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Iron upregulates melanogenesis in cultured retinal pigment epithelial cells

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Abstract

The purpose of our studies was to examine the relationship between iron and melanogenesis in retinal pigment epithelial cells, as prior observations had suggested that iron may promote melanogenesis. This relationship has potential clinical importance, as both iron overload and hyperpigmentation are associated with age-related macular degeneration (AMD). Human fetal retinal pigment epithelial cells and ARPE-19 cells were treated with iron in the form of ferric ammonium citrate, after which quantitative RT-PCR and electron microscopy were performed. Melanogenesis genes tyrosinase, tyrosinase-related protein 1, Hermansky-Pudlak Syndrome 3, premelanosome protein and dopachrome tautomerase were upregulated, as was the melanogenesis-controlling transcription factor, microphthalmia-associated transcription factor (*MITF*). Iron-treated cells had increased pigmentation and melanosome number. Multiple transcription factors upstream of *MITF* were upregulated by iron.

Keywords

Iron; Retinal Pigment Epithelium; Human Fetal Retinal Pigment Epithelial Cells; ARPE-19 Cells; Melanosome; *MITF*; *LEF 1*

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1. Introduction

Retinal pigment epithelial (RPE) cells are melanized cells derived from the outer layer of the optic cup neuro-ectoderm. In human RPE cells, melanosomes first appear during the 7th week of gestation (Breathnach and Wyllie. 1966, Hogan *et al.* 1971, Marmor *et al.* 1998, Mund *et al.* 1972). Like melanosomes in other pigmented cells of the body, RPE melanosomes undergo a four stage maturation process which can be observed ultrastructurally (Feeney-Burns. 1980, Marmor *et al.* 1998, Nordlund. 2006). In Stage I, vesicles are formed (Nordlund. 2006). In Stage II, a protein called premelanosome protein (PMEL) assembles into a scaffold of amyloid-like fibrils, creating a striated appearance (McGlinchey *et al.* 2011, Nordlund. 2006). In Stage III, proteins important for melanin synthesis, such as tyrosinase (TYR), tyrosinase related protein (TYRP1), and dopachrome tautomerase (DCT), are transported to the melanosome and begin to deposit melanin on the PMEL fibrils (Nordlund. 2006). In Stage IV, the melanosome is completely melanized, so that the fibrils are no longer visible (Nordlund. 2006).

In the human RPE cell a mixture of all four stages of melanosomes is seen between fetal weeks 7–14 (Marmor *et al.* 1998, Stroeve and Mitashov. 1983). Generation of new melanosomes then stops, and the already formed melanosomes continue maturing (Stroeve and Mitashov. 1983). By 27 weeks gestation, all of the melanosomes are melanized and are Stage IV granules (Stroeve and Mitashov. 1983). Although the melanosomes have matured to Stage IV by fetal week 27, melanin continues to be deposited in them until the age of 2 years (Marmor *et al.* 1998, Stroeve and Mitashov. 1983). It has long been held that no new melanosomes are synthesized in the RPE cells after the age of 2 years in humans; however, some argue that there is a low level of melanosome turnover and melanogenesis in adult life (Schraermeyer and Heimann. 1999).

Melanosomes within RPE cells serve multiple roles. They aid in visual function by absorbing and preventing the backscattering of light (Land *et al.* 2004, Marmor *et al.* 1998), they play a role in the development of the visual system (Guillery. 1996, Marmor *et al.* 1998), they bind metals, including calcium, zinc, copper and iron (Hong and Simon. 2007, Kaczara *et al.* 2012, Rozanowski *et al.* 2008), and they scavenge free radicals (Rozanowska *et al.* 1999, Rozanowski *et al.* 2008). With age, RPE cells accumulate lipofuscin granules containing pro-oxidant compounds such as A2E, which contribute to cell ageing and damage (Sparrow and Boulton. 2005). It is thought that by binding free radicals and metals, the melanosomes may protect the RPE cells from reactive oxygen species and may enhance lysosomal function in RPE cells (Rozanowski *et al.* 2008). Although intact melanosomes act as anti-oxidants, photodegraded melanosomes may become pro-oxidant, likely by releasing the metals that they were binding (Rozanowski *et al.* 2008). In certain diseases, such as age-related macular degeneration and aceruloplasminemia where RPE cells contain increased levels of iron (Hahn *et al.* 2003, Wolkow *et al.* 2011), melanosomes may initially play a protective role by sequestering iron. However, as iron can cause physical damage to melanosomes and contribute to their degradation (Korytowski and Sarna. 1990, Wolkow *et al.* 2011), with time melanosomes may degenerate, releasing iron and contributing to disease progression.

