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Abstract

Population-based and interventional studies have shown that elevated zinc levels can reduce the progression to advanced age-related macular degeneration. The objective of this study was to assess whether elevated extracellular zinc has a direct effect on retinal pigment epithelial cells (RPE), by examining the phenotype and molecular characteristics of increased extracellular zinc on human primary RPE cells. Monolayers of human foetal primary RPE cells were grown on culture inserts and maintained in medium supplemented with increasing total concentrations of zinc (0, 75, 100, 125 and 150 μM) for up to 4 weeks. Changes in cell viability and differentiation as well as expression and secretion of proteins were investigated. RPE cells developed a confluent monolayer with cobblestone morphology and transepithelial resistance (TER)>200 Ω*cm2 within 4 weeks. There was a zinc concentration-dependent increase in TER and pigmentation, with the largest effects being achieved by the addition of 125 μM zinc to the culture medium, corresponding to 3.4 nM available (free) zinc levels. The cells responded to addition of zinc by significantly increasing the expression of Retinoid Isomerohydrolase (RPE65) gene; cell pigmentation; Premelanosome Protein (PMEL17) immunoreactivity; and secretion of proteins including Apolipoprotein E (APOE), Complement Factor H (CFH), and High-Temperature Requirement A Serine Peptidase 1 (HTRA1) without an effect on cell viability. This study shows that elevated extracellular zinc levels have a significant and direct effect on differentiation and function of the RPE cells in culture, which may explain, at least in part, the positive effects seen in clinical settings. The results also highlight that determining and controlling of available, as opposed to total added, zinc will be essential to be able to compare results obtained in different laboratories.

Keywords	zinc; age related macular degeneration; retinal pigment epithelium
Manuscript category	Pathobiochemistry

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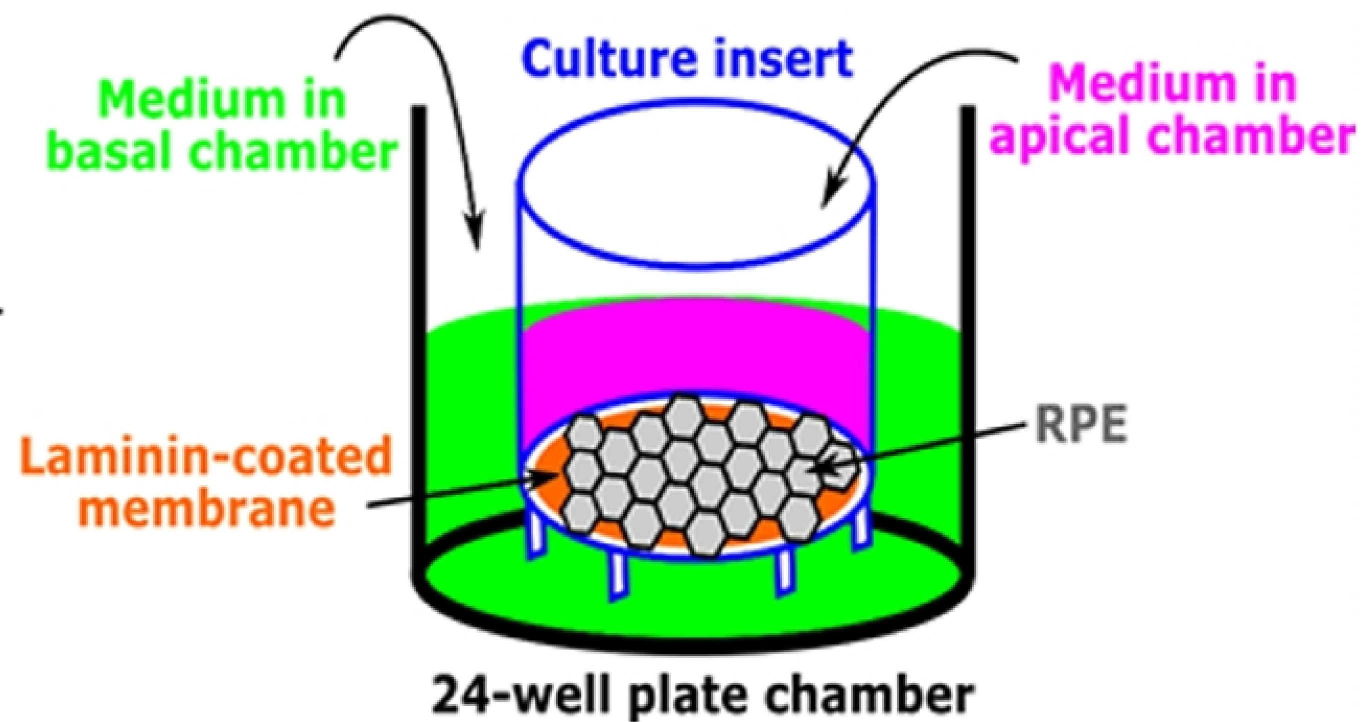
Data for: The effects of zinc supplementation on primary human retinal pigment epithelium

These table contain the fill proteomics data for the apepr by Po-Jung at al submitted JTEMB.

Highlights

- Free and not total zinc concentration is important for the interpretation of biological function
- Zinc supplementation affected RPE phenotype
- Zinc supplementation can modulate the secretion of AMD-associated protein
- Regulation of zinc availability could be key for retinal function
- The positive effect of zinc supplementation in AMD is, at least partly, due to an effect on RPE


Culture insert
Medium in
basal chamber
Medium in
apical chamber
RPE
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24-well plate chamber

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Title: The effects of zinc supplementation on primary human retinal pigment epithelium

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Short title: Zinc supplementation and the RPE

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ABSTRACT

Population-based and interventional studies have shown that elevated zinc levels can reduce the progression to advanced age-related macular degeneration. The objective of this study was to assess whether elevated extracellular zinc has a direct effect on retinal pigment epithelial cells (RPE), by examining the phenotype and molecular characteristics of increased extracellular zinc on human primary RPE cells. Monolayers of human foetal primary RPE cells were grown on culture inserts and maintained in medium supplemented with increasing total concentrations of zinc (0, 75, 100, 125 and 150 μM) for up to 4 weeks. Changes in cell viability and differentiation as well as expression and secretion of proteins were investigated. RPE cells developed a confluent monolayer with cobblestone morphology and transepithelial resistance (TER)>200 Ω*cm^2 within 4 weeks. There was a zinc concentration-dependent increase in TER and pigmentation, with the largest effects being achieved by the addition of 125 μM zinc to the culture medium, corresponding to 3.4 nM available (free) zinc levels. The cells responded to addition of zinc by significantly increasing the expression of Retinoid Isomerohydrolase (RPE65) gene; cell pigmentation; Premelanosome Protein (PMEL17) immunoreactivity; and secretion of proteins including Apolipoprotein E (APOE), Complement Factor H (CFH), and High-Temperature Requirement A Serine Peptidase 1 (HTRA1) without an effect on cell viability. This study shows that elevated extracellular zinc levels have a significant and direct effect on differentiation and function of the RPE cells in culture, which may explain, at least in part, the positive effects seen in clinical settings. The results also highlight that determining and controlling of available, as opposed to total added, zinc will be essential to be able to compare results obtained in different laboratories.

