Ultrastructural characterisation of melanogenesis in adult human retinal pigment epithelial cells after adenoviral transduction with the tyrosinase gene

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Summary

Until recently, it was widely accepted that melanogenesis does not occur in the adult retinal pigment epithelium (RPE), since the typical hallmarks of melanogenesis, the premelanosome and the expression of melanogenic proteins like tyrosinase and melanocyte-associated protein 17 (PMEL17), were absent post-natal. In the meantime, active tyrosinase has been observed in the adult RPE of different animal species, e.g. after phagocytosis of retinal photoreceptor outer segments (ROS). The aim of this thesis was to investigate whether melanogenesis can be induced in adult human RPE cells in response to ROS phagocytosis or after transduction with a tyrosinase vector. The role of the melanogenic proteins PMEL17 and TRP1 and the classical melanosomal stages, known from pre-natal melanin synthesis, were also to determine.

As a model system tyrosinase transduced amelanotic RPE cells were used to study tyrosinase function and melanogenesis and the influence of phagocytosis in adult RPE. The presences of the melanogenic proteins tyrosinase, tyrosinase-related protein 1 (TRP1) and PMEL17 were investigated using immunocytochemistry. Tyrosinase activity and loclisation was further studied with electron microscopical DOPA histochemistry. The ultrastructural morphology of melanogenic stages was compared to that of pigmented melanoma cells (MNT-1), which were used as a positive control for typical melanogenesis. Melanin synthesis was detected with HPLC analysis. MTT tests confirmed that viability was not affected after tyrosinase transduction and melanin synthesis. Post-natal RPE melanogenesis was also studied in animal experiments after subretinal injection of the tyrosinase vector in rats and rabbits.

Compared to controls, tyrosinase active cells had a redifferentiated cobblestone morphology, were pigmented and had an improved phagocytosis rate. Tyrosinase trafficking was different to the classical model found in MNT-1 cells, since a DOPA reaction was not observed in Golgi-derived vesicles, but as membrane-less, small DOPA granules free-floating in the cytoplasm. Melanogenesis occurred without the involvement of TRP1, PMEL17 and typical striated premelanosomes and melanogenic stages. In contrast, melanin was synthesised in lysosome-like organelles. Thus, a new pathway of melanogenesis is described for this model system. Transduced RPE cells of living rats and rabbits and cultured cells of human

donors showed also a similar morphology of melanogenesis as the ARPE-19 cells. Interestingly, control MNT-1 cells contained similar melanosomes in addition to the classical stages of melanogenesis.

Although a transport of ingested material to newly-formed ARPE-19 melanosomes was not observed, phagocytosis led to an improved tyrosinase activity and to an accelerated melanogenesis, compared to non-fed transduced cells.

In conclusion, tyrosinase transduction in combination with phagocytosis led to a morphological reorganisation and functional improvement of cultured ARPE-19 cells. Additionally, melanogenesis has been induced, which is independent of premelanosome formation. It can be transferred to the *in vivo* situation by gene therapy.

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I. Glossary and Abbreviations

AdTyr: an adenoviral vector, carrying the human tyrosinase cDNA under a hCMV-promoter in the E1 (envelope 1)-deleted region

AMD: Age-related macular degeneration is the main cause of blindness in the elderly population (60+) of the western world: In early stages, lipofuscin and drusen accumulate between the retinal pigment epithelium (RPE) and Bruch's membrane. Later stages can be divided in to two different types, the dry and the wet form: 1) "dry", non-exudative AMD: Atrophic changes in the macula region lead to visual impairment, but the lesions are not as severe as the lesions in "wet" AMD. Therapies for dry AMD are still lacking. 2) The "wet", exudative form affects about 15% of AMD patients. Neovascularisation of leaking vessels from the choriocappilaris into the retina leads to enhanced RPE and retinal detachment. These vessels have a greater tendency to leakage and bleeding into the macula, ultimately leading to irreversible damage to the photoreceptors. Laser coagulation and medication that minimises oxidative stress and blood vessel outgrowth build the main strategies of treatment, but they can only attenuate vision loss.

AP: the adaptor-protein complexes transport coated vesicles from the Golgi apparatus (AP-1, 3, 4), or from the cell membrane (AP-2) to their destination organelles.

ARPE-19: is an immortalised, highly differentiated amelanotic (pigment-less) RPE cell line, spontaneously arisen from primary pigmented RPE cells of a 19-year-old male human donor. ARPE-19 cells express typical RPE marker proteins like CRALBP (cellular retinaldehyde-binding protein), MERTK (mer-tyrosine kinase) and RPE65 (retinal pigment epithelium-specific protein with 65kDa), are highly polarised and keep their epithelial and phagocytic functions even with high passages.

Bruch's membrane: is the border between the retina and the choroid; it is formed by the basal membranes of the RPE and the vessels of the choriocapillaris and contains several layers of collagen and elastic fibres.

Choriocapillaris: highly fenestrated blood vessels at the apical border of the choroid

DHI: 5,6-dihydroxyindol is a melanin precursor in eumelanogenesis.

DHICA: 5,6-dihydroxyindol-2-carboxylic acid is a melanin precursor in eumelanogenesis.

DOPA: L-dihydroxyphenylalanine is a melanin precursor and the second substrate in eu- and phaeomelanogenesis.

DOPA reaction: an electron microscopical method, in which fixed tissue/cells can be checked for tyrosinase activity. Incubation of the fixed material with the substrate L-DOPA leads to melanin formation at sites where active tyrosinase is present. Thus, the localisation of the enzyme can be documented ultrastructurally.

Endosome: a class of clathrin-coated vesicles; e.g. phagosomes and Golgi-derived vesicles

ER: endoplasmic reticulum, **rER** rough ER; **sER** smooth ER

Eumelanin: black or brown melanin composed of variable amounts of DHI and DHICA monomers

hRPE: primary human RPE cells

LAMP-1: lysosome-associated membrane-glycoprotein 1; a lysosomal marker enzyme, also found in melanosomes

Lipofuscin: age pigment, which accumulates in lysosomes of postmitotic cells with highly oxidised undegradable material

Lysosome: a degradative organelle of the cell, which fuses with phagosomes to digest ingested material

Lysosome-related organelle (LRO): are a family of cell type-specific organelles that include melanosomes, platelet-dense bodies, and cytotoxic T cell granules. All of these organelles contain subsets of lysosomal proteins (e.g. LAMP-1) in addition to cell type-specific proteins (e.g. TRPs for melanosomes).

Macula (*macula lutea*): the centre of the visual field, containing the highest density of photoreceptors in the whole retina; the macula is important for the recognition of faces and for reading.

MART1: also known as melan A, a melanogenic protein with unknown function

Melanocyte: neural crest-derived pigment cell

Melanogenesis: synthesis of the pigment melanin catalysed by the enzyme tyrosinase inside specialised pigment cells

Melanolipofuscin: product of the “fusion” of melanin and lipofuscin granules

MERTK: mer-tyrosine kinase: a receptor at the apical surface of RPE cells, which is involved in photoreceptor outer segment phagocytosis

MNT-1: a pigmented melanoma cell line, continuously showing typical stages of melanogenesis

MVBs, MLBs: multi-vesicular/ -lamellar bodies of mostly unknown origin, which occur in many cells often in association with digested material

Neural crest: stage in the development of the neuroepithelium; after closure of the neural tube, neural crest-derived cells migrate to their destinations, differentiating into multiple cell types (e.g. melanocytes, smooth muscles, sympathetic neurons).

Neural tube: stage in the development of the neuroepithelium, which differentiates into the central nervous system (CNS)

Neuromelanin: kind of melanin, which resides in dopaminergic neurons; it is not synthesised by tyrosinase but by tyrosine-hydroxylase or by auto-oxidation of dopamine or epinephrine.

Phaeomelanin: kind of melanin, composed of tyrosine and thiol-compounds (e.g. cysteine) with catalytic action of tyrosinase

Phagocytosis: ingestion of extracellular material by a cell; here: ingestion of retinal photoreceptor outer segments by the RPE; the endocytic vesicle is called phagosome

PMEL17: melanocyte-associated protein 17 is a structural protein, essential for the synthesis of premelanosomes

Premelanosome: initial stage of melanin synthesis in pigment cells

Pre-natal/ post-natal: occurrences before and after birth, respectively

ROS: retinal photoreceptor outer segments: they form the apical parts of rods and cones, which receive light from the lens and convert it into electric signals. In rods, the visual pigment is called rhodopsin. It can be used for the immuno-localisation of ROS after phagocytosis by RPE cells.

RPE: retinal pigment epithelium: epithelial monolayer of neural tube origin; together with the endothelial cells of the choriocapillaris it builds the blood-retinal barrier between choroid and retina

Tyrosinase: the main catalytic enzyme of melanogenesis

Tyrosinase gene family: family of proteins, which are close to tyrosinase and also function in melanin synthesis; members are called tyrosinase-related proteins (TRP).

Tyrosine: the amino acid L-tyrosine, which is the main substrate of melanogenesis

Uvea: the pigmented, middle layer of the eye; it contains the choroid, iris, ciliary body and pars plana.

II. Introduction

The pigment melanin is synthesised in specialised neurons of the brain, in melanocytes of the skin, hair, ear and the connective tissue of inner organs. Uveal melanocytes and pigment epithelial cells of the iris, ciliary body and retinal pigment epithelium (RPE) are the pigment cells of the eye (Marks & Seabra, 2001). Figure 1 illustrates the structure of a vertebrate eye and highlights the position and morphology of the RPE and the choroidal melanocytes. Among other functions, the pigment cells of the eye are responsible for iris colour and the absorption of scattered light in the background of the eye (Dayhaw-Barker, 2002; Roberts, 2002).

This chapter gives a short overview of the pigmentary system of the retinal pigment epithelium (RPE), with regard to cell morphology, melanogenesis, and age-related changes in the eyes of elderly individuals. Finally, a review of the findings leading to the objectives of this thesis is presented.

1. The retinal pigment epithelium

The RPE is a monolayer of pigmented cells of neural tube origin in the background of the eye (Figure 1 A, B). Together with the endothelial cells of the choriocapillaris it forms the blood-retinal barrier (Konari et al, 1995). RPE cells are cubical and highly polarised. Basal infoldings and apical microvilli serve as enlargement of the surface. The typical oval shaped melanosomes of the RPE are located in the microvilli, and round shaped melanosomes can be found in the cytoplasm near the nucleus (Figure 1 C). Besides its barrier function, which is facilitated by tight junctions, the RPE has several properties essential for vision (Marmorstein et al, 1998), including the balanced nutritional supply of the retina (Bialek & Miller, 1994). One of the most important functions of the RPE is the phagocytosis and degradation of retinal photoreceptor outer segment discs (ROS), which are shed during the light perception process (Bosch et al, 1993; Young & Bok, 1969). After ingestion, the RPE degrades the material and recycles certain lipids and 11-*cis*-retinal, the reactive component of the visual pigment of the rods (Flannery et al, 1990; Rando, 1992). Loss of phagocytic activity leads to retinal damage (Nandrot & Finnemann, 2008), due to the accumulation of undegraded ROS in the sub-retinal space. In RCS (Royal College of Surgeons) rats for example, a mutation in the surface receptor Mertk (mer-tyrosine

kinase) leads to loss of specific phagocytosis of ROS and to loss of the visual function of the retina (D'Cruz et al, 2000).

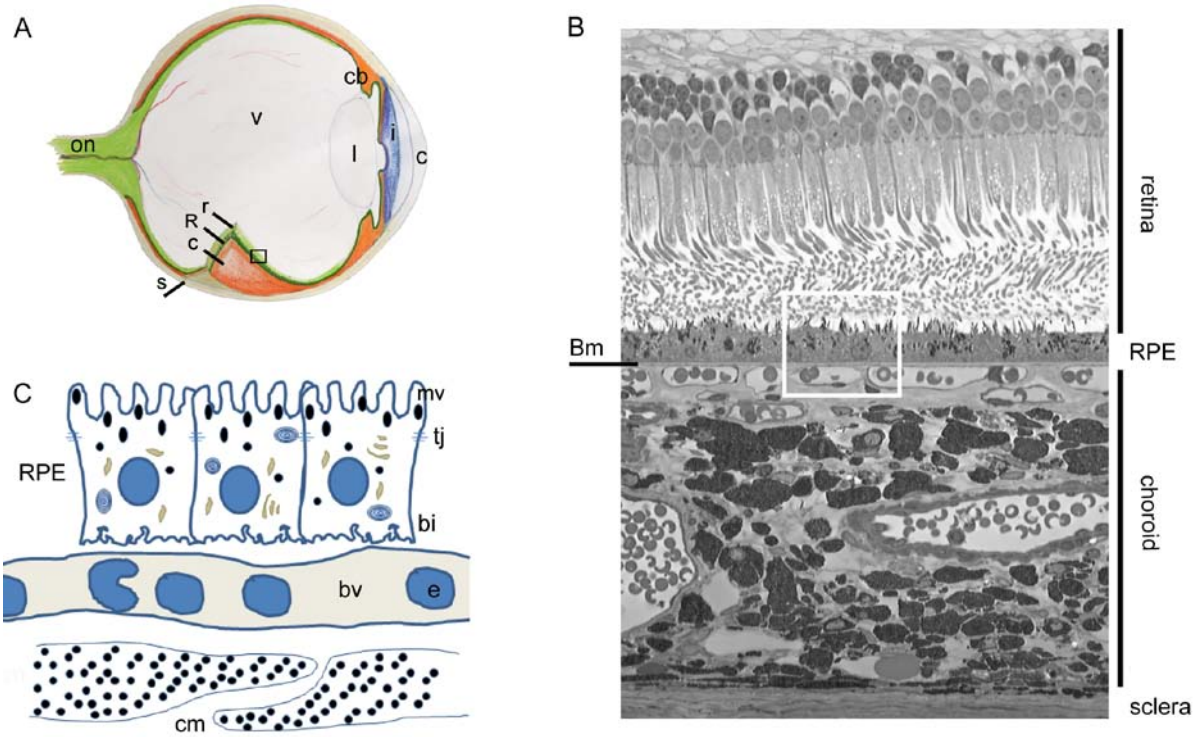


Figure 1: Histology of the retinal pigment epithelium (RPE) A) A scheme of the vertebrate eye shows the RPE (R) lying between the retina (r) and the choroid (c); sclera (s), c cornea, cb ciliary body, i iris, l lens, on optic nerve, v vitreous body; the highlighted square is magnified in B. B) Cross section of a monkey eye: the apical part of the RPE is closely related to the photoreceptors of the retina. Bm Bruch's membrane; C) Schematic diagram of the RPE, as highlighted in B: basal infoldings (bi) and apical microvilli (mv) enlarge the surface; tight junctions (tj) of the RPE together with the blood vessels (bv) of the choriocapillaris build the blood-retinal barrier; choroidal melanocytes (cm) are densely packed with round melanosomes (black spots), while RPE melanosomes are more oval shaped, e erythrocyte;

Other RPE functions are assumed to be related to their melanin content (reviewed by (Sarna, 1992). The melanosomes of the RPE absorb scattered light, which would otherwise disturb visual acuity (Peters et al, 2006) and, together with antioxidative enzymes and non-enzymatic antioxidants (Newsome, 1994), melanosomes protect against oxidative stress in the background of the eye (Schraermeyer & Heimann, 1999). They accumulate heavy metals, free radicals and toxic substances, like gentamycin (Zemel et al, 1995), which would otherwise be harmful to the cell. Additionally, the melanosomes are responsible for short-term storage of metal ions like Ca^{2+}, K^{+} and Zn^{2+} (Kokkinou et al, 2005; Samuelson et al, 1993) that are needed for cell metabolism. However, aged melanosomes may also have prooxidative functions (reviewed by (Boulton et al, 2001)).

2. Melanogenesis

Melanocytes of the skin and hair are able to produce melanin for their whole lifetime inside specialised organelles, the melanosomes (Jimbow et al, 1976). Frequently, they transfer the pigment granules to neighbouring keratinocytes, where the melanosomes function as antioxidative scavengers, and absorber of light and toxic substances (reviewed by (Sarna, 1992)). In contrast, melanosomes of the RPE and other melanocytes are not recycled under normal conditions and are not transferred to neighbouring cells. It is surmised that RPE melanosomes are synthesised only before birth (Carr & Siegel, 1979; Miyamoto & Fitzpatrick, 1957; Sarna, 1992), although post-natal melanogenesis has been described by several groups in the meantime (Dorey et al, 1990; Herman & Steinberg, 1982; Peters et al, 2000; Schraermeyer & Heimann, 1999; Schraermeyer & Stieve, 1994; Thumann et al, 1999).

Melanocytes and RPE cells have a different embryonic origin and development (Smith-Thomas et al, 1996): While RPE cells emerge from the neural tube, melanocytes are neural crest-derived cells. Consequently some differences exist between melanocyte and RPE melanogenesis (Bharti et al, 2008; Bharti et al, 2006; Murisier et al, 2007), which affect, for example the transcription of melanogenic genes in the embryo by using different transcription and growth factors. Also the shape of mature RPE melanosomes differs from that of melanocytes in being oval (Schraermeyer & Heimann, 1999). Despite this, melanogenesis in RPE cells has been described as sharing a common pathway with melanocytes. They use the same melanogenic proteins and the ultrastructural characterisation of morphologically distinct melanosomal stages I to IV has been confirmed for both melanocytes and pre-natal RPE cells (Lopes et al, 2007). RPE pigmentation is still not fully understood, thus findings from melanocyte pigmentation are summarised below to give a small introduction regarding the melanising machinery of pigment cells.

Melanogenesis is the synthesis of melanin from tyrosine residues by the enzyme tyrosinase in pigment cells. Different types of melanin can be distinguished, eumelanin, phaeomelanin and neuromelanin. Eu- and phaeomelanin can be found in melanocytes and pigment epithelial cells, while **neuromelanin** is located only in specialised brain neurons (reviewed by (Breathnach, 1988)). For the formation of **eumelanin**, which is black to brown, the proteins tyrosinase, tyrosinase-related

protein (TRP) 1 and 2 and the structural protein melanocyte-associated protein 17 (PMEL17) are necessary. The main precursors of eumelanin are 5,6-dihydroxyindol (DHI) and 5,6-dihydroxyindol-2-carboxyilc acid (DHICA). Details are described in 2.2. The yellow to red **phaeomelanin** is composed of tyrosine together with cysteine or other thiol-compounds. Originally, it was believed that melanin granules contained only polymers of one monomer (Mason, 1967). It is now accepted that most melanin granules are mixed melanosomes, composed of eumelanin (Oetting and King 1994; (Prota, 1992) and phaeomelanin (reviewed by (Ito & Wakamatsu, 2003; Ito & Wakamatsu, 2008)). Nevertheless, RPE and choroidal pigmentation contain mostly eumelanin.

2.1 Melanosomal proteins

2.1.1 Tyrosinase and Tyrosinase-related proteins (TRPs)

The tyrosinase gene family members tyrosinase, TRP1 and TRP2, are the main enzymes and regulatory proteins of melanogenesis (Tsukamoto et al, 1992). They are type 1 membrane-glycoproteins and share a high nucleotide sequence homology and a similar protein structure throughout the animal kingdom (Camacho-Hubner et al, 2002). The transcriptional regulation of the tyrosinase gene family is rather complex and is reviewed in several publications (Murisier & Beermann, 2006; Sturm, 2009; Tachibana, 2000). Tyrosinase and TRPs are metallo-proteins and have two putative histidine binding sites for metal ions (Huber & Lerch, 1988; Jackman et al, 1991). Tyrosinase binds copper, while the other two family members have other cofactors, zinc for TRP2 (Solano et al, 1994) and possibly iron or copper for TRP1 (Martinez-Esparza et al, 1997).

Tyrosinase, initially translated as a 60 kDa polypeptide and heavily glycosilated in the rough ER (rER) to a 70-74 kDa form, has seven glycosilation sequons (Ujvari et al, 2001). It contains a bicubric active site (2 regions CuA and CuB) each with three highly conserved histidines, responsible for metal binding and redox-reactions. Glycosilation, folding and copper acquisition are regulated by glycosilation enzymes and ER chaperones (Branza-Nichita et al, 1999). In addition, the substrates L-DOPA and L-tyrosine (Halaban et al, 2001) and TRP1 (Francis et al, 2003; Kobayashi et al, 1998) apparently facilitate the maturation and dimerisation of tyrosinase in the rER.

Tyrosinase undergoes sequential modifications of the sugar residues first in the ER and then in the Golgi apparatus and is shuttled through the latter via glycosphingolipids (Sprong et al, 2001). Before this, copper binding at the CuB site facilitates initial processing of tyrosinase into a form capable of being transported out of the rER (Olivares et al, 2003). Upon reaching the trans-cisterns of the Golgi apparatus, the mature, copper-ladden tyrosinase is a catalytically active enzyme (Maul, 1969).

Tyrosinase (Huizing et al, 2001) and TRP1 (Jimbow et al, 1997; Orlow et al, 1993) contain a dileucine-based motif with flanking regulatory amino acids at their cytoplasmic tail that interact with adaptor-protein complexes (AP-3 and AP-1, respectively), which facilitate the shipment from the Golgi apparatus to late endosomal and lysosomal compartments (Bonifacino, 2004) and finally to the melanosome (Calvo et al, 1999; Simmen et al, 1999). In AP-3 mutants, tyrosinase does not reach the melanosome, while TRP1 is transported properly (Huizing et al, 2001).

Integrated into the melanosomal membrane, tyrosinase facilitates the first two steps of melanogenesis. In humans, it also catalyses the last steps from DHICA monomers to eumelanin polymers. Besides its function as a rate limiting enzyme in melanogenesis, tyrosinase also has a low catalase function (Wood & Schallreuter, 1991) and seems to be involved in the antioxidative defence of pigment cells (Valverde et al, 1996a; Valverde et al, 1996b). Aberrations in the composition and structure of the enzyme result in degradation of the molecule by endoplasmic reticulum and proteasome-associated mechanisms (Berson et al, 2000; Halaban et al, 1997; Mosse et al, 1998). This may lead to hypopigmentation and oculo-cutaneous albinism (OCA) type 1 (Halaban et al, 2000; Spritz et al, 1990).

TRP1 has a low “pseudo-tyrosinase” activity (Jimenez-Cervantes et al, 1993) and it has also been found to assist as a chaperone-like protein in tyrosinase dimerisation and in heat denaturation defence (Francis et al, 2003). Absence of TRP1 leads to OCA type 3 (Boissy et al, 1996). **TRP2** (also called dopachromtautomerase) catalyses the non-decarboxylative tautomerisation of dopachrome to DHICA in eumelanogenesis (Kroumpouzos et al, 1994).

2.1.2 Other melanogenic proteins

PMEL17 localises to early stages of melanogenesis (Kobayashi et al, 1994; Raposo et al, 2001) and there it builds the typical structures needed for melanin accumulation (Berson et al, 2003). It also binds the eumelanin-intermediates DHI and DHICA (Chakraborty et al, 1996; Fowler et al, 2006; Lee et al, 1996) and it has been suggested that it serves as a sink within melanosomes for the detoxification of cytotoxic intermediates (Fowler et al, 2006; Theos et al, 2006). The transport pathways of the PMEL17 protein are still not fully understood, but trafficking by AP transporters AP-1, AP-2 and AP-3 are discussed (Valencia et al, 2006). Further proteins, involved in the formation of functional melanosomes are the **OA1 protein** (ocular albinism protein 1), **MART1** (also known as melan-A), the **P-protein** (pink eyed dilution homolog protein; OCA 2) and **MATP** (membrane-associated transporter protein; OCA 4) which either facilitate trafficking of proteins and other molecules to the melanosomes or have other yet unknown functions (Hearing, 2005). A high molecular weight complex in the melanosomal membrane, consisting of tyrosinase, TRP1, TRP2 and the lysosomal protein LAMP-1 (lysosomal-associated membrane protein 1), seems to be responsible for the conversion of tyrosine to eumelanin (Hearing et al, 1982; Jimenez-Cervantes et al, 1998). Other proteins like PMEL17 (Lee et al, 1996), P-protein, MART1 and melanogenic inhibitors may be connected (Hearing et al, 1992). The melanogenic complex is still not understood, but it seems to stabilise tyrosinase conformation and action in the melanosome.

2.2 The Raper-Mason Scheme of melanin formation

The Raper-Mason-Scheme describes the reaction cascade for tyrosinase-catalysed eu- and phaeomelanin synthesis (Figure 2 for details). Eumelanin synthesis involves a complex series of oxidations and rearrangements of tyrosine, which result in the formation of an indole-quinone ring-structure that readily polymerises to high-molecular weight biopolymers (reviewed by (Riley, 1993)). The melanin polymers formed differ in length, absorption and physical and chemical properties dependent on the ratio of the DHI and DHICA monomers (Aroca et al, 1992; Pawelek, 1991).

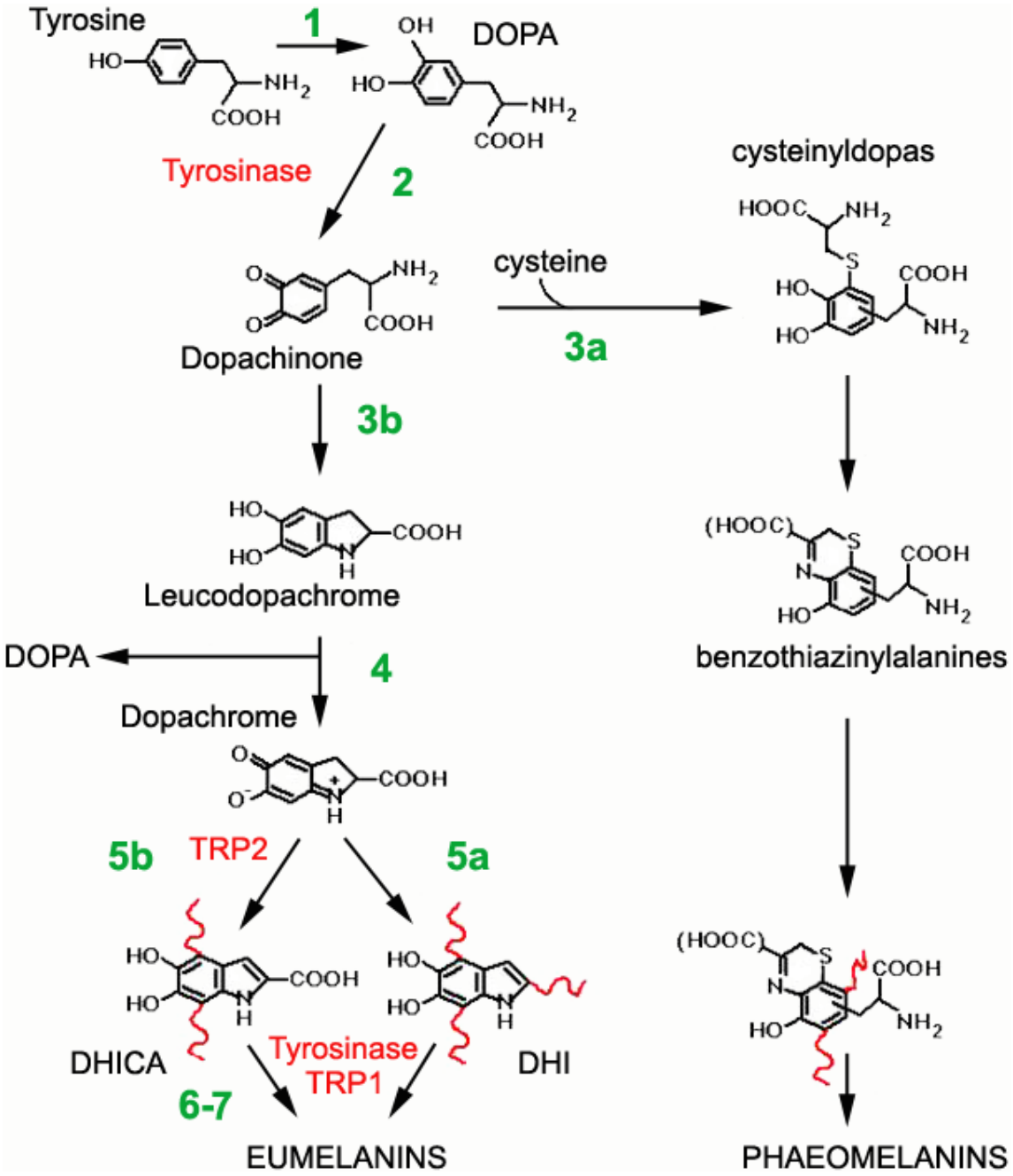


Figure 2: The Raper-Mason Scheme (adapted from http://omlc.ogi.edu/spectra/melanin/melaninsynth.gif (Prota, 1988)). 1+2) The first two steps, hydroxylation of tyrosine to DOPA and the oxidation from DOPA to Dopaquinone are catalysed by tyrosinase. 3) From Dopaquinone two different reactions are possible (Formation of eu- or pheomelanin): a) In presence of compounds with free thiol groups (glutathione, cysteine) and low pH rapid spontaneous reaction to sulfour-containing compounds (cysteinyldopas, benzothiazinylalanines) leads to phaeomelanin production. This reaction is favoured. b) In absence of thiol-compounds, Dopaquinone is transformed into eumelanin (continue with 4). 4) At neutral pH, the slow reaction from Dopaquinone to Leucodopachrome which is highly unstable, can be achieved, but spontaneously collapses into DOPA and Dopachrome. 5) From Dopachrome two different reactions can occur, leading to different eumelanin species. This reaction is regulated by TRP2: a) spontaneous decarboxylation to DHI b) with action of TRP2, the production of non-decarboxylated DHICA is favoured. 6) DHI and DHICA are transformed into 5,6-indolchinones (with and without carboxyl-group). DHI oxidizes spontaneously (or faster with tyrosinase action); DHICA transformation is slower but needs TRP1 catalysis in mice or tyrosinase in humans (Boissy et al., 1989). 7) Indolchinones react with dihydroxyindoles forming semichinones that polymerize spontaneously to eumelanin.

2.3 Cellular actions– the 4-stage model of eumelanogenesis

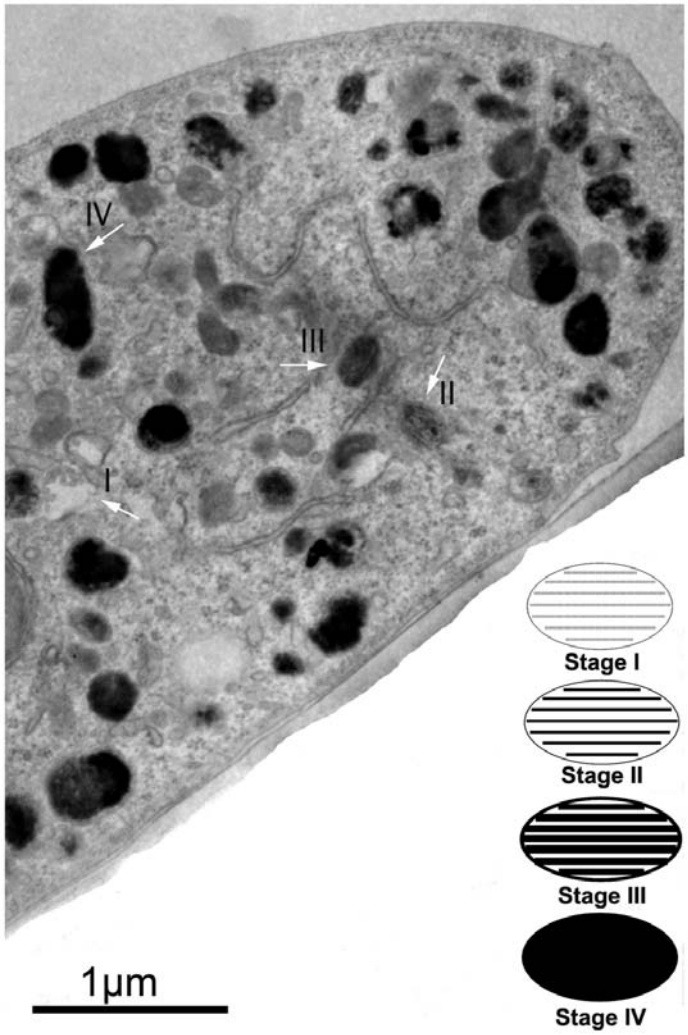


Figure 3: Classical stages of melanogenesis: The electron micrograph shows different stages of melanogenesis in MNT-1 melanoma cells. A scheme, describing the four possible stages is given on the right.

The cellular procedure of pigment synthesis (=melanogenesis) has been described first by Seiji (Seiji et al, 1963). This model still serves as the standard scheme for melanocytes and pre-natal RPE cells. Meanwhile, it has been extended by other authors and is actually described as follows (Hearing, 2005) (Figure 3): The melanogenesis starts with the occurrence of an amorphous vesicle, the premelanosome, which is positive for the protein PMEL17. PMEL17 is responsible for the building of internal, fibrillar striations that give the vesicle a typical structure (stage I pre-melanosome). After the intra luminal arrangement is completed (stage II), Golgi-derived vesicles, filled with melanogenic proteins, can enter the premelanosome. After integration in the melanosomal membrane, tyrosinase synthesises melanin, which is deposited along the internal striations of the organelle (stage III). When the whole organelle is filled with melanin, it is termed stage IV or mature melanosome (=melanin granule).