The relationship between melanosomes and iron may extend beyond the physical effects of iron on melanin, as iron may also alter signaling pathways that contribute to melanosome biosynthesis and degradation. For example, when a microarray analysis was performed on the RPE cells of *Cp*^{-/-}, *Heph*^{sla/sla} mice, which accumulate iron in the RPE cells, the gene with the most significant changes was Hermansky-Pudlak Syndrome 3 (*HPS3*) (Dunaief lab, unpublished data). *HPS3* is part of BLOC (biogenesis of lysosome-related organelles complex), which sorts and traffics proteins such as tyrosinase (TYR) and tyrosinase-related protein-1 (TYRP1) to developing melanosomes (Di Pietro *et al.* 2004, Raposo and Marks. 2007). Similarly, microarray studies on mice with mutations in the master melanogenesis transcription factor, microphthalmia-associated transcription factor (MITF), have demonstrated changes in the iron transporter *Nramp1* (*Slc11a1*) in RPE cells (Gelineau-van Waes *et al.* 2008).

Additional evidence suggests that iron homeostasis is linked to melanogenesis. Patients with iron overload, as well as with overload by other metals, have increased skin pigmentation resulting from increased melanin levels (Nordlund. 2006). In the disease hemochromatosis, where multiple organs are overloaded with iron, the darkening of skin is due to increased melanin levels and not to iron deposition (Cawley *et al.* 1969, Nordlund. 2006, Perdrup and Poulsen. 1964). Although a link between iron and melanogenesis has been observed, the mechanism for this association has not been previously described (Cawley *et al.* 1969, Nordlund. 2006). The purpose of our current studies was to examine the relationship between iron and melanogenesis in the RPE at the molecular level.

2. Materials and Methods

2.1. Cell culture

2.1.1 Human Fetal Retinal Pigment Epithelial (hfRPE) Cell Culture—Eyes from 16–19 week old donors were obtained from Advance Bioscience Resources (Alameda, CA), and cultures were established as previously described (Maminishkis *et al.* 2006, Maminishkis and Miller. 2010). Briefly, eyes were rinsed in antibiotic-antimycotic plus gentamicin solution (Invitrogen, Grand Island, NY), and eyecups were made. Eyecups were incubated in dispase-I (Roche Diagnostics, Indianapolis, IN) solution in 5% serum hfRPE media for 30 minutes (Maminishkis *et al.* 2006, Maminishkis and Miller. 2010). Single cell RPE layers were peeled off in sheets and collected in 5% hfRPE medium. Cells were washed, separated into single cells and put into T25 Primaria flasks (Fisher Scientific, Pittsburgh, PA) in 15%-serum containing media (Maminishkis *et al.* 2006, Maminishkis and Miller. 2010). After one day, media was changed to 5% serum hfRPE media. Media was then changed three times per week. After at least 4 weeks in T25 flasks, the hfRPE cells were trypsinized with 0.25% trypsin (Invitrogen, Grand Island, NY) and seeded onto cell culture transwell inserts (cat 3460, Corning-CoStar, Corning, NY) that were coated with human extracellular matrix (cat 354237, BD Biosciences, San Diego, CA), as previously described (Maminishkis *et al.* 2006, Maminishkis and Miller. 2010). Cells from 7 different donors were used to establish the cultures used for these experiments, they were grown for at least 4 weeks on transwell inserts prior to use. All cells used in experiments had a transepithelial resistance of >300 $\Omega \cdot \text{cm}^2$.

2.1.2 ARPE-19 cell culture—ARPE-19 cells obtained from ATCC (Manassas, VA) and were grown as previously described (Dunn *et al.* 1996a). Cells from passages 24–29 were used for current experiments. Confluent flasks were split in a 1:4 ratio. Freshly passaged ARPE-19 cells were allowed to reach full confluence in high serum media D10-F12, after which they were switched to iron treatment conditions. Cells were maintained at confluence without further splitting until the end of the experiment. Cells were treated with or without 250 μ M FAC for one week or 2 months as described below. Cells in high serum conditions were maintained in high glucose D10-F12 media during the experiment (the 10 in D10 refers to 10% Fetal Bovine Serum, and the DMEM had 4.5g/L glucose), while cells in low serum conditions were grown in low glucose D1-F12 media (the 1 in D1 refers to 1% Fetal Bovine Serum, and the DMEM had 1g/L of glucose).

