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Developmental Regulation of the Nuclear Ferritoid-Ferritin Complex of Avian Corneal Epithelial Cells: Roles of Systemic Factors and Thyroxine

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Abstract

Previously we observed that avian corneal epithelial cells protect their DNA from oxidative damage by having the iron-sequestering molecule ferritin – normally cytoplasmic – in a nuclear location. This localization involves a developmentally-regulated ferritin-like protein – ferritoid – that initially serves as the nuclear transporter, and then as a component of a ferritoid-ferritin complex that is half the size of a typical ferritin and binds to DNA. We also observed that developmentally, the synthesis of ferritin and ferritoid are regulated coordinately – with ferritin being predominantly translational and ferritoid transcriptional. In the present study we examined whether the mechanism(s) involved in this regulation reside within the cornea itself, or alternatively involve a systemic factor(s). For this, we explanted embryonic corneas of one age to the chorioallantoic membrane (CAM) of host embryos of a different age – all prior to the initiation of ferritin synthesis. Consistent with systemic regulation, the explants initiated the synthesis of both ferritin and ferritoid in concert with that of the host. We then examined whether this systemic regulation might involve thyroxine – a hormone with broad developmental effects. Employing corneal organ cultures, we observed that thyroxine initiated the synthesis of both components in a manner similar to that which occurs in vivo (i.e. ferritin was translational and ferritoid transcriptional).

Keywords

ferritin; ferritoid; thyroxine; CAM; corneal epithelium

Introduction

In the adult, reactive oxygen species (ROS) can be generated by UV light and molecular oxygen (O₂) (Shimmura et al., 1996); in the embryo ROS are present in amniotic fluid (Longini et al., 2007) – presumably resulting from metabolism. ROS can damage a wide variety of macromolecules ranging from DNA to proteins to lipids. The damage to DNA is potentially carcinogenic; in skin, for example, UV-induced damage to DNA is thought to be a major factor in the increasing incidence of epidermal cancers (Hart et al., 1977). Corneal epithelial (CE) cells, however, seem to be refractory to such damage. Primary cancers of these cells are

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extraordinarily rare, even though this tissue is transparent and constantly exposed to ROS-generating UV light and O₂ (Smolin and Thoft, 1987). In addition, within the central CE are multipotent progenitor cells that can repopulate the CE in normal tissue homeostasis, without activation of the pigment granule-containing limbal stem cells (Cotsarelis et al., 1989; Majo et al., 2008). This suggests that CE cells have evolved defense mechanisms – not involving pigmentation – that prevent damage to their DNA. Previous studies in our laboratory suggest that one mechanism for such protection involves having iron-sequestration molecule ferritin in a nuclear location rather than the cytoplasmic location it has in most other cell types. This nuclear ferritin seems to greatly diminish the effects of ROS on DNA, which are effected, at least in part, by the presence of free iron (Fe²⁺) that can exacerbate oxidative damage through the Fenton reaction-mediated formation of hydroxyl radicals – the most energetic and damaging ROS (Henle et al., 1996; Luo et al., 1996; Stohs and Bagchi, 1995).

For the nuclear transport of ferritin in CE cells, we previously identified a novel protein that binds to ferritin and translocates it into the nucleus. This protein, that we have termed ferritoid for its similarities to the ferritin-H chain, consists of three domains. The largest domain is ferritin-like and is involved in the binding of ferritoid to ferritin; the second domain is NH₂-terminal and contains an SV40-type nuclear localization signal that is involved in nuclear translocation (Millholland et al., 2003), and the third is a COOH-terminal that regulates the association of ferritoid and ferritin (Beazley et al., 2008).