Keywords: zinc, age related macular degeneration, retinal pigment epithelium, mass spectrometry

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INTRODUCTION

123 According to the population-based study conducted in Rotterdam, those with the
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125 highest quartile of zinc nutrition have a lower risk of progression in age-related
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127 macular degeneration (AMD) [1]. Decreased levels of total zinc in the peripheral
128 blood serum and the retinal pigment epithelium (RPE)/choroid [2-6] have also been
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130 associated with development of AMD. Accordingly, the Age-Related Eye Disease
131 Study (AREDS) trial showed that the progression to advanced AMD was significantly
132
133 reduced in patients who received daily supplementation of 80 mg zinc, especially in
134 combination with a cocktail of antioxidants [7]. The mechanism behind this beneficial
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136 zinc effect remains elusive, but understanding the processes affected by zinc may lead
137 to improved or novel treatment strategies for AMD.
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141 Zinc is the second most abundant trace element in the human body [8-10]. Zinc
142 toxicity is rarely observed *in vivo* [11, 12], although in cell culture experiments zinc
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144 overdose can trigger cell death [13-16]. In the eye, zinc is present in high
145 concentrations [17-19] where the majority of ocular zinc is localised to the
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147 RPE/choroid complex [20]. Zinc deficiency or zinc overload in the RPE can lead to a
148 variety of problems [21-25]. However, the actual concentration of biologically active
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150 or available zinc ions (e.g., the small fraction of total zinc ions not tightly bound by
151 protein and other ligands) is not determined; therefore, comparison of results between
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153 laboratories is usually difficult. Active uptake and prolonged retention of zinc has
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155 been shown in RPE *in vivo* [26] and the presence of exchangeable zinc in RPE has
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157 been demonstrated [27, 28] and was localized to the Golgi apparatus [29],
158 melanosomes, and lysosomes [30-33].
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160 Based on protocols for culturing and differentiating human RPE cells [34-36] there
161 are abundant high affinity zinc-binding proteins such as serum albumin in the culture
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163 medium and it is hypothesized that RPE cells in culture are in a potentially zinc-
164 deficient environment. In this report, we cultured primary human RPE cells in a zinc-
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166 enriched environment by supplementing the culture medium with different
167 concentrations of added zinc sulphate and found that under these conditions 3.4 nM
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169 free zinc could accelerate RPE differentiation, alter gene expression, and modify
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171 secretion of AMD-specific proteins.
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MATERIALS AND METHODS

RPE Cell Culture.

Primary human foetal RPE cells were purchased from ScienCell™ Research Laboratories at passage 1 (P1). Cells were propagated and frozen as P2 cells. For the experiments below, P3 cells were seeded onto laminin-coated 24 well plastic culture plates with glass cover slip or porous cell culture inserts (Millipore Millicell-HA Culture Plate Inserts, PIHA 01250) with the density of 125,000 cells per square centimetre and cultured in Epithelial Cell Medium (EpiCM, ScienCell™ Research Laboratories) for one week to allow optimal propagation, following the manufacturer's instructions. Following this period cells were differentiated in the so-called "Miller medium"[35] with or without 75, 100, 125, 150 or 200 μM added zinc sulphate (Sigma-Aldrich) apically, and when the cell culture insert was used, non-supplemented medium basally for 28 days. Medium was changed twice a week and cells were maintained at 37°C and 5% CO_2. Prior to change of culture medium, trans-epithelial resistance (TER) was measured using the EVOM2 Epithelial Voltohmmeter and STX2 electrodes (World Precision Instruments). Both apical and basal media were collected at the time of the change of medium for toxicity measurements using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) to monitor cell viability. Pigmentation was imaged using a Nikon LZM1500 binocular microscope at 1x magnification and the coverage of pigment at different zinc concentrations was determined by ImageJ software (ImageJ v.1.51p; background subtraction and thresholding then analysing particle size distribution).

Determination of free zinc in the medium

Zinc concentrations in the Miller medium were calculated using MINEQL, a chemical equilibrium program for personal computers [37],as well as measured using fluorescent sensors essentially as described [13]. Briefly, the known affinities [23, 38] and concentrations of the 25 most important zinc ligands were included in the calculation; other potential ligands (e.g. vitamins, growth factors) were not included in the calculation due to their low concentrations (< 10 nM) and/or weak affinities (K_D> 1 μM). Bioavailable ("free") zinc was held to include Zn^{2+} together with its labile complexes with weak, rapidly exchangeable ligands such as water, hydroxide, or chloride: e.g., ZnOHCl, $ZnCl_2$, etc. The results were corrected for temperature,

ionic strength, and pH, and the presence of dissolved CO_2 in the medium was included. The accuracy of this calculation method was previously demonstrated for other media[39] and sea water[40]. We used a variation on our previously described fluorescence-based zinc biosensors [41-43] to measure the free zinc in the growth medium**.** The sensor employed one of two variants of apocarbonic anhydrase II to provide sensitivity to the appropriate free zinc concentrations, and a polymeric form of ABDN (7-amino-(2'-hydroxyethyl)-benz-2-oxa-1,3-diazole-4-sulfonamide) which exhibits a 50 nm blue shift in its peak fluorescence emission when bound to holocarbonic anhydrase compared to its unbound form which is observed in the absence of zinc, when both are entrapped in a porous gel. The ratio of fluorescence emission at 550 to 600 nm is thus a measure of the proportion of carbonic anhydrase with zinc bound, which in turn is a simple function of the free zinc concentration[41].The variants of the carbonic anhydrase were wild type bovine CA II and H94N human CA II, which exhibited apparent K_D's under the conditions of the experiment of 0.10± 0.033 and 0.71± 0.06 nM, respectively (data not shown).