2.1.3 Treatment with Ferric Ammonium Citrate—All cells were treated with 250 μ M Ferric Ammonium Citrate (Sigma-Aldrich, St. Louis, MO) for 1 week or 2 months. Prior to each treatment, fresh 50 mM ferric ammonium citrate stock solution was made in 5% hfRPE media, D1-F12 media (containing 1% serum), or D10-F12 media (containing 10% serum) and sterile filtered. The stock solution was further diluted to 250 μ M in 5% hfRPE media, D1-F12, or D10-F12.

2.2 Quantitative RT-PCR analysis

RNA was extracted from treated cells using the QIAGEN RNeasy Mini Kit and QIAshredder Spin columns (Valencia, CA) as described by the manufacturer. Reverse transcription was performed using the Taqman Reverse Transcription Reagents (Applied Biosystems, Carlsbad, CA). For quantitative PCR all materials and reagents were obtained from Applied Biosystems (Carlsbad, CA) and reactions were run on a 7500 Fast Real-Time PCR System Thermocycler (Applied Biosystems, Foster City, CA). The following probes from Applied Biosystems were used: human Eukaryotic 18S rRNA (Hs99999901_s1), *TFRC* Hs00174609_m1, *TYR* Hs01099965_m1, *TYRP1* Hs00167051_m1, *HPS3* Hs00289968_m1, *DCT* Hs01098278_m1, *PMEL* Hs00173854_m1, *MITF* Hs01117294_m1, *DICER1* Hs00229023_m1, *XPC* Hs01104206_m1, *PAX3* Hs00240950_m1, *SOX10* Hs00366918_m1, *SOX9* Hs00165814_m1, *LEF1* Hs01547250_m1, *OTX2* Hs00222238_m1 and *NRF2/NFE2L2* Hs00975961_g1. For fRPE cells, triplicate culture wells from each of two different donors were used for all experiments. From each well, the qPCR measurements were performed in triplicate. For ARPE-19 experiments, the 1 week high serum result was generated from 9 wells, the 1 week low serum from 6 wells, and the 2 months low serum from 3 wells, again with each well tested using 3 separate qPCR reactions.

Western analysis for TYRP1—Cultured and treated ARPE-19 cells were lysed in 1 $\times$ Laemmli-SDS sample buffer plus protease/phosphatase inhibitor cocktail (Cell Signaling Technology, Danvers, MA). Thirty micrograms of total protein, boiled at 95°C for 5 minutes, was used in each sample. The protein lysates were separated on a 4% to 12% gradient SDS-PAGE gel and transferred onto nitrocellulose membranes. Blocking was achieved by incubation for 1 hour in Odyssey blocking buffer (Licor Biosciences, Lincoln, NE) with 0.1% Tween-20. Membranes were incubated overnight at 4°C with 1:200 mouse

anti-TYRP1 antibody (Santa Cruz Biotechnology). After washes, membranes were incubated with donkey anti-mouse IRDye® 800CW secondary antibody (Licor) at 1:7500 dilution. Anti-GAPDH antibody (Cell Signaling) was used as loading control. Bands were detected with a Licor Odyssey imager. Densitometry analysis for relative abundance was performed with ImageJ software version 1.46r.

2.3 Electron microscopy

Human fetal RPE cells were fixed on transwells with 2.5% glutaraldehyde/2% paraformaldehyde in 0.1 M sodium cacodylate buffer for at least one day (Electron Microscopy Sciences, Hatfield, PA). Samples were post-fixed with 2% osmium tetroxide, infiltrated and embedded in EMbed 812 (Electron Microscopy Sciences, Hatfield, PA). Samples were masked, and sections were examined by a masked observer with a JEOL1010 Transmission Electron Microscope. Images of the first ten intact cells were acquired from each section with a Hamamatsu camera and AMT Advantage Image Capture software. Cells from three different donors were used. The total number of Stage I, Stage II, Stage III and Stage IV melanosomes in each cell was counted by a masked observer. Results were analyzed separately for each donor.