2.4 The premelanosome

The premelanosome is the essential starting point of melanogenesis. Absence of the protein PMEL17 leads to the loss of premelanosome formation, to mistargeting and degradation of melanogenic enzymes and to defective pigmentation in affected individuals (Theos et al, 2006). The origin of the amorphous vesicle, forming the premelanosome is still not understood. Earlier hypotheses proposed that the

premelanosome of melanocytes (reviewed by (Jimbow et al, 1976)) and RPE cells (Eppig, 1970; Eppig & Dumont, 1972; Mishima et al, 1978; Stanka, 1971; Stanka et al, 1981) is formed out of cisterns or vesicles of the endoplasmic reticulum or the Golgi-Endoplasmic Reticulum-Lysosome zone (GERL), which is part of the smooth ER (Novikoff et al, 1968). Observations, resembling the ultrastructural similarity of melanocyte melanosomes to lysosomes (Schraermeyer, 1995) and the occurrence of lysosomal enzymes in the melanosome (Diment et al, 1995; Orlow et al, 1993), indicated that the melanocytes melanosome might well be a specialised lysosome (reviewed by (Orlow, 1995)). Additionally, RPE melanosomes have been associated with phagosomes and lysosome-like organelles (Schraermeyer & Stieve, 1994), further indicating that the RPE melanosome is also a lysosome-like organelle (reviewed by (Schraermeyer & Heimann, 1999)). Recent ultrastructural studies, using immuno-gold labelled antibodies, have clarified that the premelanosome of melanocytes and melanoma cells is an endosome (Raposo & Marks, 2002). Thus, in melanocytes, PMEL 17 travels to endosomes, where it builds internal striations and matures to stage II melanosomes. For RPE cells, corresponding studies are still lacking.

3. Age-related changes in the RPE - Lipofuscin

In young adult people, another pigment, lipofuscin, accumulates in the cells of the retinal pigment epithelium (Roberts, 2002). It is also found in the liver and the brain of aged persons. The composition of lipofuscin in the RPE is still not fully understood (Ng et al, 2008), but it has been found to contain undegradable remnants of phagocytosed photoreceptor outer segments (Feeney-Burns & Eldred, 1983; Feeney-Burns et al, 1988; Warburton et al, 2005). Heterogeneous groups of lipids and protein aggregates that are highly oxidised and thus auto-fluorescent, build the main components of this age pigment (Eldred, 1989; Eldred et al, 1982). From the fourth decade onwards, melanolipofuscin granules become visible (Feeney-Burns et al, 1984). They have a melanin core and a lipofuscin envelope, but it is unknown how these granules develop. Since undegradable phagocytosed material can be integrated into the melanosomal membrane (Schraermeyer & Stieve, 1994), and tyrosinase has been found in association with phagosomes (Julien et al, 2007; Schraermeyer et al, 2006), the following is speculated: tyrosinase scavenges radicals

in phagosomes of young RPE cells. Thus lysosomal enzymes are protected, ROS is degraded and tyrosine residues are used as substrate for melanogenesis. In contrast, tyrosinase activity is reduced in aged RPE (as also found in greying hair melanocytes), ROS are incompletely degraded and lipofuscin accumulates in lysosomes and around melanin granules, building melanolipofuscin.

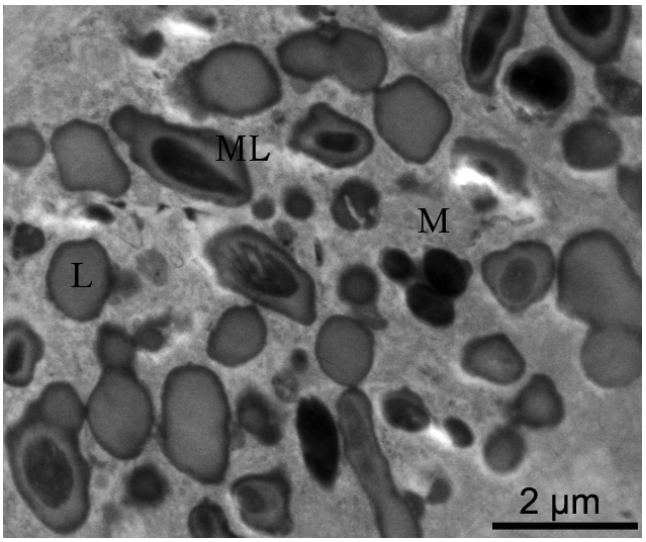


Figure 4: Typical pigments of an aged human RPE cell: Only few melanin granules (M) are left. Instead, melanolipofuscin (ML) and lipofuscin (L) granules are prevalent (from Biesemeier et al., 2008).

In aged people, almost all the pure melanin granules have gone and melanolipofuscin and lipofuscin granules are left (Figure 4).

Compared to the melanosomes of young individuals, aged melanins are laden with toxic substances and thus have prooxidative properties (Boulton & Dayhaw-Barker, 2001). Also the age pigments accumulating in the cytoplasm are highly oxidative. Together with the elevated oxygen pressure and the light exposure in the eye, they amplify the oxidative status in the RPE, facilitating ocular phototoxicity (Boulton et al, 2004). Elevated stress leads to diminished and perturbed degradation of phagocytosed material (Terman & Brunk, 1998) due to the disruption of lysosomal membrane stability (Eldred, 1995). To some extent, the undegraded material gets excluded and accumulates in the subretinal space between the Bruch's membrane and the RPE, where it builds drusen. The drusen also amplify oxidative reactions, leading to further lipofuscinogenesis (Glenn et al, 2009) creating a vicious circle. Geographically, the greatest accumulation of lipofuscin and melanolipofuscin granules occurs at the posterior pole of the eye, especially in the macula region (Feeney-Burns et al, 1984; Weiter et al, 1986). This distribution pattern correlates well with the distribution of degenerative changes associated with age-related macular degeneration (AMD), and the higher the amount of drusen and melanolipofuscin in the background of the eye, the higher the risk of developing AMD later on (Dorey et al, 1989; Holz et al, 2007). Melanolipofuscin accumulation has been reported to more closely reflect the onset of AMD than the accumulation of lipofuscin (Feeney-Burns et al, 1984; Warburton et al, 2007). Therefore,

melanolipofuscin deposition is widely accepted as being one major cause of retinal degeneration and blindness in the elderly population of the Western world (Bird et al, 1995; von Ruckmann et al, 1997). Thus a better understanding of the mechanisms causing (melano-) lipofuscin formation is desirable.

4. Previous findings

Many pigment cell functions are related to their melanin content, including light absorption and scavenging of oxidative reactions (Sarna, 1992). Age-related changes to eye pigmentation in elderly people create a prooxidative environment and can cause diseases that may result in blindness. Consequently, it is highly questionable that melanin is only synthesised pre-natally and not renewed over a lifetime. Meanwhile, some authors have demonstrated that there are intermediate stages of melanogenesis, occurring spontaneously in the adult RPE of opossums (Herman & Steinberg, 1982) and cattle (Schraermeyer, 1992) and also in cell culture (Dorey et al, 1990). They could be upregulated under certain conditions: e.g. after stimulation with melanogenesis activating agents such as lactic acid (Mishima, 1994) and Ca^{2+}(Rak et al, 2006). *In vivo*, the induction of photic stress in hamsters, led to the occurrence of striated melanosomal stages in the RPE (Schraermeyer, 1992). Furthermore, tyrosinase expression has been found to occur in adult RPE tissue (Dryja et al, 1978), or has been induced in cell culture by stimulation with growth factors, like the melanocyte-stimulating hormone (Abul-Hassan et al, 2000). After phagocytosis of retinal outer segments, both tyrosinase (Schraermeyer et al, 2006) and melanogenic stages were observed in cell culture of bovine RPE cells (Schraermeyer, 1995; Schraermeyer et al, 1999b; Schraermeyer & Stieve, 1994) and in rabbit (Thumann et al, 1999) and rat eyes (Peters et al, 2000). Residues of the phagocytosed material were found inside the melanosomal membrane (Figure 5), indicating that melanin granules are able to take up undegradable phagocytosed material. Additionally, phagosomes and lysosome-like organelles were found in close association with tyrosinase, melanin granules and melanogenic stages.

These findings lead to the assumption that new melanin was synthesised in response to phagocytosis, forming new granules or covering the original melanosomes.

On the other hand, a defective degradation of the ingested material can lead to lipofuscinogenesis inside the melanosome and to the production of melanolipofuscin granules. Accordingly, the renewal of RPE melanin or the prevention of melanolipofuscinogenesis could prevent age-related diseases in the background of the eye.

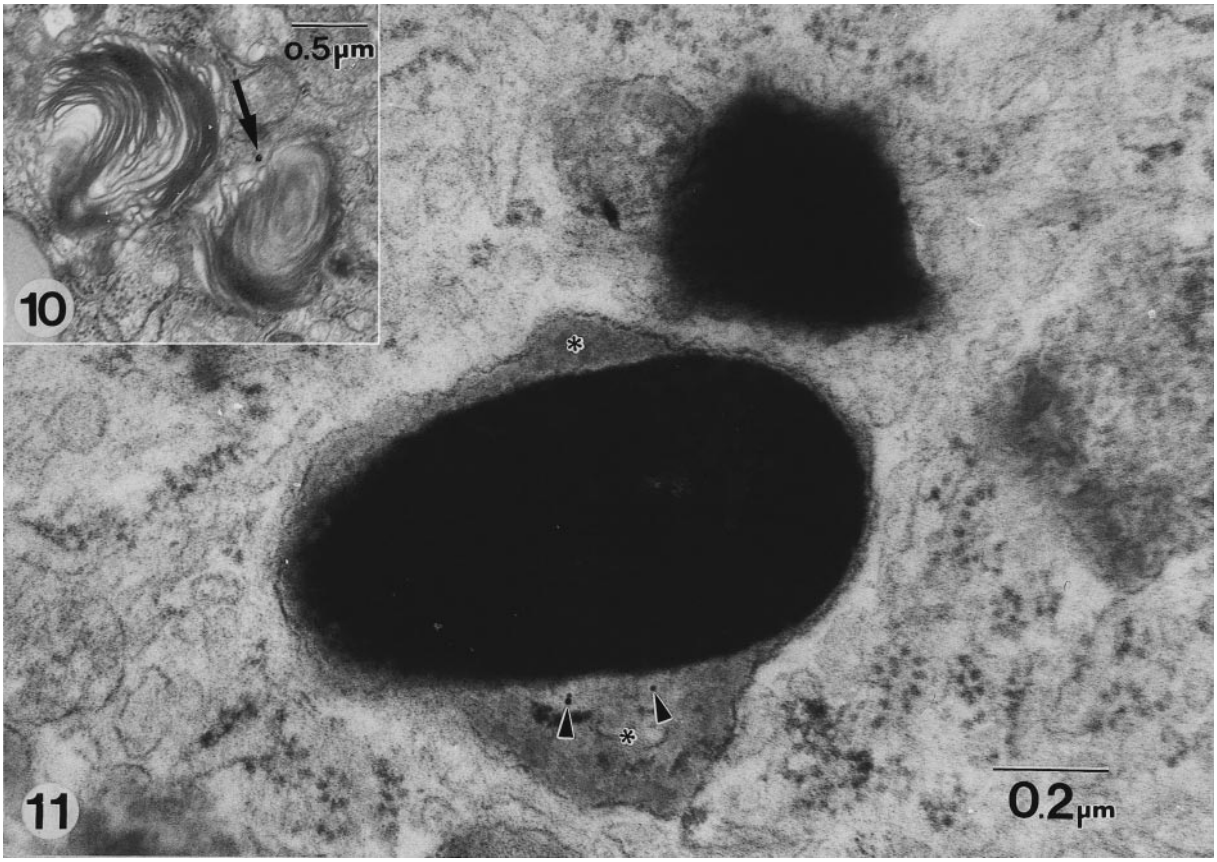


Figure 5: Phagocytosed material is integrated into the melanosomal membrane: The inset (former figure 10) shows two typical phagosomes of an RPE cell, which contain ROS material that is labelled with gold granules. The main picture (former figure 11) shows an RPE melanosome, five days following after phagocytosis of gold-labelled ROS: the space between the limiting membrane and the melanin matrix is enlarged and contains gold granules (arrowheads) and homogeneous electron opaque material (asterisk) (adapted from Schraermeyer et al., 1999b).

New findings concerning the origin of melanosomes in melanocytes (Raposo & Marks, 2002) and that melanogenic proteins share transport mechanisms with endosomes, lysosomes and other lysosome-related organelles (Bonifacino, 2004), correlate well with the findings from the RPE cells mentioned above. These also showed a relation between the degradative pathway and melanisation. However, in the former experiments, no quantitative evidence has been obtained that new melanin was synthesised in RPE cells. The association of melanosomes and lysosome-like organelles might as well be a sign of melanin degradation (Sarna, 1992). Although tyrosinase has been observed in adult tissue, the presence of PMEL17 in adult RPE has not yet been reported (Lopes et al, 2007).

In fibroblasts, melanogenesis has been found to take place in lysosomes after tyrosinase transfection (Borovansky et al, 1997; Winder et al, 1993). Melanogenesis occurred without the involvement of other melanosomal proteins and led to a melanosomal structure, which was different to the classical model (Borovansky et al, 1997), suggesting an alternative pathway of melanin formation inside lysosomes, as has been proposed for post-natal RPE melanogenesis.

Probably, post-natal melanin synthesis occurs without the involvement of typical PMEL17-positive striated premelanosomes inside lysosome-like organelles.

On the other hand, it is possible that tyrosinase expression in adults is not correlated with melanogenesis but serves a yet unknown function, maybe in the defence against oxidative stress, as assumed for melanocytes (Kim & Han, 2003; Perluigi et al, 2003; Wood & Schallreuter, 1991). The background of the eye is a highly oxidative environment due to the high oxygen pressure from the choroid, the diurnal turnover of retinal material and light exposure. As in melanocytes, melanosomes of the RPE have been found to scavenge oxidative reactions (Rozanowski et al, 2008a; Rozanowski et al, 2008b; Zadlo et al, 2007). However, aged and photosensitised melanin has a prooxidative function (Rozanowska et al, 1997) and tyrosinase and melanogenic intermediates (Heiduschka et al, 2007) are discussed as having anti- or prooxidative capacity depending on their concentration and the surrounding circumstances (Seagle et al, 2005a; Seagle et al, 2005b; Urabe et al, 1994). Nevertheless, Schraermeyer found melanogenic stages after induction of light stress in hamster RPE (Schraermeyer, 1992), suggesting an upregulation of tyrosinase activity.

Possibly, tyrosinase machinery is activated to scavenge free radical building due to oxidative reactions after light induction or phagocytosis of outer segments.

Thus the function of tyrosinase and melanin in the adult retinal pigment epithelium is still unknown and many theories have been raised, which require further investigation.

5. Aim of the thesis

Regarding previous studies, mostly done on animal cells and tissues, the aim of this thesis was to investigate the relationship of tyrosinase, pigmentation and phagocytosis in adult human RPE, as set out in the following questions:

1) Are tyrosinase and melanisation involved in the defence against oxidative stress in aged human RPE cells?
2) Can melanogenesis be induced in non-pigmented adult human RPE cells?
    a. Is the classical scheme of melanogenesis true for adult human RPE melanogenesis or can an alternative pathway be described?
    b. Is the typical striated premelanosome essential for melanogenesis?
    c. Is the trafficking of adenovirally transduced tyrosinase altered compared to the pathway described in the literature? Can it occur at free ribosomes in the cytoplasm?
3) How does ROS phagocytosis influence tyrosinase activity and melanogenesis in this system?
4) Are remnants of phagosomal degradation transported to or do they fuse with melanosomes, generating melanolipofuscin?
5) Is the expression of tyrosinase useful or toxic for adult RPE cells?

To answer the first question, a method was established to induce UV-A light-mediated oxidative stress in human retinal pigment epithelial cell culture (primary pigmented human donor RPE cells (hRPE) versus the amelanotic retinal pigment epithelium 19 cell line (ARPE-19)). Then the antioxidative capacity, apoptosis and viability of the cells were tested. It was found that the antioxidative capacity of melanised donor RPE cells was highly affected by the amount of prooxidative age pigments in the cells.

Before studying the effects of tyrosinase and melanisation any further, a system had to be established where pigmented/ tyrosinase-active cells were generated without the disturbing presence of aged donor pigments. Since ARPE-19 cells lack an overall pigmentation on the one hand, but are highly differentiated RPE cells with typical phagocytic function on the other hand, they presented an ideal system to study the new synthesis and function of melanin without the interference of old melanin granules.

To answer the questions 2 to 5, ARPE-19 cells were transduced with an adenoviral vector allowing the expression of the human tyrosinase gene (AdTyr). Afterwards, tyrosinase production, activity and melanin synthesis were analysed and quantified at the immunocytochemical, light microscopical and ultrastructural level. The viability of RPE cells after transduction was also evaluated. The influence of specific (ROS) and unspecific (autofluorescent latex beads) phagocytosis was studied simultaneously using immunocytochemistry.

Additionally, pigmented human donor RPE cells were fed with outer segments and tyrosinase expression and activity of the cells with and without transduction with the vector AdTyr were evaluated. Animal experiments should reveal the changes in RPE cells of rats and rabbits after transduction with the AdTyr vector.

Using these methods, new aspects in the melanogenesis of adult cultured RPE cells were obtained and it was possible to define characteristics for the occurrence of an alternative pathway for tyrosinase trafficking.

In the following, manuscripts that have been published and are under examination are summarised and discussed to answer the questions listed above.

III. Summarised results of the manuscripts enclosed

For a better understanding, the manuscripts are not listed in chronological order, but according to their logical relevance to this thesis.

Manuscript 1: Biesemeier A., Kokkinou D., Julien S., Heiduschka P., Berneburg M., Bartz-Schmidt K.U., Schraermeyer U. **UV-A induced oxidative stress is more prominent in naturally pigmented aged human RPE cells compared to non-pigmented human RPE cells independent of zinc treatment.** J Photochem Photobiol B. 2008 Feb 27;90(2):113-20. Epub 2007 Dec 4

Age-related macular degeneration is the leading cause of blindness in the elderly population of the western world (Bird et al, 1995). Changes in RPE pigmentation play a key role in age-related oxidative stress and disease (Boulton et al, 2004). While melanin in young RPE is assumed to have an antioxidative function, the age-pigments lipofuscin and melanolipofuscin are prooxidative and lead to an increase in oxidative stress reactions due to radical building. Dark skinned people have fewer age-related eye diseases than Caucasians (Ambati et al, 2003), and it is discussed if this is due to their higher melanin/lipofuscin ratio in the RPE (Gregor & Joffe, 1978). Zinc treatment together with a vitamin diet is one treatment strategy for AMD (Age-related Eye Disease Study Research Group, 2001). Zinc, which can be stored by melanins (Kokkinou et al, 2005; Potts & Au, 1976), is used as a cofactor in many antioxidative enzymes and is thus involved in cell protection (Bray & Bettger, 1990; Brewer et al, 1983; Deng et al, 2000). In this study, it was investigated whether melanised donor RPE cells have more antioxidative capacity against UV-light induced oxidative stress compared to non-pigmented ARPE-19 cells. In addition, cells were supplemented with 100 µM zinc chloride to analyse whether the antioxidative action of zinc and its storage in melanosomes were able to support the antioxidative action in melanised cells.

Cells of the non-pigmented ARPE-19 cell line and human donor RPE cells (hRPE; age of donors: 60-70 years) were supplemented with 100 µM zinc chloride and they were then illuminated with UV-A light (330-440 nm) for 15 to 60 minutes. Afterwards, oxidative stress (DCFDA assay of H_2O_2 production), apoptotic events (TUNEL assay) and viability (MTT test) were investigated.

Without irradiation, hRPE cells showed less oxidative stress when supplemented with zinc compared to their control. Despite its significance, this effect was very small. After irradiation, all samples had significantly increased their H_2O_2 production compared to controls. No zinc-related effect was observed in either ARPE-19 or hRPE cells. Apoptosis and viability were also affected by irradiation independent of zinc treatment. In contrast to the theory, pigmented hRPE cells showed a lower viability after irradiation compared to the non-pigmented ARPE-19 cells. Analogous effects were found also in the TUNEL assay. The non-pigmented cells showed less apoptosis than the pigmented cells.

The results obtained were unexpected since it was supposed that pigmented cells would have a higher antioxidative capacity due to their melanin content than non-pigmented cells. The human donor RPE cells used in the study contained not only melanin but also high amounts of melanolipofuscin and lipofuscin. Hence, the protective effects of melanin and the supplemented zinc were nullified by the prooxidative effects of the age pigments. As expected, zinc had no effect on ARPE-19 cells, since these cells lack melanin, which is responsible for storage of trace metal ions.

However, the results of this study are only valid for aged human RPE, possibly resembling the oxidative state of pre-AMD persons. This study cannot describe the effects of UV-light on RPE cells from young adults or children, which do not contain the prooxidative melanolipofuscins. In future research, also cells from younger donors (<20 years), containing no melanolipofuscin and less lipofuscin granules, will also be used to investigate the different oxidative properties of RPE pigments, according to their age and composition.

Manuscript 2: Julien S., Kociok N., Kreppel F., Kopitz F., Kochanek S., **Biesemeier A.**, Blitgen-Heinecke P., Heiduschka P., Schraermeyer U. **Tyrosinase biosynthesis and trafficking in adult human retinal pigment epithelial cells.** Graefes Arch Clin Exp Ophthalmol. 2007 Oct;245(10):1495-505. Epub 2007 Feb 21.

Tyrosinase, the catalysing enzyme of melanogenesis, is found predominantly in pre-natal RPE (Carr & Siegel, 1979; Miyamoto & Fitzpatrick, 1957) and it is still not clear if it also appears in adult RPE (Schraermeyer, 1993; Schraermeyer et al, 2006). Here it is demonstrated that tyrosinase can be upregulated by phagocytosis of retinal outer segments (ROS) in human donor RPE (hRPE) cultures.

Primary cultures of hRPE cells were fed with bovine outer segments and latex beads for five and twenty hours respectively, and then tyrosinase expression and activity were analysed with different methods: tyrosinase mRNA expression was evaluated with quantitative real time RT-PCR. Fluorescence immunocytochemistry with anti-human tyrosinase antibody and light and electron DOPA histochemistry showed the location of tyrosinase in the cell. Tyrosinase activity was analysed by measuring the [^{3}H]-tyrosine hydroxylase activity of the enzyme after phagocytic events. As controls, cells were cultured with assay medium only. Additionally, tyrosinase staining of cells with the tyrosinase vector AdTyr is presented.

Fluorescence immunocytochemistry showed immunoreactivity of tyrosinase in hRPE cells only after phagocytosis of ROS and not under control conditions. Transduction with AdTyr, an adenoviral vector allowing the expression of the human tyrosinase gene, led to an intense tyrosinase staining also in non-fed hRPE cells. Tyrosinase presence and activity in fed cells were also tested using the DOPA-oxidase assay in light and electron microscopical samples. It was found that a positive reaction took place in small granular vesicles (DOPA granules) throughout the cytoplasm five and twenty hours after feeding. In addition, the tyrosinase was located inside phagosomes (20 h), which contained either lamellar material, resembling ROS residues or ingested latex beads (Figure 6). Without feeding, no DOPA reaction was observed with light and electron microscopy. The intracellular location of tyrosinase was also tested with antibodies and a co-localisation of rhodopsin (from ROS) and tyrosinase was observed in some organelles, twenty hours after feeding.

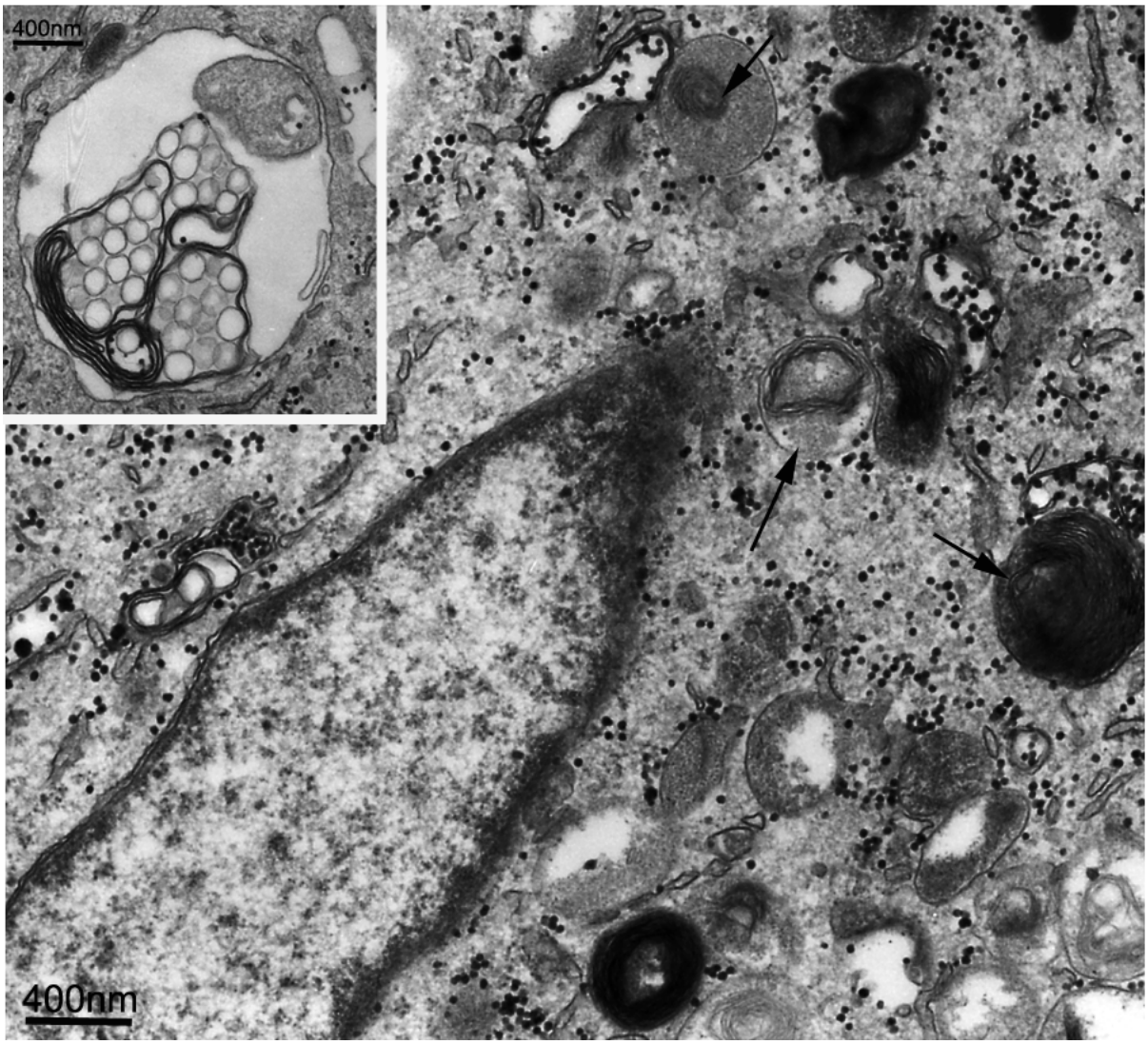


Figure 6: DOPA histochemistry of a fed donor RPE cell: DOPA granules are spread over the cytoplasm and fuse with multi-lamellar bodies, resembling phagosomes (arrows). The inset shows a huge phagosome with ingested latex beads, which has also incorporated DOPA granules.

The mRNA levels of tyrosinase and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) in control and fed cells were compared using real time RT-PCR. The level of tyrosinase mRNA was calibrated to the amount of GAPDH in control cells. Five and twenty hours after feeding, the mRNA level of tyrosinase was reduced compared to the controls. However, it had re-increased above the control-level forty hours after feeding. Additionally, tyrosine-hydroxylase activity of tyrosinase was measured five, twenty and forty hours after phagocytosis of ROS in monkey RPE cells. Tritiated L-tyrosine was used as a substrate and the release of tritium from tyrosine was measured in homogenised samples. The absence of the enzyme tyrosine-hydroxylase, which would give unspecific results in the activity-assay, was also confirmed by immunocytochemistry. It was found that ROS fed cells had a higher hydroxylase activity than controls (x 180). Human melanoma cells were used

as positive control and cells from the amelanotic region of monkey eyes were used as negative control. Pure ROS were almost free of tyrosinase contamination as shown by a very low hydroxylase activity.

This study showed that generation of the tyrosinase protein and its activity can be stimulated by feeding cultured hRPE cells with outer segments. Active tyrosinase has been found to reside in electron-dark granules all over the cytoplasm and in association with phagosomes. The tyrosine-hydroxylase activity of the enzyme was also confirmed to be higher when cells were fed with ROS. However, the mRNA levels were reduced at both five and twenty hours after feeding and re-increased after forty hours. It is surmised that tyrosinase mRNA is always present in adult human RPE cells, but effective translation and protein synthesis is inhibited under normal conditions. When cells have phagocytosed ROS, the inhibition is lifted and tyrosinase protein can be synthesised without post-translational degradation.

Manuscript 3: Biesemeier A., Kreppel F., Kochanek S., Schraermeyer U. **The classical premelanosome, known from pre-natal melanogenesis, is not essential for melanogenesis in adult RPE cells** (Cell & Tissue Research, in print).

The pathway of organelle formation in melanogenesis was characterised by (Seiji et al, 1963). The first structure described is the premelanosome. It has a body of typically striated fibrils, which are formed by the protein PMEL17. After the internal structure is completed, tyrosinase enters the premelanosome and catalyses the formation of melanin polymers, which gather at the internal striations, forming electron-dark stripes, which grow bigger and finally cover the whole organelle. The organelle is now called a mature melanosome and it shows neither an internal structure nor further tyrosinase activity. It is proposed that melanogenesis does not occur when PMEL17 is absent and premelanosomes cannot therefore be formed (Theos et al, 2006). However, overexpression of tyrosinase in non-pigment cells showed melanin synthesis in lysosomes without the occurrence of typical premelanosomes (Borovansky et al, 1997; Winder, 1991; Winder et al, 1995; Winder et al, 1993).

To obtain new melanogenesis without the interference of old melanin granules in the cell, non-pigmented ARPE-19 cells were transduced with the adenoviral vector

AdTyr. The expression of different melanogenic proteins (tyrosinase, TRP1, PMEL17) was investigated using fluorescence immunocytochemistry, and the presence of active tyrosinase was analysed using ultrastructural DOPA histochemistry. Occurring melanogenesis was investigated with light and electron microscopy. An MTT assay confirmed the viability of the cells after transduction. Non-transduced ARPE-19 cells served as negative controls. Additionally, cells from a pigmented melanoma cell line (MNT-1) were used as positive controls for melanogenesis occurring in the classical way (Raposo et al, 2001).

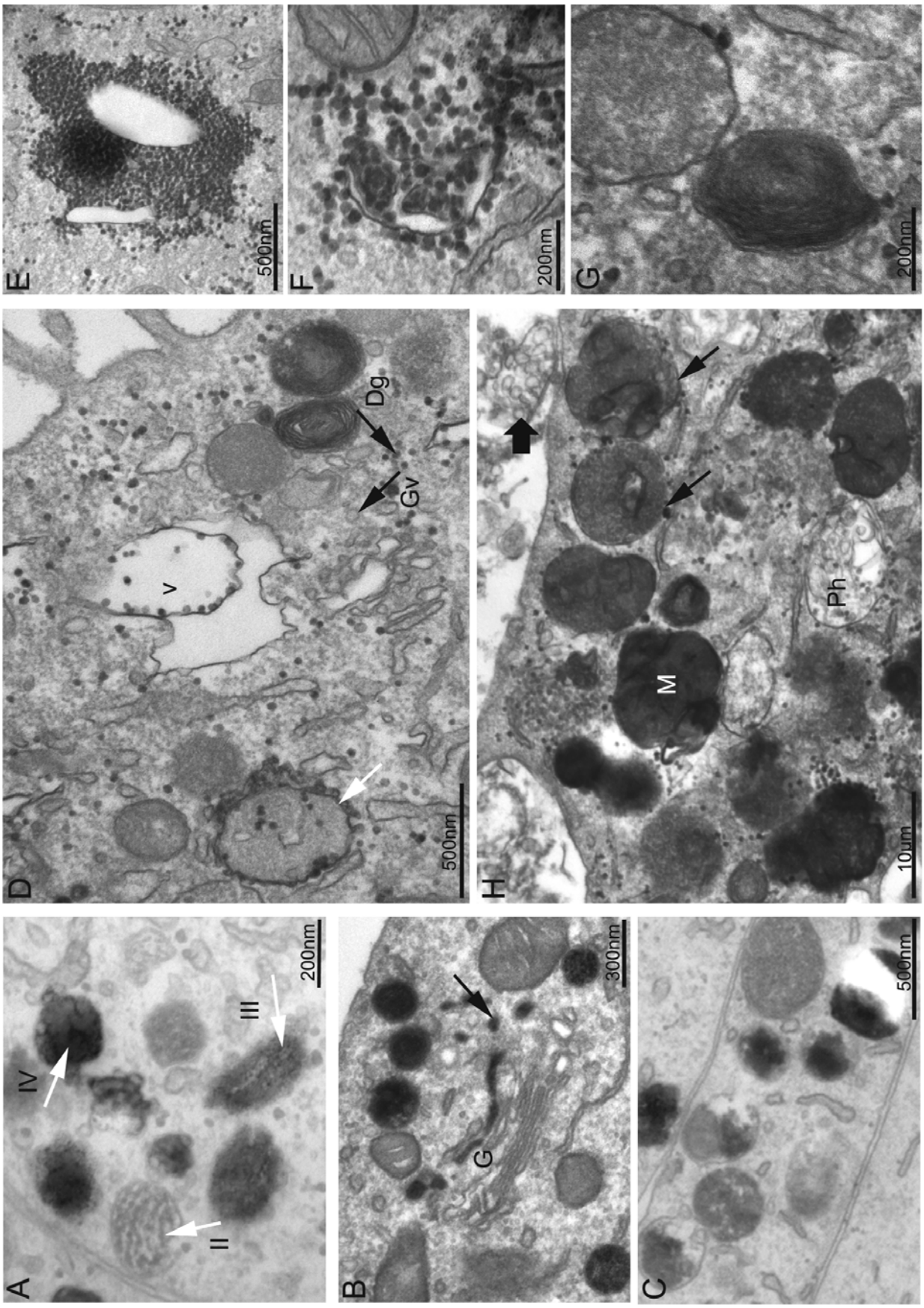
MNT-1 cells showed the typical melanogenic stages I to IV (Figure 7 A) and expressed all proteins tested. The DOPA reaction showed tyrosinase in trans-Golgi stacks and in Golgi-derived coated vesicles (Figure 7 B). Additionally, atypical melanosomes were observed, which did not have the striated internal structure of classical melanosomes (Figure 7 C).