Immunohistochemistry (IHC) and confocal microscopy

After 28 days of zinc supplementation, specimens were rinsed in PBS (3×5 min), fixed for 10 min in 4% (v/v) paraformaldehyde in PBS, and stored in 0.4% paraformaldehyde pending sectioning and immunohistochemical analysis. The membrane inserts were carefully excised, then cryopreserved by immersion in 30% (w/v) sucrose (in PBS) overnight at 4˚C, followed by 30% sucrose plus Tissue-Tek® O.C.T.™ compound (Sakura) at 50% : 50% (vol:vol) for 2 hr, and finally in 100% (v/v) O.C.T. for 1 hr. Cryopreserved samples were embedded in OCT and 20 µm sections were generated on a cryostat (Bright Instruments). Sections were rinsed with PBS and blocked with 5% (v/v) donkey serum in PBT (PBT: PBS containing 0.5% (w/v) bovine serum albumin and 0.1% (v/v) Triton X-100) for 1 hr at room temperature. The sections were co-labelled with polyclonal goat anti-human Apolipoprotein E (1/500 in PBT, Millipore) and monoclonal mouse anti-human PMEL17 (clone HMB45, 1/2000 in PBT, Dako) antibodies for 1 hr at room temperature. Sections were rinsed for 3×5min in PBS, after which they were incubated in Alexa Fluor 546-conjugated donkey anti-goat IgG and Alexa Fluor 488-conjugated donkey anti-mouse IgG (both 1/200 in PBT, Life Technologies) for 1hr at

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299 room temperature. After removal of the secondary antibodies by rinsing the sections
300 with 3x5 min in PBS, nuclei were stained with Hoechst 33342 (1.5 µg/ml in PBS,
301 Life Technologies) and mounted on slides with Vectashield antifade mounting
302 medium (Vector Laboratories). Immunofluorescence was imaged using a Zeiss
303 LSM700 confocal microscope and analysed by ZEN 2 software (Carl Zeiss
304 Microscopy GmbH).
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309 *Transmission electron microscopy (TEM)*
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311 After 28 days of zinc supplementation, specimens for TEM were fixed in a solution of
312 1% (v/v) glutaraldehyde and 1.5% (v/v) paraformaldehyde in 0.1 M PBS at pH 7.2.
313 Specimens were post-fixed with 1% (w/v) osmium tetroxide in 0.1 M PBS for 50 min,
314 dehydrated and embedded in Araldite. For EM, ultra-thin sections were cut and
315 stained with 1% (w/v) uranyl acetate and Reynolds' lead citrate. Semi-thin sections
316 for light microscopy were also cut and stained with Toluidine Blue. EM was
317 performed using a JEOL JEM-1010 Transmission Electron Microscope. Images were
318 collected using a Gatan Orius CCD camera and converted from Digital Micrograph
319 DM3 format to 8-bit TIFF images for analysis at 4008 X 2762-pixel resolution.
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327 *Mass spectrometry and label-free quantitative analysis*
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329 To analyse the secreted proteins with or without 125 µM added zinc at day 28 after
330 zinc supplementation, cells were washed with 1x5 min PBS then kept in serum-free
331 "Miller medium" for 24 hr, after which the apical secretome and the filter insert for
332 basal secretion were collected and frozen immediately, except when part of the
333 membrane insert was fixed for immunolabeling. Proteins absorbed by the membrane
334 during culturing representing the basally secreted protein pool were directly
335 proteolyzed on the membrane by incubation with ammonium bicarbonate buffer,
336 reduction with dithiothreitol for 30 minutes at 60°C, followed by
337 carbamidomethylation of cysteines with iodacetamide for 30 minutes at room
338 temperature under constant agitation. Proteins contained in the apical secreted
339 compartment were digested by a filter-aided sample preparation method as described
340 previously [44]. Trypsin (1µg) was added and samples were digested overnight at
341 37°C. Peptides were acidified and directly used for mass spectrometry.
342 LC-MS/MS analysis was performed on an Orbitrap XL mass spectrometer
343 (ThermoFisher Scientific) online coupled to an Ultimate 3000 RSLC nano-HPLC
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(Dionex) as described [44]. Acquired raw data was loaded onto the Progenesis QI software for MS intensity-based label-free quantification (Nonlinear Dynamics, Waters). After alignment to achieve a maximum overlay of peptide features, filtering of singly charged features and features with charges >7 as well as normalization to correct for systematic experimental variation, all MSMS spectra were exported and searched against the Ensembl human database (100158 sequences) using the Mascot search engine with the following search settings: enzyme trypsin, one missed cleavage allowed; 10 ppm peptide mass tolerance and 0.6 Da fragment mass tolerance. Carbamidomethylation was set as fixed modification, methionine oxidation and asparagine or glutamine deamidation were allowed as variable modifications. A Mascot-integrated decoy database search calculated an average false discovery of <0.1% when searches were performed with a mascot percolator score cut-off of 18 and significance threshold of 0.01. Peptide assignments were re-imported into the Progenesis QI software. The abundances of all unique peptides allocated to each protein were summed up and the resulting normalized abundances were used for calculation of fold-changes of proteins and significance values by a Student's T-test.

RESULTS

Determination of free zinc concentrations in the Miller medium

While publications usually report only the added zinc concentration to an experiment, the concentration of added (or total) zinc is typically much greater than that readily available for biological activity. Most of the added zinc is tightly bound to proteins, amino acids and other components of the culture medium. Thus, the concentration of zinc that cells will actually be exposed to depends critically on the composition of a culture medium [13] and therefore, the level of free, rapidly exchangeable zinc was modelled in the RPE culturing "Miller" medium, used for our zinc supplementation. With the known amounts and zinc affinities of the principal components of the medium, free zinc levels were calculated and measured to be at least one thousand-fold lower (in the nM range) than the total levels present in the medium (Figure 1). Comparable results had been obtained with other media and cells or cell lines previously [39]. Also, our model was validated by measuring the free zinc as a function of added zinc in the medium with two different fluorescence sensors (Figure 1); the good agreement between measured and calculated levels of available zinc in

this and other media suggests that MINEQL accurately predicts the level of available zinc present for a given level of added (total) zinc. Henceforward, the zinc effects are also reported in terms of the free zinc concentration.

The effects of zinc on the differentiation and maturation of RPE cells: Development of transepithelial resistance (TER)

RPE cells *in vivo* are highly differentiated both morphologically and functionally. One indicator of RPE cell differentiation is the development of TER. In the absence of added zinc (Figure 2, the maximum TER value after 4 weeks in culture was 208.6Ω*cm^2. Cells cultured with 75 µM-added zinc (2.0 nM free zinc) showed no effect on TER compared with no added zinc. However, both 100 µM and 125 µM added zinc (2.7 nM and 3.4 nM free zinc, respectively) produced a robust and statistically significant increase in the TER which plateaued at 343.2 Ω*cm^2. Addition of 150 µM zinc (4.0 nM free zinc) produced no change in TER at any of the time points compared with no added zinc. None of these concentrations of added zinc was toxic to the RPE cells as no change in LDH release was detected (Figure S1). However, addition of 200 µM zinc resulted in round-shaped RPE cells and detachment from the insert membrane. Based on the results of the TER measurement in subsequent experiments we used 125 µM added zinc, equivalent to 3.4 nM free zinc.

