkers to detect the senescence phenotype [24, 25].

Oxidative stress originating from reactive oxygen species is the foremost causes of the RPE cell dysfunction that triggers AMD. In particular, oxidative stress has been shown to induce RPE cell senescence and degeneration [26-29]. In addition, we demonstrated that oxidative stress leads to formation of RMPs, which in turn give rise to RPE dysfunction (unpublished data).

Extracellular vesicles (EVs) are categorized primarily into exosomes, microvesicles, and apoptotic bodies, depending on their size and approach they have for release. Microvesicles (also termed microparticles (MPs) or ectosomes) form by changing the phospholipids of the membrane and shed as blebs; they are 100–1000 nm in diameter. MPs can be released to the extracellular milieu by different cells from various tissues, including epithelial cells in the retina [26, 30, 31].

Microvesicles contain varied groups of biomolecules, including proteins, lipids, and nucleic acids such as DNA, RNA, mRNA, and miRNA [32]. miRNAs are small regulatory RNAs implicated in post transcriptional regulation [33]. Signalling pathways regulating aging phenotypes may be a potent axis affected by miRNAs. Moreover in the response to cellular stress injury, miRNAs can switch the cellular program from replication to senescence while targeting specific mediators [5, 34, 35].

One of the well-known miRNAs families is let-7, which is thought to potentially function in many tissues such as the retina [36]; let-7 has preserved activity in aging

development and is highly expressed in aged tissues [6]. Let-7 family members play a key role in many disorders such as cardiovascular disease, cancers, and obesity-related and age-related diseases [6, 8, 36-38].

Wagner et al. have shown that among the let-7 family members, let-7f is one of the senescence-associated miRNAs [39, 40]. Several studies have shown that let-7f downregulates cells by suppressing proliferation and leads to cell cycle arrest and senescence, thus suggesting that it may be used as a biomarker in some diseases [41-44]. The discovery of these short sequences of nucleic acids may open new windows into therapeutic opportunities via identifying the signaling pathways involved in pathophysiological fate [45]. In conclusion, our findings indicate that the detrimental effects of MPs on RPE cells may be attributable to the miRNA let-7f. This hypothesis may contribute to improving the treatment strategies beyond those that already exist. However, this finding must be further examined in vivo to determine its clinical relevance.

Author contributions statements:

S.S designed experiments, prepared all figures (1-5) and wrote main manuscript text.

C.Y designed experiments and edited main manuscript text.

C.G helped for figure 5 preparation and analysis.

P.H designed experiments and edited the manuscript.

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LEGENDS

Figure 1. Let-7f dose-dependently reduced RPE cell viability. (A) RPE primary cells were co-incubated with different concentrations of let-7f for 48 h. Cell viability was measured with MTT assays and is reported as a percentage of nonexposed (control) cells. A 50 nM concentration, compared to control, significantly decreased cell viability. **(B)** RPE primary cells were incubated with let-7f (effective dose: 50 nM) or RMPs (10 µg/ml) with or without let-7f inhibitor for 48 h, cell viability was evaluated with MTT assays, and the results were compared. Let-7f and RMPs resulted in a significant decrease in viability, whereas let-7f inhibitor neutralized the effect of RMPs. ****p <0.0001, ++++p <0.0001, ~~~p <0.0003 vs. control.

Figure 2. Let-7f suppressed RPE cell proliferation in a dose dependent manner. RPE primary cells were incubated with different concentrations of let-7f for 48 h, after which cell