In immunocytochemistry experiments, ARPE-19 controls never showed any tyrosinase, PMEL17 or TRP1 staining, indicating that melanogenic enzymes were completely absent. Also after tyrosinase transduction, TRP1 and PMEL17 were still absent, while tyrosinase staining was distributed over the whole cytoplasm. Comparing the ultrastructural morphology and tyrosinase distribution in MNT-1 cells (Figure 7 A-C) and the transduced ARPE-19 cells (Figure 7 D-H), it was found that the localisation of tyrosinase in the transduced cells was different to the classical scheme. Small DOPA granules were spread all over the cytoplasm (Figure 7 D). They were associated with vacuoles (v, in Figure 7 D) or crowded around vacuoles (Figure 7 E) of unknown origin. Some granules were also associated with or found inside tubules of the ER (not shown, see manuscript for detail) or at cytoplasmic membranes of unknown origin (Figure 7 F). DOPA granules were also associated with multi-vesicular (MVB) and multi-lamellar (MLB) bodies (Figure 7 G). However, they localised very rarely to tubules that could either belong to the GERL part of the smooth ER or to the Golgi apparatus. Tyrosinase reactivity was not observed in Golgi-derived vesicles. In comparison, the DOPA positive granules observed were much smaller (Figure 7 D) and lacked a visible covering membrane (Figure 7 F). It is assumed that synthesis and transport of tyrosinase occurred in a completely different way to the known pathway. Typical premelanosomes and melanogenic stages were also not observed. Six days after transduction, the first brown melanin granules

accumulated in the transduced cultures. Ultrastructurally, these melanin granules (as in Figure 7 H) resembled electron-dark, cloudy material inside multi-vesicular and multi-lamellar bodies. While the MVBs and MLBs were unmelanised in negative controls and in cells up to 48 h after transduction, these bodies were almost completely filled with electron-dark, melanin-like material two weeks after transduction. Additionally, multi-shaped organelles (mixed organelles) filled with lamellar, vesicular and electron-dark material were observed, which seemed to build a linking stage between MVBs and MLBs and the first stage of melanogenesis. The multi-vesicular, multi-lamellar and mixed bodies resembled organelles, which have been recognised as (auto-) phagosomes (Novikoff, 1973) and degradative organelles (Peters et al, 2000; Schraermeyer & Stieve, 1994) before). Hence, it is assumed that melanogenesis can take place inside lysosome-like organelles without the occurrence of typical premelanosomes inside these bodies. Interestingly, MNT-1 cells also showed similar electron-dark bodies to some degree (Figure 7 C).

Since premelanosomes are thought to be necessary for a safe melanogenesis, preventing oxidation of cytoplasmic proteins by the highly oxidative melanogenic reactions, the viability of the transduced cells was investigated with an MTT assay. It showed no differences between control and transduced cells during the first two weeks after transduction.

In summary, it was shown that melanogenesis can take place without the formation of typical premelanosomes and without support from the proteins PMEL17 and TRP1 inside multi-vesicular and multi-lamellar organelles. Additionally, an alteration in tyrosinase transport seems possible, due to the lack of typical DOPA staining in Golgi-derived vesicles. Instead, DOPA granules, which lack a covering membrane, supported tyrosinase transport. The results indicate the existence of an alternative pathway of melanin formation, which might also occur to some degree in MNT-1 cells.


A
200nm
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200nm

Figure 7: Ultrastructural observation of MNT-1 (A-C) and transduced ARPE-19 cells (D-H): A) The classical stages of melanogenesis and B) typical DOPA reaction of trans-Golgi cisterns (G) and Golgi-derived vesicles (arrow) are frequently seen in MNT-1 cells. C) Electron-dark organelles, which resemble the atypical melanogenic stages of transduced ARPE-19 cells, are also observed in MNT-1 cells. D) Twenty four hours after transduction, DOPA positive granules (Dg) are spread over the cytoplasm of a transduced ARPE-19 cell. Frequently, they fuse with vesicles (white arrow) or vacuoles (v) of unknown origin and they can clearly be distinguished from Golgi-derived vesicles (Gv). E) Hundreds of DOPA granules are clustered together and accumulate close to a huge membrane-bound vacuole of unknown origin. F) Often, DOPA granules accumulate around intracellular membranes. Here, it can be seen that the DOPA granules lack a covering membrane, while the double membrane of the mitochondrion at the upper right can clearly be seen. G) Multi-vesicular and multi-lamellar bodies are also associated with DOPA granules. H) Six days after transduction, DOPA granules are associated with phagocytosed ROS material (Ph) and electron-dark organelles (arrows). Some ROS material is still not ingested (bold arrow). A mature melanin granule is also present (M). (Figures 7 A, D, E, and F are adapted from manuscript 3).

Manuscript 4: Biesemeier A., Blitgen-Heinecke P., Kreppel F., Kochanek S., Schraermeyer U. **Tyrosinase in conjunction with phagocytosis of retinal outer segments influences the morphology and melanogenesis in cultured human ARPE-19 cells** (submitted to Exp Eye Res).

Findings from Manuscript 2 and from earlier studies (Peters et al, 2000; Schraermeyer et al, 2006) indicate the following: 1) Tyrosinase expression is upregulated after phagocytosis of retinal outer segments in donor RPE cells and 2) in adult RPE, melanin granules can be colocalised with remnants of phagocytosis, suggesting incorporation of the ingested material in the melanosome and eventually synthesis of new melanogenic stages. The purpose of this study was to investigate further interrelations between phagocytosis and tyrosinase expression.

Changes in the morphology and functions of tyrosinase transduced ARPE-19 cells were analysed after phagocytosis of retinal outer segments (specific phagocytosis) and latex beads (unspecific phagocytosis). Therefore, fluorescence immunocytochemistry, ultrastructural DOPA histochemistry, and light and electron microscopy were used. Melanin synthesis was confirmed with HPLC analysis. Cells without phagocytosis and transduction were used as controls. Labelling of ROS with rhodopsin antibodies visualised phagocytic events.

In contrast to control cells (Figure 8 A), transduced cells appeared “redifferentiated” showing a typical cobblestone morphology (Figure 8 B) and melanin pigmentation (Figure 8 C). Additionally, transduced cells appeared to be higher than neighbouring non- transduced cells (Figure 8 D). This might be due to a rearrangement of the cell structure into the cubic form they have *in vivo*.

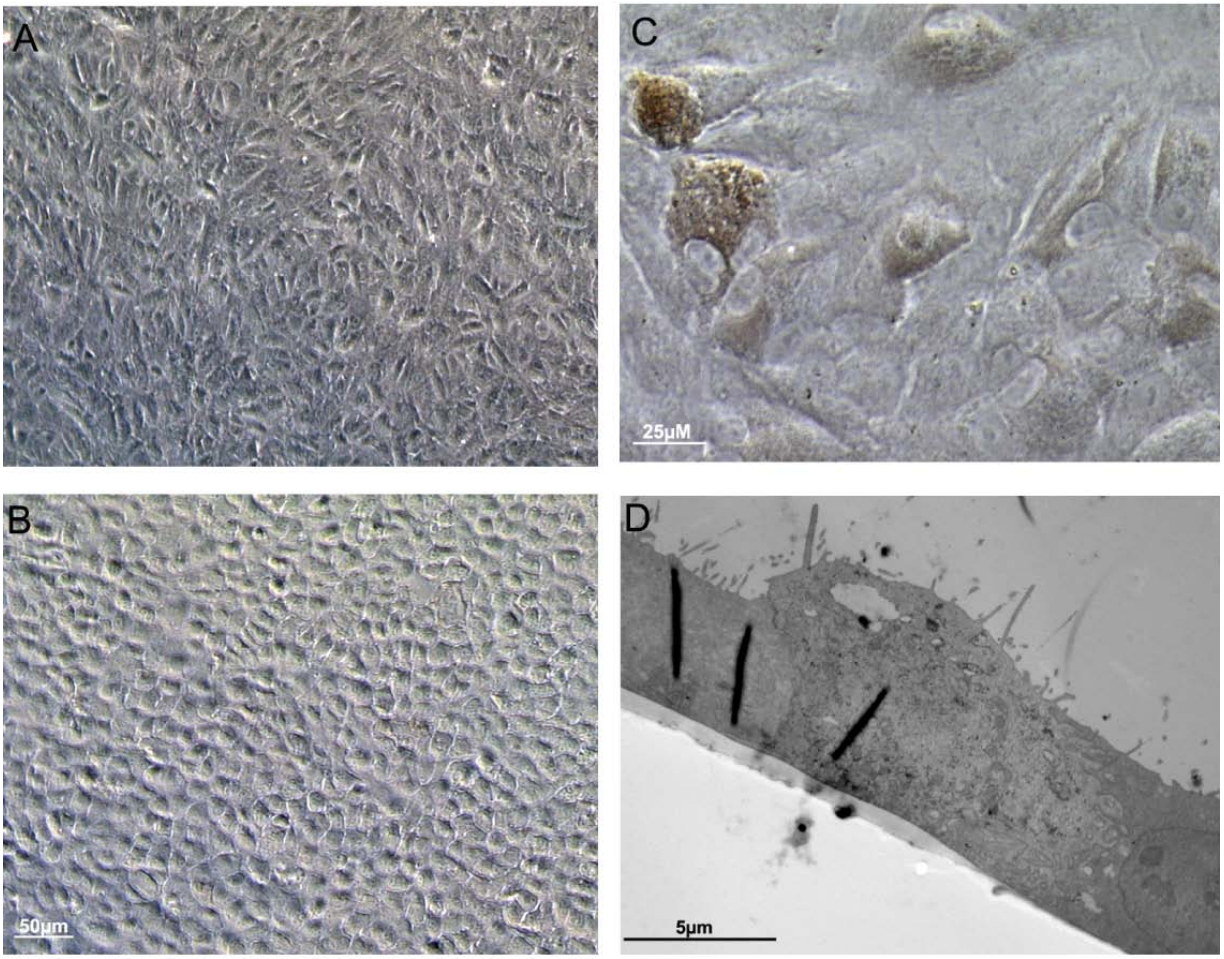


Figure 8: Morphology of ARPE-19 cells: A-C light microscopy, D electron micrograph: A) Control ARPE-19 cells had a mostly spindle shaped, non-pigmented appearance (here: day 7). B) In the first days after transduction, cells developed a hexagonal morphology, grew bigger and appeared more prominent than control cells (here: day 6). C) After one week of transduction, first melanin granules were observed. The light micrograph shows a transduced and fed sample, 11 days after transduction. D) Electron micrograph of DOPA reaction at low magnification: the transduced cell in the middle, DOPA positive vesicles can clearly be recognised, is to 1/3 higher than neighbouring cells, which lack DOPA staining and thus might not be transduced (Figure from manuscript 4).

Comparing the influence of tyrosinase on phagocytosis and vice-versa it was observed that while the transduced cells were able to phagocytose four times more ROS than non-transduced cells, the fed transduced samples had significantly more tyrosinase positive cells than non-fed transduced samples. It was also observed that fed transduced samples produced visible amounts of melanin more rapidly than non-fed cells. Possibly this was due to an increase in substrate availability by phagocytosis of outer segments, since the unspecific phagocytosis of latex beads had no additional effect on tyrosinase expression. Hence only the specific phagocytosis of outer segments had an effect on melanising machinery. Besides, non-transduced control cells showed a base level of DOPA positive organelles, which was not observable with fluorescence immunocytochemistry.

In conclusion, tyrosinase expression caused a more differentiated morphology resembling the *in vivo* state, including pigmentation, epithelial morphology, and an improved function (increased phagocytic activity) compared to the control. In addition, the specific phagocytosis of outer segments was able to increase both tyrosinase expression and function leading to an accelerated melanogenesis in transduced and fed ARPE-19 cells.

IV. Discussion

Age pigments impair the antioxidative capacity of melanin and zinc in aged RPE cells

The aim of this work was to evaluate the function of tyrosinase and melanin in adult human RPE cells. For this purpose, it was first investigated whether melanin has an antioxidative function in human RPE **(Manuscript 1)** by analysing the response of human donor RPE cells to UV-A induced oxidative stress in culture. Typical donors had an age above 50 years, implicating that the RPE cells contained not only granules with antioxidative melanin but also aged melanin, melanolipofuscin and lipofuscin, which lack antioxidative capacity and even nullified the effects of the supplemented antioxidant zinc chloride. Aged melanin (Rozanowski et al, 2008b; Zareba et al, 2006) and melanolipofuscin (Warburton et al, 2007) have been found to act prooxidatively and can increase the stress in the eye when illuminated with UV and blue light. Consequently, the non-pigmented ARPE-19 cells showed a better survival and less apoptosis than the pigmented donor RPE cells. Since it was difficult to obtain younger donor-RPE cells, which lack age pigments, this project was halted and a system had to be generated, in which melanin pigmentation could be studied without disturbing age pigments. As a consequence, the main part of the work concentrated on the question of how melanogenesis can be induced in human RPE cells.

Involvement of ROS phagocytosis in tyrosinase expression of adult human RPE cells

First, it was investigated whether tyrosinase can be induced in cultured human RPE cells after phagocytosis of retinal outer segments, as published by Schraermeyers group for bovine RPE cells (Schraermeyer et al, 2006). It was found that pigmented donor RPE cells were able to express the tyrosinase protein in response to phagocytic events, and that the enzyme was closely associated with remnants of the phagocytosed material, as observed ultrastructurally (colocalisation of DOPA reaction with latex beads) and in immunocytochemistry experiments (colocalisation of rhodopsin and tyrosinase antibodies) **(Manuscript 2)**. It was not surprising that tyrosinase expression can be affected by ROS phagocytosis, since ROS phagocytosis has been found to affect the expression of multiple genes of many

different functions in the RPE (Chowers et al, 2004). Hence, pathways yet unknown may have been activated or silenced to make melanogenesis possible in the present experiments.

Since it was difficult to observe tyrosinase activity and the renewal of melanosomes in pigmented tissues, the experiments were repeated with non-pigmented ARPE-19 cells as a next step. In cell culture, RPE cells lose their original pigmentation, due to ongoing mitosis without restoration of the pigment granules. Immortalised ARPE-19 cells are thus more or less unpigmented but retain the ability to produce small amounts of melanin (Dunn et al, 1996) as can be observed ultrastructurally and with DOPA histochemistry **(Manuscript 4)**, but this pigmentation has only minor significance.

ARPE-19 cells acted completely differently compared to donor RPE cells, since they always showed a small number of DOPA granules, but in an amount which was not observable with fluorescent microscopical methods, and did not express more tyrosinase in response to feeding **(Manuscript 4)**. This might be due to changes in differentiation and protein expression of the immortalised ARPE-19 cells compared to primary donor RPE cells. Differences in the gene expression of primary RPE cells and ARPE-19 cells have been shown before by (Cai & Del Priore, 2006). Thus tyrosinase expression had to be increased by transduction with the adenoviral vector AdTyr. Subsequently, a phagocytosis-induced upregulation of tyrosinase activity was also observed, as anticipated **(Manuscript 4)** and likewise the formation of melanosomes was accelerated.

Phagocytosis of ROS enhances melanogenesis in tyrosinase transduced ARPE-19 cells

In transduced ARPE-19 cells, purely the ingestion of ROS, not the ingestion of latex beads, enhanced tyrosinase expression and activity. Moreover, the observed melanin synthesis was further enhanced only by phagocytosis of retinal outer segments. This suggests that merely the specific phagocytosis of ROS via MERTK receptors (which is reviewed by (Strauss, 2005)) and downstream effectors is able to interact with tyrosinase activity in ARPE-19 melanogenesis. Possibly, tyrosine residues from the ingested material were used for melanin synthesis, since biochemical experiments have shown that peptides and proteins can be converted

into melano-protein by tyrosinase (Ito et al, 1984; Marumo & Waite, 1986; Schraermeyer, 1994).

However, the transport of phagocytosed material to newly formed melanosomes was not confirmable, since the labelling of ROS with gold granules (data not shown) was not successful. It was also difficult to recognise the ROS inside phagosomes of routine EM experiments, because the ingested material often did not have the typical membranous layers as has been previously observed (Schraermeyer et al, 1999b). Due to the threat of bovine spongiform encephalitis (BSE), it is no longer permitted to use bovine eyes and therefore porcine material was used. Since porcine eyes are smaller than bovine eyes, much more material was needed to gather enough ROS for the experiments. Thus they had to be frozen after preparation and thawed later for the phagocytosis tests. Possibly the freezing process affected the ultrastructural morphology of the ROS and their binding ability to immuno-gold labelling. Nevertheless, melanin was synthesised in vesicles, resembling degradative organelles by ultrastructure, as discussed below. This melanogenesis was enhanced after phagocytosis of ROS.

Interestingly, phagocytosis-related auto-fluorescence or lipofuscinogenesis have not been observed either. In contrast, earlier cell culture studies showed an increase in phagocytosis-related auto-fluorescence in RPE and to a higher extent in IPE (iris pigment epithelium) cells after ROS feeding, which was contributed to the formation of undegradable material. The effect was greater in IPE cells, since IPE cells do not phagocytose ROS under natural conditions (Dintelmann et al, 1999). It is known that the accumulation of undegradable material in the RPE leads not only to lipofuscinogenesis but also to further impairment of degradation and to a prooxidative atmosphere in the background of the eye. The improved phagocytic capacity of tyrosinase transduced ARPE-19 cells, which was also observed in the present work (four times more phagosomes compared to control cells), leads to the assumption that a better degradation is possible in the presence of tyrosinase.

Tyrosinase protects against oxidative stress after phagocytosis

The upregulation of tyrosinase after phagocytosis on the one hand and the upregulation of phagocytosis after tyrosinase expression on the other hand, can be explained as follows. Possibly, tyrosinase acts in defence against phagocytosis-

related oxidative stress, either by production of antioxidative melanin or by scavenging the stress itself. Thereupon, phagocytosis was upregulated without increasing oxidative reactions. In melanocytes, tyrosinase has been found to interact with oxidants like H_2O_2 (Wood & Schallreuter, 1991), but it is still unclear whether it has pro- or antioxidative function here. RPE melanosomes have also been found to have peroxidase activity, when they fused with phago-lysosomes (Schraermeyer & Stieve, 1994). Thus, they might be able to decrease peroxide production of ingested material. Furthermore, dark skinned people suffer less oxidative stress in the background of the eye than Caucasians, resulting in a reduced prevalence for AMD (Ambati et al, 2003; Gregor & Joffe, 1978; Pauleikhoff & Holz, 1996; Weiter et al, 1986). Whether this is due to the antioxidative action of tyrosinase, melanin itself or its precursors remains unknown. Additional data (not shown in the manuscripts; n=1, 6 wells) showed that transduced, pigmented ARPE-19 cells had the ability to survive more than 38 days in cell culture without passaging, while control cells died earlier.

The results gathered do not prove, but strongly indicate, a function of tyrosinase in reducing oxidative stress in the adult RPE. Future experiments, using tyrosinase transduced ARPE-19 cells in the UV-A light set up, as introduced in **Manuscript 1**, might clarify the hypothesis completely.

Improvement of RPE characteristics after tyrosinase transduction

Like other cell types, RPE cells also undergo senescence in culture. They lose typical RPE characteristics like their epithelial morphology (Grisanti & Guidry, 1995), their pigmentation and phagocytic function (Carr et al, 2009). Likewise, immortalised ARPE-19 cells dedifferentiate with ongoing passages and gain a spindle-shaped, fibroblastic appearance, although they keep many functions for a long time (Dunn et al, 1996). However, it was found that ARPE-19 cells, which were transduced with AdTyr, appeared “redifferentiated” compared to control cells, and regained their hexagonal, epithelial morphology and pigmentation, and they also showed an enhanced phagocytic activity. It is assumed that tyrosinase activated downstream effectors of differentiation, leading to changes in morphology and improved functioning of RPE cells. This is also indicated by findings from Gargiulo et al., who observed that retinal pigmentation and vision were regained in a mouse model of oculo-cutaneous albinism after tyrosinase transduction (Gargiulo et al, 2009).

The premelanosome and the classical pathway of melanogenesis are not involved in ARPE-19 cell melanogenesis

In the experiments for **Manuscript 3,** it was observed that melanogenesis can occur without the formation of premelanosomes. The lack of essential melanogenic proteins (PMEL17 and TRP1) together with the complete absence of typical melanogenic stages in ARPE-19 cells revealed that an alternative pathway of melanin formation does exist in this artificial system. Tyrosinase did not show the typical distribution inside trans-Golgi cisterns and Golgi-derived vesicles (approx. 59 nm in diameter), as described for melanocytes (Hearing, 2005; Raposo et al, 2007) and pre-natal RPE in the literature (Stanka, 1971). Instead, it was transported from the ER to smaller DOPA granules (approx. 34 nm in diameter), which had no membrane (Figure 9) and further to multi-shaped organelles of unknown origin, where melanin was synthesised later on. Hence, an alternative pathway of tyrosinase trafficking is proposed. It is assumed that tyrosinase was synthesised at the ER and transported to the melanogenic stages without processing in the Golgi apparatus, as indicated by the frequent DOPA staining of cytoplasmic membranes of unknown origin and the staining of typical ER tubules. On the other hand, the high occurrence of free tyrosinase in the cytoplasm indicates a production of the enzyme at the free ribosomes, as proposed by (Moyer, 1963). Normally, aberrations in processing and transportation of tyrosinase lead to degradation of the protein inside proteasomes (Berson et al, 2000; Halaban et al, 1997) and lysosomes (Chen et al, 2009; Wasmeier et al, 2006). Here, proper glycosilation and processing of the enzymes was not evaluated, but the presence of eumelanin inside former multi-lamellar and multi-vesicular bodies in the present work indicates a suitable processing.

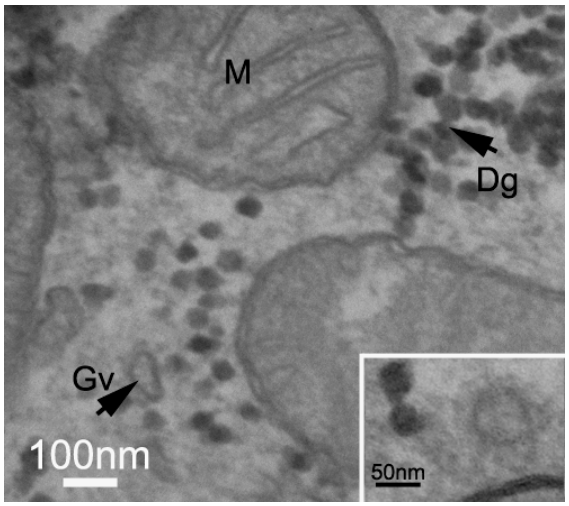


Figure 9: DOPA granules of a transduced APRE 19 cell: DOPA granules (Dg) have the half diameter of Golgi-derived vesicles (Gv) and lack a covering membrane. In contrast, the double membrane of the mitochondria (M) can clearly be observed as two independent lines. Inset: as in 9, higher magnification

For comparison to the classical stages I to IV, the melanogenic stages in ARPE-19 cells were termed stages 1 and 2 for early stages and stage 3 and 4 for later stages. Table 1 summarises the occurrence of MVBs, MLBs and mixed bodies as precursors of the melanogenic stages 1-4 at different time points after transduction. The resulting mature melanin granule (stage 4) was morphologically similar to the round melanosomes of melanocytes, but not to typical oval RPE melanosomes.

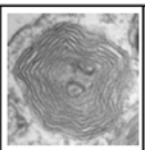
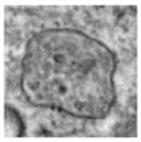
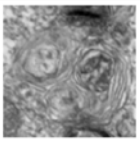
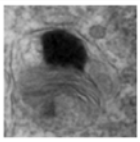
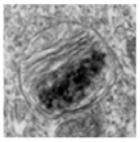
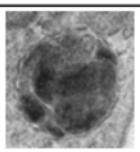
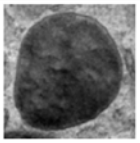
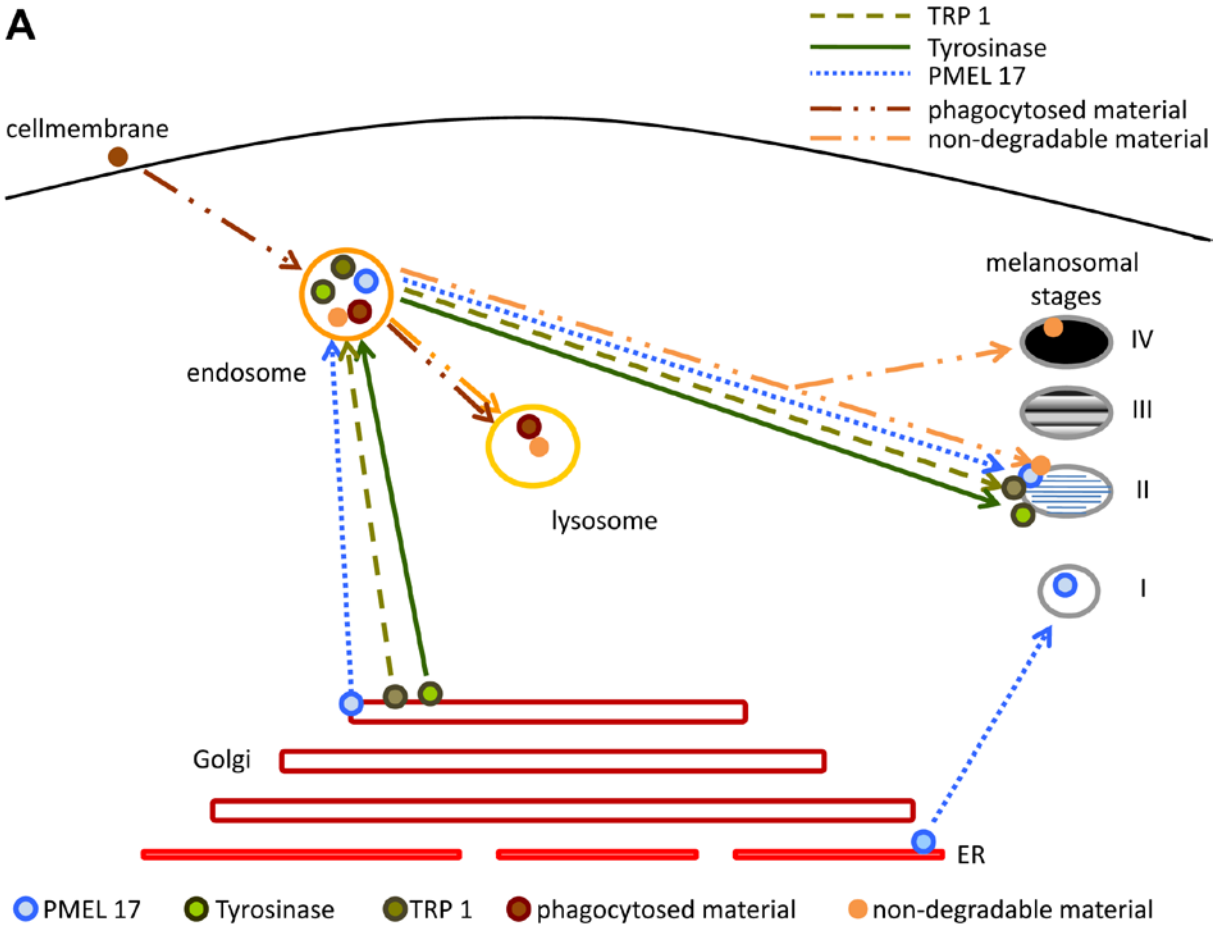
				melanogenic stages 1-4			
							
	MLB	MVB	Mixed Body	low melanised	medium melanised	highly melanised	full melanised
c	++++	+	++	-	-	-	-
48h	++++	++++	+++	+	-	-	-
6d	++	+++	++++	++	++	+	-
14d	+	++++	++	++	++	++	++

Table 1: Occurrence of unusual melanogenic stages 1 to 4 in controls (c) and at different time points after transduction: - rarely seen (less than 3% of counted organelles), + present (5-7%), ++ frequently seen (-15%), +++ often seen (-25%), ++++ mainly seen (>26%). In addition, multi-vesicular bodies (MVB), multi-lamellar bodies (MLB) and mixed bodies are included, which are suggested to be the precursors of melanogenesis in ARPE-19 cells (Figure from manuscript 3).

Since DOPA vesicles were found inside phagosomes (Figure 7 H) and the ultrastructure of the melanised multi-shaped organelles was similar to the phagocytic vesicles of **Manuscript 2**, and earlier studies (Novikoff, 1973; Schraermeyer & Stieve, 1994) it is speculated whether the MVBs and MLBs documented in this work belong to the phago-lysosomal pathway. Although it is still not confirmed that phagocytosed material was used for melanin synthesis, it is assumed that melanogenesis occurred in lysosome-like organelles without the additional involvement of premelanosomes, Golgi-derived vesicles and the typical stages of melanin formation.

Interestingly, similar electron-dark and cloudy organelles were also found in the MNT-1 cells, together with the typical melanogenic stages. It is assumed that the pathway of melanin synthesis which has been described for the transduced ARPE-19 cells, does also occur in MNT-1 cells. A comparison of the classical pathway of melanogenesis and the alternative pathway, described here is given in Figure 10.


A
TRP 1
Tyrosinase
PMEL 17
phagocytosed material
non-degradable material
cellmembrane
melanosomal
stages
IV
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ER
PMEL 17
Tyrosinase
TRP 1
phagocytosed material
non-degradable material

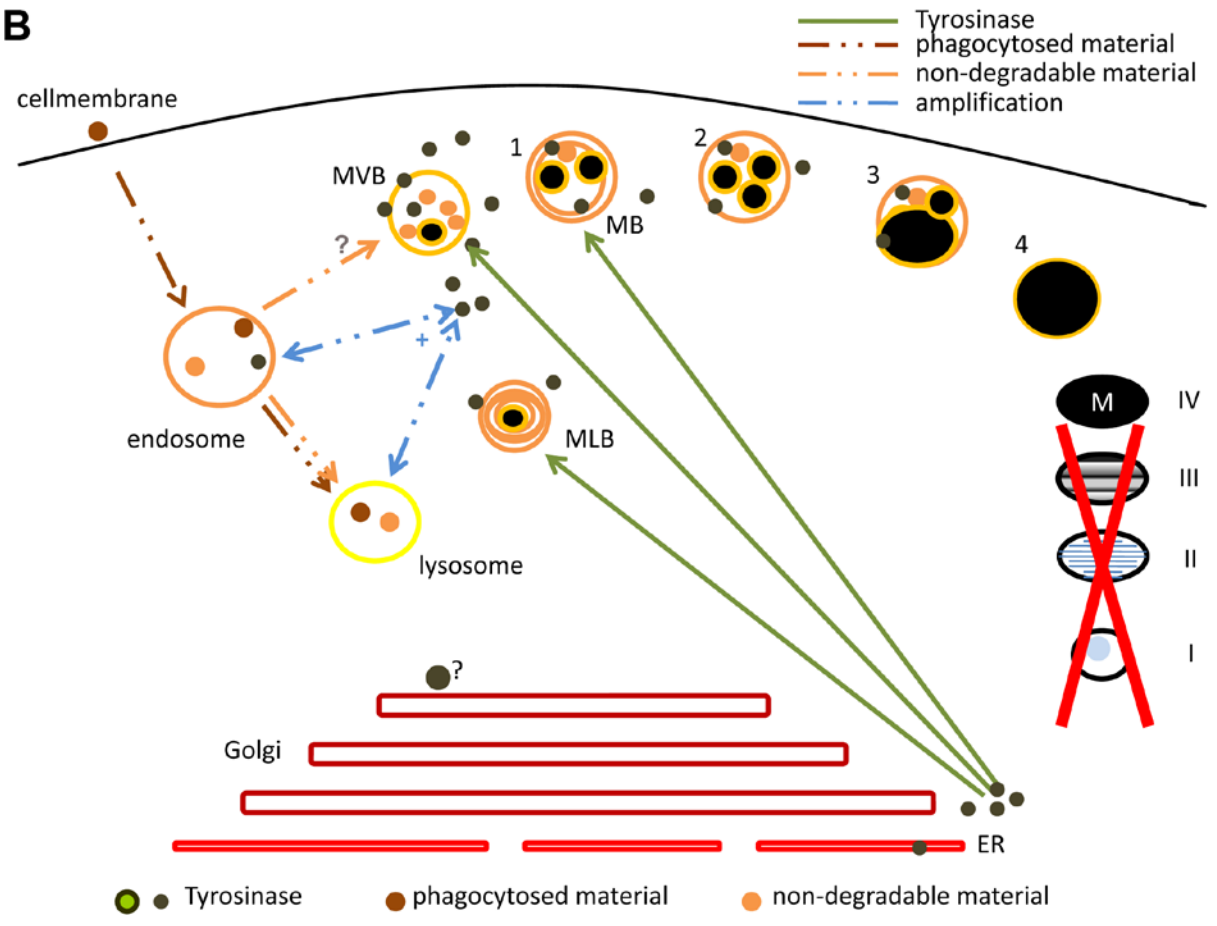
B
Tyrosinase
phagocytosed material
non-degradable material
amplification
cellmembrane
MVB
1
MB
2
3
4
?
+
endosome
MLB
M
IV
III
II
I
lysosome
?
Golgi
ER
Tyrosinase
phagocytosed material
non-degradable material

Figure 10: Comparison of the typical pathway of melanogenesis in pigment cells (A) and a newly discovered pathway for adult ARPE-19 cells found in this work (B): A) The scheme describes the typical transport pathways of melanogenic proteins to the classical stages of melanogenesis (scheme adapted from Hearing 2005): Tyrosinase, TRP1 and PMEL17 travel inside coated Golgi-derived vesicles first to endocytic organelles (early and late endosomes) and later to stage II melanosomes. PMEL17 is also able to travel directly to stage I melanosomes. Additionally, the transport of phagocytosed material to endocytic organelles and finally to lysosomes and to melanosomes (non-degradable material) in RPE cells is described.
B) Alternative pathway of melanin synthesis in tyrosinase-transduced ARPE-19 cells. Only tyrosinase, without support from Pmel17 and TRP1, is needed for melanogenesis. Premelanosomes and typical stages of melanogenesis (I-IV) have not been found; instead, melanin formation takes place in multi-lamellar (MLB), multi-vesicular (MVB) and mixed (MB) lysosome-like organelles. The assumed sequence of melanogenic phases in this system is indicated by the numbers 1-4. Tyrosinase trafficking is also different to the classical scheme, since small DOPA granules, which lack a covering membrane, are observed in the cytoplasm instead of typical Golgi-derived vesicles. Actually, it is not evident if the Golgi apparatus and related vesicles are involved in this pathway (black ?) and it is still not confirmed whether phagocytosed material is integrated in the melanosomal matrix (gray ?). Phagocytosis and tyrosinase presence amplify each other's occurrence and activity (blue ✚).