2.4 Statistical Methods

Statistical analysis was performed and graphs were created using GraphPad Prism 5 (La Jolla, CA). All experiments were performed in triplicate on cells from at least two different biological donors. For the analysis the t-test was used, with $p < 0.05$ considered significant.

3. Results

3.1. Transferrin receptor is down-regulated by iron in hfRPE and ARPE-19 cells

Transferrin receptor mRNA expression is regulated by free iron levels, and decreases when cellular iron levels are increased (Hadziahmetovic *et al.* 2011, Muckenthaler *et al.* 2008). Human fetal RPE (hfRPE) cells, ARPE-19 cells in high serum conditions and ARPE-19 in low serum conditions had decreased levels of transferrin receptor expression after 1 week treatment with ferric ammonium citrate (Figure 1), indicating that the cells were iron-loaded.

3.2 Melanogenesis effector genes and MITF are upregulated by iron in hfRPE

Iron-loaded hfRPE cells up-regulated expression of several melanogenesis effector genes, including tyrosinase, tyrosinase-related protein 1, and Hermasky-Pudlak Syndrome 3 (Figure 2A–C). Dopachrome tautomerase (DCT) and premelanosome protein (PMEL) were expressed but not upregulated (2D–E). The master transcription factor of melanogenesis, MITF (Bharti *et al.* 2006, Fuhrmann. 2010), was up-regulated after iron treatment (Figure 2F).

3.3 Melanosome number is increased in hfRPE after iron treatment

Electron microscopy was performed on hfRPE cells that had been treated with ferric ammonium citrate (FAC) for one week to determine whether the upregulation of melanogenesis gene expression after iron treatment corresponds to an increase in

melanosome number. The total number of melanosomes was increased after iron treatment in two out of three donors (Figure 3B). This increase could be attributed to the formation of new melanosomes, as the percentage of Stage I melanosomes in the iron-treated cells was increased in the two donors (Figure 3C). Additionally, there was an increase in the percentage of completely melanized (Stage IV) melanosomes in the two donors, suggesting that upregulation of melanogenesis gene expression may be accompanied by increased melanization in hRPE cells of certain donors (Figure 3F).

3.4 Melanogenesis effector genes and MITF are upregulated in ARPE-19 cells by iron

ARPE-19 cells that were treated with iron for one week in high serum (10% FBS) conditions upregulated melanogenesis genes TYR, TYRP1, HPS3, PMEL and MITF (Figure 4). DCT, however, did not amplify until the very late cycles and thus iron's effect on its expression could not be determined.

3.5 ARPE-19 cells in long-term low serum conditions upregulate melanogenesis genes and become pigmented

Prior studies by Tian *et al.*, suggest that ARPE-19 cells in serum-withdrawn media have expression profiles closer to native RPE cells than those in high serum conditions (Tian *et al.* 2005a). With this in mind we treated ARPE-19 cells with iron in low serum conditions (1% FBS) for 1 week or 2 months. ARPE-19 cells that were treated with iron in low serum conditions for 1 week upregulated TYR and TYRP1, but not PMEL. MITF and HPS3 were down-regulated (Figure 5A–E). After two months of iron treatment in low serum conditions, however, ARPE-19 cells had significant increases in the expression of all melanogenesis genes and MITF (Figure 5F–K). In addition, after two months of treatment, similar to TYRP1 mRNA, TYRP1 protein levels were increased by greater than 2-fold (Figure 5L). And, by 5 weeks of iron treatment in low serum conditions, iron-treated ARPE-19 cells had visible pigmentation by light microscopy (Figure 5M).

3.6 Multiple upstream regulators of MITF are upregulated by iron in hRPE and ARPE-19 cells

Multiple transcription factors and pathways regulate *MITF* expression and melanogenesis (Liu and Fisher. 2010), including transcription factors PAX3, LEF1, SOX9, SOX10 and OTX2, as well as genes involved in the tanning response.