Pigmentation

Well-differentiated RPE cells develop pigmentation, basally located nuclei, and numerous apical microvilli[36]. Pigmentation was visualized by light microscopy (Figure 3) and quantified at the end of 28 days of zinc supplementation. Both controls and zinc-treated samples exhibited pigments, however in the presence of zinc the extent of pigmentation was much more widespread (Figure 3A and D, respectively). In our hand, in the absence of zinc, pigmentation appeared first close to the edge of the inserts (Figure 3A). Our observations were validated qualitatively by comparing TEM images from 0 and 125 µM added zinc. We found higher numbers of pigment granules and apical microvilli in the presence of 125 µM added zinc (Figure 3B and E). Immunolabeling with anti-PMEL17, which labels pre-melanosomes, showed increased immunoreactivity after zinc treatment, supporting the EM observations (Figure 3C and F, respectively). The increase in pigmentation was assessed

quantitatively by analysing the level of pigmentation at different concentrations of added zinc (Figure 3G).

Basal and apical secretion of proteins

Polarised RPE cells have been shown to secrete a variety of proteins both apically and basally [45]. Apolipoprotein E (APOE) had been used to monitor basal secretion and sub-RPE accumulation of materials [46]. The immunoreactivity of this protein was therefore used to examine the effects of zinc in basal secretion in our experiments. As expected, APOE accumulated in the cell culture inserts (Fig 3C, magenta) in a fashion identical to that reported by Johnson et al.[46]. In the presence of 125 μM added zinc we detected an increase in APOE immunoreactivity in the filter inserts (Figure 3F; magenta). This qualitative observation was then confirmed by MS analysis (Table 1) in which a significant increase in secretion of APOE and other proteins was detected upon zinc treatment in membrane inserts (Table 1). While most changes in protein levels increased after zinc treatment, Histone cluster 1, H2ae secretion showed a significant decrease (Table 1 and Supplementary table T1).

We also examined the effects of zinc supplementation on protein secretion in the apical compartment of the RPE (Table 2 and Supplementary table T2). There were several proteins that showed increased secretion after zinc supplementation in the apical compartment (Table 2). Particularly interesting were the increased secretion of complement factor H (CFH) [47-49] and high temperature requirement serine protease A1 (HTRA1) [50, 51] which are genetically associated with AMD [52-55].

DISCUSSION

An outstanding question in relation to AMD is how zinc supplementation exerts its observed beneficial effect [56-59]. Here we report that one of the ways is through direct effects on the RPE cells. Our results suggest that the availability of zinc can directly and significantly affect the RPE, and therefore may have influence on the interactions of the RPE with the photoreceptors and the choroidal micro vessels.

Zinc is indispensable for a multitude of molecular functions including, but not restricted to, regulating transcription factors, hundreds of enzyme activities, and protein structure and stability, as well as signaling [60]. Experiments with other tissues showed that zinc is involved in maintaining membrane integrity and tight junction formation [61-65]. In fact, zinc supplementation had a direct effect on zona ocludens-1 and occludin [66], which are both present and part of tight junctions in RPE cells; this may explain the significant change in TER in our experiments (Figure 2). In addition, zinc may affect the cytoskeletal network [67] and thereby exert its effect or contribute to changes in TER through the reorganization of the cytoskeleton. One of the important functions of RPE cells is to regulate the transport in and out of the neural retina. Therefore, regulation of TER by zinc will have important consequences *in vivo* as the RPE monolayer is part of the outer blood-retinal barrier.

Pigmentation is used as a differentiation marker for RPE cells [68, 69]. Melanin is thought to play a role in both normal and pathological behavior of RPE cells [70]. Melanin production is a complex process directly involving three enzymes: tyrosinase and tyrosinase-related proteins 1 (TYRP1) and 2 (TYRP2). Zinc directly binds to tyrosinase and TYRP2 [71-74], which could be the reason for the high zinc content of melanosomes [75-77]. In addition, zinc directly affects the activities of tyrosinase and TYRP2, which may explain why the zinc-enriched environment increased the cell pigmentation, leading to increased immunoreactivity of PMEL17 in our experiments (Figure 3).The increased melanin content and TER values were accompanied by increased density of apical microvilli and melanosomes (Figure 3B and E), all of which are characteristic of RPE cell maturation [34-36, 78]. Therefore, increased availability of zinc appears to be directly involved in the development and probably maintenance of a mature and healthy RPE.

RPE cells transport and secrete a variety of proteins, lipids and other chemicals bidirectionally [45, 79-81] and there is an apical/basal difference in this process *in vivo* as well as in cell culture [45]. Perhaps not surprisingly, we identified several proteins in the apical and basal secretome (Supplementary table 1 and 2). Amongst the basally secreted proteins was APOE, which has been associated with AMD [82], and had been shown to be secreted from and accumulated under primary human RPE cells in culture [46, 83]. We not only found that APOE behaves the same way in our experiments, but also showed that zinc increases the secretion of this protein (Table 1 and Figure 3). The association between APOE and zinc appears to be at multiple levels. APOE isoforms bind zinc directly [84], and this binding can increase their stability [85]. In addition, it is believed that zinc can increase cellular APOE levels by directly affecting transcription [86] and/or secretion [87]. Which of these lead to the increased accumulation of APOE in our experiments will need to be investigated further, especially in light of the involvement of APOE in sub-RPE deposit formation [88]. Apart from APOE we identified a number of other components of sub-RPE deposits [89] in the basal secretome (Supplementary table T1) and some of these were significantly affected by the addition of extracellular zinc (Table1). Apart from changes in basal secretion, apical secretion was also affected by zinc supplementation (Table 2). We found increases in secretion of CFH and HTRA1, both of which have been previously reported in RPE secretomes [54, 55]. The exact role of these apically secreted proteins is yet to be determined, but it was suggested that both might protect photoreceptors from effects of inappropriate complement activation and/or amyloid deposition [54, 55].

Previously we reported that on thin polycarbonate inserts sub-RPE deposits can readily form drusen-like deposits [83]. We did not see such deposits in this study. This might be due to the use of 100 μm thick mixed cellulose esters in the inserts, into which proteins can diffuse and become trapped in the fiber meshwork. Entrapment within the insert was observed in another study [46], suggesting that this type of filter insert is useful to analyze the basal secretome.

The concentration of total zinc that was added to the cell culture medium in our experiments was several orders of magnitude higher than the resulting free or bioavailable zinc concentration that is available for biological functions (125 μM vs

3.4 nM, respectively). This is routinely seen due to the complex mixtures containing many zinc buffering components in the culture medium [13]. For example, fetal bovine serum, comprised of a mixture of serum albumin, amino acids, growth factors, protease inhibitors, proteins, lipids, and minerals [90], has both a large zinc-binding capacity and affinity: albumin and alpha-2-macroglobulin together bind more than 98% of serum zinc [91-93]. As a result, only a small fraction of zinc in serum is really available to be biologically active [13]. For this very reason, if cells would release zinc this zinc would be rapidly captured by the buffers and would not affect biological activity. Thus, the free zinc concentration is effectively "clamped" in these experiments.