Findings from animal experiments

For the animal experiments (which are not included in the manuscripts), the vector AdTyr was injected sub-retinally into the eyes of albino rats (n=4 Wistar, n=1 Sprague Dawley) and one albino rabbit (New Zealand White). The eyes were investigated with DOPA and routine electron microscopy at different time points after transduction. DOPA staining was observed in ER tubules, but was lacking in Golgi cisterns and related vesicles. Membrane-bound electron-dark organelles, resembling melanin granules, were frequently found (Figure 11). Whether these electron-dark organelles are composed of melanin polymers has not been studied yet. Nevertheless, the experiments implicate that the findings from the ARPE-19 cells can also be transferred to the *in vivo* system. Future experiments, using novel electron microscopical methods like EDX (Energy Dispersive X-ray spectroscopy) and EELS (Electron Energy Loss Spectroscopy) on the one hand and cryo-electron microscopy and labelling of retinal outer segments with gold-labelled antibodies or ^{3}H-labeleld protein on the other hand will determine respectively the chemical composition of RPE pigments and whether degraded material can be incorporated in newly formed melanin granules.

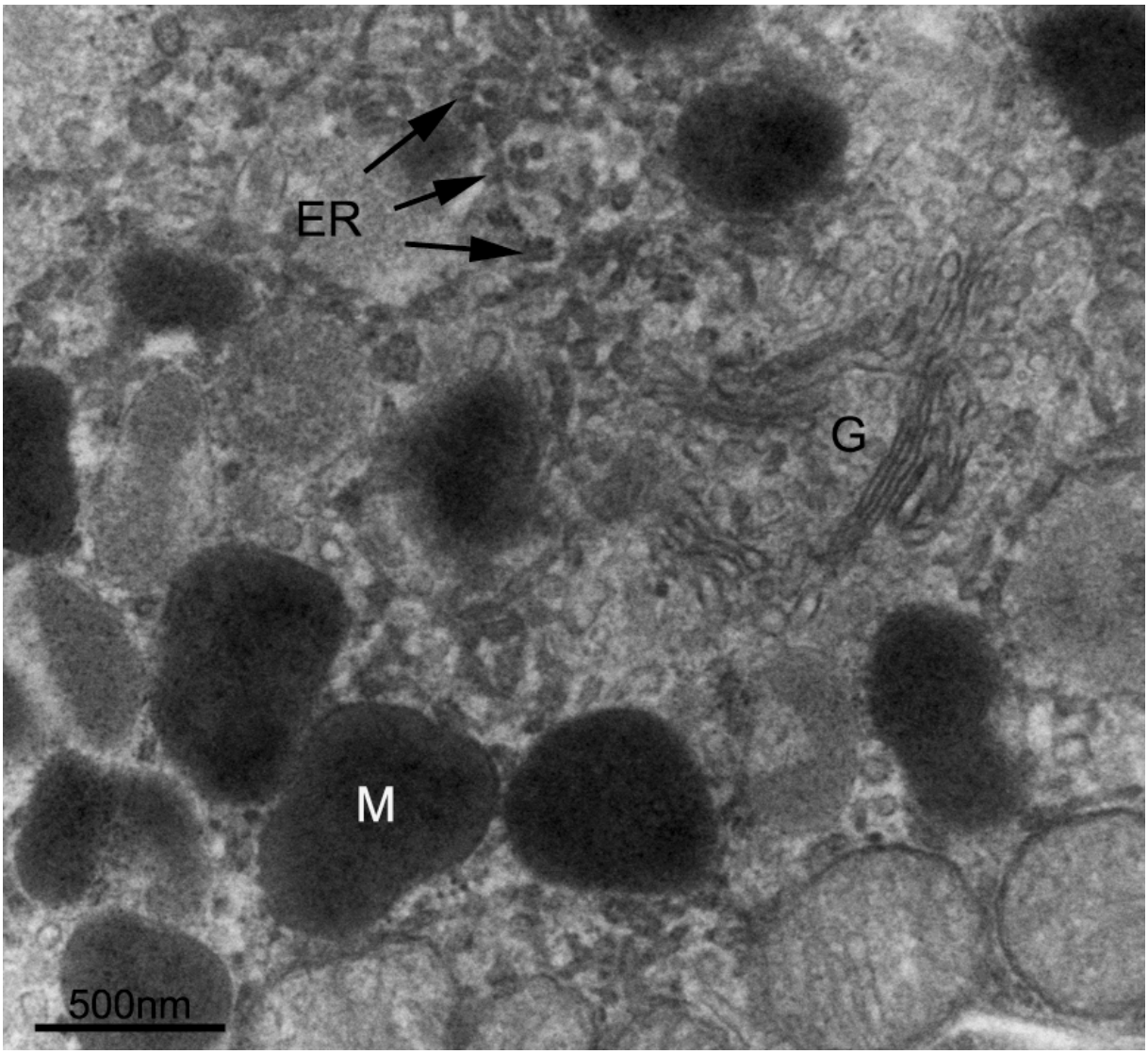

Figure 11: DOPA reaction, showing a tyrosinase-transduced RPE cell of a SD rat (5 million virus particles in 20 µl PBS were injected; the eyes were fixed nine days after transduction): ER tubules (ER) are DOPA positive (arrows), while the Golgi apparatus (G) is not stained. Additionally, membrane-bound electron-dark granules, resembling melanosomes (M) can be observed.

Tyrosinase as a possible application in AMD therapy

Changes in adult RPE pigmentation lead to oxidative stress and associated disorders. First approaches in the transplantation of healthy pigment epithelial cells from the iris in rats (Schraermeyer et al, 1999a; Semkova et al, 2002) and humans (Lappas et al, 2000) and transplantation of bone marrow-derived stem cells (Otani et al, 2004) give hope for the renewal of RPE and photoreceptors (Leveillard et al, 2007; MacLaren et al, 2006) as a new strategy in the prevention and early treatment of AMD. Undegradable material from ROS phagocytosis is the substrate for lipofuscinogenesis and it is proposed that melanolipofuscin is generated by the attachment of lipofuscin to mature melanosomes. In the present work, it has been

shown that tyrosinase expression enhances phagocytosis of RPE cells, leads to a morphological redifferentiation of cultured cells and to repigmentation. It has also been observed that phagocytosis of ROS enhances melanogenesis in this system without the generation of prooxidative lipofuscin. The properties of the tyrosinase vector bear the possibility of rescuing RPE cells of elderly people. Future experiments, with more improved tyrosinase vectors might help in creating a gene therapy for the prevention of age-related maculopathies.

In conclusion, the question whether tyrosinase and melanisation are involved in defence against stress has not been fully answered. Unfortunately, the influence of melanin in primary RPE cells could not be investigated, because lipofuscin accumulation in aged cells distorted the results. Nevertheless, some findings indicate a protective role for tyrosinase, since transduced cells appeared morphologically reorganised and had improved functions. In addition, the viability of ARPE-19 cells after transduction was not altered compared to non-transduced controls, demonstrating that tyrosinase and newly formed melanin were not toxic to the cells.

The hypothesis that melanogenesis can be induced in non-pigmented adult human RPE cells has been verified, since melanin synthesis was measured quantitatively with light and electron microscopy and qualitatively with HPLC analysis after transduction with the vector AdTyr. Furthermore, a pathway of melanogenesis is presented, which is different to the classical scheme described in the literature. This pathway lacks both typical premelanosomes and tyrosinase trafficking using Golgi-derived vesicles. Instead, a formation of tyrosinase at free ribosomes and trafficking of the soluble protein to melanosomal stages are indicated.

Evaluating the influence of ROS phagocytosis on tyrosinase activity and melanogenesis, diverging results have been obtained for primary donor RPE cells and ARPE-19 cells, respectively. While primary cells were able to activate tyrosinase in response to phagocytosis, non-transduced ARPE-19 cells did not produce tyrosinase in response to feeding alone. Nevertheless, after induction of tyrosinase with the adenoviral vector AdTyr, they synthesised more tyrosinase in response to ROS feeding than merely transduced cells. Additionally, they were also able to build more melanin. The transfer of ingested material to melanosomes could not be confirmed for ARPE-19 cells. Nevertheless, in the present experiments, no lipofuscin

was generated, demonstrating that the degradation machinery was not inhibited by the experiments. Moreover, the absence of lipofuscin indicates a beneficial role for tyrosinase in ROS degradation.

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1. Erklärung zum Eigenanteil an den Manuskripten

Biesemeier A., Kokkinou D., Julien S., Heiduschka P., Berneburg M., Bartz-Schmidt K.U., Schraermeyer U. **UV-A induced oxidative stress is more prominent in naturally pigmented aged human RPE cells compared to non-pigmented human RPE cells independent of zinc treatment.** J Photochem Photobiol B. 2008 Feb 27;90(2):113-20. Epub 2007 Dec 4.

20% der konzeptionellen Planung und Anfertigung des Manuskriptes und 20% der Versuchsvorbereitung wurden von meiner Vorgängerin Frau Dr. med. Despina Kokkinou durchgeführt. Nach ihrem Weggang wurden sie von mir weiterbearbeitet, angepasst und zur Publikation gebracht. 80% der konzeptionellen Planung und Anfertigung des Manuskriptes, 80% der Versuchsvorbereitung und 100% der Versuchsdurchführung und Auswertung wurden von mir geleistet. Herr Prof. Dr. Berneburg hat uns die UV-A-Bestrahlungs-Einheit zur Verfügung gestellt und Herr Dr. Heiduschka und Frau Dr. Julien haben Korrektur gelesen.

Julien S., Kociok N., Kreppel F., Kopitz F., Kochanek S., **Biesemeier A.**, Blitgen-Heinecke P., Heiduschka P., Schraermeyer U. **Tyrosinase biosynthesis and trafficking in adult human retinal pigment epithelial cells.** Graefes Arch Clin Exp Ophthalmol. 2007 Oct;245(10):1495-505. Epub 2007 Feb 21.

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Das Manuskript wurde von Frau Dr. Julien geplant und geschrieben. Ich habe die Isolation der RPE-Zellen und deren Kultur durchgeführt und weitere Techniken (Phagozytosetest, Transfektionsstudien) unserer Arbeitsgruppe dabei erlernt. Die adenoviralen Vektoren wurden von Herrn Prof. Dr. Kochanek und Herrn Dr. Kreppel (Institut für Gentherapie, Universität Ulm) hergestellt. Die RT-PCR wurde von Herrn Prof. Dr. Kociok (Netzhautlabor, Köln), die Tyrosin-Hydroxylase-Aktivitäts-Studien wurden von Herrn Prof. Kopitz (Institut für Pathologie und Neurochemie, Universität Heidelberg) durchgeführt.

Biesemeier A., Kreppel F., Kochanek S., Schraermeyer U. **The classical premelanosome, known from pre-natal melanogenesis, is not essential for melanisation in adult RPE cells.** (Cell & Tissue Research, in print).

Die Originalveröffentlichung wird erscheinen auf www.springerlink.com.

Die vollständige konzeptionelle Planung und Anfertigung des Manuskriptes und 100% der Versuchsvorbereitung, Durchführung und Auswertung der Experimente wurden von mir geleistet. Die Standard-Einbettung der Zellen für die Elektronenmikroskopie wurde von mir für die Verwendung in Zellkulturplatten leicht abgewandelt. Die adenoviralen Vektoren wurden von Herrn Prof. Dr. Kochanek und Herrn Dr. Kreppel (Institut für Gentherapie, Universität Ulm) hergestellt und die zu verwendende Menge an infektiösen Partikeln für die Verwendung an humanen RPE Zellen von mir etabliert.

Biesemeier A., Blitgen-Heinecke P., Kreppel F., Kochanek S., Schraermeyer U. **Tyrosinase in conjunction with phagocytosis of retinal outer segments influences the morphology and melanogenesis in cultured human ARPE-19 cells.** (submitted to Exp Eye Res).

Ich habe die Versuche vollständig selbst geplant, durchgeführt und ausgewertet und das Manuskript selbst geschrieben. Nur die HPLC Analysen wurden von Frau Dr. Petra Blitgen-Heinecke durchgeführt. Die adenoviralen Vektoren wurden von Herrn Prof. Dr. Kochanek und Herrn Dr. Kreppel (Institut für Gentherapie, Universität Ulm) hergestellt.

Bei keinem der angeführten Manuskripte ging der Anteil von Prof. Dr. Schraermeyer sowie Prof. Dr. Mallot über das im Rahmen eines Betreuungsverhältnisses übliche Maß hinaus.

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Biesemeier A, Blitgen-Heinecke P, Kreppel F., Kochanek S., Schraermeyer U. Tyrosinase in conjunction with phagocytosis of retinal outer segments influences the morphology and melanogenesis in cultured human ARPE-19 cells. Submitted to Exp Eye Res.

Biesemeier A, Kreppel F, Kochanek S, Schraermeyer U. The classical premelanosome, known from prenatal melanogenesis, is not essential for melanisation in adult RPE. Cell & Tissue Research, in print.

Kokkinou D, Julien S, **Biesemeier A**, Schraermeyer U. Zinc deficiency leads to lipofuscin accumulation in pigmented but not in albino rat eye. Submitted.

Vorträge

Biesemeier A, Schraermeyer U. Das klassische Prämelanosom - Organell der Pigmentsynthese im Melanozyten - ist für die Melanogenese des RPE nicht essentiell notwendig. Symposium des Netzwerks Elektronenmikroskopie Tübingen (NET), Tuebingen, 26.05.2008

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Poster

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3. Manuskripte

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UV-A induced oxidative stress is more prominent in naturally pigmented aged human RPE cells compared to non-pigmented human RPE cells independent of zinc treatment

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Abstract

To investigate the effects of zinc supplementation on human amelanotic (ARPE-19) and native pigmented retinal pigment epithelial cells (hRPE) under normal light conditions and after ultraviolet A light exposure.

hRPE cells, containing both melanin and lipofuscin granules, were prepared from human donor eyes of 60–70 year old patients. Cells of the amelanotic ARPE-19 cell line and pigmented hRPE cells were treated with zinc chloride and subjected to oxidative stress by UV-A irradiation. Intracellular H_2O_2 formation was measured using a fluorescence oxidation assay. Additionally, apoptosis and viability assays were performed. Control cells were treated identically except for irradiation and zinc supplementation.

Under normal light conditions, zinc treated hRPE cells produced less H_2O_2 than unsupplemented hRPE cells. Viability and apoptosis events did not change.

After UV-A irradiation, ARPE and hRPE cells were greatly impaired in all tests performed compared to the non-irradiated controls. No differences were found after zinc supplementation. hRPE cells showed a higher apoptosis and mortality rate than non-pigmented cells when stressed by UV-A light.

ARPE cells never showed any zinc related effects. In contrast, without irradiation, zinc supplementation reduced H_2O_2 production in pigmented hRPE cells slightly. We did not find any zinc effect in irradiated hRPE cells. After UV light exposure, pigmented cells showed a higher apoptosis and mortality than cells lacking any pigmentation. We conclude that cells with pigmentation consisting of melanin and lipofuscin granules have more prooxidative than antioxidative capacity when stressed by UV light exposure compared to cells lacking any pigmentation.

Keywords: Human retinal pigment epithelial cells; Melanin; Melanolipofuscin; Lipofuscin; Oxidative stress; UV-A irradiation

1. Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly population of the Western world [1]. While some population-based studies postulate that there is no association between skin/ eye colour and AMD development [2–4], others showed that AMD is more pronounced in white than in black people [5–8]. Furthermore, accumulation of age pigments (lipofuscin) was found to occur more in Caucasians than in Afro-Americans [7].

The functional integrity of the retina depends on the maintenance activity of the pigmented retinal epithelium [9]. High pigmentation, mainly melanin, is believed to play

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a protective role for the retina by sequestering heavy metals that might otherwise catalyze undesired oxidative reactions [10], or by trapping free radicals produced by photochemical reactions [11]. In contrast, melanin is also known to produce free radicals and to oxidize physiological substrates during ultraviolet and visible light exposure [12–15].

Defects in RPE (retinal pigment epithelium) melanin enhance sensitivity to light damage [16,17]. Moreover, formation of melanolipofuscin with age causes changes in metal binding capacity [18]. High levels of melanolipofuscin have been associated with various pathologies including ocular disorders like AMD and retinitis pigmentosa [19]. Lipofuscin is an auto-fluorescent, granular pigment that accumulates in the cytoplasm of postmitotic cells. It has an excitation maximum within the range of UV-A and visible blue light, while it is emitting in the yellow–orange range [20]. Therefore, it is expected to have photo-oxidative capacity [21], reviewed in [19], which can be counteracted by the application of zinc and antioxidants.

Currently, application of zinc is one possible treatment of dry AMD. Zinc with its direct and indirect antioxidant effects [22,23] has been shown to delay AMD progression and reduces vision loss [24]. Zinc is associated with melanin in pigmented tissues [25] and is known to participate as a cofactor of several antioxidative enzymes [26,27]. Zinc deficiency increases oxidative stress in the retina [28], and lack of metal ions like zinc and copper is associated with lipopigment accumulation in the aging RPE [29].

However, zinc has also been shown to induce oxidative stress under special conditions [30]. Lengyel et al. found that high amounts of zinc were located especially in sub-RPE deposits of AMD maculae, and they postulated that an overdose of zinc promotes progression of AMD in the aging eye [31]. High concentrations of zinc may cause RPE and photoreceptor cell death through zinc-induced lipid peroxidation [32]. It seems that the zinc effect is concentration dependent. While low doses protect cells from oxidative stress, higher amounts have the opposite effect [33].

Oxidative stress, a disturbance in the prooxidant–antioxidant balance in favour of the prooxidants with potential damage, is believed to be involved in the pathogenesis of AMD [34–36], although this assumption remains unproven [37]. The high oxygen pressure in the tissue and light exposure are discussed as being the reason for prooxidative reactions in the receptor and RPE layer [34].

The aim of this study was to investigate whether pigmentation, zinc or a combination of both have a photoprotective effect in human retinal pigment epithelial cells after UV-A light exposure. For comparison, non-pigmented ARPE-19 cells were used.

2. Materials and methods

2.1. Human non-pigmented RPE cells

Human amelanotic retinal pigment epithelial cells (ARPE-19 cell line) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and used between passages three and thirteen. Cells were grown in DMEM medium with 10% fetal calf serum (FCS) and 1% antibiotics (containing 5 units/ml Penicillin G and 5 mg/ml Streptomycin) in 250 ml culture flasks (Greiner, Frickenhausen, Germany). All cell culture reagents were obtained from GIBCO (Invitrogen, Karlsruhe, Germany). Experiments were performed in 96-well culture plates (Costar Corning, Bodenheim, Germany).

2.2. Human natural pigmented RPE cells

The eyes were obtained from five human organ donors aged between 60 and 70 years. The eyes were delivered without the cornea. The vitreous was removed. The retina was gently detached and removed with tweezers after incubation with PBS for 5 min. The remaining choroid/RPE complex was incubated with 0.25% Trypsin-EDTA for 30 min at 37 °C. The floating RPE cells were collected by pipetting with a 1 ml pipette tip. After resuspension in complete medium (DMEM medium with 10% FCS + 1% antibiotics), the cells were plated and grown until confluency in $35 \times 10\ mm^2$ culture plates (Greiner, Frickenhausen, Germany).

2.3. Additional material

MTT reagent, DCFDA (2′,7′-dichlorofluorescein diacetate) and $ZnCl_2$ were purchased from Sigma, Deisenhofen, Germany; Dimethylsulfoxyd (DMSO), Triton X-100 and paraformaldehyde (PFA) from Merck, Darmstadt, Germany, and the TUNEL Assay (In Situ Cell Death Detection Kit, Fluorescein) from Roche Diagnostics GmbH, Mannheim, Germany.

2.4. Irradiation

Irradiation was performed using a Sellasol 1200 irradiation device (Sellas Medizinische Geräte GmbH, Ennepetal, Germany) emitting light with wavelengths from 330 to 440 nm (maximum at 375 nm). Light intensity was measured with an electronic power device connected to an optical sensor for UV-A (RM12, Dr. Groebel UV-Elektronik, Ettlingen, Germany). The cells were irradiated with 20 mW/cm^2 for 15 min in the H_2O_2 assay and for one hour in the TUNEL and MTT assay.

2.5. Experimental procedure

Cells were cultured in 96-well culture plates (15,000 cells/well). After twenty four hours, one group of cells was incubated with 100 μM zinc chloride in complete medium for two hours. The other group was incubated in complete medium only. Twenty four hours later, samples of both groups were incubated in PBS and irradiated with UV-A light (20 mW/cm^2). Control groups were kept under normal light conditions without zinc supplementation.

2.6. Measurement of intracellular H_2O_2 formation in ARPE-19 and hRPE cells using DCFDA

The fluorescence substance DCFDA was used for imaging the intracellular formation of H_2O_2 after UV-A irradiation. DCFDA emits fluorescence light when oxidized by H_2O_2.

Twenty four hours after zinc addition, cell cultures of zinc-treated and untreated cells were incubated with 60 μM DCFDA in PBS (instead of PBS alone) for 15 min at 37 °C. The samples were then exposed to UV-A light for another 15 min. After UV-A light exposure, the fluorescence intensity of the supernatants was measured in a fluorescence microplate reader (excitation 495 nm, emission 530 nm). Cell cultures prepared under the same conditions without UV-A irradiation were used as controls. We also confirmed that there was no auto-fluorescence of the dye-solution without cells when exposed to UV-A light.

2.7. Measurement of cell viability via MTT test 48 h after UV-A irradiation

In the viability tests, the cells were irradiated for one hour in PBS only and transferred afterwards to complete medium. Forty eight hours after exposure to UV-A light, the viability of the cells was tested with an MTT assay. The medium was removed and new medium with 10% MTT reagent (5 mg/ml in PBS) was added. After three hours of incubation at 37 °C in a humid dark chamber, cells were lysed with DMSO, and absorbance was measured at 570 nm in a microplate reader. UV-A dose was adjusted to a lethal dose of 50–60% for the ARPE cells.

2.8. Apoptosis in ARPE-19 and hRPE cells 48 h after UV-A irradiation

TUNEL staining (Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labelling) was used for detection and quantification of apoptosis as a result of oxidative stress, based on labelling of DNA strand breaks.

The cells were treated as in the MTT test. Forty eight hours after UV-A exposure, the TUNEL assay was performed as described in the manual. Briefly, cells were fixed with 4% PFA at room temperature for 20 min and subsequently washed with PBS. Permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate buffer) was applied, and the cells were kept on ice for two minutes. After two washes with PBS, the cells were incubated with TUNEL reaction mixture (enzyme solution and label solution) for one hour at 37 °C in a humid dark chamber. Label solution was applied only for negative controls. For a positive control, cells were treated with DNAse. After incubation, the cells were washed three times with PBS. The cells were examined under a fluorescent microscope (Axiovert 135, Zeiss) with an excitation of 450–490 nm and an emission of 515 nm LP filter. Pictures of equal sized areas (20× magnification) were taken for the counting of apoptotic nuclei.

2.9. Routine electron microscopy

Tissue pieces were fixed overnight at 4 °C in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 100 mM sucrose. Then the specimens were postfixed with 1% OsO_4 at room temperature in 0.1 M cacodylate buffer for 3 h, blockstained with uranyl acetate, dehydrated in a graded series of ethanol, and embedded in Spurr's resin. Ultra thin sections were post stained with uranyl acetate and lead citrate and investigated under an electron microscope (EM 902A; Zeiss).

2.10. Statistical analysis

Statistical evaluation was based on Student's *t*-test, and *p*-values were considered not significant when exceeding 0.05. All groups were compared to the ARPE-19/hRPE cells without zinc and without UV-A exposure.

3. Results

3.1. H_2O_2 formation in ARPE-19 and hRPE cells

When H_2O_2 is produced inside living cells, the DCFDA substance shows a green fluorescence when excited at 495 nm, the intensity proportional to H_2O_2 production. DCFDA fluorescence intensities of non-irradiated ARPE and hRPE cells without zinc were set 100% and used as controls. Fluorescence intensities of cells with or without zinc and UV-A exposure were compared (Fig. 1).

Irradiated ARPE cells with zinc ($p = 0.01$) and without zinc supplementation ($p = 0.006$) produced more H_2O_2 than control cells. No zinc effect could be shown for irradiated and non-irradiated ARPE cells, respectively ($n = 24$).

Similarly, irradiated hRPE cells with and without zinc treatment showed an increase in H_2O_2 production compared to non-irradiated cells ($p < 0.0001$). Cells treated with UV-A light showed no zinc related effect amongst each other. In contrast to non-pigmented ARPE cells, non-irradiated hRPE cells treated with zinc showed slightly less H_2O_2 production ($p = 0.03$) than their control hRPE cells ($n = 36$). Despite its significance, this effect is very small.

3.2. Viability of ARPE-19 and hRPE cells after UV-A irradiation

Cell viability was tested using an MTT assay. The test-substance becomes violet when metabolised by the cell's mitochondria. Viability of untreated control cells was set to 100% (Fig. 2).

Irradiated ARPE cells lost about 60% of their viability ($p < 0.0001$). There was no effect of zinc on the viability of non-irradiated and irradiated ARPE cells ($n = 30$).

The pigmented hRPE cells lost about 90% of their viability after UV-A irradiation ($p < 0.0001$) and showed an approximately three times lower viability compared to irradiated, unpigmented ARPE cells ($p = 0.01$ for cells with

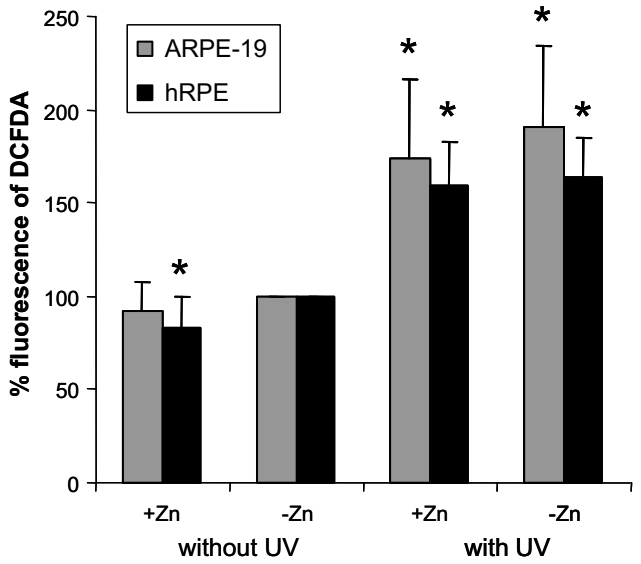

Fig. 1. Intracellular H_2O_2 formation in ARPE-19 and hRPE cells. H_2O_2 production increases significantly in ARPE-19 ($p < 0.01$) and hRPE cells ($p < 0.0001$) after UV-A irradiation. Zinc treatment has no pro- or antioxidative effect on irradiated and non-irradiated ARPE cells. Zinc-treated hRPE cells show a slight decrease in H_2O_2 production under normal light conditions ($p = 0.03$). ($n = 24$ for ARPE; $n = 36$ for hRPE cells). Asterisks mark p-values <0.05 compared to control cells.

zinc; $p = 0.003$ for cells without zinc supplementation). Zinc supplementation had no additional effect on either irradiated or non-irradiated hRPE cells ($n = 21$).

3.3. Apoptosis of ARPE-19 and hRPE cells after UV-A irradiation

Using the TUNEL assay, apoptotic nuclei show a green fluorescence when excited at 495 nm light. The number of apoptotic nuclei was counted using pictures of equal sized areas of each well (Fig. 3).

Non-irradiated ARPE and hRPE cells with or without zinc, respectively, showed no apoptotic pattern. Irradiated ARPE cells with and without zinc were highly apoptotic (both $p < 0.0001$). However, zinc treatment had no effect on ARPE cells ($n = 35$). hRPE cells with and without zinc supplementation ($p < 0.0001$) were also highly apoptotic after UV-A irradiation and showed no zinc effect. The apoptosis rate of hRPE cells after UV-A exposure was higher than that of irradiated ARPE cells ($p = 0.02$ for cells without zinc supplementation and $p = 0.001$ for cells with zinc supplementation; $n = 28$).

All results are summarized in Table 1, including mean values and p-values of the t-tests.

4. Discussion

Age-related macular degeneration (AMD) is a major international socio-economic problem with more than 1.75 million individuals affected currently in the United States and almost 3 million individuals at substantial risk of developing AMD by 2020 [38]. Primary lesions associated with loss of vision are believed to be located in the retinal pigment epithelium [39]. Melanin constituting the pigmentation of the retinal epithelium acts as a scavenger of free radicals produced by oxidative stress resulting from photoreactions [40]. Defects in RPE melanin enhance light sensitivity and increase light damage [9]. Blue light is believed to play the major role in retinal light damage [41], and UV-A light as well. Although absorbed in high amounts by the lens [42], UV-light can damage the DNA of the cells indirectly through the action of photosensitizers [43,44]. Melanin and RPE cells have been found to show

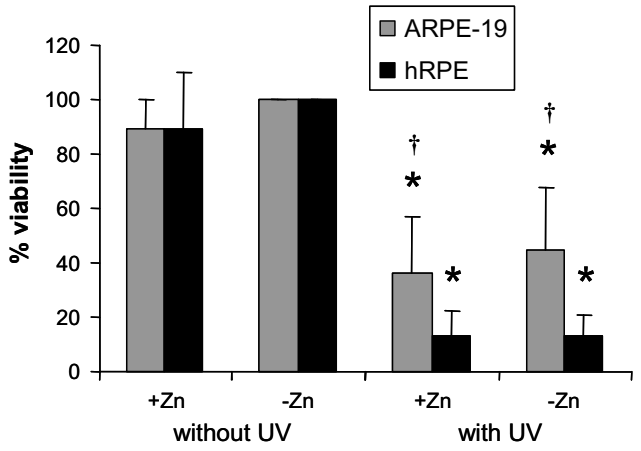

Fig. 2. Viability of ARPE-19 and hRPE cells. After irradiation, cell viability has decreased to about 40% in ARPE-19 cells, independent of zinc supplementation ($p < 0.0001$; $n = 30$). Only 10% of hRPE cells survive after UV-A irradiation ($p < 0.0001$). Zinc supplementation has no antioxidative effect ($n = 21$). Additionally, pigmented cells are more impaired than amelanotic cells ($p = 0.01$ for cells with zinc; $p = 0.003$ for cells without zinc supplementation; marked by †). Asterisks mark p-values <0.05 compared to control cells.

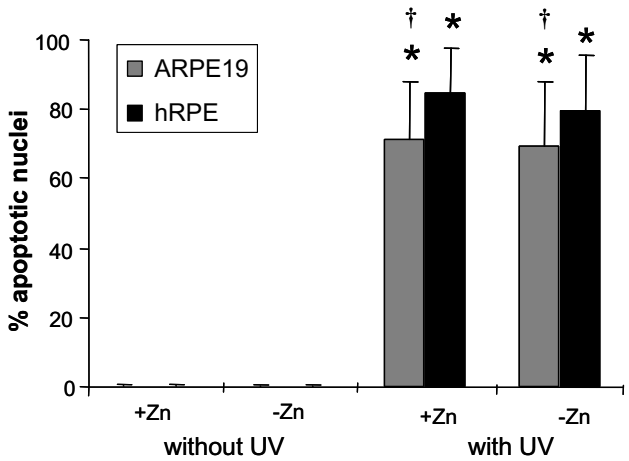

Fig. 3. Apoptosis in ARPE-19 and hRPE cells. ARPE-19 cells undergo apoptosis after UV-A irradiation independent of zinc treatment ($p < 0.0001$; $n = 35$). There is a high increase in apoptosis in hRPE cells after UV-A exposure ($p < 0.0001$; $n = 28$), also higher than in irradiated ARPE cells ($p = 0.001$ for cells with and $p = 0.02$ for cells without zinc supplementation marked by †). Consequently, no zinc effect could be shown in UV-A irradiated cells. Non-irradiated cells showed no apoptosis.