When skin is exposed to UV light, DNA damage occurs, upregulating DNA damage repair genes, ultimately leading to increased production of melanin (Cui *et al.* 2007, Liu and Fisher. 2010). In a similar fashion, elevated free iron in RPE cells may increase production of DNA-damaging free radicals, activating the tanning response. The DNA repair gene Xeroderma pigmentosum *complementation* group C gene (XPC) was upregulated in hRPE and ARPE-19 cells treated with FAC (Figure 6). Similarly, the endoribonuclease DICER, which is involved in DNA repair (Tang and Ren. 2012), is regulated by MITF (Cheli *et al.* 2010), and whose levels are altered in age-related macular degeneration (Kaneko *et al.* 2011), was upregulated by FAC in hRPE and ARPE-19 cultured in low serum for 1 week and for two months, but not in ARPE-19 cultured in high serum (Figure 6). The mRNA of

the antioxidant response transcription factor, NRF2, was only elevated in the two month low serum ARPE-19 group (Figure 6).

Transcription factor PAX3 could not be detected in any hFRPE or ARPE-19 samples. SOX9 and SOX10 were up-regulated in hFRPE that were treated with iron for one week and in ARPE-19 that had been treated for 2 months with iron in low serum conditions (Figure 7). In ARPE-19 cells treated with iron in high or low serum for 1 week, SOX10 mRNA levels were very low and it amplified too late to analyze. SOX10 and OTX2 were upregulated in ARPE-19 that had been treated with iron for two months (Figure 7). LEF1 and MITF were significantly elevated after iron treatment in hFRPE, ARPE-19 in high serum, and ARPE-19 in low serum for 2 months.

4. Discussion

In our current studies we have demonstrated that iron upregulates the expression of melanogenesis genes in both hFRPE cells and ARPE-19 cells, and causes increased pigmentation. Additionally, multiple upstream transcription factors that contribute to melanogenesis are upregulated by iron treatment.

Iron upregulated expression of most melanogenesis genes in hFRPE cells, except for DCT and PMEL, at the one week time point, and increased the number of melanosomes. We found that in hFRPE cells, the number of both stage I and IV melanosomes was increased, but not that of Stage II or III. It is possible that under these conditions, melanosomes may transition through Stage II and III at a faster rate than through the other stages. Examining the EM images, we did not detect any melanosomes of unusual size or structure in these cells, as would have been expected if DCT or PMEL were not present (Biesemeier *et al.* 2010, Theos *et al.* 2006), and both DCT and PMEL were detectable by qPCR (cycle 24 for DCT, cycle 21 for PMEL); thus, it is likely that DCT and PMEL were already present in sufficient quantities in hFRPE cells for normal melanosome production, or that their transcriptional levels increase in response to iron at a different time point. ARPE-19 cells had much lower levels of PMEL and DCT at the one week time point, with PMEL amplifying at cycles 31–32, and DCT at cycles 35 or later. By the 2 month time point, both PMEL and DCT were detectable in ARPE-19 at cycles 26–30 and were robustly upregulated by iron, PMEL 5-fold and DCT 12-fold (Figure 5). Thus, as ARPE-19 cells become more differentiated and more similar to hFRPE, they increase PMEL and DCT expression, with iron upregulating their expression even further.

We tested ARPE-19 cells under various serum conditions, as studies by other labs had suggested that certain serum levels may make ARPE-19 cells more similar to native RPE than others. In the original studies by Dunn *et al.* describing ARPE-19 cells, cells were grown in D10-F12 media (identical to our high serum media), the cells were said to be “fully differentiated” by approximately 12 weeks (Dunn *et al.* 1996b), and would eventually develop pigmentation. A study by Handa *et al.*, however, found that the expression profiles of ARPE-19 cells in no serum were more similar to native RPE than ARPE-19 in high serum conditions (Tian *et al.* 2005b). This study prompted us to check ARPE-19 cells in low serum conditions as well as high serum levels. Interestingly, we found that ARPE-19 cells

increased expression of melanogenesis genes more robustly and completely when grown in high serum than in low serum at the 1 week time point. This suggests that there may be a factor in the serum that works together with iron to induce melanogenesis gene expression even more strongly than iron alone. Additionally, cells at the two month time point had melanogenesis gene expression changes and levels that were much more similar to hRPE cells than at the one week time point. It appears that prolonged culturing in low serum does make ARPE-19 cells more differentiated and more similar to hRPE cells. Thus, in future studies it would be advantageous to use differentiated ARPE-19 cells that have been in culture for at least 2 months in low serum. We also found that ARPE-19 cells treated with iron developed pigmentation by 5 weeks, much sooner than ARPE-19 without iron treatment.