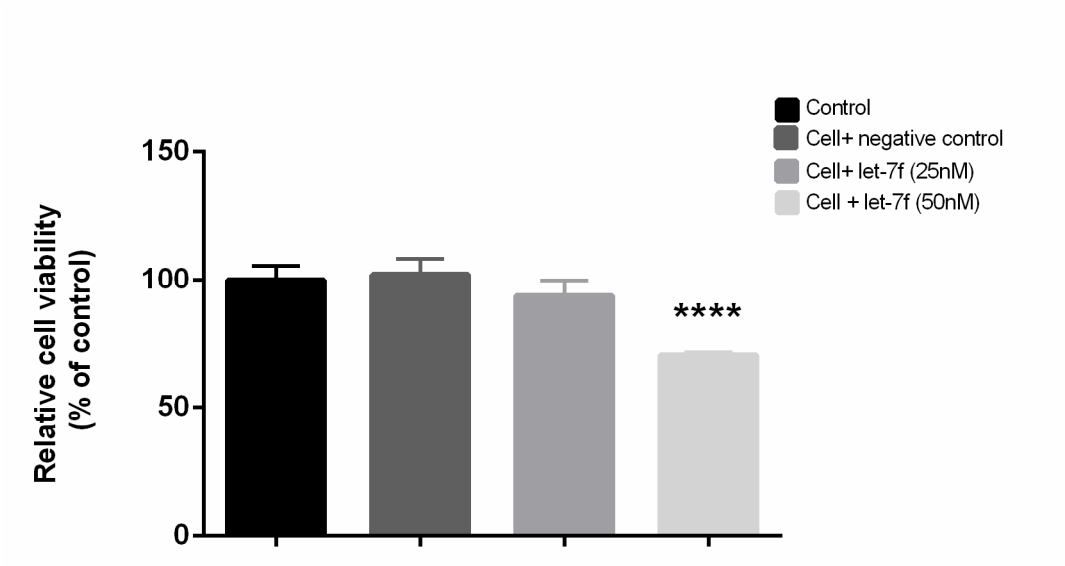
proliferation was evaluated with [3H]-thymidine incorporation assays. Cell proliferation rates were determined and are presented as a percentage of the control. As indicated, let-7f at 50 nM suppressed proliferation. **p<003 vs. control.

Figure 3. Let-7f resulted in cell cycle arrest at G0/G1. Representative graphs depicting cell-cycle progression in RPE primary cells transfected with different doses of let-7f. The percentage of cells in each cell cycle phase is presented as the mean ± SEM. Let-7f at 50 nM arrested the RPE cells in G0/G1 phase and decreased the number of cells in S phase. *P < 0.011, **P < 0.0076 vs. control.

Figure 4. Let-7f induced senescence in RPE cells. (A) Representative images of mouse senescent primary RPE cells identified by senescence-associated β-galactosidase (SA-β-Gal) staining after 48 h exposure to let-7f (50 nM). Senescent cells were identified according to blue color formation, as marked by arrows. (**B**) SA-β-Gal positive cells were counted with phase-contrast (DIC) microscopy and are depicted as a percentage of total cells in the bar graph. **p<0.0062 vs. control.

Figure 5. Expression of cyclin dependent kinase inhibitor proteins (CDKI; p15 and p21) increased in RPE cells exposed to let-7f. (A) ARPE-19 cells were exposed to 50 nM of let-7f for 48 h, and p15 and p21 were characterized by western blotting. The protein levels were normalized to those of β-actin, and the control condition was set to 100%. **p < 0.003, ***p < 0.0002 vs. control. p15 and p21 are the cyclin-dependent kinase inhibitors p15INK4b and p21Cip1, respectively. **(B)** ARPE-19 cells were transfected with RMPs (10 μg/ml) with or without let-7f inhibitor (50 nM) for 48 h, and p15 and p21 expression was measured by western blotting. The protein levels were normalized to those of β-actin, and the control condition was set to 100%. **p <0.0078, *p <0.0208, ****p <0.0001, ++p <0.0058 vs. Control.

A



B

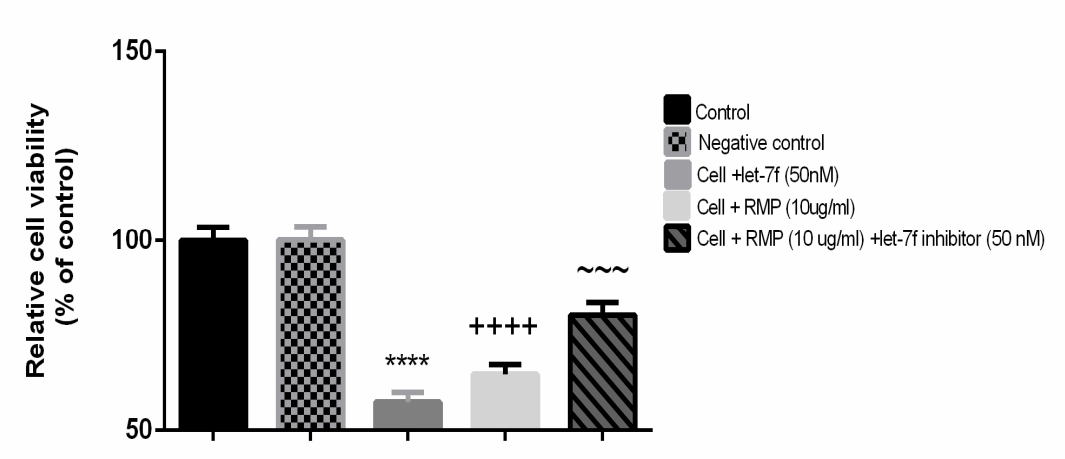


Figure1. Dose dependent effect of let-7f in RPE cell viability.