Table 1
Mean values of the different tests and their p-values compared to control samples

	Sample name	H_2O_2 production(I)	Viability(II)	Apoptosis(III)
1	ARPE cells (control 1)	100%	100%	0%
2	ARPE cells with zinc	91.7% ± 15.7%	89.2% ± 10.9%	0%
3	ARPE cells with UV	190.4% ± 44.3% $p = 0.006$ to control 1	44.7% ± 23.2% $p < 0.0001$ to control 1	69.0% ± 19.2% $p > 0.0001$ to control 1
4	ARPE cells with zinc and UV	174.2% ± 42.0% $p = 0.01$ to control 1	36.4% ± 20.6% $p < 0.0001$ to control 1	71.15% ± 16.6% $p > 0.0001$ to control 1
5	hRPE cells (control 2)	100%	100%	0%
6	hRPE cells with zinc	82.6% ± 17.3% $p = 0.03$ to control 2	89.3% ± 20.5%	0%
7	hRPE cells with UV	163.1% ± 21.6% $p < 0.0001$ to control 2	12.9% ± 8.0% $p < 0.0001$ to control 2	79.4% ±16.4% $p > 0.0001$ to control 2
8	hRPE cells with zinc and UV	158.9% ± 24.1% $p < 0.0001$ to control 2	12.9% ± 9.6% $p < 0.0001$ to control 2	84.4% ± 13.3% $p > 0.0001$ to control 2

Additional parameters:

(I) H_2O_2-production (t-test):
Sample 4 compared to 2: $p = 0.01$;
Sample 8 compared to 6: $p < 0.0001$
(II) Viability: t-test:
Sample 4 compared to 2: $p < 0.0001$
Sample 8 compared to 6: $p < 0.0001$
Sample 7 compared to 3: $p = 0.003$(†)
Sample 8 compared to 4: $p = 0.01$(†)
(III) Apoptosis: t-test:
Sample 4 compared to 2: $p < 0.0001$
Sample 8 compared to 6: $p < 0.0001$
Sample 7 compared to 3: $p = 0.02$(†)
Sample 8 compared to 4: $p = 0.001$(†).

oxidative reactions, including hydrogen peroxide production when exposed to light [45,46].

The UV-light device used in this study emitted light with a maximum in the UV-A range, approaching the near blue light. Although we presumed a protective effect of pigmentation in hRPE cells after irradiation, naturally pigmented hRPE cells showed a lower vitality and a higher apoptosis than non-pigmented cells. The donors of the hRPE cells, aged between 60 and 70 years, showed an RPE pigmentation consisting of melanin and high amounts of lipofuscin (Fig. 4). We assume that lipofuscin pigmentation in combination with light exposure had a toxic effect on the hRPE cells that could not be counteracted by the remaining melanin. Other groups have published that exposure of lipofuscin-fed cells to blue light (400–550 nm) leads to a time-dependent loss of cells from the monolayer with the remaining cells exhibiting a change in cell morphology, increased vacuolation and blistering of the cell membrane [47], also seen in our experiments (data not shown). Thus, cumulative toxic damage over a lifetime may contribute to a variety of changes in the outer retina, which may be causal factors in the development of AMD [40,41,48–51]. Our findings are consistent with the findings that lipofuscin has prooxidative capacity. Hence, we can postulate that cells without pigmentation are less stressed by UV-A irradiation than cells with lipofuscin pigmentation.

Zinc has antioxidative capacity, and zinc supplementation in RPE cells has been shown to enhance catalase activity [27]. The ability of melanin to bind metal ions including zinc was widely examined in the past [25]. Borovansky et al. reported a high zinc concentration in isolated melanosomes [52,53]. It has also been reported that its concentration in pigmented ocular tissues is high compared to others [54,55]. The number of functional melanosomes in the fundus [56] and iris [57] is most likely important for the storage, transport or release of zinc. However, high amounts of zinc have been shown to induce oxidative reactions in amelanotic RPE cells, because melanin is absent [58]. Cytotoxicity of zinc has also been reported for normal pigmented eyes [30,59] and depends mainly on its concentration [60]. Zinc is also found to induce cell death and apoptosis after retinal ischemia [61].

This is the first study focused on a direct comparison of amelanotic and naturally pigmented human cells in combination with zinc uptake experiments and UV-A induced oxidative stress. Non-irradiated and irradiated ARPE cells showed no zinc related effects at all, whereas non-irradiated hRPE cells produced slightly less H_2O_2 when treated with zinc, compared to their controls. A protective effect of zinc could not be proved for the irradiated hRPE cells. Pigment granules (lipofuscin, melanin) in human diseased RPE lose the ability to bind metal ions, including zinc [62]. Melanin undergoes age-related changes, to which photooxidation can contribute, playing a central role in pathogenesis [63,64]. Aerobic photoreactivity increases with age and decreasing wavelength and so it is expected to lead to the development of aging disorders like AMD. Melanin and RPE cells have been shown to produce hydrogen peroxide

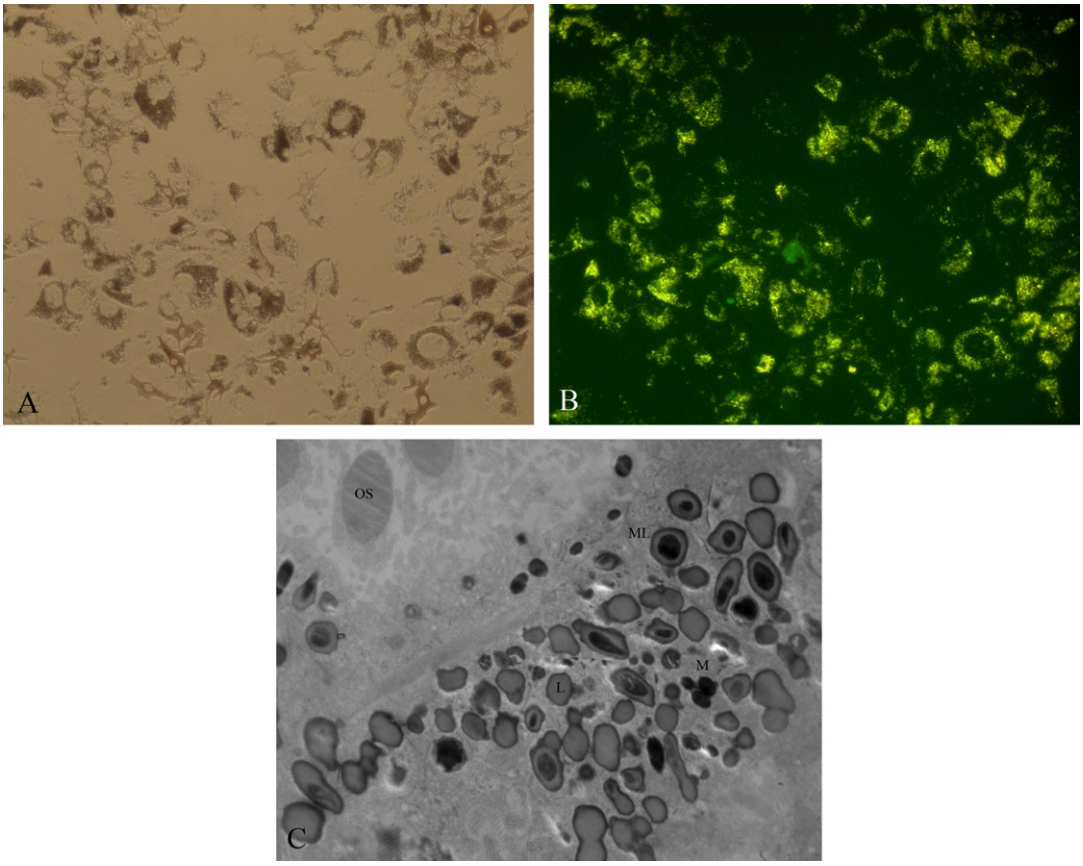

Fig. 4. Pigmentation of hRPE cells. (A) Light image of melanin pigmentation in hRPE cells. (B) Auto-fluorescence of lipofuscin pigmentation of the same area as in (A). Magnification 40×, excitation 450–490 nm LP filter, emission 530 nm. (C) Electron micrograph of an aged human RPE, showing many melanolipofuscin (ML) and pure lipofuscin (L) granules. Hardly any melanin (M) granules are left. This picture demonstrates how the pigmentation in the eye has changed with age. The donor had no obvious eye disease and was the same age as the donors of the irradiated cells.

when exposed to light. Melanin can photogenerate superoxide anions and hydrogen peroxide [46] and has the ability to bind several chemicals and drugs leading to the development of ocular or inner ear lesions [65]. Nevertheless, phototoxicity of melanin is lower than that of lipofuscin and photoprotective effects are of greater importance. It was also shown previously, that the formation of melanolipofuscin rather than the loss of melanin and the increase in lipofuscin has toxic effects on RPE cells and is closely related to the onset of AMD [66].

Our results are consistent with this observation. Pigmented cells showed a higher oxidative damage than unpigmented cells. The high melanolipofuscin amount in our donor eyes was probably the reason we obtained a prooxidative effect after UV-A exposure, rather than an antioxidative effect as a result of the melanin.

The hRPE cells used in this study were isolated from five different donors. Cells of one eye were grown to confluence and then split in three parts for the three different tests to avoid too many passages, and to obtain heavily pigmented cell cultures (Fig. 4A). Individual differences in the pigmentation were present, therefore results differed strongly. Even small differences in pigmentation and cell number, despite confluence, can result in wide differences in the effect of drugs and stress [67]. Additionally, the appropriate concentration of zinc was hard to select in this study, because various groups have found different effects of zinc on RPE cells dependent on conditions and methods. Tate et al. found that 100 μM $ZnCl_2$ protect zinc deficient RPE cells from oxidative stress [27,68]. Ha et al. found that concentrations of zinc chloride and zinc sulphate up to 150 μM were tolerated by normally fed ARPE-19 cells and could induce antioxidative pathways, while concentrations over 200 μM were toxic and resulted in cell death, whereas others stated that zinc concentrations exceeding 10 μM cause apoptosis in RPE cells [33]. In our experiments, zinc supplementation to cells with and without pigmentation led neither to an anti nor to a prooxidative effect. Although we used similar methods, there were some differences compared to the formerly named studies. In our experiments, the cells did not suffer from zinc deficiency, and we used zinc chloride instead of zinc sulphate or other salts. In addition, our donor eyes, obtained from 60–70 year old Caucasians, contained high amounts of lipofuscin.

5. Conclusion

In the present study, we investigated the toxic effect of UV-A light on human amelanotic and naturally pigmented RPE cells. Additionally, the effect of zinc was investigated.

UV-A irradiation led to an increase in H_2O_2 production and apoptosis and a significant decrease in viability in both hRPE and ARPE-19 cells. The antioxidant capacity of the cells could not be enhanced by addition of zinc in either amelanotic or naturally pigmented RPE cells. However, the results of this study are only valid for aged RPE cells and it is unknown whether they are also true for RPE cells of young people. Here, we demonstrate that pigmentation does not always guarantee a better survival of cells. When eyes of donors aged 60–70 years are used, the age pigment lipofuscin is present in high amounts, and the antioxidative capacity of the cells cannot be improved by zinc supplementation when stressed by UV-A light.

Acknowledgements

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LABORATORY INVESTIGATION

Tyrosinase biosynthesis and trafficking in adult human retinal pigment epithelial cells

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Abstract
Background Tyrosinase (EC 1.14.18.1) is the key enzyme of melanin pigment formation and it is unclear whether it is synthesized in human postnatal retinal pigment epithelium (RPE). In this study, we investigated if phagocytosis of rod outer segments (ROS) can increase tyrosinase expression in vitro.
Methods Primary cultures of human RPE cells were fed with isolated ROS from cattle and with latex particles. After phagocytosis, RPE cells were tested for tyrosinase presence and activity with several independent methods: (1) immunocytochemistry with anti-tyrosinase antibodies and (2) ultrastructural as well as light microscopic DOPA histochemistry; (3) mRNA was isolated from human RPE before incubation with ROS and 5, 20 and 40 h after feeding with ROS. The amount of tyrosinase mRNA was determined quantitatively by real-time reverse transcription polymerase chain reaction (RT-PCR), and the tyrosinase activity was investigated by measuring tyrosine hydroxylase activity using [^{3}H]tyrosine.
Results Tyrosinase was found in fed RPE cells using these methods, but was absent without feeding. Furthermore, we showed co-localization of rhodopsin and tyrosinase in the fed RPE cells. Contrary to tyrosinase activity, the mRNA for tyrosinase was clearly present in the cultured RPE cells which had not been exposed to ROS, decreased significantly from 5 h after exposure to ROS and returned to its original non-fed level 40 h after ROS feeding.
Conclusion Our study does not present new evidence that de novo melanogenesis takes place in the adult differentiated RPE. However, in contrast to the classic hypothesis, which states that tyrosinase is only detected in embryos, we provide evidence with several independent methods that the expression of tyrosinase and its enzymatic activity are induced in cultured human adult RPE by phagocytosis of ROS.

Keywords Tyrosinase · Biosynthesis · Retinal pigment epithelium · Human · Phagosome

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Introduction

In humans, melanin pigment is biosynthesized in the neuroectodermic retinal pigment epithelium (RPE) and neural crest-derived melanocytes [27]. Melanin-producing cells contain specific organelles, the premelanosomes in which the binuclear copper enzyme tyrosinase catalyses melanin biosynthesis [28, 48].

The RPE is located between the retina and the choroid of the eye and plays an essential role in ocular metabolism.

Basic adult RPE functions are the formation of the outer blood-retina barrier, transepithelial transport, regulated retinoid storage, scavenging of light-induced reactive oxygen species and digestion of the shed rod outer segment disk membranes [45]. The disk shedding and succeeding phagocytosis processes are regulated by circadian rhythm in RPE cells [23]. Differentiated RPE cells do not divide and remain functional throughout the life of an individual. Tyrosinase is the rate-limiting enzyme of melanin biosynthesis and catalyses three reactions of melanin synthesis: the hydroxylation of L-tyrosine to 3,4-dihydroxyphenyl-L-alanine (L-dopa), the oxidation of L-dopa to dopaquinone [24] and the oxidation of 5,6-dihydroxyindole to 5,6-dihydroxyquinone [21]. It is assumed that RPE melanogenesis is only found prenatally, since tyrosinase, the key enzyme in melanin biosynthesis, was detected in human early stage embryos only and was not detectable after gestation stopped [5, 29, 37, 49].

However, premelanosomes and early-stage melanosomes have been found in adult RPE [10, 41, 45, 55], which led to the hypothesis questioned here. Tyrosinase activity has been found in adult cultured bovine [3, 42], porcine [10], mouse [33], rabbit [52], rat [53] and human RPE cells, too [2]. Additionally, tyrosinase activity was also demonstrated in adult bovine RPE obtained from an eye cup [11].

Tyrosinase promoter activity was significantly upregulated in cultured human RPE cells treated with pigment epithelium-derived factor (PEDF), basic fibroblast growth factor (bFGF), α-melanocyte-stimulating hormone (α-MSH) or L-tyrosine compared with control cells [1]. In conclusion, the tyrosinase gene is not only expressed but can be regulated in response to different chemicals in cultured human RPE cells. However, tyrosinase enzymatic activity was not found in this study [1]. In addition, a recent study shows that phagocytosis of rod outer segments (ROS) induces expression of various genes in RPE cells [6].

The aim of our study was to examine the presence of tyrosinase protein as well as its enzymatic activity in adult human RPE. One of the most important functions of the RPE is phagocytosis of shed rod photoreceptor outer segment disks. Therefore, we investigated whether phagocytosis of ROS can increase tyrosinase expression in vitro.

Materials and methods

Culture of human RPE cells

Human RPE cells were harvested from the eyes of two male keratoplasty donors aged 50 and 61 years within 8 h post mortem. The cells were maintained in minimal essential medium (MEM) containing 5% (v/v) fetal calf serum (FCS). Each third-passage RPE cell preparation was checked by cytokeratin staining. Cell counts were obtained after trypsination. Viability was assessed by crystal violet staining of adherent cells or trypan blue dye exclusion of trypsinized cells.

Isolation of rod outer segments

The procedure was carried out as specified by Schraermeyer and Stieve [42]. In brief, isolated bovine retinae were agitated for 2 min in KCl buffer [0.3 M KCl, 10 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES), 0.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 48% (w/v) sucrose] at pH 7.0 and then centrifuged at 2,000 rpm in a tabletop centrifuge (type UJ 1, Heraeus-Christ, Osterode, Germany) for 5 min. The supernatant was filtered through a tube gauze finger stall, diluted with KCl buffer (1:1) and then centrifuged for 10 min at 2,500 rpm. The pellet, containing ROS fragments, was washed and centrifuged in complete culture medium without serum before feeding to the RPE.

Phagocytosis of rod outer segments/latex particles by human RPE cells

Human RPE cells were exposed to rod outer segments that had been isolated as described above in full culture medium containing serum for 5 h on a microscope slide. As controls, cells were cultured with assay medium only and with latex beads (diameter 0.1 μm, 5×10^7 particles/ml, Sigma, Deisenhofen, Germany) for 5 and 20 h.

Electron-microscopic localization of tyrosinase

The enzyme tyrosinase was localized by electron-microscopic histochemistry [39, 40]. In brief, third-passage RPE cells that had been exposed to fragmented rod outer segments as described above were fixed in 0.1 M sodium cacodylate buffer (pH 6.8) with 2.5% glutaraldehyde for 1 h. RPE cells without feeding were treated in the same manner. Specimens were washed twice in sodium cacodylate buffer and kept at 2-8°C overnight in this buffer, containing 5 mM L-dihydroxyphenylalanine (L-dopa, obtained from Sigma-Aldrich, Taufkirchen, Germany). Thereafter, the solutions were renewed, and the RPE cells were incubated for a further 5 h at 37°C. The RPE cells were washed in sodium cacodylate buffer and immersed for 1 h at room temperature in the same buffer containing a mixture of osmium tetroxide (1%) and potassium ferrocyanide (1.5%). Finally, the RPE cells were dehydrated and embedded in Spurr's resin for routine electron microscopy. Ultrathin sections were stained with uranyl acetate and lead

citrate and examined with a Zeiss EM 902A electron microscope.

Generation of Ad-Tyr

To generate the Ad-Tyr vector, the plasmid 123.B2 (kind gift from T. Woelfel, Mainz) was digested with *Eco*RI to release a 1,906-bp fragment containing the human tyrosinase cDNA [4]. The fragment was blunt ended with Klenow and cloned into the *Eco*RV site of pCMVPac(+) under generation of pVI01. pCMVPac(+) is based on pCMVβ (Invitrogen, Carlsbad, CA, USA) with two modifications: (1) the β-galactosidase coding sequence (*Not*I fragment) is replaced with a polylinker containing a unique *Eco*RV site and (2) upstream of the hCMV promoter and downstream of the SV40 polyA *Pac*I sites are inserted which allow for release of the expression cassette. This plasmid serves as a tool to construct *Pac*I-flanked expression cassettes driven by the hCMV promoter and containing an SV40 late 19s intron for strong ubiquitous expression of cDNAs (further details about pCMVPac(+) can be obtained from the authors upon request). The *Pac*I fragment from pVI01 containing the hCMV promoter, SV40 intron, human tyrosinase cDNA and SV40 polyA was isolated from pVI01 and cloned into *Pac*I of pGS70 [38] under generation of pVI02. Finally, the *Pac*I fragment from pVI02 was cloned into *Pac*I of pGS66 [38] under generation of pVI03, an infectious adenovirus plasmid coding for the E1-deleted Ad vector Ad-Tyr.

The virus backbone was released from pVI03 by digestion with SwaI and transfected into N52E6 cells [38]. After appearance of a cytopathic effect, the virus vector was amplified on N52E6 cells and purified by double CsCl banding and desalting with PD-10 columns (Amersham Biosciences, Freiburg, Germany). The infectious and total particle titres were determined on A549 cells as described by Kreppel et al. [22]. Vector genome integrity was confirmed by restriction analysis of DNA prepared from purified virions. Expression of tyrosinase was confirmed in Western transfer experiments with cell lysates from A549 cells transduced with different amounts of Ad-Tyr.

Transfection of human RPE cells with Ad-Tyr

Optimal gene transfer conditions were determined for each method using 100,000 cells per well in 24-well plates. Tyrosinase positivity was evaluated 5 days after gene transfer by immunodetection using the mouse anti-tyrosinase monoclonal antibody AB-1 as primary antibody. Ad-Tyr with 10^8 infectious units/μl were added to the human RPE cell culture and the amount of the vector DNA varied from 1 μl, 2 μl and 5 μl to 1 μl diluted 10 times. Optimal results were obtained using 1 μl of Ad-Tyr with 10^8 infectious units/μl.

Antibodies

The mouse anti-tyrosinase monoclonal antibody AB-1 was purchased from NeoMarkers (Fremont, CA, USA), the mouse anti-tyrosinase monoclonal antibody 2G10 from Chemicon Int. Inc. (Temecula, CA, USA) and is described by the manufacturer as an antibody against human tyrosinase [7]. The secondary purified goat Cy3-conjugated anti-mouse IgG antiserum was obtained from Rockland Immunochemicals Inc. (Gilbertsville, PA, USA). This antiserum had been raised against mouse IgG. The monoclonal anti-tyrosine hydroxylase antibody, clone TH-2 and IgG1 isotype, was from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany) and was used as a control. This antibody was raised against an epitope of rat tyrosine hydroxylase, which is also present in human tyrosine hydroxylase.

Immunodetection of tyrosinase and tyrosine hydroxylase

Antibodies (3–8 μg/ml) were used on paraformaldehyde-fixed RPE monolayers as described elsewhere [44]. Primary antibodies were diluted at 1:200 and incubated in buffer containing 0.01 M sodium phosphate and 0.25 M NaCl for 30 min at room temperature. The secondary antibodies were diluted 1:1000 and incubated in the same buffer for 1 h. Cells were photographed using an Axioplan 2 microscope (Zeiss, Oberkochen, Germany). Image processing was performed using an Orca camera (Hamamatsu Photonics, Herrsching,Germany) and Openlab 4 software (Improvision, Tübingen, Germany).

Histochemical evaluation of tyrosinase-active RPE cells

Bioactive tyrosinase in confluent ROS-fed human RPE cells plated on microscope slides was determined by light microscopic (LM) histochemistry [10]. The cells were washed twice with phosphate-buffered saline (PBS) and fixed in 0.1 M cacodylate buffer (pH 7.2) with glutaraldehyde (1%). After 15 min, the cells were washed three times with PBS and incubated for 60 h at room temperature in 0.05 M sodium phosphate buffer (pH 7.0) with 5 mM L-dopa, 5 mM D-dopa, or without dopa. Subsequently, the cells were rinsed with sodium phosphate buffer and photographed using an AxioCam color camera (Zeiss, Oberkochen, Germany).

Localization of rhodopsin and tyrosinase in human RPE cells

Human RPE cells (P0) were isolated from donor eyes for keratoplasty (aged 51–61 years). RPE phenotype was examined by pan-cytokeratin immunostaining. The cells were fed with rod outer segments for 5 h on a microscope slide. Immunostaining of the RPE monolayer was done

24 h after feeding with an anti-rhodopsin antibody (clone RET-P1) as primary and a Cy3-conjugated as secondary antibody. Tyrosinase was detected with a mouse monoclonal antibody (2G10) and a Cy2-conjugated secondary antibody. Briefly, we first performed the indirect immunolabelling of rhodopsin and before the second indirect immunolabelling of tyrosinase, the cells were incubated with bovine serum albumin (BSA) 5% for 1 h. To verify the specificity of the double indirect labelling, we performed a sequential double labelling again, but this time we detected first tyrosinase and secondly rhodopsin. We obtained the same results.

A merged image was made for verification of possible co-localization of rhodopsin and tyrosinase expression (orange) in ROS phagosomes. The nuclear staining was performed with Hoechst dye (blue).

Real-time reverse transcription polymerase chain reaction (RT-PCR)

The mRNA levels for tyrosinase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in unfed cells were compared to ROS-fed cells by real-time RT-PCR using SYBR Green I (Molecular Probes, Eugene, OR, USA) on an iCycler (Bio-Rad Laboratories, Hercules, CA, USA). Using the primer analysis software OLIGO 4.1 (National Biosciences, Plymouth, MN), we selected gene-specific primers suitable for real-time RT-PCR (tyrosinase: ACT TAC TGG GAT AGC GGA TGC CTC T and TGC ATT GGC TTC TGG ATA AAC TTC TT, GAPDH: TCA ACG ACC ACT TTG TCA AGC TCA and GCT GGT GGT CCA GGG GTC TTA CT). The T_m of the primers was chosen between 58.5 and 59.5°C. The expected fragment length ranges between 119 and 199 bp. With these primers, the mRNA expression of tyrosinase together with GAPDH as calibrator were analyzed simultaneously in double reactions in three separate experiments. The analysis was repeated once. Aliquots of the diluted cDNA corresponding to 15 ng initially used total RNA were mixed with 10× reaction buffer containing Tris-HCl and KCl, $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$ (Qiagen, Hilden, Germany), 0.2 mM of each dNTP, 0.2 μM of each specific primer, 0.1× SYBR Green I and 1 U of HotStarTaq DNA polymerase (Qiagen, Hilden, Germany) in a volume of 50 μl. The following PCR cycle parameters were used: hot start polymerase activation for 15 min at 95°C and up to 50 cycles at 95°C for 20 s, 57°C for 20 s and 72°C for 20 s. Detection of the fluorescence product was carried out during the last 10% of the cycles. To confirm amplification specificity, the PCR products from each primer pair were subjected to agarose gel electrophoresis and melting curve analysis (data not shown). Genomic DNA contamination was excluded by choosing primers hybridizing to different exons. Amplicons with introns were not detected. Moreover, control amplification reactions that were performed with non-transcribed RNA as templates gave only background fluorescence. The quantification data were analyzed with the iCycler iQ system software (Bio-Rad Laboratories, Hercules, CA, USA) as described [30]. After PCR baseline subtraction performed by the software, the log-linear portion of the fluorescence vs cycle plot was extended to determine a fractional cycle number at which a threshold fluorescence was obtained (threshold cycle, C_T) for each analyzed gene and GAPDH as the reference. The comparative C_T method was used for quantification of the target genes relative to GAPDH. The C_T of GAPDH was constant.

Tyrosine hydroxylase activity of tyrosinase

Samples of cultured monkey RPE cells were homogenized (using a Potter-Elvehjem homogenizer at 10 strokes up-and-down at 1,200 rpm) in 100 mM potassium phosphate buffer, pH 7.4, before and after feeding with purified ROS. Then 25 μl of the homogenate were mixed with 25 μl assay buffer containing 100 mM potassium phosphate, pH 7.4, 18.5 MBq L-[3,5-^{3}H] tyrosine (specific radioactivity 1.83 GBq/μmol; Moravek Biochemicals, Brea, CA, USA) and 1 μM L-dopa (Sigma-Aldrich Chemie, Taufkirchen, Germany) and incubated for 24 h at 37°C. The reaction was stopped by adding 100 mg Celite 545 (Merck KGaA, Darmstadt, Germany) and 100 mg activated charcoal suspended in 1 ml 0.1 N HCl solution. After 1 h shaking at room temperature, the samples were centrifuged (15,000 *g*, 5 mn), 500 μl of the resulting supernatant were mixed with 10 ml of Ultima Gold scintillation cocktail and radioactivity was determined by a Packard Tri-Carb 2900TR liquid scintillation counter. As a control, the tyrosinase activity of 0.1 mg of purified ROS was determined.

Protein concentration of the samples was measured according to the method of Lowry et al. [25], using BSA as standard.

Results

Electron-microscopic localization of tyrosinase by DOPA histochemistry

Tyrosinase activity was investigated by electron-microscopic (EM) histochemistry. Dopa oxidase activity of tyrosinase was determined.

Five hours after feeding with ROS (Fig. 1a) or latex particles (Fig. 1b), tyrosinase activity was demonstrated in human RPE cells by dopa oxidase assay. Dopa-positive

electron-dense vesicles, indicating the presence of tyrosinase, were present throughout the cytoplasm as shown in an electron micrograph. Interestingly, single dopa-positive vesicles containing tyrosinase exist all over the cytoplasm after 20 h exposure to latex particles, and, in addition, merged dopa-positive material is present as well inside the large vacuole containing the latex beads (Fig. 1c). Early endosomes containing latex beads also stained electron dense after the dopa reaction (Fig. 1c). Phagocytosis of latex beads also induces tyrosinase activity in adult human RPE. Dopa-positive vesicles were not found in the RPE cells without feeding (Fig. 1d).

Antibody staining of tyrosinase in human RPE cells

In a monolayer of cultured human ROS-fed RPE cells, control immunocytochemical experiments performed using antibodies against L-tyrosine 3-monooxygenase (EC 1.14.16.2) were negative (Fig. 2a). Corresponding experiments using non-fed cells and antibodies against L-tyrosine 3-monooxigenase were negative, too (Fig. 2d). Without feeding with ROS, using anti-tyrosinase antibodies, no specific staining different from background levels was detected (Fig. 2c). However, low specific staining was found 5 h after feeding (Fig. 2b) corresponding to the

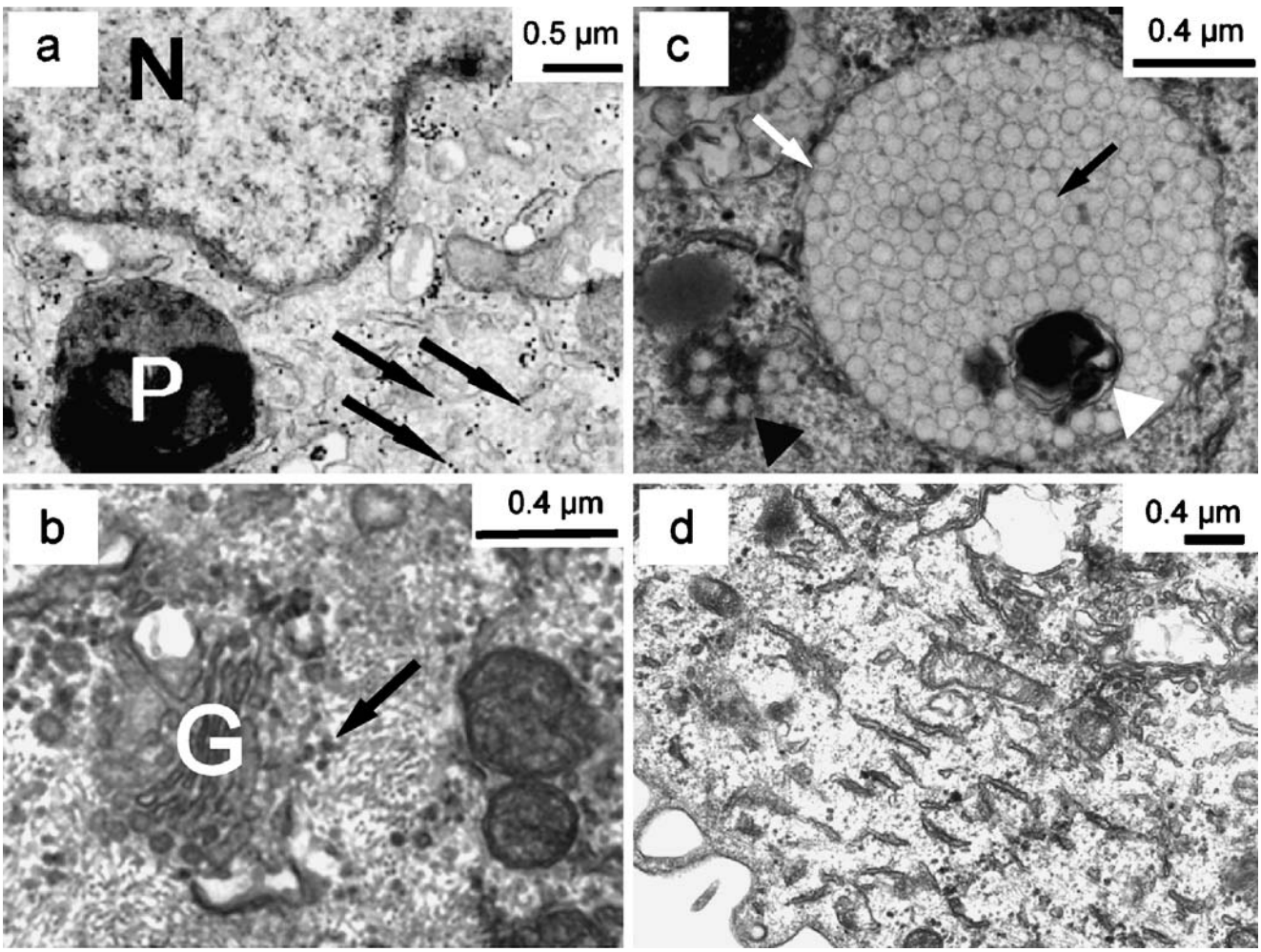
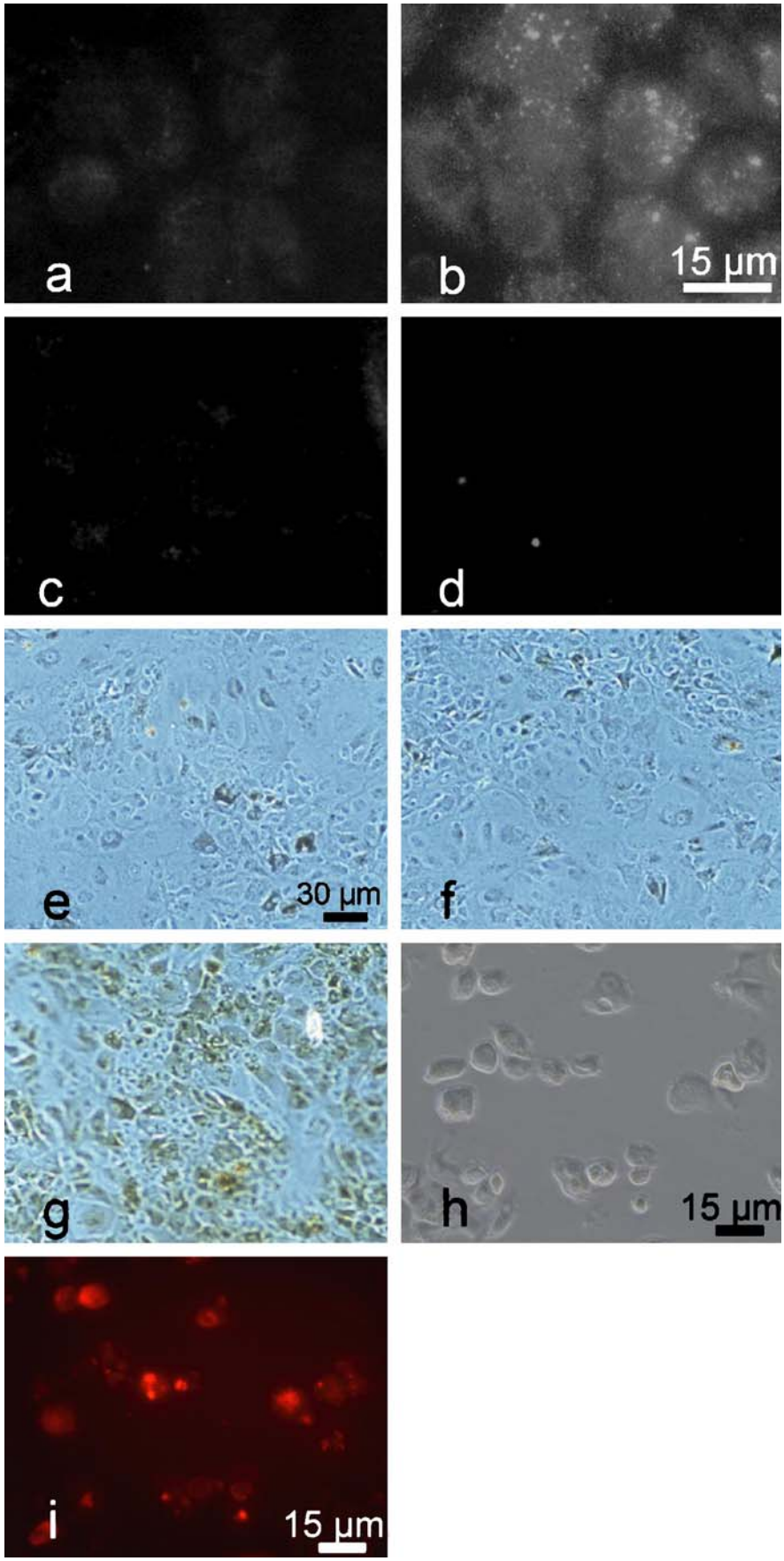


Fig. 1 **a** EM histochemistry of human adult retinal pigment epithelial cells. Activity of tyrosinase was detected by dopa oxidase bioactivity. Dopa-positive vesicles (*arrows*) containing tyrosinase exist all over the cytoplasm close to a phagosome (*P*) as shown in an electron micrograph suggesting classic biosynthesis of the enzyme 5 h after feeding with rod outer segments (ROS). The phagosome also is stained electron dense after the dopa reaction. Phagocytosis of ROS induces tyrosinase activity in the adult human retinal pigment epithelium (RPE) in vitro (*N* nucleus). **b** Dopa-positive Golgi (*G*) bodies are seen 5 h after exposure to latex beads. Golgi-derived vesicles suggest classic biosynthesis of this enzyme 5 h after feeding with latex beads (*black arrow*). **c** Twenty hours following exposure of human adult RPE cells to latex particles the beads (*black arrow*) are located within a large late endosome (*white arrow*). Single dopa-positive vesicles localizing tyrosinase exist all over the cytoplasm and merged dopa-positive material is present inside the large vacuole containing the latex beads (*white arrowhead*) and inside an early endosome (*black arrowhead*). Phagocytosis of latex beads also induces tyrosinase activity in adult human RPE. **d** Electron micrograph taken from control adult human RPE that were cultured for 20 h without latex particles and without ROS. Dopa-positive vesicles were not observed in these cultures


a
b
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◀ **Fig. 2** **d** Adult human retinal pigment epithelial (RPE) cells were prepared and cultured with rod outer segments (ROS) for 5 h for immunocytochemistry. Afterwards, no staining was visible with anti-tyrosine hydroxylase control antibodies. Without feeding with ROS, no staining was detectable with the anti-tyrosine hydroxylase antibodies (**c**) or anti-tyrosinase antibodies (**a**), too. **b** Five hours after feeding with ROS, distinct staining was found using anti-tyrosinase antibodies corresponding to the dopa-positive vesicles or cells in Figs. 1a, 2g. After a 5-h ROS feeding and a subsequent 60-h incubation of human adult RPE cells with 5 mM L-dopa, numerous buff dopa-positive cells were verifiable (**g**). In control experiments using 5 mM D-dopa (**f**) or without dopa (**e**), respectively, only a few single cells were dopa-positive. Phagocytosis of ROS induces tyrosinase (dopa oxidase) activity in the adult human RPE in vitro. **h**, **i** Tyrosinase expression in human RPE cells transfected with Ad-Tyr. Cells were transfected 5 days with 1 ml of the vector DNA with 108 infectious units/ml. Differential interference contrast (**h**), fluorescence image (**i**)

electron-microscopic dopa staining shown in Fig. 1a,b. In addition, we have shown that almost all human RPE cells transfected with the Ad-Tyr virus for 5 days were stained using anti-tyrosinase antibody (Fig. 2h,i).