Different cell lines require different culture media, therefore it is important to report not only the added zinc but also the resulting bioavailable zinc concentrations [13]. It is also important to note that based on previous work by Bozym, et al. [13, 39], the effects in terms of free zinc are transferrable to other systems and media whereas the effects of added zinc are not. While this might not be true for all media, MINEQL calculation of zinc accurately reflected the measured available zinc levels in our experiment (Figure 1.). Based on this, we also calculated the buffering capacity of DMEM/F12, another medium often used to culture RPE cells, and found that one can reach ~3 nM free zinc at the concentration of <40 μM added zinc, a three to four-fold lowered buffering capacity compared to the Miller medium used in this report. While direct determination is always preferred, it appears that modelling buffering capacity can give a good estimate for potential biological activity.

In summary, increased zinc availability had a multitude of effects on the RPE cells in our experiments. In addition to our findings, zinc has been shown to mediate oxidative stress[94], phagocytic and lysosomal function [70, 95], macromolecule synthesis- and caspase-dependent apoptosis[96], increased photic injury [97], and DNA damage[98]. Zinc can also kill RPE cells in culture if there is too much or too little of it [25, 96], although *in vivo* toxicity is rarely reported [11, 12]. Interestingly, the range of free zinc concentrations that RPE cells are able to handle appears to be relatively narrow, <5 nM, at least in culture. It is unclear whether *in vivo* the extracellular milieu has bioavailable zinc identical or similar to these values. However, the fact that culture media have a capacity to keep available zinc levels in

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712 our experiments in the nanomolar range even up to 150 μM added zinc suggests that
713 zinc buffering capacity will need to be considered when *in vitro* and *in vivo*
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715 supplementations are considered. The challenge now is to determine the "normal" free
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717 zinc concentration for the RPE *in vivo*. The RPE directly interacts with the
718 photoreceptors and the Bruch's membrane, and indirectly with the choroidal
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720 circulation. Given that total zinc levels are altered in human eyes with signs of AMD
721 [6, 99-101], all the reported changes suggest that determining and maintaining a
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723 "healthy" concentration of available zinc will be important for the maturation and
724 health of the RPE, therefore intervening the development and progression of AMD.
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FIGURE LEGENDS

FIGURE 1. Determination of free zinc concentrations in the Miller medium. Free zinc concentration in the Miller medium was determined based on changes of fluorescence intensity ratio as a function of total zinc added. Black circles (●) represent the calculated concentration of free zinc using MINEQL [13]. Open circles (○) indicate free zinc concentrations measured by emission intensity ratios using a fluorescent biosensor composed of wild type bovine apo-carbonic anhydrase II and polymeric ABDN, and open squares (□) represent free zinc levels measured using human H94N apo-CA II plus poly-ABDN. Under these conditions the zinc binding K_D's of wt bovine and human H94N CA-II's are measured as 0.10 ± 0.03 and 0.71 ± 0.06 nM, respectively (results not shown). The double logarithmic plot estimated that at 125 µM of added zinc the free zinc concentration is approximately 3.4 nM (dashed line).

FIGURE 2. Transepithelial (TER) measurements of the RPE cultures supplemented with 0, 75, 100, 125, and 150 µM zinc. TER measurements of RPE grown with 0 µM (-●-), 75 µM (-○-), 100 µM (-□-), 125µM (-■-), and 150 µM (-Δ-) added zinc were measured with an epithelial volt-ohm meter. 100 µM (*) and 125 µM (#) added zinc demonstrated statistically significant ($p < 0.05$) increases in TER compared to 0 µM.

FIGURE 3. Effects of added zinc on RPE pigmentation and cell differentiation. RPE cultures developed pigmentation and this was observed under light microscopy (panels A&D). TEM (B&E) showed characteristics of RPE cell differentiation including pigment maturation (M) and microvilli (MV) development. IHC images (C&F) indicated melanosome (green), ApoE (magenta) and nuclei (blue). (G) Zinc concentration dependent increase in pigmentation was analysed by ImageJ and coverage was determined as percentage of total image area of three inserts in each condition; # labels values that are significantly different from no added zinc ($p<0,05$). Asterisks indicate Millicell-HA Culture Plate Inserts; scale bar is 10 µm.

FIGURE S1. Determination of toxicity of added zinc to RPE cultures. Cell culture medium was collected from the apical (A) and basal (B) compartments of the culture