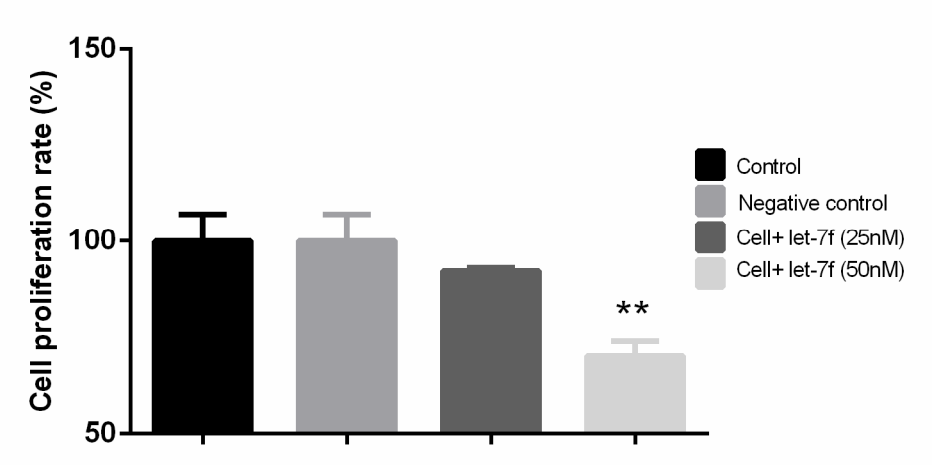


Figure2. Let-7f suppressed RPE cell proliferation in dose dependent manner.

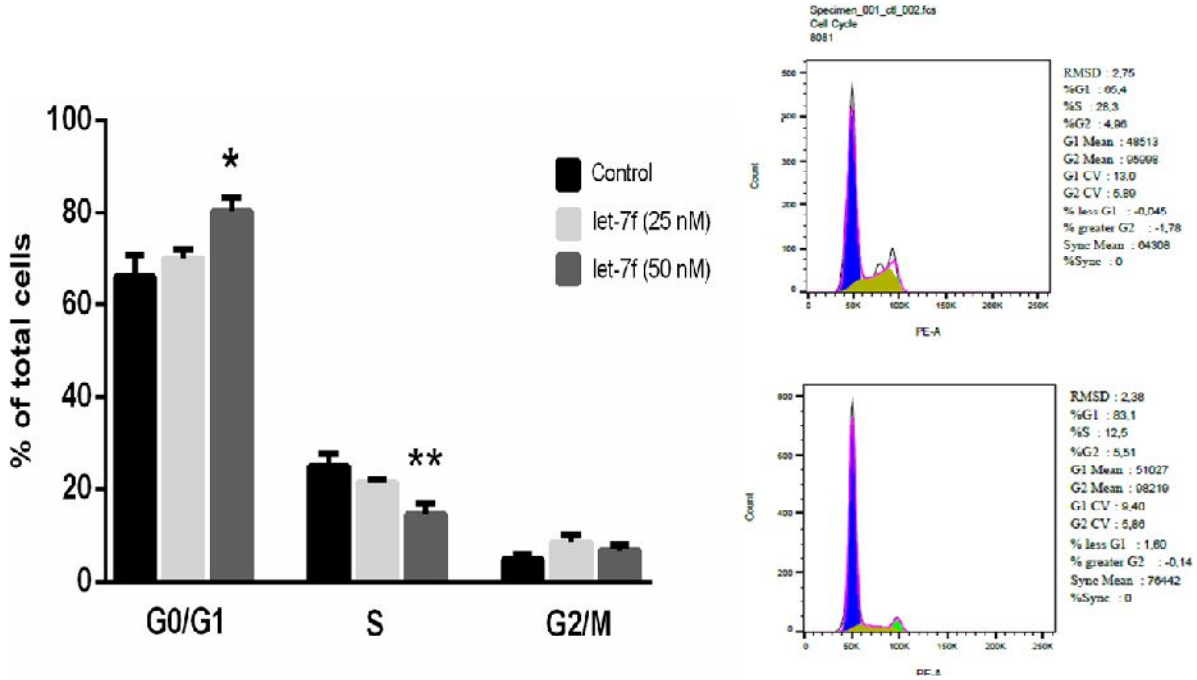


Figure3. Cell cycle arrested at G0/G1 under the effect of let-7f.