Tyrosinase activity in ROS-fed human RPE cells

Tyrosinase activity was determined by light microscopic histochemistry. Dopa oxidase activity of tyrosinase was analyzed.

Five hours after feeding with ROS, tyrosinase activity was verified in human RPE cells in the presence of 5 mM L-dopa by dopa oxidase assay (Fig. 2g). Corresponding tests with 5 mM D-dopa (Fig. 2f) or without dopa (Fig. 2e) indicated only marginal dopa oxidase activity.

Co-localization of rhodopsin and tyrosinase in human RPE cells

To test the hypothesis that tyrosinase protein is localized within phagosomes of human RPE cells, we performed immunohistochemistry with both antibodies against tyrosinase and rhodopsin. Rhodopsin staining that indicates the localization of phagosomes processing rod outer segments was present 24 h after feeding with ROS (Fig. 3a, red). Tyrosinase protein was also expressed at this time point (Fig. 3b, green). However, tyrosinase protein was not detected without feeding with ROS (data not shown). In monolayers of cultured human RPE cells, we observed many cells that co-localize both proteins (Fig. 3c, orange).

ROS feeding regulates tyrosinase mRNA level in human RPE cells

The mRNA levels for tyrosinase and GAPDH in unfed cells were compared to ROS-fed cells by real-time RT-PCR.

Five hours after exposure to ROS, the GAPDH calibrated tyrosinase mRNA level was reduced to 66%, $p_{0.05}$= 0.038, compared to the GAPDH calibrated tyrosinase mRNA level in the control. Twenty hours after exposure to ROS, the GAPDH calibrated tyrosinase mRNA level was still decreased compared to the control ($p_{0.05}$=0.01), and by 40 h after exposure to ROS, the tyrosinase mRNA level had increased to the level of the cells not exposed to ROS ($p_{0.05}$=0.16) (Fig. 4).

Tyrosine hydroxylase activity of tyrosinase

Using a highly specific radioactive assay, L-tyrosine 3-hydroxylase activity was determined in cultured adult monkey RPE cells before incubation with ROS, and 5, 20 and 40 h after feeding with ROS or in isolated ROS (Fig. 5).

By measuring the release of tritium from tritiated L-tyrosine in homogenized samples, we demonstrated that feeding with ROS increases tyrosinase enzyme activity in comparison to non-challenged RPE (1,649.4±142.4 after 20 h to 9.2±17.5 fmol/mg per h, Fig. 5) by a factor of 180. The increase of tyrosinase activity between the ROS-fed and non-fed RPE cells was significant (n=3, mean of 3 measurements). As a positive control, human melanoma tissue, and as a negative control, RPE from the amelanotic area of monkey eyes, were used (data not shown). Detectable tyrosinase activity in 0.1 mg of purified ROS was very low (0.9±3.2 fmol/mg per h).

Discussion

In a very recent publication, we have already demonstrated by using three independent methods that the expression of tyrosinase and its enzymatic activity are induced in cultured adult RPE from cattle by phagocytosis of ROS in vitro [47]. The results of our new study prove that phagocytosis of ROS can induce tyrosinase expression in human RPE cells. Here, we provide LM and EM histological evidence for the presence and induction of tyrosinase activity after phagocytosis of ROS in adult human RPE.

The relevance of phagocytosis-induced tyrosinase expression is not well known. Several studies indicate that melanin biosynthesis occurs within lysosomes [32, 33, 39, 42, 43], since melanosomes belong to the same lineage of organelles. Furthermore, melanosomes comprise many lysosomal enzymes [8], and it was argued that the melanosome was a specialized lysosome [34].

It is not surprising that tyrosinase biosynthesis is induced by phagocytosis of rod outer segments because ROS uptake is linked to regulation of many genes in the RPE [6]. Additionally, lipids, particularly sphingolipids, induce melanogenesis by augmenting the expression of tyrosinase and its related proteins in vitro [26]. Thus, we aim at

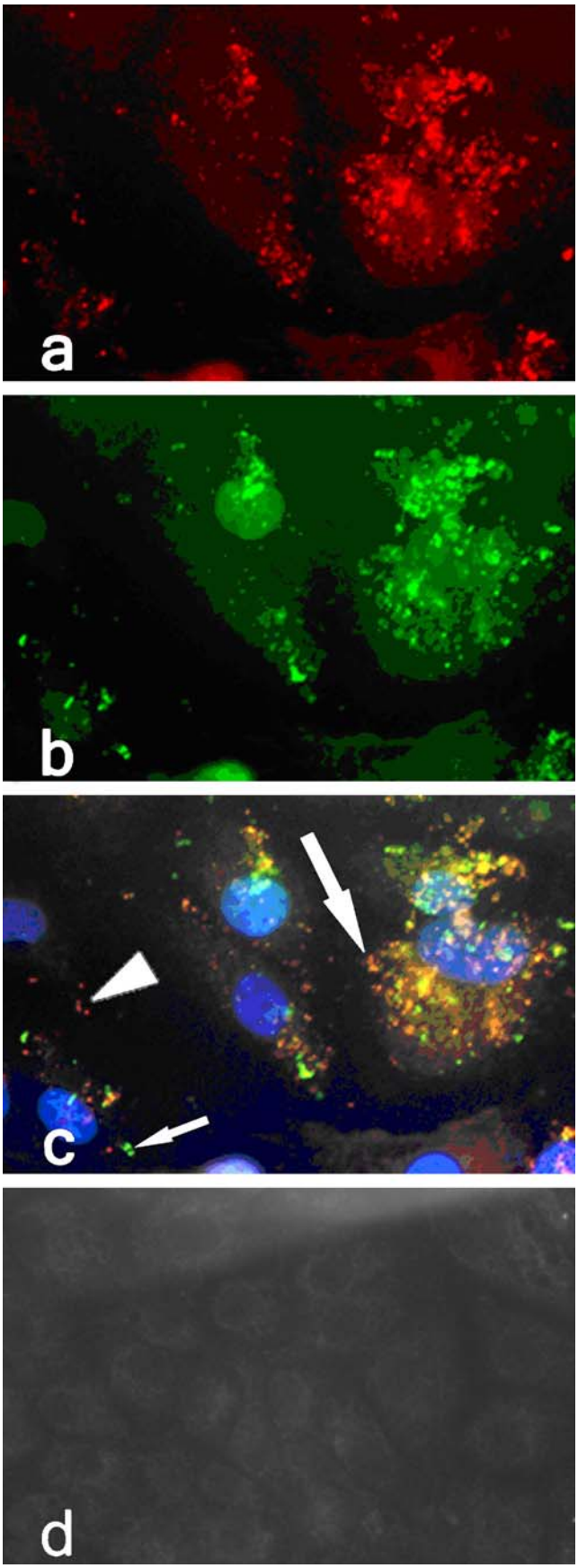

Fig. 3 Co-staining of rhodopsin and tyrosinase in adult human retinal pigment epithelial (RPE) cells in vitro 24 h after incubation with rod outer segments (ROS). **a** The fluorescence microscopy-based image taken from the RPE monolayer shows immunostaining with an anti-rhodopsin antibody detected with a Cy3-conjugated secondary antibody (*red*). Rhodopsin was highly expressed in ROS phagosomes 24 h after feeding with ROS. **b** The same fluorescent image after labelling of tyrosinase with a monoclonal antibody and a Cy2-conjugated secondary antibody (*green*). **c** A merged image of **a+b** that demonstrates colocalization (*large arrow*, *orange* fluorescence) of rhodopsin (*arrowhead*) and tyrosinase (*small arrow*) expression in ROS phagosomes. The nuclear staining was performed with Hoechst dye (*blue*). No expression of tyrosinase was found without feeding with ROS (**d**)

researching in subsequent studies whether ROS lipids induce expression of tyrosinase and melanogenesis.

The electron-microscopic results of tyrosinase activation after feeding with ROS match the classic model of melanogenesis with Golgi-derived dopa-positive vesicles widespread in the cytoplasm of pigment cells. Vesicles incorporating tyrosinase may fuse with phagosomes. Tyrosinase may detoxify reactive oxygen species inside phagosomes, e.g. superoxide anions [51], or may have another as yet unknown function. This model complies with results of this and recent studies, in which tyrosinase was found in partly degraded phagosomes [42]. In addition, there is an interrelation between phagosomal degradation pathways and melanosome biogenesis in RPE cells due to the fact that phagosomal material is transported to the melanosomes or melanogenesis occurs inside phagosomes [46].

Recent experimental results and the findings of this study are in line with the current model of tyrosinase trafficking in melanocytes assuming that phagosomes are related to endosomes. Tyrosinase is transported from the trans-Golgi network to early endosomes and attains via late endosomes lysosomes and premelanosomes in melanocytes [16, 35].

There is a causal interrelationship between the phagocytosis of ROS and the production of reactive oxygen intermediates such as superoxide anion radicals $\left(O_2^{-\bullet}\right)$, hydrogen peroxide ($H_2O_2$) and hydroxyl radicals ($OH^{\bullet}$) [9]. Hydrogen peroxide increases tyrosinase activity in cultured human melanoma cells [19], is a competitive inhibitor of tyrosinase [54] and induces tyrosinase [15, 19]. Moreover, superoxide anion radicals augment activity of human tyrosinase [54]. Tyrosinase may protect against oxidative stress by diverse mechanisms: first, by oxidation of tetrahydroisoquinones, tyrosinase may promote their conversion into inert insoluble polymers; second, by utilizing the superoxide anion as substrate. Hence, the L-dopa: oxygen oxidoreductase could act as a free radical trapping enzyme [51]. Moreover, it has been shown in retinal diseases with an oxidative stress component like age-related macular degeneration that black people are more protected

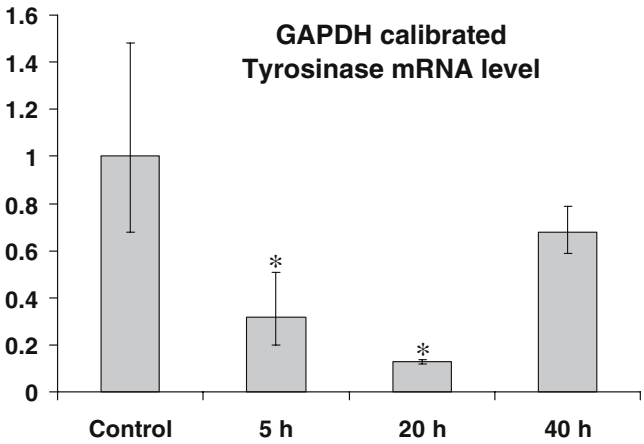

Fig. 4 Quantitative real-time RT-PCR. The mRNA levels for tyrosinase and GAPDH in unfed cells were compared to ROS-fed cells by real-time RT-PCR using SYBR Green I on an iCycler. The quantification data were analyzed with the iCycler iQ system software (see "Materials and methods"). Tyrosinase mRNA is present in cultured human RPE cells. The amount of tyrosinase mRNA decreases slightly 5 h after ROS exposure and increases again at 40 h after exposure. Statistical differences between control (tyrosinase mRNA level in unfed cells) and tyrosinase mRNA level in ROS-fed cells are designed by * ($p<0.05$, paired t-test, $n=3$)

than Caucasians [14]. It is not yet known whether this phenomenon is caused by tyrosinase itself and/or its metabolites. Although the antioxidant properties of melanin have been demonstrated largely in model systems (see references 2 and 10–24 in the very recent study of Zareba et al. [56]), in most cases using synthetic melanin, results from these models cannot be directly extrapolated to biological systems such as RPE cells in which melanin is found in melanosomes. Moreover, no evidence was obtained in the study by Zareba and colleagues [56] to indicate that melanosomes confer cytoprotection against

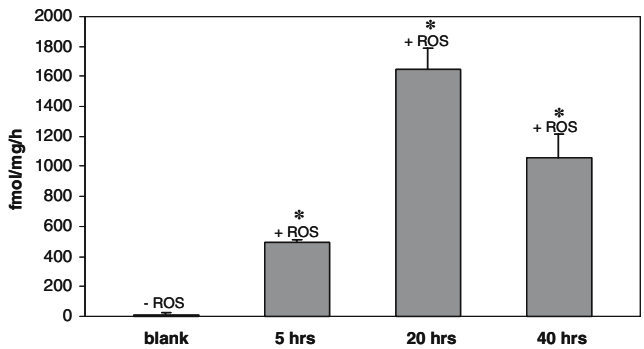

Fig. 5 Phagocytosis of ROS increased the activity of tyrosinase. Organ cultures consisting of monkey RPE cells were exposed to ROS for 4 h. Then 5, 20 and 40 h after feeding with ROS the cultures were homogenized and used for determination of tyrosinase activity. Without feeding with ROS tyrosinase activity was almost not detectable (*$p\leq0.05$). These results clearly demonstrate that tyrosinase activity was induced only after feeding with ROS

oxidative stress induced chemically or photically in ARPE-19 cells.

Tyrosinase mRNA has been analyzed in melanocytes but not in RPE of adult vertebrates. Cloudman S-91 mouse melanoma cells respond to α-MSH by upregulation of tyrosine mRNA transcription followed by an increase in tyrosinase activity. Contrary to our results, which show a decrease in mRNA expression followed by a return to the pre-stimulated state following stimulation by exposure to ROS, maximal increase in tyrosinase mRNA was delayed in Cloudman S-91 mouse melanoma cells and occurred 60 h after hormone stimulation [36]. Our results showing the presence of tyrosinase mRNA but no tyrosinase activity in unstimulated cells suggest that unstimulated RPE cells have a constitutive level of tyrosinase mRNA that is inhibited from being translated or that the translated tyrosinase is immediately hydrolyzed. Stimulation of the RPE by ROS phagocytosis allows the translation of the tyrosinase mRNA by removing an unknown inhibitor or prevents degradation of translated tyrosinase. The decrease in tyrosinase mRNA we observed following stimulation could be the result of degradation of the mRNA made accessible to RNAses during translation of the tyrosinase mRNA, followed by upregulation of transcription to restore the pool of tyrosine mRNA.

Ershov and co-workers [12] have shown that the expression of the transcription factors AP-1 and AP-2 is increased in RPE cells after ROS phagocytosis. These transcription factors may then bind the tyrosinase gene promoter at their corresponding binding sites [13] and thereby upregulate transcription of tyrosinase. However, studies in other systems have shown that tyrosinase activity is not regulated at the level of transcription but it is regulated post-translationally. Results from studies with mammalian melanoma cells and melanocytes parallel those obtained from RPE suggesting that regulation of tyrosinase in RPE cells occurred post-transcriptionally. Kitano and Hu [20] working with melanoma cells in vitro found an inverse correlation between melanin synthesis and tyrosinase synthesis. Moreover, no correlation was found between tyrosinase message levels and melanin content using Northern blot analysis and in situ hybridization, suggesting that post-transcriptional regulation of tyrosinase and/or other events determine the rate of pigment synthesis in human melanocytes [31]. Although it was suggested that melanosome complexes were formed by fusion of melanosomes with phagosomes derived from outer segments of photoreceptors [17], this study does not present new evidence that de novo melanogenesis takes place in the adult differentiated RPE. Therefore, upregulation of tyrosinase activity is possibly not related to melanogenesis but has an unknown function as is the case in neurons in vitro [18] or in vivo [50]. Nevertheless, this study shows for the

first time using several independent methods that tyrosinase activity as well as its expression is induced by phagocytosis of rod outer segments in adult human RPE cells.

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The classical pathway of melanogenesis is not essential for melanin synthesis in the adult retinal pigment epithelium

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Summary

Premelanosomes are presumed to be essential for melanogenesis in melanocytes and pre-natal RPE cells. In this study, we analysed melanin synthesis in adenoviral-transduced, tyrosinase gene expressing ARPE 19 cells to find out whether premelanosome formation is needed for post-natal melanogenesis.
Synthesis of melanogenic proteins and melanin granules was investigated with immunocytochemistry, light and electron microscopy. The occurrence of tyrosinase was analysed ultrastructurally, using DOPA histochemistry. The viability of transduced cell cultures was analysed using an MTT assay.
We found active tyrosinase in small granule-like vesicles all over the cytoplasm and in the ER and the nuclear membrane. Tyrosinase was also associated with multi-vesicular and multi-lamellar organelles. Typical premelanosomes, the structural protein PMEL17, and tyrosinase-related protein 1, as well as the melanosomal stages I-IV, as described in the literature, were not detected. Instead, melanogenesis took place inside multi-vesicular and multi-lamellar bodies of unknown origin. Viability was not affected up to ten days after transduction. Here we demonstrate a pathway of melanin formation, which lacks the typical hallmarks of melanogenesis, as they are described in the literature.

Keywords: ARPE 19, tyrosinase, premelanosome, melanogenesis, alternative pathway

Introduction

Many differences between melanocyte and RPE melanisation are due to their different embryonic origin and development (Bharti et al. 2006; Bharti et al. 2008; Murisier et al. 2007), and affect, for example, the regulation of melanogenesis and the transcription of melanogenic genes by using different transcription and growth factors. While skin melanocytes produce melanosomes continuously, RPE cells are presumed to create melanosomes only prenatally. In addition, the shape and size of mature RPE melanosomes differs from that of melanocytes (Schraermeyer and Heimann 1999). Despite this, melanogenesis in RPE cells has been described as sharing a common pathway with melanocytes. They use the same melanogenic proteins and the ultrastructural characterisation of morphologically distinct melanosomal stages I to IV has been confirmed for both melanocytes and pre-natal RPE cells (Lopes et al. 2007). The formation of melanosomes in both cell types has been described as follows: An amorphous vesicle, called the premelanosome, which accumulates typical intraluminal striations, is believed to build the first phase of melanogenesis (stage I). After the intraluminal arrangement is completed (stage II), tyrosinase-filled coated vesicles, which have emerged from the Golgi apparatus, can enter the premelanosome. Tyrosinase and other melanising proteins are integrated into the melanosomal membrane and synthesise melanin, which is deposited along the internal striations (stage III). When the whole organelle is filled with melanin, it is termed stage IV or a mature melanosome (Seiji et al. 1963).
There are two criteria essential for melanogenesis in melanocytes and pre-natal RPE cells: the presence of premelanosomes and the synthesis of tyrosinase and other melanogenic proteins. Without the enzymatic action of tyrosinase, the formation of melanin is not possible

(Oetting and King 1999). Since tyrosinase and melanin precursors have a high oxidative potential and can facilitate radical building and oxidative stress reactions in the cell, they have to be separated inside a closed organelle, the premelanosome (Theos et al. 2006). The premelanosome is characterised by having typical internal striations, which are used for melanin deposition. These striations are composed of the structural protein PMEL17 (Berson et al. 2001; Berson et al. 2003) and probably other components, as yet unknown. The absence of PMEL17 leads to the loss of premelanosome formation, to mistargeting and degradation of melanogenic enzymes and to defective pigmentation in mutant individuals (Theos et al. 2006).
While some authors have found tyrosinase in adult RPE (Schraermeyer et al. 2006; Julien et al. 2007; Basu et al. 1983; Abul-Hassan et al. 2000; Varela et al. 1995), the presence of PMEL17 has not yet been reported (Lopes et al. 2007). Therefore, a melanin renewal in the adult RPE has long been doubted (Carr and Siegel 1979; Dorey et al. 1990; Miyamoto and Fitzpatrick 1957; Sarna 1992).
In the few publications existing about post-natal RPE melanogenesis (Herman and Steinberg 1982; Thumann et al. 1999; Schraermeyer et al. 1999; Peters et al. 2000; Schraermeyer and Stieve 1994; Dorey et al. 1990), opinions differ concerning the occurrence of typical striated premelanosomes. Goldman-Herman and Steinberg (Herman and Steinberg 1982) only found the melanogenic stages II-IV in the RPE of opossums. Others showed that striated premelanosomes were associated with phago-lysosomal organelles (Thumann et al. 1999; Schraermeyer et al. 1999; Peters et al. 2000; Schraermeyer and Stieve 1994; Young 1977) or observed melanogenesis taking place in organelles of the degradative pathway inside multi-vesicular and multi-lamellar bodies (Novikoff et al. 1979) and without the formation of striated premelanosomes (Dorey et al. 1990). The morphological diversity of post-natal melanogenic stages observed indicates the presence of an alternative pathway of melanosome formation in adult RPE cells, which might lack typical PMEL17 striations or which might at least be morphologically different to the classical model.
Here we demonstrate that melanogenesis can occur without the formation of typical premelanosomes inside multi-vesicular and multi-lamellar organelles of ARPE 19 cells.

Methods

Human amelanotic retinal pigment epithelial cells

Human amelanotic retinal pigment epithelial cells (ARPE 19 cell line) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and used between passages three and thirteen. Since ARPE 19 cells are a highly differentiated, immortalized RPE cell line, lacking an observable pigmentation due to passaging and the very low ability to form new pigments (Dunn et al. 1996), they provide the ideal system to study new pigment synthesis of adult RPE cells. Cells were grown in DMEM medium with 10% FCS and 1% antibiotics (containing 5 units/ml Penicillin G and 5 mg/ml Streptomycin) in 250 ml culture flasks (Greiner, Frickenhausen, Germany). All cell culture reagents were obtained from GIBCO (Invitrogen, Karlsruhe, Germany). The experiments were performed in 24-well culture plates (Costar Corning, Bodenheim, Germany) for the light and electron microscopy and in 16-well chamber slides (nunc, Wiesbaden) for fluorescence immunocytochemistry.

MNT-1 melanoma cells

The MNT-1 cells were a kind gift from Prof. P.G. Natali, Dep. of Immunology and Molecular Pathology Laboratories, "Regina Elena" National Cancer Institute, Rome, Italy. They were cultured in RPMI 1640 medium +10% FCS without the supplementation of antibiotics.

Additional material:

Chemicals were purchased from Sigma, Deisenhofen, Germany; dimethylsulfoxid (DMSO) and paraformaldehyde (PFA) from Merck, Darmstadt, Germany.

Generation of AdTyr

The adenoviral vector AdTyr carries the human tyrosinase insert in its E1 deleted region. It was generated using a method that has been described previously (see (Julien et al. 2007)).

Experimental procedure

Cells were cultured in 24-well culture plates ($1x10^5$cells/well) for light and electron microscopy and in 16-well chamber slides ($5x10^3$cells/well) for immunocytochemistry experiments. Twenty-four hours after seeding, the cells were transduced with 50 infectious units of the AdTyr vector per cell in complete medium for another twenty-four hours. Then the medium was changed and the cells were cultured until fixation. Controls without the tyrosinase vector were treated in the same way.

Fluorescence immunocytochemistry

Mouse anti-human tyrosinase antibody clone T311 was purchased from BIOZOL (Eching, Germany). Mouse anti-human PMEL17 antibody was purchased from ENZO (Farmingdale, NY) and mouse anti-human TRP1 antibody from Santa Cruz (Santa Cruz, California). Cy3-labelled goat-anti-mouse secondary antibody was purchased from Rockland Immunochemicals Inc. (Gilbertsville, PA, USA). Twenty-four hours after transduction, the cells were fixed with 4% PFA and lysed with 0.2% Triton X 100b and 1% DMSO. The cells were then labelled with primary antibody (1:50) at 4°C over night and with the secondary antibody (1:1000) for 45 minutes at room temperature.

Measurement of cell viability via MTT test 48 hours and ten days after transduction

To test the viability of cells after transduction with the tyrosinase vector (48 h) and after melanin production had started (10 days), an MTT assay was used. The medium was removed and MTT reagent (5 mg/ml in PBS) was added. After three hours of incubation at 37°C in a humid dark chamber, cells were lysed with DMSO and absorbance was measured at 570 nm in a microplate reader. Non-transduced ARPE 19 cells were used as a control.

Routine electron microscopy (EM)

Cells were fixed overnight at 4°C in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) one, six and fourteen days after transduction. The specimens were postfixed with 1% OsO_4 and 1.5% potassium ferrocyanide in 0.1 M sodium cacodylate buffer at room temperature for three hours, blockstained with uranyl acetate overnight, dehydrated in a graded series of ethanol, and embedded in EPON. Ultrathin sections were poststained with lead citrate and investigated under a transmission electron microscope (EM 902A; Zeiss).

DOPA histochemistry

After fixation for EM, the localisation of tyrosinase is still possible by incubation of the fixed specimen with the substrate L-DOPA (L-dihydroxyphenylalanine). Electron-dense material will appear at those sites where tyrosinase has metabolised the substrate. DOPA histochemistry was performed as has been described earlier (Schraermeyer 1992). In short, twenty-four hours after transduction, cells were fixed for one hour in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 6.8) on ice. They were washed three times in sodium cacodylate buffer and incubated in a freshly prepared 5 mM L-DOPA suspension (in 0.1 M sodium cacodylate buffer, pH 6.8) at 4°C overnight. After renewal of the DOPA suspension, specimens were incubated for another 5 h at 37°C. Samples were postfixed with 1% OsO_4 and 1.5% potassium ferrocyanide at room temperature in 0.1 M sodium cacodylate buffer for three hours, blockstained with uranyl acetate overnight, dehydrated in a graded series of ethanol, and embedded in EPON resin. Ultrathin sections were poststained with lead citrate and investigated under the TEM.

Results

Light microscopic observations

Cell cultures were examined and photographed by light microscopy every two days to visualise any melanogenesis occurring. Confluent control ARPE 19 cells contained no melanin. Transduced cells synthesised melanin, which appeared six to ten days after transduction. Nineteen days after transduction, half of the cells contained a great number of melanin granules (Figure 1).

Fluorescence immunocytochemistry of melanogenic enzymes

To confirm transduction with AdTyr and to evaluate if other melanogenic enzymes are induced after tyrosinase gene overexpression, fluorescence immunocytochemistry was performed. For a positive control, MNT-1 cells were used. MNT-1 is a pigmented melanoma cell line, obligatorily expressing melanogenic genes and continuously forming all stages of melanosomes (Raposo et al. 2001). 100% of the cells synthesised the tested proteins (Figure 2 a-c).

At no time point were TRP1 and PMEL17 immunoreactions detectable in ARPE 19 cells, whether with or without transduction (Figure 2 d, e for 24 h after transduction; also tested on days two and three after transduction, not shown). Non-transduced ARPE 19 cells showed no tyrosinase staining either. Twenty-four hours after transduction, 50% of the transduced ARPE 19 cells showed a clear tyrosinase staining all over the cytoplasm, in vesicles, cisterns and tubules, with strong labelling of the tubular network near the nucleus. The nuclear membrane formed by the endoplasmic reticulum (ER) was also frequently stained, but not in all transduced ARPE 19 cells (Figure 2 f).

DOPA reactivity and melanogenic stages in MNT-1 cells

In the DOPA reaction experiments, MNT-1 cells showed the typical tyrosinase staining of trans-Golgi cisterns and budding vesicles, and also showed the typical ultrastructure of melanogenic stages, as described by Seiji (Seiji et al. 1963) and others. Additionally, cloudy bodies filled with electron-dense, amorphous material, were observed, which resembled melanin granules, as later described for ARPE 19 cells (Table 1).

DOPA histochemisty of transduced ARPE 19 cells

As indicated in the fluorescence immunocytochemistry, ultrastructural DOPA staining of active tyrosinase in transduced ARPE 19 cells showed the following: tyrosinase was spread all over the cytoplasm, mostly packed in small vesiculo-globular bodies (DOPA granules), but also in tubules and cisterns of the ER and the GERL (Golgi-Endoplasmic Reticulum-Lysosome zone) (Figure 3 a) and sometimes inside the nuclear membrane (not shown). Figures 3 b-h show the typical DOPA-positive structures, which were found frequently in transduced ARPE 19 cells. DOPA granules were associated with membrane-bound bodies of unknown origin (Figure 3 b, c). Figure 3 c shows DOPA granules inside a multi-vesicular body with a double-membrane, resembling an autophagosome. We also found DOPA granules inside multi-lamellar bodies (not shown) or crowded along and inside huge vacuoles (Figure 3 d, e). The DOPA granules were not dispersed homogeneously throughout the cytoplasm, but often formed clusters, which covered great areas of the cell. The DOPA granules stayed in close contact to tubules and cisterns of the ER (Figure 3 f) and to intracellular membranes of unknown origin (Figure 3 g). The distribution of the DOPA-positive structures was compared to that in MNT-1 cells. Typical DOPA staining of trans-Golgi cisterns and budding vesicles (59 ± 13 nm in diameter) were only rarely seen in transduced ARPE 19 cells. Instead, ARPE 19 cells contained these smaller granule-like DOPA-positive bodies (34 ± 7 nm in diameter), which lacked a visible covering membrane (Figure 3 h) and were significantly smaller than the Golgi vesicles ($p < 0.0001$). Similar DOPA granules were not functionally detected in MNT-1 cells. The granules seen in ARPE 19 cells can also be distinguished from ribosomes (ca. 22 nm in diameter) and intracellular matrix components like glycogen, which forms rosette-like structures. They only appeared in DOPA stained samples. Table 1 summarises the observed differences between MNT-1 and transduced ARPE 19 cells according to the findings in the DOPA reaction and the occurrence of melanogenic stages by routine EM, as described below.