Following nuclear transport, ferritoid remains associated with ferritin as a unique nuclear ferritoid-ferritin complex (Nurminskaya et al., 2009). This complex has properties that distinguish it from a typical cytoplasmic ferritin molecule, including its size – which is approximately half that of a cytoplasmic ferritin – and its ability to bind to DNA – which could position it where iron sequestration would be most effective in protecting DNA from ROS-mediated damage (Cai et al., 1998; Cai et al., 2008; Linsenmayer et al., 2005), and where it could afford additional protection through physical interaction with the DNA (Grant et al., 1998). In these characteristics, the nuclear ferritoid-ferritin complex(s) of CE cells is strikingly similar to a class of small, ferritin-like bacterial proteins (the DNA-binding Proteins of Starvation) that are synthesized under adverse environmental conditions and which protect DNA from oxidative damage (Grant et al., 1998).

As ferritoid and ferritin assemble as a complex, it is likely that their developmental synthesis is concomitant. Consistent with this hypothesis, we recently observed that the initiation of ferritoid synthesis occurs less than six hours before that of ferritin (at E10.75 and E11, respectively) (Beazley et al., 2008).

Developmentally there are two ways in which the temporal initiation of the synthesis of ferritoid and ferritin could be regulated. One is that the mechanism(s) responsible reside within the developing cornea itself. The other is that their initiation is triggered by a systemic factor(s) produced elsewhere in the embryo. In the present study, to distinguish between these two possibilities, we employed heterochronic grafting of embryonic corneas to the chorioallantoic membrane (CAM) of host embryos. The CAM is an extraembryonic site that supports a full range of developmental processes in grafted tissues – including corneas, as we have shown previously (Zak and Linsenmayer, 1985a; Zak and Linsenmayer, 1985b). As tissues explanted to the CAM are nourished by the host this should provide the explant with systemic factor(s) produced by the host embryo. For these experiments, corneas from various ages of embryos (all pre-ferritin/ferritoid) were grafted to the CAM of different ages of host embryos (also pre-ferritin/ferritoid). Then, at intervals following explantation, the appearance of ferritin and ferritoid in the CE of the explant and host were assayed by immunofluorescence. If systemic factors are responsible for the temporal initiation of synthesis of ferritin and ferritoid, these molecules should appear in the graft and host at the same time – irrespective of the age of the

cornea used for explantation. As will be described in the results, the experiments show this to occur.

Also in the present study we examined whether thyroxine is one systemic factor responsible for the initiation of synthesis of ferritoid and ferritin. Thyroxine was chosen as a candidate for two reasons. One reason is that studies on another cell type, hepatocytes, have shown that: (1) thyroxine amplifies the synthesis of cytoplasmic ferritin (Deshpande and Nadkarni, 1992); (2) this effect of thyroxine involves translational regulation; and (3) the presence of low levels of iron is required for this effect (Leedman et al., 1996). Similarly, for CE cells our previous observations show that initiation of synthesis of ferritin and ferritoid are at least partly under translational control and that iron is required [(Beazley et al., 2008) and see Discussion]. The other reason we considered thyroxine to be a candidate is that in corneal development it is known to have a profound effect. The formation of the thyroid gland occurs around E10 (Thommes et al., 1992), shortly before the time we detect the appearance of CE nuclear ferritin and ferritoid (E11), and analyses employing RIA for thyroxine show an increase in thyroxine in the developing eye at that time (Prati et al., 1992). In addition, Conrad et al. (2006) have recently shown that thyroxine alters the expression of a number of corneal genes. In the present study we examined the effects of thyroxine on the initiation of synthesis of ferritoid and ferritin in cultured corneas. The results show that thyroxine does initiate the synthesis of both molecules in a dose-dependent manner that recapitulates their development *in vivo*.

Results and Discussion

Heterochronic grafting of corneas to the CAM of a different age host

As described in the Introduction, the developmental initiation of the synthesis of ferritoid and ferritin could be regulated either by mechanism(s) that reside within the cornea itself, or involve systemic factor(s). To distinguish between these alternatives, corneas from different ages of pre-ferritin/ferritoid stage embryos were explanted to the chorioallantoic membrane (CAM) of host embryos that were also pre-ferritin/ferritoid, but of an age that differed from the cornea donor (i.e., heterochronic combinations). Then, at various times following explantation, the corresponding host and donor corneas were assayed for the presence of ferritin and ferritoid by immunofluorescence.