1240
1241
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1243 inserts and assessed by a colorimetric assay. Maximum LDH (dotted lines) was
1244 measured by complete lysis of confluent RPE. LDH release was measured after
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1246 incubating the cells for different length of time with 0 μM (-●-), 75 μM (-○-), 100 μM
1247 (-□-), 125μM (-■-), and 150 μM (-Δ-) added zinc.
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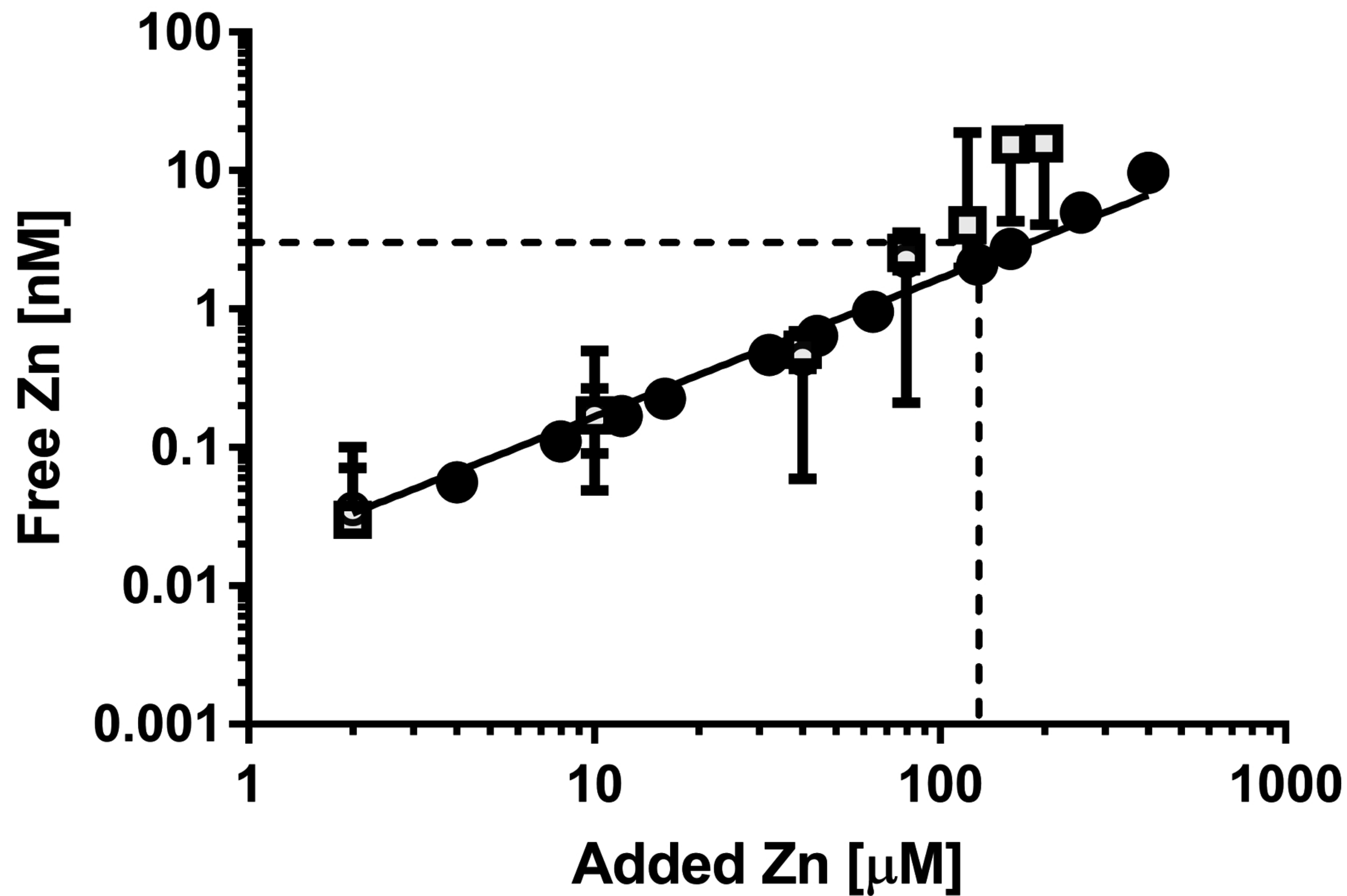
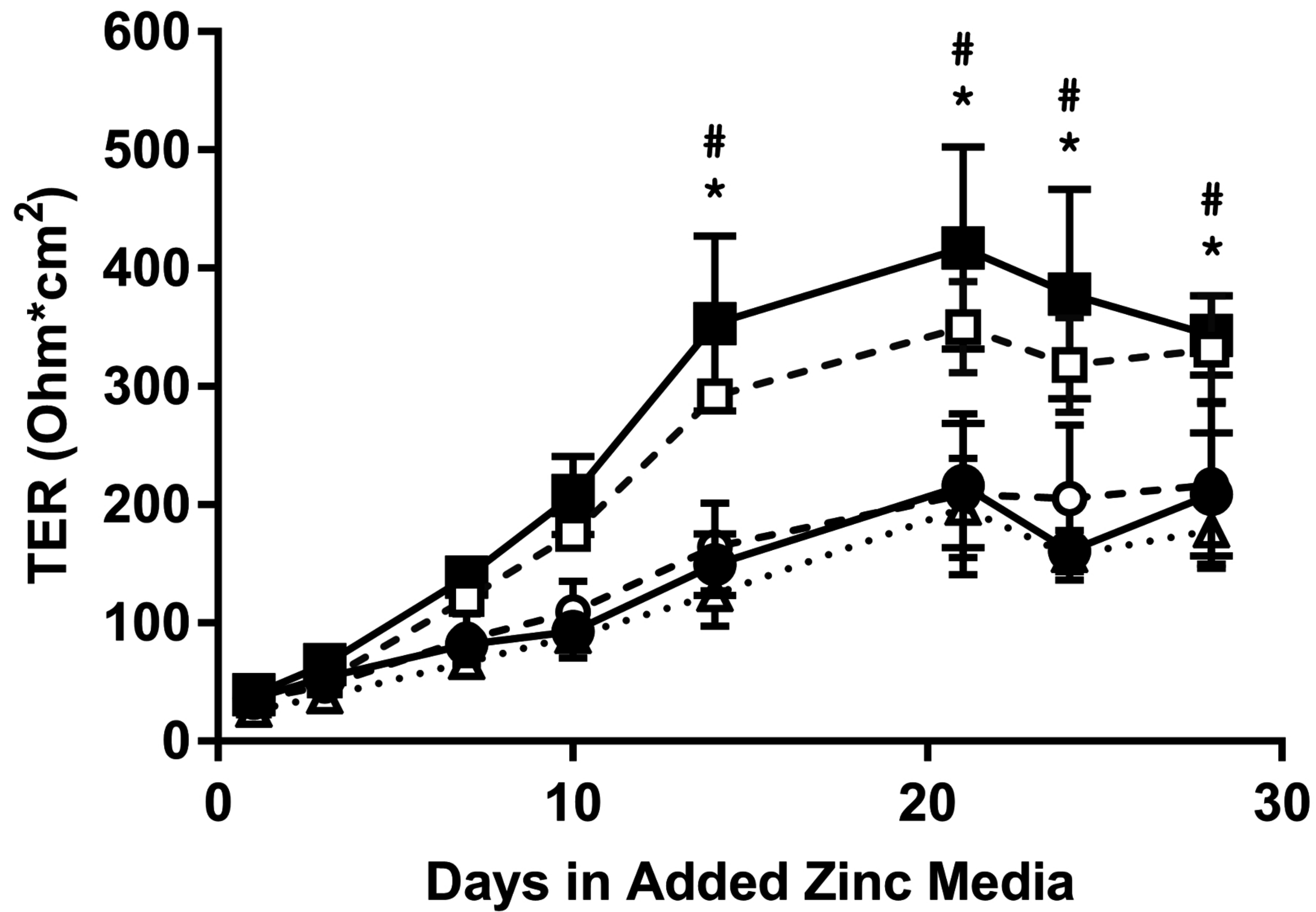
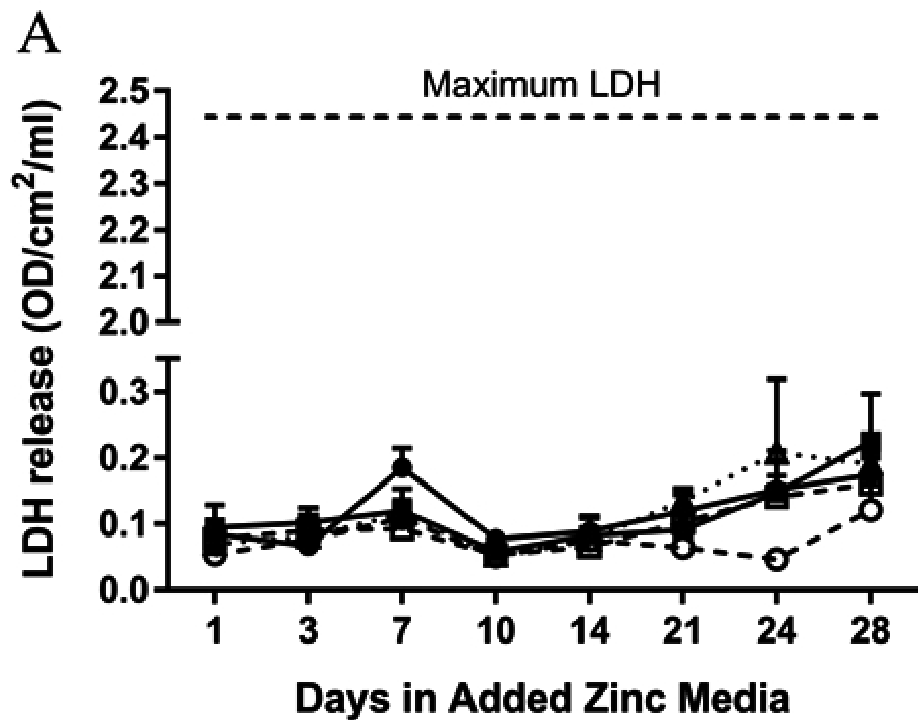
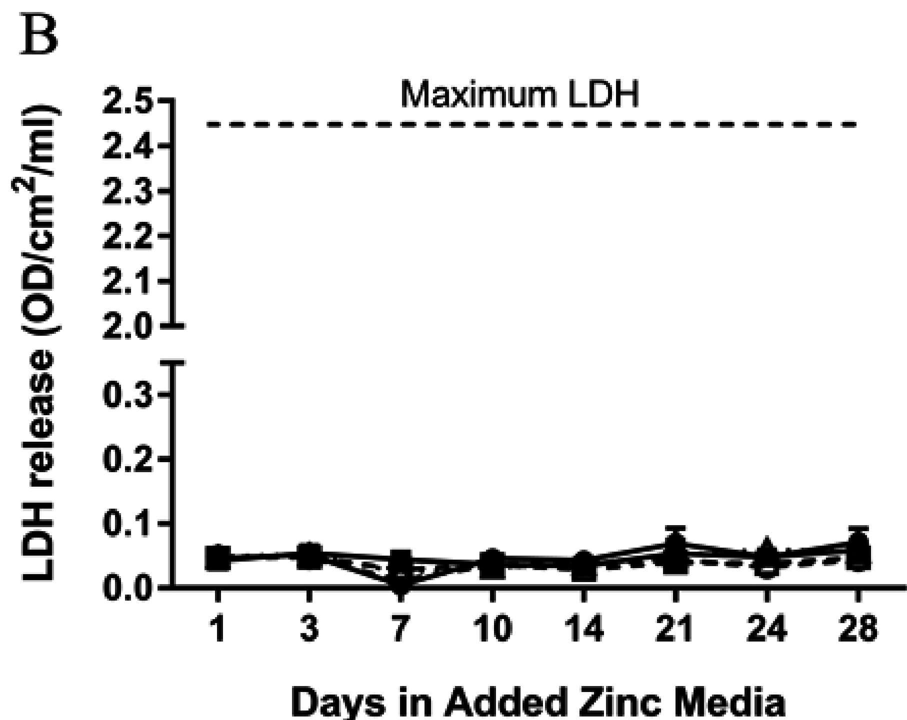