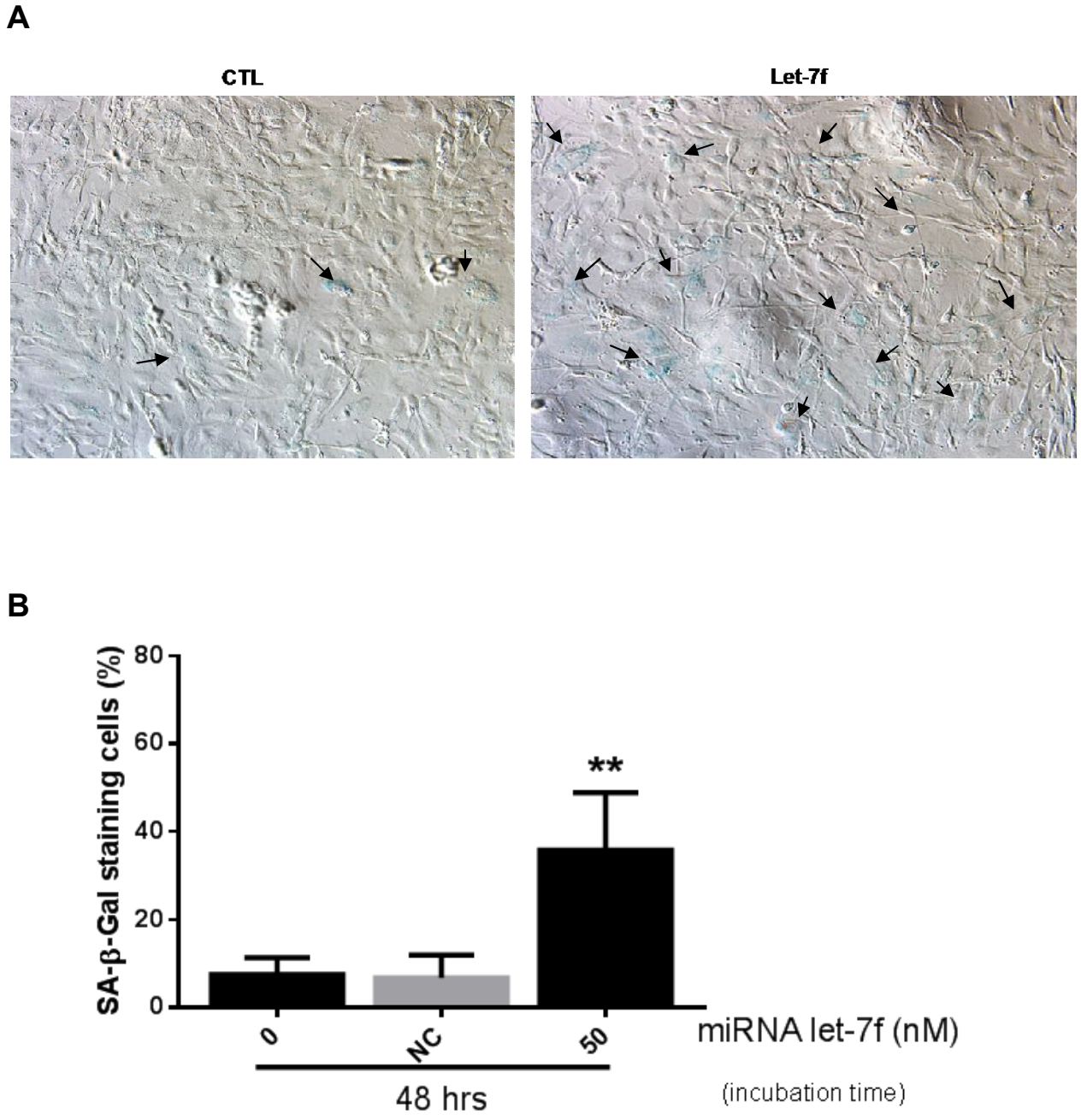


Figure4. Let-7f induced senescence in RPE cells.