The heterochronic combinations we examined are schematically shown in figures 1A and 2A; also shown figures 1 and 2 are immunofluorescence micrographs for ferritoid and ferritin in the resulting explanted corneas and host corneas. The combinations consisted of explanted corneas from donors that, at the time of explantation, were either 3 days younger than the host [Fig. 1A, explant (E6); host (E9)], or 3 days older than the host [Fig. 2A, explant (E9); host (E6)]. Also shown in these schematic diagrams are the number of additional days, following explantation, that the hosts were allowed to develop before the corneas were analyzed (Fig. 1A: +1d, and +3d; Fig. 2A: +2d, +4d, and +6d), and the day at which the host would normally initiate ferritoid (FTD) and ferritin (FTN) synthesis (E11) (Beazley et al., 2008). In all experiments, for each time point at least three explants and three host corneas were evaluated.

Younger Corneas to Older Hosts (Fig.1)

In the first series of experiments (Fig.1), corneas from E6 embryos were explanted to the CAM of embryos that were three days older (E9), and then after one and three days of incubation both the host and explanted corneas were examined for the presence of ferritoid and ferritin (Fig. 1A, +1d and +3d). Consistent with our previous studies showing that ferritin and ferritoid are not present in the CE until ~E11 (Beazley et al., 2008), the E9 host embryos, after one day of incubation had neither molecule [as they had progressed only to E10 (Fig. 1B, host E10)].

However, after three days of incubation (i.e. when the host was now E12) their CE now had both molecules in the nucleus (Fig.1C, host E12), as expected.

For the explants, if the temporal synthesis of ferritin and ferritoid is inherently regulated within the cornea itself, after neither length of incubation should they have initiated synthesis of the molecules. After one day of incubation they chronologically would be E7 [Fig. 1C (E6+1d)], and at 3 days of incubation they chronologically would be E9 [Fig. 1C (E6 + 3d)], still two days before synthesis should be initiated.

At one day of incubation [Fig. 1B, explant (E6+1d)], neither the explants nor the hosts showed ferritoid or ferritin. However, as neither the host nor the donor corneas had reached the time when the initiation of synthesis would be expected to occur (E11), no conclusions could be drawn. So these essentially served as negative controls.

However, after three days of incubation both the hosts (Fig. 1C, host E12) and explants [Fig. 1B, explant (E6+3d)] had initiated the production of ferritoid and ferritin. Although chronologically the explants were only E9 (E6+3d), with respect to the synthesis of ferritin and ferritoid they had differentiated past the point where synthesis would normally occur (i.e. E11). This suggests that developmentally they had progressed at least two days beyond their chronological age – and in concert with the host embryo – which is consistent with regulation involving the stimulatory action of a systemic factor(s) produced by the host embryo.

A potential alternative to this conclusion is raised by observations made in other of our experiments employing in vitro culture of either pre-ferritin stage corneas (Beazley et al., 2008) or CE cells (Cai et al., 1997). We have observed that under both of these conditions the initiation of synthesis of ferritin and ferritoid occurs precociously. Therefore, in these present experiments it was possible that for the corneas explanted to the CAM, the precocious synthesis observed did not result from a temporally-produced, stimulatory cue(s) from the host embryo. Instead it could reflect release from an inhibitory environment that is operative when a cornea is in situ, but when a cornea is placed in an ectopic site – in this case the CAM – it is released from this inhibitory mechanism. If so, the observed concomitant initiation synthesis of ferritin and ferritoid observed in this experiment could simply be coincidental.