Although both ARPE-19 and hRPE responded by increasing MITF and most melanogenesis effector genes in response to iron, the responses of genes upstream of MITF were more varied. Many genes and pathways regulate MITF expression, and based on our studies, several of them may be involved in upregulating melanogenesis in response to iron. One mechanism by which iron upregulates melanogenesis gene expression may be through the same pathways that UV light induces in skin cells. In support of this, XPC, which is upregulated in the UV-light induced melanogenesis pathway, was upregulated in hRPE and ARPE-19 cells exposed to iron; however this pathway will need to be examined more closely.

Other transcription factors which are known to upregulate MITF: SOX9, SOX10, LEF1, OTX2, were also increased in response to iron to varying degrees. LEF1 was the most consistently elevated gene across the different conditions. The link between iron and these transcription factors has not been studied closely in the past.

SOX9 and *SOX10* are highly homologous transcription factors, belonging to Group E of the Sry-box (SOX) family of transcription factors (Wegner. 2005). SOX 9 and 10 are expressed in neuroectodermal and neural crest cells and perform many diverse functions at different stages of development and during adult life (Wegner. 2005). Under or overexpression of these is associated with pigmentary changes in mice, and they have been shown to interact with and influence *MITF* expression (Harris *et al.* 2010, Sznajder *et al.* 2008, Wegner. 2005). The regulation of SOX9 and SOX10 is not as well understood; both seem to be regulated by multiple other transcription factors and pathways, one of which is the Wnt pathway (Wegner. 2005). Interestingly, the Wnt pathway is also known to regulate LEF1 (Takeda *et al.* 2000, Yasumoto *et al.* 2002), a basic helix-loop-helix and leucine zipper transcription factor (Takeda *et al.* 2000, Yasumoto *et al.* 2002). Takeda *et al.* have previously demonstrated that addition of exogenous Wnt to cultured murine melanocytes results in MITF upregulation through LEF1 (Takeda *et al.* 2000). Similarly, LEF1 knockout mice have deficiency of pigment in their melanocytes among other embryologic abnormalities (Yasumoto *et al.* 2002).

Previously, there were no studies in which *SOX9*, *SOX10* or *LEF1* were shown to be regulated by iron levels, especially in melanin-containing cells. There are studies, however, demonstrating that Wnt signaling can be regulated by iron levels in cells lines from the

colon(Brookes *et al.* 2008). Brookes *et al.* demonstrate that in the Caco2 cell line iron can induce Wnt signaling(Brookes *et al.* 2008). There are no similar studies in pigmented cells. Perhaps in RPE cells, iron may increase Wnt signaling, which may then lead to upregulation of *SOX9*, *SOX10* and *LEF1*, which in turn upregulate *MITF* expression and melanogenesis. Further studies will need to be performed to determine if this is indeed the case.

Iron-induced TYR and TYRP1 expression may not be completely dependent upon MITF, as, under low serum, 1 week FAC exposure conditions, TYR and TYRP1 are modestly upregulated, yet MITF is downregulated. Interestingly, HPS3 is also downregulated under these conditions, so it may be more dependent upon MITF than TYR and TYRP1.

It is of interest that DICER mRNA levels can be affected by iron, as DICER has been implicated in AMD pathogenesis. Iron can impair DICER's ability to cleave toxic Alu sequences by binding to the Alubinding protein PCBP2 (Wright *et al.*, ARVO meeting 2014, abstract 2187).

5. Conclusions

Iron upregulates melanogenesis gene expression in hfrPE and ARPE-19 cell and results in increased pigmentation. Differentiated ARPE-19 cells grown in low serum for 2 months are more similar to hfrPE in their responses to iron than un-differentiated ARPE-19. Several genes upstream of TYR, TYRP1 and MITF are upregulated by iron, and the relative importance of each in iron-induced melanogenesis remains to be determined.

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Abbreviations

hfrPE cells	Human Fetal Retinal Pigment Epithelial Cells
FAC	Ferric Ammonium Citrate

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Highlights

- Iron upregulates melanogenesis gene expression
- Iron increases melanosome number
- Iron upregulates transcription factors upstream of melanogenesis genes

Figure 1.