Observation of melanogenic stages by EM

Classical premelanosomes with their striational structure and other typical melanogenic stages could not be observed in either control ARPE 19 cells or transduced ARPE 19 cells. Instead, we found many different kinds of electron-dense granules in the transduced ARPE 19 cells. Before analysing melanogenesis in our experiments, we had to distinguish newly-formed melanin granules from other electron-dense organelles of unknown origin. We found different types of relevant bodies and examined their content in our samples. We discarded inappropriate candidates and defined a membrane-bound, electron-dense, cloudy and amorphous filled organelle (Table 2, Figure 4) to be the newly-formed melanin granule. These organelles occurred almost only in transduced cells. Table 2 summarises the

occurrence of melanogenic organelles as they are observed at different time points. Organelles, which were filled to at least 1/5 with electron-dense amorphous material, but also contained other material, were defined as untypical melanosomal stages 1 to 4 (in contrast to the classical stages I to IV). If less than 1/5 of the granule was filled with the electron-dense material, the organelle was attributed to the mixed bodies, containing lamellar, vesicular and other shaped material. Additionally, Table 2 shows the typical distribution of multi-vesicular bodies (MVBs) and multi-lamellar bodies (MLBs), often seen in all samples independent of transduction.

We found a chronological sequence, demonstrating that in the first days after transduction no melanised stages were observable. Instead, MVBs and MLBs and a few mixed bodies (enclosing a potpourri of vesicles, lamellae and electron-dense material) were found. Later on (after 6 days), the material inside the MVBs, MLBs and mixed bodies of transduced samples appeared to merge together, harbouring melanin-like material to form melanogenic stages 1-3, showing lamellae and vesicles, but also higher amounts of electron-dense and amorphous material inside. Mixed vesicles were found particularly in six-day-old cells. After fourteen days, most of the organelles were filled completely with melanin (stage 3-4). The melanin granules were 0.3-1 µm in diameter, resembling the size of MVBs and MLBs, and related to them. Control cells (c in Table 2, day 6) contained only MLBs and some MVBs and mixed bodies, but the latter were less frequently observed.

Viability of transduced cells

Two and ten days after transduction, the viability of control ARPE 19 cells and cells transduced with AdTyr was analysed using an MTT assay. Two days after transduction, tyrosinase-transduced ARPE 19 cells (101 +/- 16%) showed the same viability as ARPE 19 cells without transduction (100 +/- 11%; Figure 5). After ten days, the tyrosinase-transduced cells (91 +/- 17%) showed a small, but insignificant decrease in viability compared to ARPE 19 cells (100 +/- 12%; $p>0.05$; n.s.).

Discussion:

Most publications dealing with RPE and melanocyte melanogenesis agree that the enzyme tyrosinase emerges from the trans-faced cisterns of the Golgi apparatus and travels inside coated vesicles to stage II melanosomes (reviewed by (Hearing 2005), but the origin of the amorphous vesicle forming the stage I premelanosome is still not understood. Findings from the late 60s to the early 80s proposed that the premelanosome of melanocytes (reviewed by (Jimbow et al. 1976) and pre-natal RPE cells (Mishima et al. 1978; Eppig and Dumont 1972; Eppig 1970; Stanka et al. 1981; Stanka 1971) is formed out of the ER or tubules of the associated GERL (Novikoff et al. 1968). Novikoff was the first who assumed that the RPE melanosome is a specialised lysosome, emerging from acid phosphatase-positive cisterns of the ER (Novikoff et al. 1979). In the 90s, further scientists confirmed that melanosomes of melanocytes (Schraermeyer 1995), pre-natal RPE (reviewed by (Orlow 1995) and post-natal RPE, reviewed by (Schraermeyer and Heimann 1999; Mishima 1994), are lysosome-related organelles, since they are closely related to phagosomes and lysosomes and have lysosomal enzymes (Diment et al. 1995). New techniques, using immuno-gold labelled antibodies, are able to clearly identify proteins and organelles in ultrastructural samples. Thus, recent studies have clarified that the premelanosome of melanocytes and melanoma cells is an endosomal structure (Raposo et al. 2001), in which the structural protein PMEL17 builds internal striations forming the stage II melanosome (Raposo and Marks 2002). For RPE cells, corresponding studies are still lacking. However, the results of Raposo and colleagues are in accordance with the findings from studies in non-pigmented cells: HELA cells that were forced to synthesise PMEL17 (e.g. by transfection), transported it to MVBs, where it formed premelanosomal striations (Berson et al. 2001). Transfection of fibroblasts (Winder et al. 1993; Ando et al. 1993) and different cell lines (Luo et al. 1994) with tyrosinase induced melanogenesis within lysosomes. Meanwhile, it is accepted that all melanosomes are at least lysosome-related organelles (Bonifacino 2004).

In this study, we compared melanogenesis in artificially melanised ARPE 19 cells, which were transduced with the tyrosinase gene, with naturally melanised MNT-1 melanoma cells. While MNT-1 cells predominantly synthesised typical stages of melanogenesis, including tyrosinase, Pmel17 and TRP1 protein synthesis and had typical DOPA-positive Golgi vesicles, melanogenesis in tyrosinase-transduced ARPE 19 cells showed a remarkably different pattern (Table 1):

PMEL17 (Theos et al. 2006) and TRP1 (Boissy et al. 1996), which were believed to be necessary for melanin synthesis, especially premelanosome formation and tyrosinase processing (Berson et al. 2000; Halaban et al. 1997; Mosse et al. 1998) were not detected in our ARPE 19 samples independent of transduction. Accordingly, typical stages of melanogenesis and proper tyrosinase trafficking were missing. Instead we found tyrosinase in tubules near the nucleus and also in the nuclear envelope, indicating retention of tyrosinase in the ER, as published by Calvo et al. (Calvo et al. 1999) for tyrosinase-transfected HELA cells. We also found tyrosinase free floating in the cytoplasm and accumulating inside huge vacuoles, possibly indicating that wrongly targeted and excess tyrosinase is degraded inside lysosomes (Chen et al. 2009; Wasmeier et al. 2006) or is captured inside the vacuoles. In contrast, the huge clusters of DOPA granules in the cytoplasm together with the lack of DOPA reactivity in trans-Golgi cisterns and budding vesicles can also indicate a synthesis of tyrosinase at free ribosomes in the cytoplasm without additional processing in the Golgi apparatus as has been proposed previously (Moyer 1963). We did not confirm proper glycosilation and processing of the enzymes, but the presence of eumelanin in our samples, as confirmed by HPLC analysis (manuscript submitted), indicates a suitable processing.

We analysed cell viability and found that transduction with our tyrosinase vector was not toxic to ARPE 19 cells, as observed 48 h after transduction when more than 50% of the cells synthesised active tyrosinase. Melanisation, starting six days after transduction, also did not affect cell viability. Consequently, the absence of premelanosomes, PMEL17 and TRP1 had no negative influence on tyrosinase protein synthesising cells during the first weeks after transduction.

The presence of DOPA-positive granules in the multi-vesicular and multi-lamellar bodies indicates the first steps of melanisation in our ARPE 19 cells. Ultrastructurally, these bodies resembled phagosomes (Novikoff et al. 1979; Schraermeyer and Stieve 1994; Peters et al. 2000; Novikoff 1973) and autophagosomes (Novikoff 1973). The same DOPA granules have been found in cultured bovine and human RPE after phagocytosis of outer segments without transduction (Julien et al. 2007; Schraermeyer and Stieve 1994).

Our findings are in accordance with previous studies, which also showed melanogenesis inside multi-vesicular (Novikoff et al. 1979) and multi-lamellar bodies (Dorey et al. 1990; Mishima 1994; Schraermeyer and Stieve 1994), in phago-lysosomes (Schraermeyer et al. 1999) of RPE cells and autophagosomes of melanoma cells (Novikoff et al. 1968) and tyrosinase-synthesising non-pigment cells (see above). Interestingly, we also found similar electron-dense, cloudy bodies (stage 1-4) in MNT-1 cells. We assume that in non-melanised cells melanogenesis is possible inside lysosome-related organelles after tyrosinase production and that these pathways are also possible in cells that are able to synthesise melanin in the classical way.

Endocytic vesicles, (auto-) phagosomes and lysosome-like organelles such as melanosomes belong to the same lineage of organelles that emerge from the GERL region (Novikoff 1973). Although it has been clearly demonstrated that melanosomes in melanocytes are closely related to endosomes (Raposo and Marks 2002; Schraermeyer and Dohms 1993), it is suggested that melanogenesis can happen inside related organelles, e.g. phagosomes, as well. Probably, pigment cells have multiple redundant ways of processing the different structural and enzymatic proteins of melanogenesis and several alternative ways of melanin formation may exist. For example, this is true for brain neurons, where neuromelanin synthesis takes place without the involvement of tyrosinase at all, and Tribl et al. found a close relationship of neuromelanin-containing granules with lysosome-related organelles originating from the endosome-lysosome lineage (Tribl et al. 2005). Thus, RPE melanogenesis might occur in adult tissue as well, but in a manner which is as yet unknown.

Latest findings (Gargiulo et al. 2009) show that subretinal injection of an AVV- mediated tyrosinase vector led to repigmentation and the recovery of visual function in albino mice, suggesting that both young and adult RPE cells and choroidal melanocytes are able to restore melanisation.

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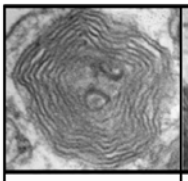
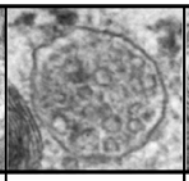
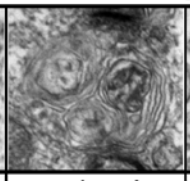
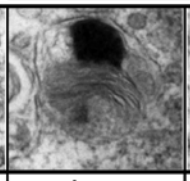
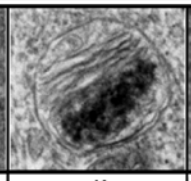
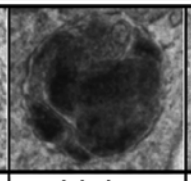
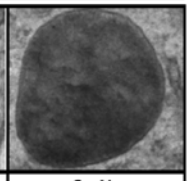
Table 1: Occurrence of DOPA-positive structures and melanogenic stages[1] as observed in MNT-1 and ARPE 19 cells.

	MNT-1	**Transduced ARPE 19**
Stage I premelanosome	**+**	**-**
Stage II premelanosome	**+**	**-**
Stage III melanosome	**+**	**-**
Stage IV melanosome	**+**	**-**
DOPA reaction in ER	**-**	**+**
DOPA reaction in GERL	**+**	**+**
DOPA reaction in Golgi apparatus	**+**	**-**
DOPA reaction in Golgi vesicles (ø ca. 59 nm)	**+**	**-**
DOPA reaction in small granules (ø ca. 34 nm)	**-**	**+**
Melanogenic stages 1-4	**+**	**+**

[1] Melanogenic stages I to IV resemble the typical stages described in the literature. Melanogenic stages 1 to 4 define unusual cloudy and amorphous-filled melanin granules as described in this study.

Table 2: Occurrence of melanogenic stages 1 to 4 in control cells (c) and at different time points after transduction:

			melanogenic stages 1-4			
						
MLB	MVB	mixed body	low melanised	medium melanised	high melanised	full melanised
c ++++	+	++	-	-	-	-
24h ++++	++++	+++	+	-	-	-
6d ++	+++	++++	++	++	+	-
14d +	++++	++	++	++	++	++

– Rarely seen (less than 3% of counted organelles), + present (5-7%), ++ frequently seen (-15%), +++ often seen (-25%), ++++ mainly seen (>26%). In addition, multi-vesicular bodies (MVB), multi-lamellar bodies (MLB) and mixed bodies are included, which are assumed to be the precursors of melanogenesis in ARPE 19 cells.

Figure legends

Fig 1 Light microscopic image, showing ARPE 19 cells, 19 days after transduction: Newly formed melanin granules can be observed all over the cytoplasm. n nucleus, * high melanised cell, ^{o} low melanised cell; scale bar 25 µm

Fig 2 Fluorescence immunocytochemistry of MNT-1 (a-c) and transduced ARPE 19 cells (d-f): a+d PMEL17; b+e TRP1; c+f tyrosinase. All MNT-1 cells show the tested proteins all over the cytoplasm. An exact localisation is not possible in these micrographs, but the proteins are accumulating in central parts of some cells (arrows in a) and at dendrite tips (arrow heads in b, c). However, only tyrosinase is present in transduced ARPE 19 cells. Inset in f: magnification, showing tyrosinase-immunoreactivity in the perinuclear space, suggesting accumulation in the ER; scale bars (a-e) 25 µm; f) 5 µm

Fig 3 DOPA-histochemistry of transduced ARPE 19 cells: a) A cell showing DOPA-positive granules spread all over the cytoplasm. They are associated with membrane-bound bodies (arrows) and cisterns (c) of unknown origin. Structures, resembling ER or GERL tubules are also stained positive (asterisk), but Golgi cisterns are stained negative (G); scale bar 1 µm. (b-h) Detailed magnifications of DOPA-positive structures, as they are frequently found in transduced ARPE 19 cells; scale bars 200 nm: b) Magnification from a: DOPA granules fuse with the membrane of a vesicular organelle of unknown origin. First granules have already arrived inside the organelle. c) A multi-vesicular organelle with a double membrane, resembling an autophagosome, has also incorporated DOPA granules (arrow). d) Hundreds of DOPA granules are clustered together and accumulate close to a huge membrane-bound vacuole of unknown origin. e) As in d: The granules seem to fuse with the covering membrane of a vacuole. f) Tubules, resembling an endoplasmic reticulum, are DOPA-positive and associated with several DOPA-positive granules. g) Often, DOPA granules accumulate around intracellular membranes of unknown origin. Here, it can be seen that the DOPA granules lack a covering membrane, while the double membrane of the mitochondrion at the upper right can be clearly seen. h) The electron micrograph shows DOPA granules (black arrows) and Golgi vesicles (white arrow). It can be clearly observed that the DOPA granules are smaller (approx. 34 nm) and lack a covering membrane. Thus they can easily be distinguished from typical membrane-bound Golgi vesicles (approx. 59 nm)

Fig 4 Melanogenic organelles as observed by EM 6 days after transduction: All relevant organelles of melanin formation can be observed. Here, multi-lamellar bodies (MLBs), a mixed body (MB) and a newly formed melanin granule (M) are presented in one cell. Scale bar 500 nm

Fig 5 Viability of ARPE 19 cells is not affected by tyrosinase (d 2) and melanin synthesis (d 10). Mean +/- SEM control: ARPE cells; +AdTyr ARPE cells, transduced with the tyrosinase vector AdTyr (p= n.s.)

Figure 1

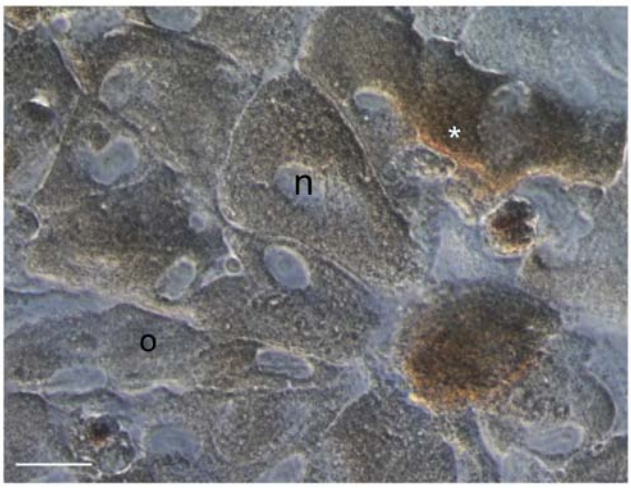


Figure 2

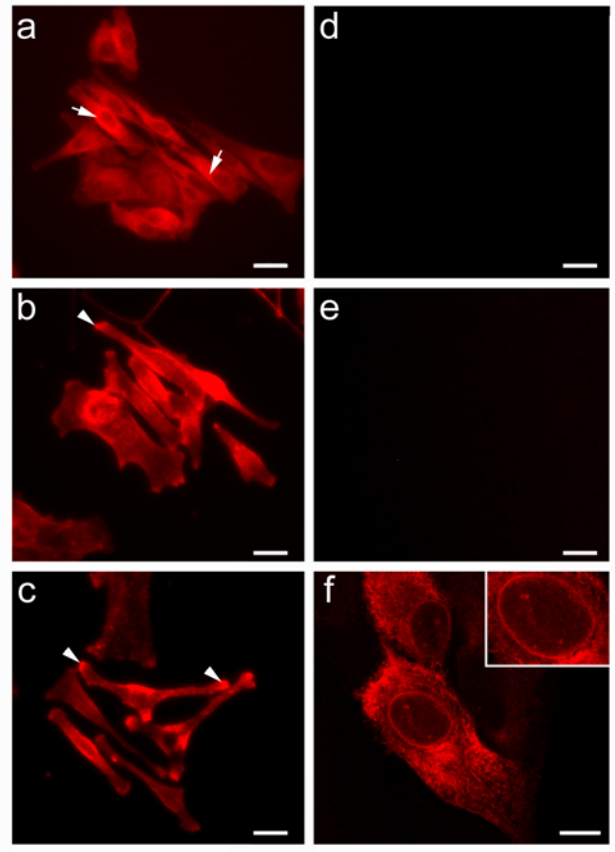


Figure 3

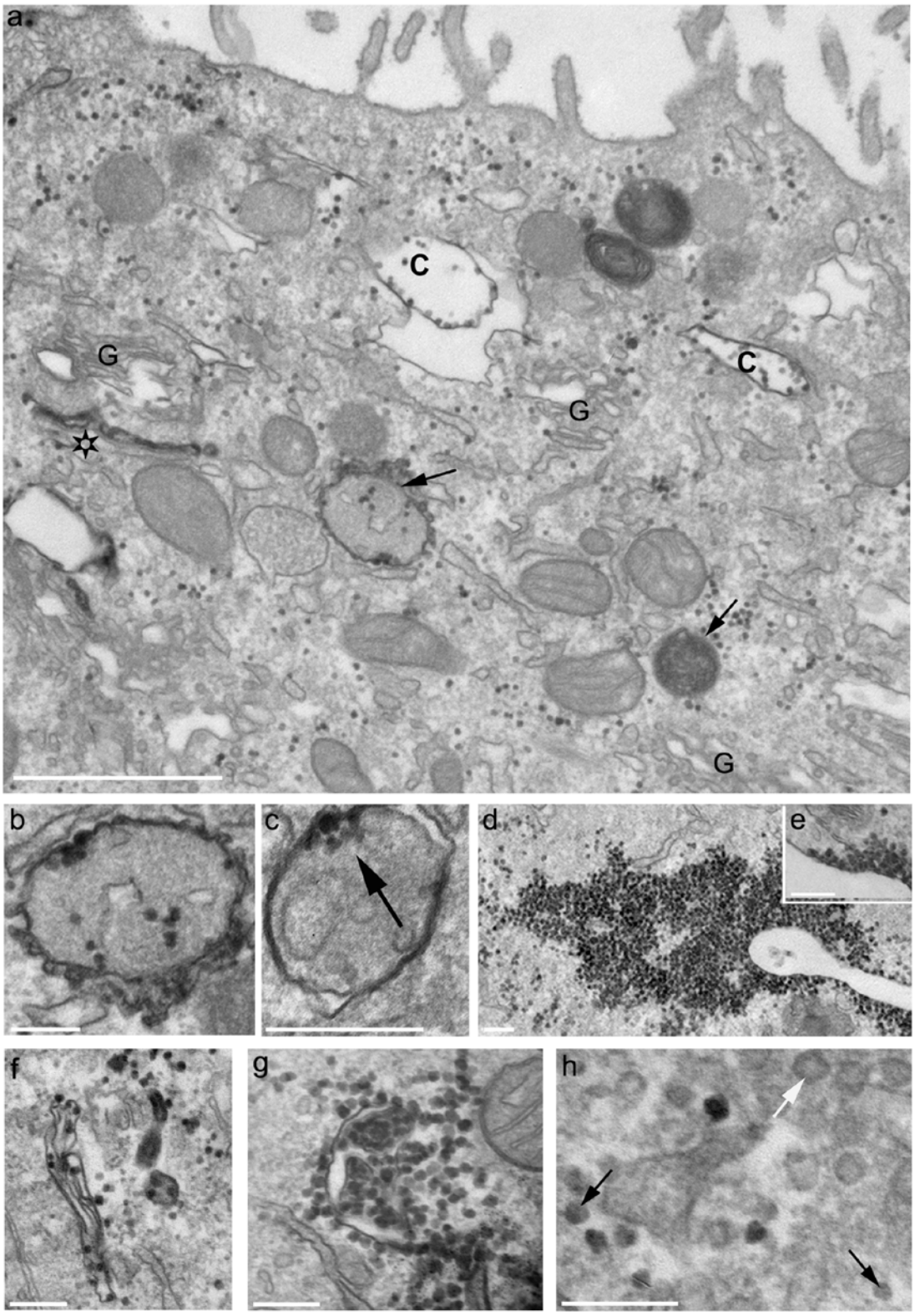


Figure 4

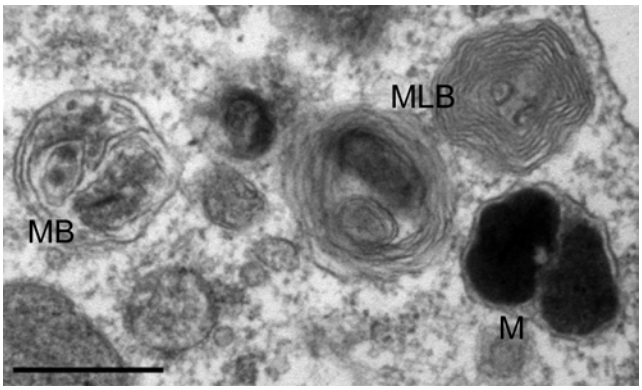
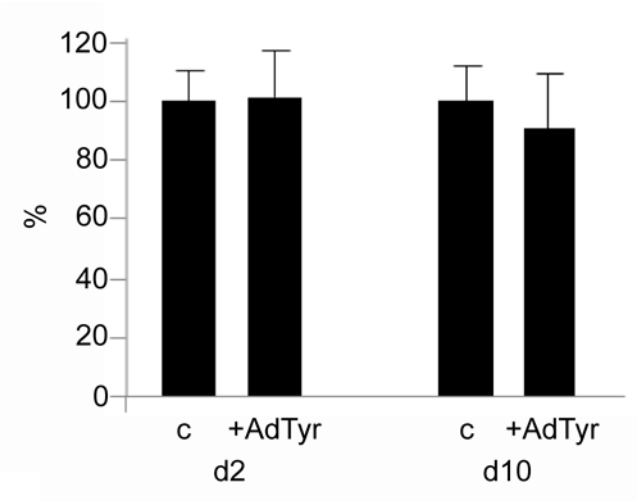


Figure 5



Tyrosinase in conjunction with phagocytosis of retinal outer segments influences the morphology and melanogenesis in cultured human ARPE 19 cells

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Abstract:
We investigated whether tyrosinase expression has an effect on cultured adult RPE morphology and function. In addition, the effect of phagocytosis on tyrosinase expression and melanisation in the same system was studied.
Cells of the non-pigmented ARPE 19 cell line, which lack tyrosinase, were transduced with an adenoviral tyrosinase vector and analysed for changes in morphology, specific and unspecific phagocytic activity and melanin synthesis with light and electron microscopy, fluorescence immunohistochemistry, DOPA histochemistry and HPLC analysis. Cultured cells without any treatment were used as controls.
Control ARPE 19 cells had a dedifferentiated, spindle-shaped morphology and a low phagocytosis rate. In contrast, transduced cells showed a typical epithelial appearance, produced melanin and were able to phagocytose four times more ROS than the controls.
Furthermore, tyrosinase expression and DOPA-oxidase activity were found to be significantly higher in transduced cells additionally fed with ROS than in non-fed transduced cells. Fed transduced cells were also able to synthesise melanin more rapidly. They contained twice as many melanised cells after one week of transduction than cells which were only transduced but not fed. Phagocytosis of latex beads had no effect on tyrosinase action and vice versa, showing that only the specific phagocytosis of ROS was able to increase the amount of melanin formation.
In conclusion, tyrosinase expression enhanced phagocytic uptake of retinal outer segments, led to repigmentation and induced a hexagonal rearrangement of the ARPE 19 cell layer, resembling an in vivo morphology. Additionally, the phagocytosis of retinal outer segments resulted in a faster tyrosinase expression and melanisation in tyrosinase transduced ARPE 19 cells.

Keywords: RPE, retinal outer segment, phagocytosis, tyrosinase, ultrastructure, melanogenesis

Abbreviations: AMD age-related macular degeneration; ARPE 19 amelanotic retinal pigment epithelial cells: A control ARPE 19 cell; AT ARPE+ AdTyr vector; AR ARPE+ ROS; ATR ARPE +AdTyr+ROS; DMSO dimethylsulfoxid; EM electron microscopy; FCS foetal calf serum, IHC immunohistochemistry; L-DOPA L-dihydroxyphenylalanine; LM light microscopy;

MERTK mer tyrosine kinase; MOI multiplicity of infection; PFA paraformaldehyde; PTCA pyrrole-2,3,5-tricarboxylic acid; ROS retinal outer segments; RPE retinal pigment epithelium;

Introduction:

The retinal pigment epithelium (RPE) is an epithelial monolayer located between the retina and the choroid and performs several functions that are critical for vision and the viability of the retina. The main ones are the phagocytosis and degradation of shed photoreceptor outer segments (Bok, 1993; Strauss, 2005) and the absorption of stray light and toxic compounds, preventing vision impairment and oxidative reactions (Mecklenburg & Schraermeyer, 2007; Rozanowski et al, 2008; Sarna, 1992).

Age-related changes to the melanin and loss of melanin granules, together with the generation of melanolipofuscin and lipofuscin, consisting of indigestible residues of phago-lysosomes (Feeney-Burns & Eldred, 1983; Feeney-Burns et al, 1988), in elderly people can lead to diseases of the eye that may result in blindness. In age-related macular degeneration (AMD), destruction of the RPE (Zarbin, 1998) leads to loss of its functions, including phagocytosis and anti-oxidative scavenging.

Cells also undergo senescence in cultured RPE, losing their differentiated appearance and function due to ongoing cell division with passages (Basu et al, 1983; Matsunaga et al, 1999; Shelton et al, 1999; Wang et al, 2004). Originally forming an epithelial monolayer with hexagonal borders, they change into a spindle- shaped mesenchymal or fibroblast-like morphology (Grisanti & Guidry, 1995). Melanin pigmentation, which is thought to be produced only at prenatal stages (Carr & Siegel, 1979), is lost during culture-passages due to the amount dividing with every mitosis circle. Expression of RPE-specific genes may also be impaired by long-time culture. ARPE 19 is an immortalised human RPE cell line, which has a normal karyology, cobblestone morphology, microvilli, tight junctions and typical RPE proteins (RPE65, CRALB). When cultured between passage 10 and 20, the ARPE 19 cells retain many RPE functions, such as barrier properties, phagocytosis and vitamin A metabolism (Dunn et al, 1996). Slow ongoing melanisation has also been observed. However, in later passages ARPE 19 cells lose their differentiated morphology and develop functional impairment (Carr et al, 2009; Dunn et al, 1996).

We demonstrate here that after induction of tyrosinase expression, ARPE 19 cells are able to redifferentiate and improve such functions as phagocytosis and melanin formation.

Materials and methods

Human non-pigmented RPE cells

Human amelanotic retinal pigment epithelial cells (ARPE 19 cell line) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and used between passages three and thirteen. Cells were grown in DMEM medium with 10% foetal calf serum (FCS) and 1% antibiotics (containing 5 units/ml Penicillin G and 5 mg/ml Streptomycin) in 250 ml culture flasks (Greiner, Frickenhausen, Germany). All cell culture reagents were obtained from GIBCO (Invitrogen, Karlsruhe, Germany). Experiments were performed in 24-well culture plates (Costar Corning, Bodenheim, Germany) and 16-well chamber slides (Nunc, Wiesbaden, Germany).

Additional material:

Chemicals and latex beads were purchased from Sigma (Deisenhofen, Germany), dimethylsulfoxide (DMSO) and paraformaldehyde (PFA) from Merck (Darmstadt, Germany). PTCA was a kind gift from Prof. Ito and used as described by Ito and Fujita (Ito & Fujita, 1985).

Isolation of retinal outer segments

The procedure was carried out as specified earlier(Dintelmann et al, 1999). In brief, isolated porcine retinae were washed in PBS until no melanin remained in the preparation. Then retinae were agitated for 2 minutes in KCl-buffer (0.3 M KCl, 10 mM HEPES, 0.5 M $CaCl_2$, 1 mM $MgCl_2$, pH 7) containing 48% sucrose, centrifuged at 500 g and filtered through a gauze fingerling. The solution was diluted with KCl buffer and centrifuged once more at 650 g. The

pellet contained retinal outer segments (ROS). To sterilise the ROS, the pellet was irradiated with 50 Gy x-ray (Gulmay RS 225).

Generation of AdTyr

The adenoviral vector with the human tyrosinase insert in the E1-deleted region (AdTyr) was generated as described earlier (Julien et al, 2007). The vectors were used in a dilution of 50 virus particles per cell (MOI50) in complete medium and incubated for 24 h.

Experimental procedure

Cells were cultured in 24-well culture plates ($1x10^5$cells/well) for electron microscopy, in 16-well chamber slides for immunohistochemistry and in 250 ml culture flasks ($3x10^6$ cells) for the HPLC analysis. One day later, the cells were transduced with the AdTyr vector in complete medium for another 24 h. The medium was changed and excess ROS (enough to cover the cells completely) were added for 4 h (IHC+EM) and 24 h for HPLC experiments. After washing three times, the cells were grown for up to 14 days. Controls without the tyrosinase vector and ROS were treated in the same way.

Immunohistochemistry (IHC) and quantification of phagocytosis

Twenty-four hours after transduction, cells were supplemented (= fed) with rod outer segments or latex beads (10µl/ml) for 4 hours. After washing, cells were fixed with 4% PFA and lysed with 0.2% Triton X 100 and 1% DMSO. Cells were labelled with a first antibody (1:50) at 4°C overnight and with a secondary antibody (1:1000) 45 minutes at room temperature. Cy3-conjugated goat anti-mouse secondary antibody was purchased from Rockland Immunochemicals Inc. (Gilbertsville, PA, USA) and ALEXA 488-conjugated secondary antibody and DAPI from (Invitrogen, Karlsruhe, Germany). Cells were labelled for tyrosinase with a mouse anti-human tyrosinase antibody (T311, BIOZOL, Eching, Germany) and for evaluation of phagocytosis with a rabbit anti-human rhodopsin antibody (Santa Cruz, Santa Cruz, CA, USA). For the quantification of phagocytosis, phagosomes labelled for rhodopsin were counted when 0.5 -1.5 µm in diameter. Cells positive for tyrosinase and or labelled for ROS were counted in fed and non-fed, transduced and non-transduced cells. The total cell number was quantified by counting DAPI-stained nuclei. Latex beads (r= 0.5 µm) were autofluorescent (green) and could be observed without extra labelling. For every experiment, seven pictures (20x) per well in at least two wells per experiment were evaluated. Results were correlated to the total count of cells per picture.

Routine Electron Microscopy

Twenty-four hours, six and fourteen days after transduction, cells were fixed overnight at 4°C in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The specimens were postfixed with 1% OsO_4 and 1,5% potassium ferrocyanide at room temperature in 0.1 M sodium cacodylate buffer for three hours, block-stained with uranyl acetate, dehydrated in a graded series of ethanol, and embedded in EPON. Ultrathin sections were post-stained with lead citrate and investigated under a transmission electron microscope (EM 902A; Zeiss). Samples were evaluated with regard to the amount of electron-dark, membrane-bound melanin granules, which occurred predominantly in transduced cells. Cells containing one or more melanosomes were counted in at least 100 cells per section in at least three sections per sample.

DOPA histochemistry

Localisation of tyrosinase in electron microscopical sections is possible by incubation of the specimen with L-DOPA (L-dihydroxyphenylalanine) after fixation with glutaraldehyde. The DOPA oxidase activity of tyrosinase will produce electron-dark material at the site where the active enzyme is present.

The procedure was performed as described earlier (Schraermeyer, 1992). In short, 24 h, six and fourteen days after transduction, cells were fixed for one hour in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 6.8) on ice. They were washed in sodium cacodylate buffer, incubated in a freshly prepared 5 mM L-DOPA suspension (in sodium cacodylate

buffer, pH 6.8) at 4°C overnight and then the DOPA suspension was renewed and specimens were incubated for another 5 h at 37°C. The specimens were postfixed with 1% OsO_4 and 1,5% potassium ferrocyanide at room temperature in 0.1 M sodium cacodylate buffer for three hours, block-stained with uranyl acetate, dehydrated in a graded series of ethanol, and embedded in EPON. Ultrathin sections were post-stained with lead citrate and investigated under a transmission electron microscope.

Eumelanin quantification
After melanisation was observed in all samples (12 days after transduction), cells were harvested by trypsination, counted in a Neubauer-chamber, washed twice in PBS, dried in a vacuum centrifuge and weighed. HPLC analysis was performed, according to the protocol of Ito (Ito & Fujita, 1985). Eumelanin can be analysed by peroxidation with permanganate. The resulting degradation product PTCA (pyrrole-2,3,5-tricarboxylic acid) can be quantified by HPLC analysis. Synthetic melanin (Sigma, Deisenhofen, Germany) and PTCA were used as standards.

Statistical analysis
Statistical evaluation was based on Student's t-test, and p-values were considered not significant when exceeding 0.05. All groups were compared to the ARPE 19 cells without treatment.