Older corneas to younger hosts (Fig. 2)

To eliminate the possibility that the precocious synthesis of ferritin and ferritoid observed in the CAM explants (above) results from their removal from an inhibitory environment, we examined the ability of younger hosts to retard the synthesis of ferritin and ferritoid by older corneal explants (Fig. 2). For this we performed the reciprocal experiment to that described above [explanting older corneas (E9) to the CAM of a younger hosts (E6)], and subsequently examined by immunofluorescence, both hosts and explanted corneas at 2-day intervals, for the synthesis of ferritin and ferritoid (as schematically diagramed in Fig. 2A).

The host embryos again behaved as expected for the appearance of ferritin and ferritoid at ~E11, as those corneas harvested at either E8 (Fig. 2B, host E8) or E10 (Fig. 2C, host E10) showed neither molecule, while those harvested at E12 had both proteins (Fig. 2D, host E12).

For the explants in this experimental series, the results again were consistent with systemic regulation from the host. For the E9 explants, after either 2 days of incubation [Fig. 2B, explant (E9+2d)], or 4 days of incubation [Fig. 2C, explant (E9+4d)], neither ferritoid nor ferritin were detected. If the appearance of these molecules were regulated inherently within the cornea itself, then the explants at both of these time points would have initiated synthesis, as chronologically both would have been at least E11 (when synthesis normally occurs). [The explant in Fig. 2B would have been E11 (E9+E2) and the explant in Fig 2C would have been

E13 (E9 + 4d)]. The hosts in these combinations had also failed to initiate the synthesis of ferritoid and ferritin; however this is to be expected as they had not reached the age when synthesis should begin, as the younger host was only E8 (Fig. 2B) and the older host was E10 (Fig. 2C).

After 6 days of incubation (Fig. 2D) when the hosts had reached E12, both ferritoid and ferritin were now present in the explants, as well as in the hosts. Thus, irrespective of the age of the explant donor, it was only when host embryo had reached an age when the synthesis of ferritoid and ferritin had been initiated that synthesis was also observed in the explants.

Therefore, taken together, the results of these experiments are consistent with ferritoid and ferritin being synthesized in the corneal explants at the same time as in the host embryo, which suggests that the synthesis of both proteins is regulated, at least in part, by a systemic factor(s) emanating from the host.

Thyroxine as a Regulator of Ferritoid and Ferritin Synthesis

As a systemic factor for regulating the initiation of ferritoid and ferritin synthesis, we considered thyroxine to be a potential candidate (as described in the Introduction). To examine the effects of thyroxine on the synthesis of ferritin and ferritoid, we employed a corneal organ culture system, recently characterized and used in other of our studies (Beazley et al., 2008). In the previous studies we observed that pre-ferritin stage (E8) corneas, when cultured in serum-containing medium (10% fetal bovine serum) initiated the synthesis of ferritoid and ferritin after as little as one day in culture. This synthesis is precocious, as chronologically these corneas are only E9 – which is two days before synthesis would normally occur *in vivo*.

In the present study we confirmed this precocious initiation of synthesis of ferritin (FTN) and ferritoid (FTD), as shown by immunofluorescence in figure 3A (+ serum). In addition we observed that one, or more, factor(s) in serum are required for initiating this synthesis, as corneas cultured in serum-free medium showed only an occasional cell to have ferritoid or ferritin, even after two days in culture – a length of time that was chosen to ensure that synthesis would be detected if it were initiated [Fig. 3A (- serum)]. Also, this absence of synthesis in serum-free medium is not due to differences in either cell death (Fig. 3B) – measured by lactate dehydrogenase activity in the culture medium – or in cell proliferation (Fig. 3C) – detected by BrdU incorporation in the CE – as these are the same in serum-containing and serum-free media. These observations demonstrate that the factor(s) necessary for the initiation of synthesis of ferritoid and ferritin are present in fetal calf serum; they also suggest the corneal organ culture system, when performed using serum-free medium, provides a means for evaluating factor(s) necessary for the initiation of synthesis of ferritin and ferritoid – as evaluated by their addition to the serum-free medium.