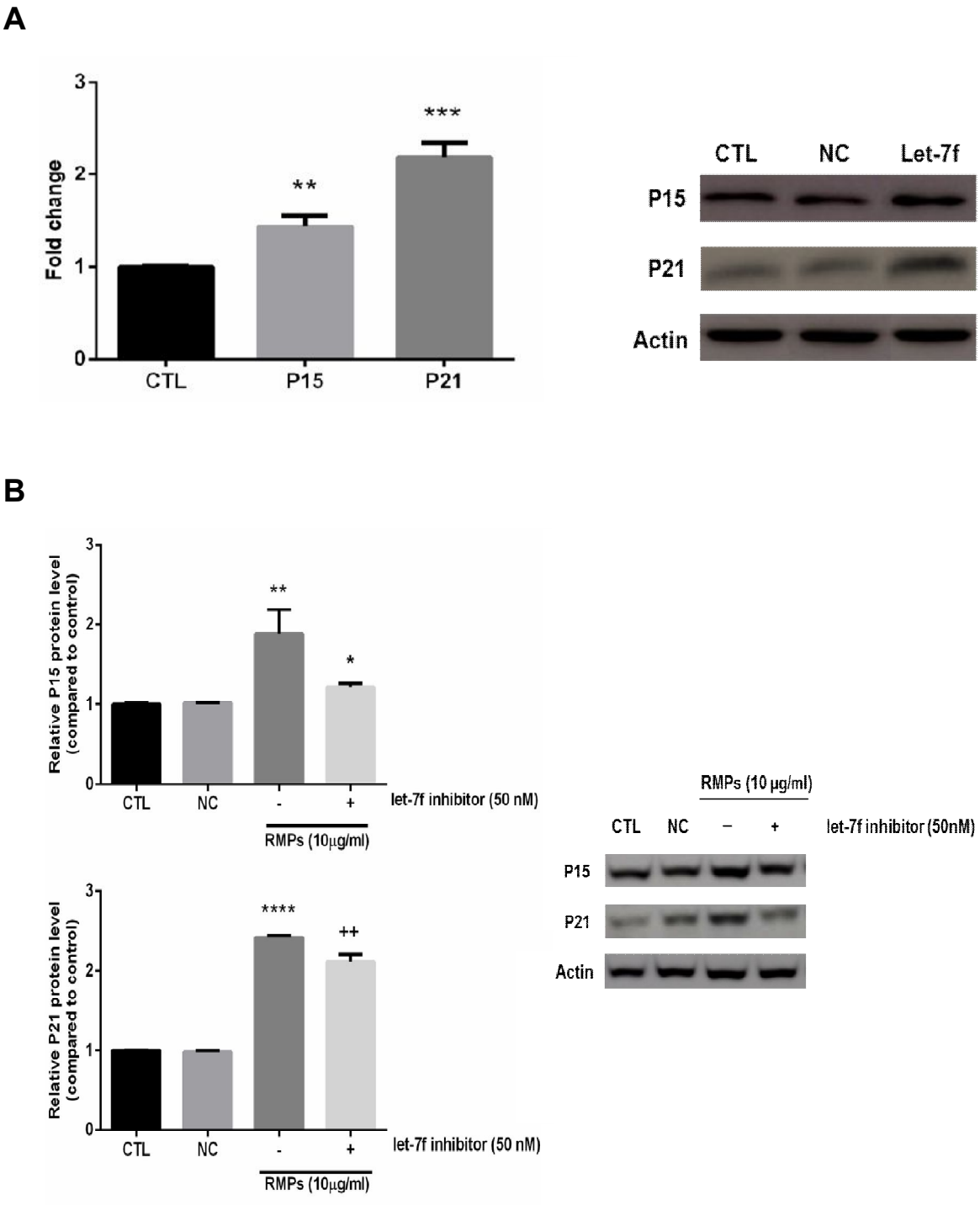


Figure5. Cyclin dependent kinase inhibitor proteins (CDKI; p15 and p21) expression increased in RPE cells exposed to let-7f.

Discussion

Aging is an inevitable process that may become a risk factor in the development of a variety of degenerative disorders and morbidities in the elderly, thus primarily resulting in the disruption of a wide range of physiological functions. Emerging evidence suggests that oxidative stress originating from reactive oxygen species is an underlying factor that is involved in the pathogenesis of early AMD and disrupts RPE cell function and promotes cell apoptosis [135]. In particular, oxidative stress has been shown to induce RPE cell senescence and degeneration [136-139].