Results:
Immunohistochemistry of tyrosinase expression
Twenty-four hours after transduction (= 4 h after ROS feeding), cells were fixed and labelled for immunohistochemistry. Negative controls without a primary antibody showed no fluorescence at all. Positive control for tyrosinase was done with MNT-1 melanoma cells, which express tyrosinase continuously (not shown).
Non-transduced cells with and without feeding showed no tyrosinase immunoreactivity at all (figure 1A, B). Transduced cells showed tyrosinase reactivity all over the cytoplasm (figure 1C, D). 53 ± 18% of the transduced cells were tyrosinase positive (n=14). ROS phagocytosis led to a significant increase in the number of tyrosinase positive cells compared to both transduced (70 ± 19%; n=9; $p<0.03$) and also latex fed cells (47 ± 9%; n=5; p=0.04). Latex phagocytosis had no influence on tyrosinase expression (figure 2).

Quantification of phagocytosis
A positive effect of tyrosinase on the ROS phagocytosis rate was observed (figure 3A): Transduced fed cells had 29 ± 11 phagosomes per cell, while non-transduced cells had only 7 ± 2 phagosomes per cell (n=7; p=0.0002).
However, unspecific phagocytosis of latex beads (figure 3B) was not enhanced by tyrosinase expression (10 ± 4 beads/ cell without transduction; 7 ± 2 beads/cell in transduced cells; p=n.s.) and vice versa (see above).

EM-DOPA-histochemistry of tyrosinase related to phagocytic events
Twenty-four hours after transduction (4 h after ROS feeding), cells were fixed for DOPA histochemistry. DOPA positive vesicles were observed in high amounts all over the cytoplasm (figure 4). Multi-lamellar (arrow in figure 4) and amorphous filled bodies were also frequently associated with DOPA vesicles. ER tubules were also stained or at least associated with DOPA positive vesicles (not shown). At least 80 cells per sample were evaluated for the presence of DOPA positive (= tyrosinase active) organelles (figure 5) and found to be positive when more than 50 DOPA vesicles were observed.
ARPE cells without treatment were used as a control. They contained a "base level" of tyrosinase positive cells (28 ± 15%; n=80). Fed ARPE cells did not have a significantly higher rate of DOPA cells than control cells (38 ± 22%; n=110).
After transduction, the majority of counted cells were DOPA positive. Without ROS feeding, it was 52 ± 19% (n=110; p=0.008 to ARPE; but not different to ARPE+ROS), and with ROS feeding they reached 89 ± 11% (n=100: $p<0.0001$ compared to non-transduced cells;

p<0.0003 compared to transduced cells). Thus, samples transduced and fed had significantly more tyrosinase active cells than all other samples.

Changes in cell morphology, observed by light and electron microscopy
Confluent control ARPE 19 cells were mostly spindle-shaped and had no observable melanin at all (figure 6A). When transduced, the cells appeared larger and exposed from the surrounding cell layer. They changed to a more cubical appearance, with hexagonal cobblestone morphology (6B-D). This happened in the first days after transduction before an initial pigmentation could be observed by LM. Transduced cells synthesised melanin, which could be visualised six to ten days after transduction (figure 6C).

Observation of melanin synthesis by light microscopy
Cultured cells were checked for the appearance of brown melanin granules every two days. Non-transduced cells never showed any pigmentation. The first melanin- containing cells could be observed after six days of transduction, predominantly in the fed samples. Pigmented cells were counted in six pictures per well and in at least two wells per experiment. The count of pigmented cells of the fed samples was set at 100% (figure 7). Values of quantification for each day observed were pooled for one week (d 7.3; n=11) and for two weeks (d 12.5; n=8) after transduction.
In the first week after transduction, transduced and fed cells (100 ± 38%) had a 2.5 times higher rate of pigmented cells than non-fed transduced cells (40 ± 47%; p<0.0001). When the cells were grown for two weeks, the non-fed samples were also highly melanised. So there were 100 ± 46% pigmented cells in the fed and 97 ± 56% in the non-fed group (p<0.0001 to one week samples).

Observation of melanin synthesis by electron microscopy six and 14 days after transduction
Cells were investigated with regard to the occurrence of electron-dark, membrane-bound melanin granules (figure 8). Six days after transduction, a significant amount of cells containing at least one melanosome, could be observed, but only in transduced and fed cells (>100 cells/section; 50 ± 17%). ARPE cells (9 ± 6%; p= 0.002 to ATR), ARPE+AdTyr (14 ± 6%; p= 0.003 to ATR) and ARPE+ROS (12 ± 4%; p=0.002 to ATR) showed only a base level of melanised cells (figure 9).
After 14 days, melanin granules could also be found in half of the non-fed transduced cells (51 ± 4%) and thus were also significantly different from the control cells with (p=0.0004) and without (p=0.001) feeding. As in the light microscopical observations, it was found that melanisation occurred more rapidly in transduced fed samples than in transduced samples without ROS feeding. The fed and transduced samples contained 57 ± 7% melanised cells (p=0.001 to fed and p=0.003 to non-fed ARPE cells). Non-transduced ARPE cells still contained only a base level of pigmentation (ARPE 6 ± 4%; fed ARPE 19 ± 3%). Fed non-transduced ARPE cells also had significantly more melanised cells than control cells (p=0.02).

Quantification of melanin content via HPLC-analysis
After high melanisation was apparent in all samples, cells were harvested and prepared for HPLC analysis using the PTCA oxidation assay by Ito (Ito & Fujita, 1985). Sepia melanin was used as a positive control. Non-transduced cells, with and without feeding, contained no observable amount of melanin. Purified retinal outer segments were free of melanin contamination. Transduced cells (n=9) showed a PTCA peak of 3 ± 1 [peak/mg], fed transduced cells (n=9) showed a peak of 4 ± 2 [peak/mg], demonstrating that both samples contained eumelanin. Figure 10 shows a typical chromatogram for transduced ARPE 19 cells (A) and for a sepia-melanin control (B).

Discussion:
The goal of this work was to analyse the effects of tyrosinase and phagocytosis on adult ARPE 19 cells. Formerly, we had shown that tyrosinase expression and activity become up-regulated when RPE cells have been fed with retinal outer segments (Julien et al, 2007;

Schraermeyer et al, 2006), although it was believed for a long time that tyrosinase and melanogenesis cannot be found in adult RPE cells (Miyamoto & Fitzpatrick, 1957). However, our findings (Schraermeyer et al, 1999; Thumann et al, 1999) and results by other groups (Dorey et al, 1990; Dryja et al, 1978) suggest that tyrosinase expression and the renewal of RPE melanin can occur under certain circumstances, but tyrosinase function in theses studies was not at all evident. Our studies clearly showed that phagocytosed undegradable material had been transported to and incorporated inside melanosomes. Thus we assume that tyrosinase and phagocytosis may be related in the adult RPE.

Here we show that artificially induced melanin synthesis can be positively influenced by retinal outer segment phagocytosis. The feeding of tyrosinase transduced ARPE 19 cells with retinal outer segments resulted in a higher occurrence and activity of tyrosinase, nearly twice as much compared to non-fed transduced cells, and in a faster melanin synthesis. Even in non-transduced ARPE 19 cells, phagocytosis of ROS led to a slight but insignificant increase in tyrosinase positive cells, as observed by DOPA histochemistry.

Surprisingly, tyrosinase expressing cells gained more remarkable properties than merely pigment synthesis. After transduction, the cells showed a more differentiated appearance with cobblestone morphology, while the control cells remained fibroblastic. Additionally, transduced cells were raised, and blown up, as if they were seeking to regain the cubic morphology they have in vivo. We also observed in a single experiment (6 wells) that tyrosinase transduced pigmented cells were able to survive longer in cell culture without passaging than control cells and cells that were transduced with the sister vector AdEGFP, which encodes the GFP protein instead of tyrosinase (data not shown). The GFP-vector did also not induce cobble-stone morphology in the transduced cells. These results indicate that it is not the transduction per se, but the expression of the tyrosinase protein which was responsible for the changes observed.

With respect to a longer survival, our data suggest that tyrosinase and melanin have a protective role in culture-dependent oxidative stress. Tyrosinase action has been shown to have antioxidative capacity before (Valverde et al, 1996a; Valverde et al, 1996b) and tyrosinase has a low catalase activity, as described by (Wood & Schallreuter, 1991). Formation of di-tyrosyl radicals can be blocked by tyrosinase action (Kim & Han, 2003), and melanin intermediates (o-di-phenols) may have antioxidative capacity as well (Garcia-Molina et al, 2005; Kim & Han, 2003; Shi et al, 2002). In melanocytes, tyrosinase has also been found to protect from “reactive oxygen species” generating compounds (Perluigi et al, 2003). In addition, melanin (Rozanowski et al, 2008) and melanin precursors have been found to have high antioxidative capacity (Memoli et al, 1997). Dihydroxyindol acts as a free radical scavenger (Schmitz et al, 1995) and a potent inhibitor of lipid peroxidation (Memoli et al, 1997) and also has a slight antioxidative effect on RPE cells in vivo (Heiduschka et al, 2007).

Moreover, transduced cells showed a phagocytosis rate four times higher than the control cells. However, tyrosinase had no effect on latex feeding (and vice versa). The definite pathways of phagocytosis are still not fully understood, but it has been found that CD36, $\alpha_v\beta_5$ and MERTK (Mer tryrosine kinase) are crucial actors in the binding and ingestion of ROS (reviewed by (Strauss, 2005). While unspecific phagocytosis of latex beads (Edwards & Szamier, 1977; Heth & Marescalchi, 1994) or melanin granules (Braun et al, 1999) is not dependent on MERTK, as observed in RCS rats, the specific ingestion of ROS is only possible when ROS come into contact with this receptor. Then, a reaction cascade starts that leads to Ca^{2+} increase and to an activation of the PKC pathway. Calcium is needed for proper binding of ROS and thus facilitates an increase in phagocytosis (Hall et al, 2002), while PKC activates the shut-off signal for ingestion by elevating the cAMP level in the cell (Hall et al, 1991). Thus ROS phagocytosis is modulated and regulated by its downstream effectors. Possibly the pigmentation genes are also affected by the same modulators e.g. cAMP has been found to elevate tyrosinase expression in cultured RPE (Abul-Hassan et al, 2000), and a Ca^{2+} switch protocol led to a more differentiated appearance of RPE cells in culture, including repigmentation (Rak et al, 2006). Also, the above mentioned PKC pathway has been found to affect tyrosinase expression in skin melanocytes via MSH regulation (Halaban et al, 1993). Thus it is speculative whether phagocytosis and tyrosinase may affect

each other positively due to post-MERTK regulators, which might be in accordance with the observation that latex fed cells react in a different way.
Phagocytosis experiments using ARPE 19 cells have been performed in the last decade with good results (Cai & Del Priore, 2006; Chen et al, 2009; Finnemann et al, 1997; Tsai et al, 2008). However, Carr et al. recently found that ARPE 19 cells (passage 22) lack phagocytic activity due to a deficiency in MERTK expression (Carr et al, 2009). In our experiments, we did not use ARPE 19 cells older than passage 13. Carr et al. discussed that immortalized cell lines like ARPE 19 might undergo dedifferentiation over time, abolishing typical RPE functions. To ascertain phagocytic function of our cells, we tested MERTK presence in our cells (passage 3, 4 and 10) with a monoclonal antibody and found them to present MERTK ubiquitously in transduced and non-transduced fed and non-fed samples (data not shown). Whether MERTK expression can be modulated by phagocytosis of ROS or the expression of melanogenic enzymes, leading to a higher phagocytosis rate, remains to be investigated in more detail.
While in the immunohistochemistry and HPLC analysis, observable amounts of tyrosinase and melanin respectively were seen only in transduced cells, the ultrastructure also showed small amounts of tyrosinase (as observed by DOPA histochemistry) and melanin granules (only observable by EM) in the non-transduced control cells. It is known from Dunn et al. that ARPE 19 cells are able to produce melanin in small amounts (Dunn et al, 1996), therefore a base level of pigmentation could be expected. We also observed weak differences between non-transduced ARPE and fed ARPE cells, concerning the amount of melanised cells, which could not be proven by HPLC analysis and light microscopy. These data will not be further considered.
However, it was only after an overexpression of tyrosinase that melanising machinery was activated, and an amount of melanin detectable with light and electron microscopy could be produced. Comparing the transduced samples, we found that melanisation was much more advanced in fed cells than in non-fed cells, as observed by LM and EM microscopy when evaluated one week after transduction. Significant amounts of electron-dark, membrane-bound melanin granules in the non-fed transduced sample were not observed before two weeks of transduction. The melanin produced was demonstrated to contain eumelanin by HPLC analysis in all transduced samples, two weeks after transduction, while control fed and non-fed ARPE 19 cells and ROS were melanin-free.
After phagocytosis, cells became pigmented more rapidly, possibly because the substrates of melanogenesis may be taken from ROS degradation products. Biochemical experiments have shown that peptides and proteins can be converted into melano-protein by tyrosinase (Ito et al, 1984; Marumo & Waite, 1986; Schraermeyer, 1994). The availability of the substrates L-tyrosine and L-DOPA is known to activate tyrosinase expression and activity (Slominski et al, 1988; Slominski & Paus, 1990) and specific transporters at the melanosomal membrane facilitate and regulate their entry (Gahl et al, 1995; Pankovich & Jimbow, 1991). Aronson found that ongoing feeding of RPE cells in culture resulted in continued melanin synthesis (Aronson, 1983). It can only be speculated whether our fed cells might have used protein-bound tyrosine residues for melanogenesis. It is also known that phagocytosis induces up and down-regulation of many genes (Chowers et al, 2004). Thus, pathways yet unknown may have been activated or silenced to make melanogenesis possible in our phagocytosis experiments. Meanwhile, a new tyrosinase-vector, derived from the adeno-associated virus 2/1 (AAV), has been found to restore melanogenesis and retinal function in the RPE and choroid of albino tyr^{c-2j} mice (Gargiulo et al, 2009). Additionally, AAV-mediated OA1-transduction increased the melanin formation by triggering stage I -endolysosome formation (Cortese et al, 2005; Surace et al, 2005). Thus, vector-induced tyrosinase expression seems to be an attractive method of restoring pigmentation and function in adult eye pigment cells.
In conclusion, we showed that phagocytosis of retinal outer segments influences the melanising machinery in adult RPE cells. We found a higher tyrosinase synthesis and activity, and phagocytosis also led to an even faster melanin formation than transduction with the tyrosinase gene only. Transduced cells regained their typical hexagonal shape and a

higher phagocytosis rate than non-transduced cells. Thus tyrosinase expression switched cultured RPE cells closer to the in vivo situation.

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Figure legends
Fig. 1: Fluorescence light micrographs taken from ARPE 19 cells, 24h after transduction (magnification 20x): A) Control ARPE 19 cells, B) ARPE 19 cells fed with retinal outer segments, C) ARPE 19 cells, transduced with the tyrosinase gene, and D) ARPE 19 cells, which were fed with retinal outer segments and transduced with tyrosinase are shown. Tyrosinase labelling (red) can be seen all over the cytoplasm, but only in the transduced cells. Phagocytic events were visualised by rhodopsin labelling (green). Phagosomes (0.5-1.5µm) are spread all over the cytoplasm.

Fig. 2: Quantification of tyrosinase immunoreactivity, 24h after transduction: Transduced ARPE cells that were additionally fed with ROS (ATR, n=9), had significantly more tyrosinase expressing cells than cells that were only transduced (AT, n=14; *$p<0.03$) or cells transduced and fed with latex beads (ATL; n=5; $p=0.04$). Latex feeding had no additional effect on tyrosinase immuno-reactivity.

Fig. 3: A) Quantification of ROS phagocytosis: Non-transduced ARPE cells (AR) phagocytosed four times less ROS than transduced (ATR) cells (n=7; $p<0.0002$). B) Quantification of latex phagocytosis: There were no significant differences in the phagocytic activity of latex fed cells (AL) and transduced, latex fed (ATL) cells (n=5).

Fig. 4: Electron micrograph taken from DOPA histochemistry of a transduced ARPE 19 cell, 6 days after transduction: The cell shows tyrosinase filled granule-like vesicles throughout the cytoplasm, sometimes in association with multi-lamellar (black arrow) and amorphous filled bodies of unknown origin (*). C: neighbouring cell, lacking DOPA positive reaction.

Fig. 5: Quantification of DOPA histochemistry, 24h after transduction: Transduced cells with (ATR) and without feeding (AT) showed significantly more DOPA positive cells than non-transduced non-fed cells (A; $^{o}p<0.008$ to AT; *$p<0.0001$ A + AR to ATR). ATR had even more DOPA positive cells than AT and non-transduced fed cells (AR $^{\bullet}p<0.0001$).

Fig. 6: Morphological observations: A-C light, D electron microscopy: A) Control ARPE 19 cells had a mostly spindle-shaped, non-pigmented appearance (here: day 7). B) In the first days after transduction, cells developed a hexagonal morphology, grew larger and appeared more prominent than control cells (here: day 6). C) After one week of transduction, first melanin granules were observed. The light micrograph shows a transduced and fed sample, 11 days after transduction. D) Electron micrograph of DOPA reaction at low magnification: the cell in the middle is transduced since DOPA positive vesicles can clearly be recognised. It is up to 1/3 higher than neighbouring cells, which lack DOPA staining and thus might not be transduced.

Fig. 7: Quantification of melanin synthesis observed one and two weeks after transduction (LM): Samples were checked for melanisation every two days. After one week of transduction, first melanised cells were observed in the transduced samples by light microscopy (ATR = 100%): Quantification of melanised cells shows that fed transduced samples (ATR) became melanised more rapidly than cells that were only transduced (AT) (n=8; *$p=<0.0001$). After two weeks, most cells in the non-fed samples were also pigmented ($^{o}p<0.0001$ to AT at one week). Non-transduced cells did not show any pigmentation when observed under the light microscope (not shown).

Fig. 8: Electron micrograph, obtained two weeks after transduction: A typical melanosome with covering membrane can be seen. About 50% of the transduced cells have synthesised melanin granules, which were 0.3 -1 µm in diameter.

Fig. 9: Quantification of melanin synthesis observed one and two weeks after transduction (EM): While transduced and fed cells (ATR) had developed a significant amount of melanised cells after one (*p<0.03 to A, AR, AT) and two (*p<0.003 to A and AR) weeks of transduction, non-fed transduced cells (AT) needed two weeks to gather enough melanin to outnumber the basal pigmentation of control cells (^{o}p<0.001 to A and AR and to AT at one week).

Fig. 10: Chromatogram of HPLC analysis, showing the peak of PTCA produced (black arrow) in A) transduced ARPE 19 cells and B) Sepia melanin control.

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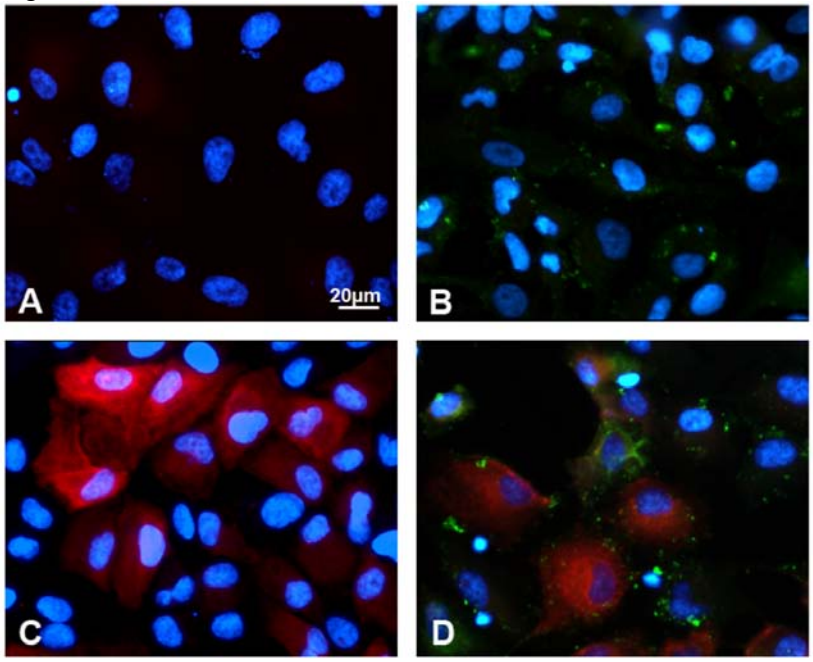
Figure 1



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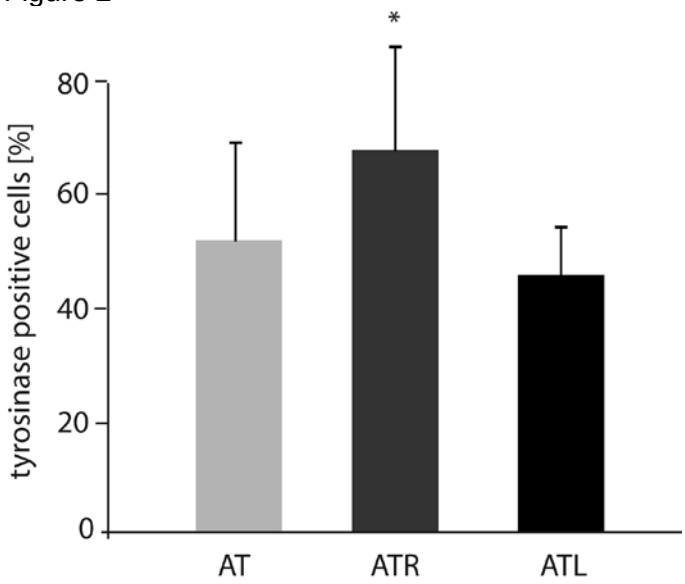

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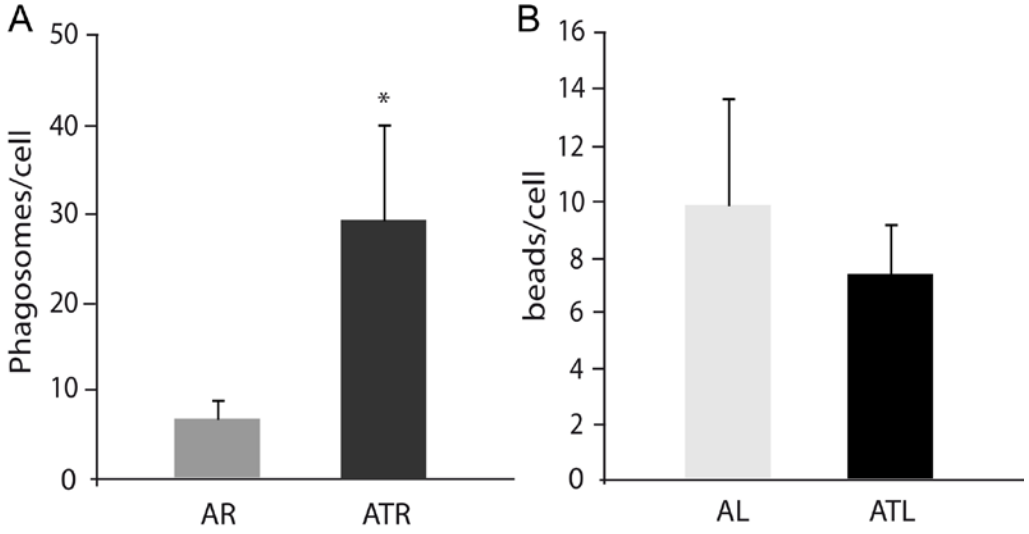

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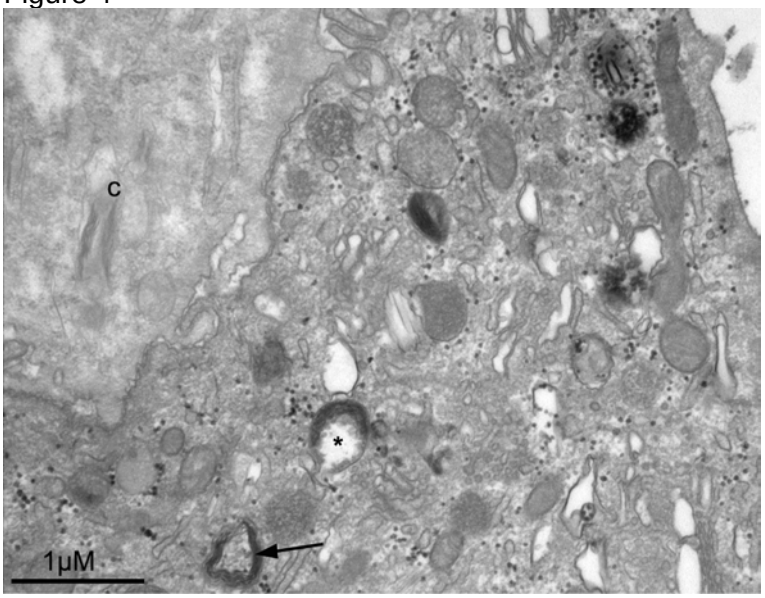

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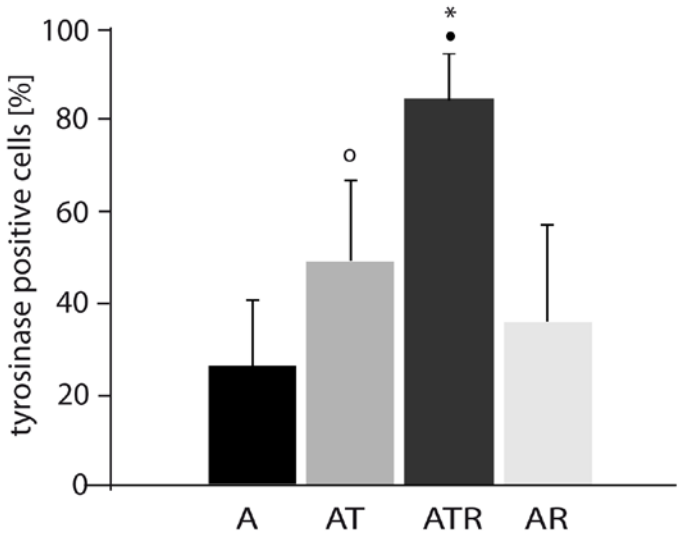

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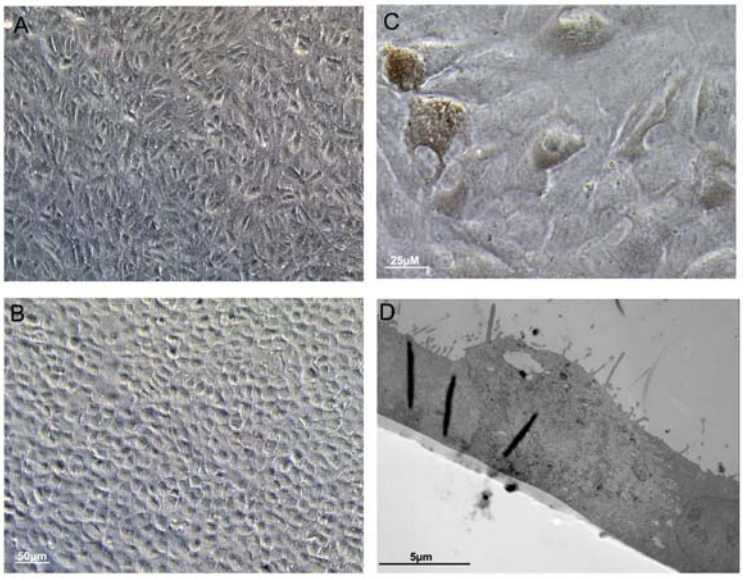

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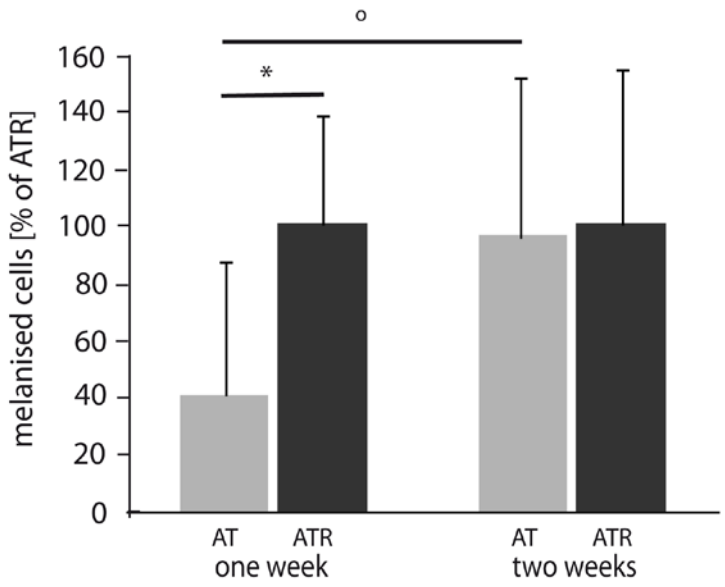

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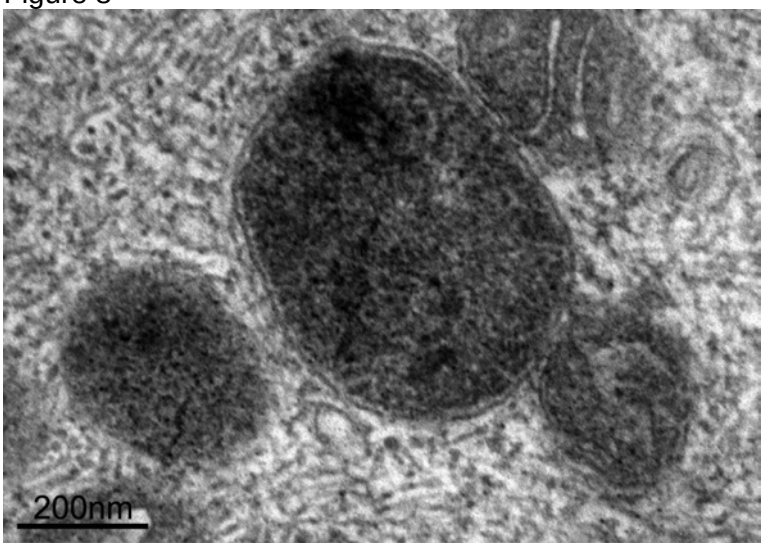

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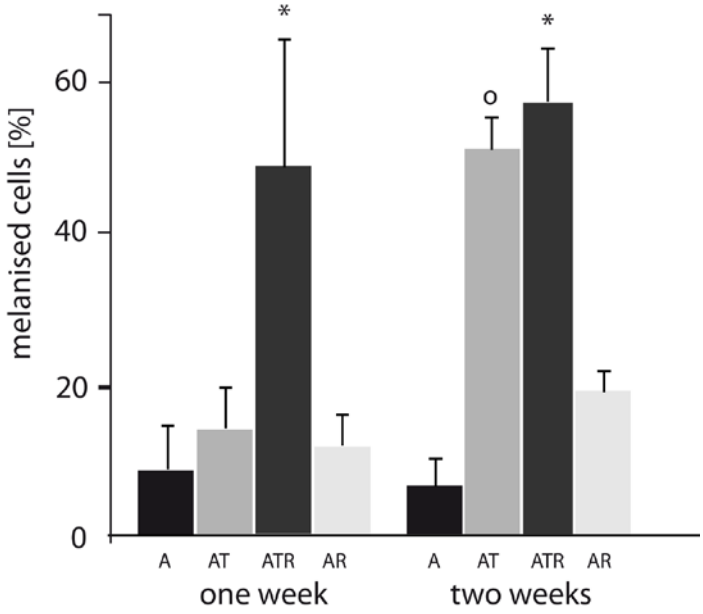

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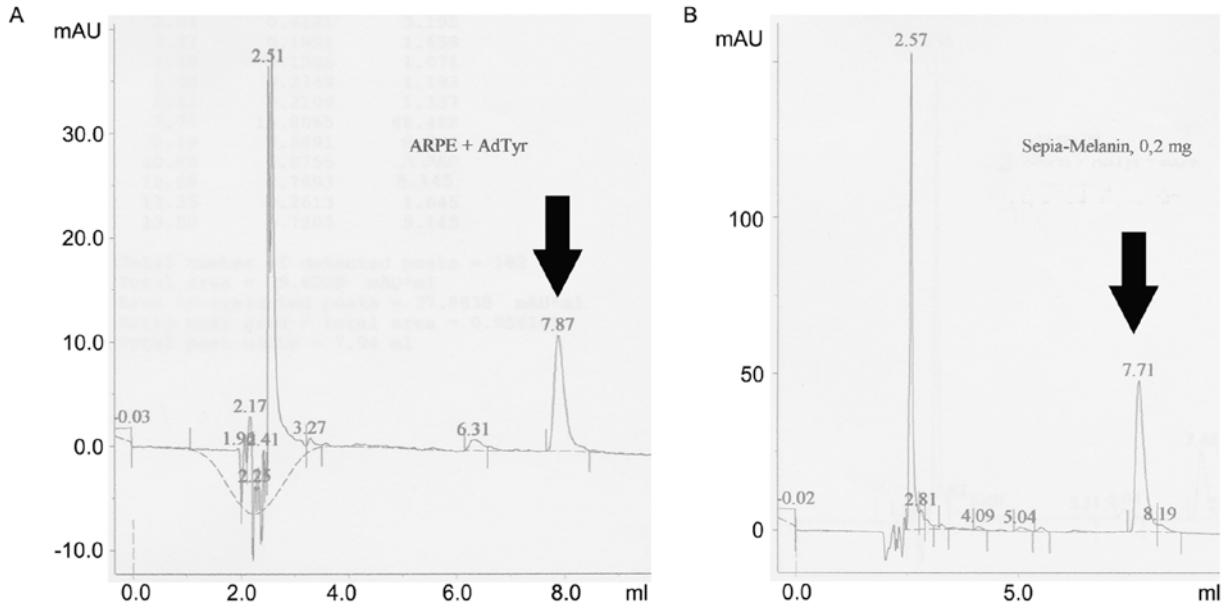
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