100
10
1
0.1
0.01
0.001
Free Zn [nM]
1
10
100
1000
Added Zn [μM]



600
500
400
300
200
100
0
0
10
20
30
TER (Ohm*cm^2)
Days in Added Zinc Media
#
*


A
Maximum LDH
LDH release (OD/cm2/ml)
2.5
2.4
2.3
2.2
2.1
2.0
0.3
0.2
0.1
0.0
1
3
7
10
14
21
24
28
Days in Added Zinc Media

B
Maximum LDH
LDH release (OD/cm2/ml)
2.5
2.4
2.3
2.2
2.1
2.0
0.3
0.2
0.1
0.0
1
3
7
10
14
21
24
28
Days in Added Zinc Media

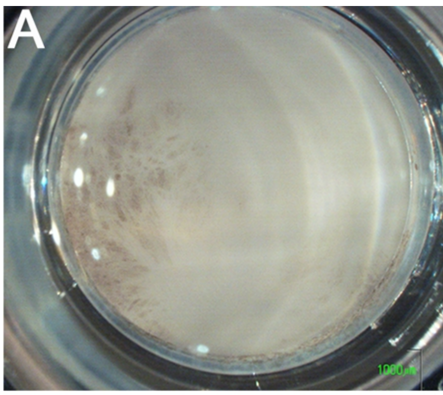
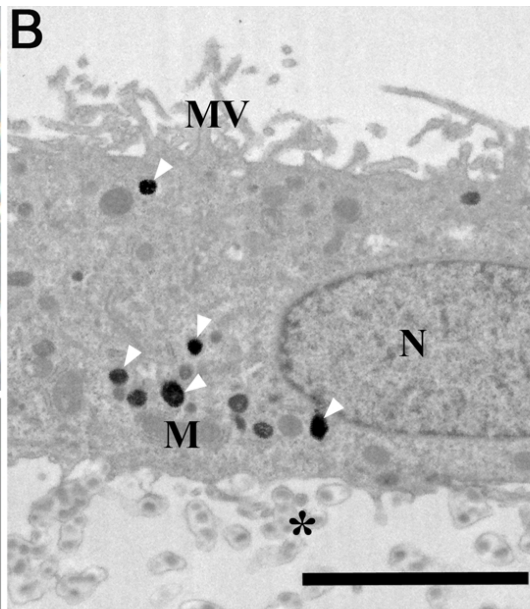
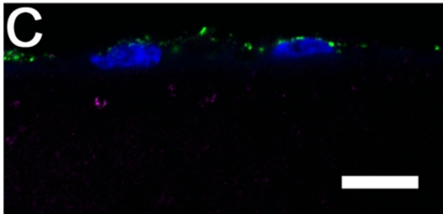
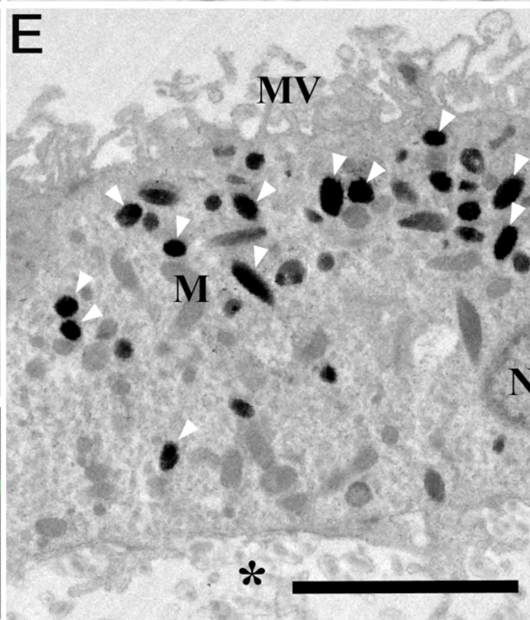
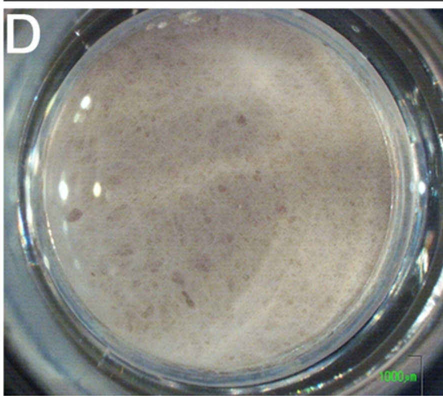
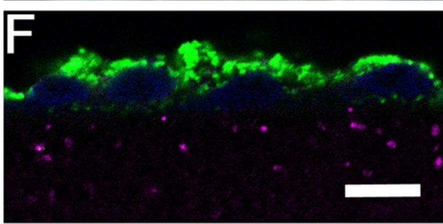
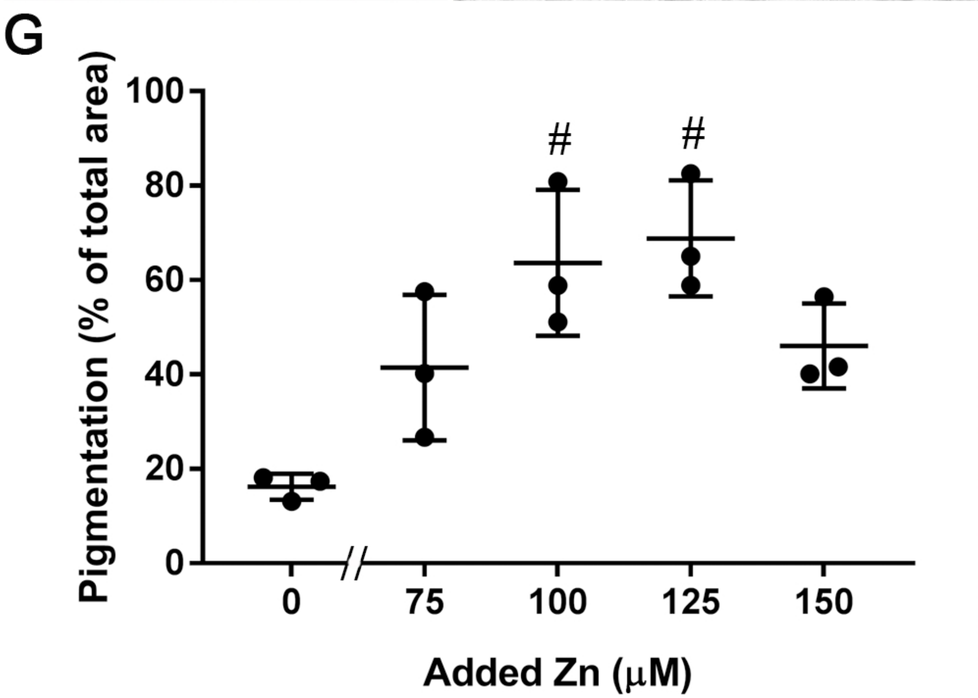