Recent research has revealed that oxidative stress also causes RPE to release EVs, thereby participating in drusen formation [115, 136] . Non-lethal oxidative injury leads to shedding of RPE-derived vesicles, which may become trapped in the RPE and basal lamina interface and gradually form RPE deposits [115]. In addition, Carver et al. have shown that oxidative stress gives rise to MPs carrying membrane complement regulatory protein MPs (mCRP-positive MPs), which have widely been postulated to contribute to drusen formation [136]. Along these lines, we demonstrated that ARPE-19 cells release EVs under a non-lethal dose of H_2O_2 stimulation as an oxidative stress; these EVs were MPs mixed with a few exosomes.

EVs, also called microvesicles, MPs, or exosomes, have been proposed to participate in diverse functions in health and disease, thus explaining the intense research focus on EVs. The study of EVs is an emerging field across many disciplines that has included many research and development efforts on their biogenesis and their roles in intercellular communication by transporting biomolecules between close and distant cells, and in normal and disease physiology. In both healthy and pathological states,

EVs remove protein waste. They also mediate intercellular communication among tissues and reflect the state of the body [87, 97, 119, 136, 139].

As we have shown in the first article, oxidative stress induces shedding of EVs, and their size distribution is similar to that of EVs derived under non-oxidative stress conditions (article 1, **Fig. 1**). Interestingly, oxidative stress caused more RMP release from old primary mouse RPE cells than that from younger RPE cells, thus revealing that aged RPE cells might be more susceptible to oxidative injury than younger cells. Because, through exposure to the antioxidant U83836 (article 1, **Fig. 2A-B**), we successfully prevented RMP release after oxidative injury, we believe that oxidative stress has a priming effect that triggers RMP release.

The *in vitro* observation of oxidative stress stimulating RMP shedding was further observed in two mouse models of oxidative stress: hydroquinone (HQ) fed mice and $Sod^{-/-}$ knock-out mice (article 1, **Fig. 2C, D**), thus suggesting a potential pathophysiological causality between oxidative stress and RMP production.

In the pathogenesis of dry AMD, RMPs have been suggested to be a likely contributor to the build-up of sub-RPE deposits, because of the largely similar content of RMPs and drusen components; moreover, oxidative stress may trigger or participate in drusen formation by releasing MPs [115, 119, 136]. Recently, we have analyzed the RMP amounts in RPE/choroidal tissue and drusen formation from 35 human cadaver eyes. The results showed a positive correlation between RMPs and drusen formation (article 1, see supplementary **Fig. S5A**). Thus, RMPs and drusen have increased prevalence in older individuals (≥ 65 years) (article 1, see supplementary **Fig. S5B-C**). However, a distinct limitation of the study on human cadaver eyes was the lack of

clinical history and ophthalmologic background for most samples. It was also difficult to determine the clinical phenotype of AMD.

In this study, we addressed another important issue that explains how oxidant-mediated injury in the RPE may be exacerbated by RMPs. EVs are potent biological agents that have been implicated in biological signals and interact with target cells to elicit responses [87, 97]. However, the pathological roles of RMPs cell in AMD are not yet well defined. We previously demonstrated that MPs derived from lymphocytes elicit potent detrimental biological effects through the endocytosis of endothelial cells [140, 141]. Likewise, here, we found that RPE cells take up RMPs in a time dependent manner (article 1, **Fig. 3A-B**). The membrane scavenger receptor CD36 expressed in RPE cells is thought to mediate the uptake of oxidized lipids and is thus implicated in the pathogenesis of AMD [142]. Given the abundance of membrane lipids in RMPs, we hypothesized that CD36 contributes to the RMPs uptake process. This hypothesis was verified by the observation that blockage of CD36 led to decreased RMP uptake (article 1, **Fig. 3C**).

RMP uptake affects RPE cell function. The RPE cell cycle is interrupted and even arrested at G0/G1 phase, and the cyclin-dependent kinase inhibitors p15 and p21 increase (article 1, **Fig. 5**); therefore RPE cell viability and proliferation might decrease (article 1, **Fig. 4**). Cell senescence is another destination for RPE cells accumulated with RMPs (article 1, **Fig. 6**). Moreover, these RMPs, once taken up, may interfere with the normal phagocytosis activity of RPE cells (article 1, **Fig. 7**). Most importantly, the effects of RMPs on RPE cell senescence and phagocytosis may suggest another implication of RMPs in early age-related pathological changes.