Quantitative RT-PCR for Transferrin Receptor in cells treated with ferric ammonium citrate for one week. (A) human fetal RPE cells. (B) ARPE-19 cells in media with high serum. (C) ARPE-19 in media with low serum.

Figure 2. Quantitative RT-PCR in human fetal RPE cells treated with ferric ammonium citrate for one week. (A) Tyrosinase. (B) Tyrosinase-related protein 1. (C) Hermansky-Pudlak Syndrome 3. (D) Premelanosome protein. (E) Dopachrome Tautomerase. (F) Microphthalmia-Associated Transcription Factor.

Figure 3.

Electron microscopic analysis of melanosomes from human fetal RPE cells that were treated with ferric ammonium citrate for one week. Cells were obtained from three separate donors. (A) The four stages of melanosome maturation. (B) Total number of melanosomes per cell. (C) Percent of Stage I melanosomes. (D) Percent of Stage II melanosomes. (E) Percent of Stage III melanosomes. (F) Percent of Stage IV melanosomes.

Figure 4. Quantitative RT-PCR in ARPE-19 cells treated with ferric ammonium citrate in media with high serum levels for one week. (A) Tyrosinase. (B) Tyrosinase-related protein 1. (C) Hermansky-Pudlak Syndrome 3. (D) Premelanosome protein. (E) Microphthalmia-Associated Transcription Factor.

Figure 5.

Quantitative RT-PCR in ARPE-19 cells treated with ferric ammonium citrate in media with low serum levels for 1 week or 2 months, and photomicrographs of cells treated with FAC in media with low serum for 5 weeks. (A–E) Cells treated for 1 week with FAC in low serum media. (A) Tyrosinase. (B) Tyrosinase-related protein 1. (C) Hermansky-Pudlak Syndrome 3. (D) Premelanosome protein. (E) Microphthalmia-Associated Transcription Factor. (F–K) Cells treated for 2 months with FAC in low serum media. (F) Tyrosinase. (G) Tyrosinase-related protein 1. (H) Hermansky-Pudlak Syndrome 3. (I) Premelanosome protein. (J)

Dopachrome Tautomerase. (K) Microphthalmia-Associated Transcription Factor. (L) Western analysis and quantification of human tyrosinase-related protein 1 (TYRP1) in control (no FAC) and 250 μ M FAC-treated ARPE-19 cells cultured in low serum conditions for 2 months. (M) Brightfield and phase contrast photomicrographs of ARPE-19 cells allowed to reach confluence in high serum media, then treated with FAC in low serum media for 5 weeks. FAC-treated cells have increased pigmentation compared to untreated cells.

Figure 6. Quantitative RT-PCR in hRPE cells or ARPE-19 cells in the indicated types of media treated with ferric ammonium for 1 week or 2 months. (A–D) XPC. (E–H) DICER1. (I–K) NFE2L2.

Figure 7. Quantitative RT-PCR in hRPE cells or ARPE-19 cells in the indicated types of media treated with ferric ammonium citrate for 1 week or 2 months. (A–D) SOX9. (E–F) SOX10. (G–I) OTX2. (J–M) LEF1.

Gene	Function	Condition			
		hRPE 1wk	ARPE-19 high serum 1wk	ARPE-19 low serum 1wk	ARPE-19 low serum 2mo
TFR3	iron import into cell	down	down	down	down
MITF	transcription factor controlling melanogenesis	up	up	down	up
TYR	melanin production	up	up	up	up
TYRP1	melanin production	up	up	up	up
HPS3	helps sort proteins to melanosomes and lysosomes	up	up	down	up
PMEL	forms amyloid scaffold of melanosomes	unchanged	up	unchanged	up
DCT	production of eumelanin	unchanged	amplified too late	amplified too late	up
LEF1	transcription factor upstream of MITF	up	up	unchanged	up
SOX9	transcription factor upstream of MITF	up	unchanged	unchanged	up
SOX10	transcription factor upstream of MITF	up	amplified too late	amplified too late	up
OTX2	transcription factor upstream of MITF	not tested	unchanged	unchanged	up
DICER1	cleaves double-stranded RNA	up	unchanged	up	up
XPC	nucleotide excision repair in DNA damage	up	up	unchanged	up
NFE2L2	transcription factor upstream of antioxidant genes	not tested	unchanged	unchanged	up