A
1000 µm

B
MV
N
M
*

C

E
MV
M
N
*

D
1000 µm

F

G
Pigmentation (% of total area)
100
80
60
40
20
0
#
#
0
75
100
125
150
Added Zn (µM)

Table 1
Effect of zinc supplementation on basal protein secretion from human foetal retinal pigment epithelium in culture

Protein name	Gene symbol	Peptide count	Unique peptide s	P values	Ratio of zinc/ control
AHNAK nucleoprotein	AHNAK	2	2	0.03110	1.35
Albumin	ALB	6	6	0.00182	1.14
Apolipoprotein A-IV	APOA4	3	3	0.00035	1.22
Apolipoprotein E	APOE	2	2	0.00230	1.33
Betaine-homocysteine S-methyltransferase	BHMT	2	2	0.01856	1.52
Cartilage oligomeric matrix protein	COMP	2	2	0.04887	1.27
CNDP dipeptidase 2 (metallopeptidase M20 family)	CNDP2	2	2	0.00805	1.32
Coagulation factor V (proaccelerin, labile factor)	F5	6	6	0.03121	1.16
Crystallin, alpha B	CRYAB	6	6	0.00095	1.32
Dihydropyrimidinase-like 2	DPYSL2	11	11	0.00127	1.2
Eukaryotic translation elongation factor 1 gamma	EEF1G	4	4	0.00717	1.41
Filamin A, alpha	FLNA	6	6	0.04472	1.33
FUS RNA binding protein	FUS	2	2	0.00596	1.23
Glucose-6-phosphate isomerase	GPI	4	4	0.00191	1.47
Heat shock 70kDa protein 1B	HSPA1B	14	4	0.02752	1.25
Heat shock 70kDa protein 4	HSPA4	2	2	0.01636	1.28
Heat shock protein 90kDa alpha (cytosolic), class B member 1	HSP90AB1	14	6	0.04500	1.59
Heterogeneous nuclear ribonucleoprotein	HNRNPD	4	3	0.04164	1.43
Histone cluster 1, H2ae	HIST1H2AE	2	2	0.02588	0.57
Inter-alpha-trypsin inhibitor heavy chain 2	ITIH2	9	9	0.00734	1.25
Oncoprotein induced transcript 3	OIT3	2	2	0.03582	1.47
Pyruvate kinase, muscle	PKM	21	2	0.00104	1.37
Ribosomal protein L22	RPL22	2	2	0.04510	1.19
Solute carrier family 9, subfamily A	SLC9A3R1	5	5	0.02962	1.47
Transgelin 2	TAGLN2	5	5	0.04890	1.12
Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	UCHL1	2	2	0.03134	1.39

Statistically significant results are listed, based on $p<0.05$ (ANOVA) and unique peptides ≥ 2.

Table 2
Effect of zinc supplementation on apical protein secretion from human foetal retinal pigment epithelium in culture

Protein	Gene symbol	Peptide count	Unique peptides	P values	Ratio of zinc/control
Biglycan	BGN	4	4	0.00959	1.53
CNDP dipeptidase 2	CNDP2	2	2	0.03747	7.84
Collagen, type XII, alpha 1	COL12A1	10	10	0.02864	2.07
Complement factor H	CFH	27	27	0.00204	1.70
Fatty acid binding protein 5	FABP5	2	2	0.03445	2.80
Fibronectin 1	FN1	36	36	0.00038	1.79
HtrA serine peptidase 1	HTRA1	3	3	0.00005	5.46
Leucine proline-enriched proteoglycan (leprecan) 1	P3H1	2	2	0.00266	2.07
Microtubule-associated protein 4	MAP4	7	7	0.01499	1.80
Myristoylated alanine-rich protein kinase C substrate	MARCKS	3	3	0.03464	3.08
Platelet derived growth factor C	PDGFC	3	3	0.02959	2.56
Pyrophosphatase 1	PPA1	2	2	0.02982	3.22
Serpin peptidase inhibitor	SERPINA3	12	11	0.03224	2.15
Serpin peptidase inhibitor	SERPINH1	3	3	0.02714	1.94
Thrombospondin 1	THBS1	38	36	0.01000	1.53

Statistically significant results are listed, based on $p < 0.05$ (ANOVA) and unique peptides ≥ 2.