When the number of senescent cells increases beyond that of normal cells, age-related diseases, such as AMD, which involve tissue structure disruption and malfunction, become likely [143, 144]. Although there is no direct clinical evidence of a relationship between RPE cell senescence and AMD, a study in a progeroid mouse model has provided evidence supporting a causal link between $p16^{Ink4a+}$ senescent cells and eye age-related morbidity [145, 146]. Disruptive phagocytosis in RPE cells may also interrupt the physiological process ensuring the health and integrity of the neural retina and choriocapillaris [122, 147, 148]. Thus, these changes in RPE cells induced by RMPs lead to the accumulation of sub-RPE deposits after oxidant injury.

Our findings strongly suggest that RMPs released by RPE cells under oxidative stress might accelerate RPE degeneration, a major feature of AMD. This study supports the RPE oxidant injury hypothesis, in which oxidative stress promotes RPE cell release of MPs, and the accumulation of RMPs leads to RPE cell dysfunction, thus consequently increasing the subretinal deposits [149]. Subsequently, we focused on the specific mechanisms by which RMPs exert their biological effects and attempted to address how these effects contribute to the pathogenesis of disease.

As explained these EVs represent an important mode of intercellular communication by serving as vehicles for transfer between cells and acting as a messenger. . Therefore, the detection of EVs and the biomolecules that they carry in body fluids offers a desirable diagnostic opportunity. These vesicles contain characteristic material such as miRNA representing the cell of originso extensive studies have investigated utilizing miRNAs as biomarkers in disease diagnosis.

miRNAs play important roles in regulating cellular processes and are critically associated with the pathogenesis of cancer and other diseases [96, 150-153]. Signaling pathways regulating aging phenotypes may be a potent axis affected by miRNAs. Moreover in responding to cellular stress injury, miRNAs can cause cells to transition from replication to senescence states, through mediator signals [154-156].

A recent study has shown that specific miRNAs known to be upregulated in elderly patients (including the miR-17-92 cluster, let-7, and miR-34a) are also capable of inducing cellular senescence [157]. Overexpression or downregulation of these miRNAs probably promotes senescence by interfering with genes in the p15–p21 pathway.

One of the well-known miRNAs families is let-7, which is thought to have a potential function in many tissues such as the retina [127]; the activity of let-7 is preserved in aging development, and let-7 is highly expressed in aged tissues. The let-7 family plays a key role in many disorders, such as cardiovascular disease, cancers, and obesity-related and age-related diseases [127, 129, 158-160].

Building on our results on the effects of RMPs in RPE cell dysfunction, we further performed RNA sequencing of RMPs and then determined let-7f abundance in RPE-derived vesicles. The results of the present study showed that let-7f represses cellular growth and proliferation in a concentration dependent manner (article 2, **Fig 1A, 2**), and is followed by cell cycle arrest in G0/G1 (article 2, **Fig 3**) and an increase in the cyclin-dependent kinase inhibitors p15 and p21 (article 2, **Fig 5**). These changes together may drive senescence as a deleterious consequence (article 2, **Fig 4**). Thus, a let-7f inhibitor can potentially attenuate the effects of RMPs in interfering with cell

cycle signals. Along this line, as expected, let-7f inhibition led to a tangible decrease in the expression of p15 and p21 proteins (article 2, **Fig 5B**). These proteins are implicated in cell senescence, the potential fate of tumor cells that fail to proliferate as well as some other cells, and thus may have diagnostic value for the senescence phenotype [161, 162].

Wagner et al. has shown that, among the lethal-7 family, let-7f is one of the senescence-associated miRNAs [163, 164]. Several studies have demonstrated that let-7f downregulates cell proliferation and leads to cell cycle arrest and senescence, thus suggesting that it may serve as a biomarker in some diseases [165-168]. The discovery of these short sequences of nucleic acids may launch novel therapeutic opportunities via finding the signaling pathway responsible for disease pathogenesis [169].

In conclusion, our findings indicated that the detrimental effect of MPs in RPE cells may be mediated by the miRNA let-7f. This possibility will be considered further if it can yield a more effective treatment strategy than those that already exist.

Importantly, the novel findings provide new information for better understanding the pathobiology of dry AMD, which may help in developing preventive and therapeutic strategies for this age-related blinding disease. However, this finding must be further examined in vivo to determine its clinical applicability.

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