To determine the potential involvement of thyroxine, the serum-free corneal organ cultures were supplemented with T3, the active form of thyroxine, and two days later were examined for the presence of ferritin and ferritoid by immunofluorescence. As can be seen in figure 3 (10 nM T3), addition of the hormone clearly induced the synthesis of both proteins in the CE.

To further evaluate this effect of thyroxine on the synthesis of ferritin and ferritoid, immunoblot analyses were performed on cell lysates of CE tissue isolated from corneas after culture (Fig. 4A). These immunoblots were then quantified by densitometry, and the amount of ferritin and ferritoid produced in serum-free cultures – with and without exogenous T3 – was compared to that serum-containing cultures (assigned a value of 100%) (Fig. 4B-C). In serum-free cultures ferritin and ferritoid were greatly reduced (Fig. 4A) with ferritin being 8% of that of the serum-containing ones (Fig. 4B), and ferritoid being 10% (Fig. 4C) (n=4). However, when the serum-free cultures were supplemented with T3 [at increasing concentrations within the physiological

range from 1nM to 100 nM (Samuels et al., 1973)] the production of ferritin and ferritoid were both greatly increased, with maximum responses being reached at 10nM (Fig. 4A). At this concentration of T3 the synthesis of ferritin increased to 70% of that in serum-containing medium (Fig. 4B), and the synthesis of ferritoid had risen to 52% of that in serum-containing medium (Fig. 4C). Thus, a large part of the stimulating activity of serum can be mimicked by thyroxine alone.

Previously, we (Beazley et al., 2008) observed that during normal development the regulation of ferritin in the CE is predominately translational – with appreciable ferritin mRNA being present in the CE of pre-ferritin stage corneas (E6) and only a modest increase occurring just before initiation of the protein (at E10 ~4 fold), and progressing only slightly thereafter (at E14 ~ 6-fold). For ferritoid synthesis, conversely, the regulation is predominantly transcriptional, with its mRNA being essentially non-detectable before synthesis of the protein (E6); a large increase occurring at the time of initiation of the protein (at E10 ~ 13-fold) and a progressive increase thereafter (at E14 ~300 fold).

We therefore examined whether in the organ cultures, the stimulation of synthesis by T3 and serum was regulated by the same mechanisms as occurs in vivo (ferritin is translational and ferritoid transcriptional). For this we employed qRT-PCR to analyze the mRNAs for ferritin and ferritoid in the CE of corneas cultured in serum-free medium versus serum-containing medium and serum-free medium containing 10 nM T3 (Fig. 5).

For ferritin, which in vivo is predominantly regulated by translational mechanisms (Fig. 5A) there was only a slight increase (not statistically significant) for the mRNA in the CE of the serum-containing cultures, or in the serum-free cultures containing T3. Thus, the increases in ferritin protein in serum-containing medium and T3-supplemented serum-free medium that are observed (described above) likely reflect an increase in translation.

For ferritoid mRNA (Fig. 5B), the T3-supplemented medium also showed a large (11.2 fold) stimulation (as compared to the serum-free medium), and the serum-containing medium showed an even greater stimulation (78.1 fold). As the magnitude of these increases is similar to those observed for the CE at progressively later days of development (described above), this reinforces ferritoid being regulated largely at the level of mRNA.

However, it is likely that ferritoid is also regulated at the translational level. Although, ferritoid mRNA is greatly stimulated in T3-supplemented medium, this is only about 14% of the level observed in serum-containing medium (as just described). For ferritoid protein, however, the level in T3-supplemented medium was 52% of that in serum-containing medium (described above), suggesting an even greater effect of T3 on ferritoid translation. Consistent with this apparent translational regulation of ferritoid, we (Beazley et al., 2008) previously observed that when iron is removed from cultures – using the iron chelator deferoxamine – ferritoid protein synthesis is completely inhibited but the level of ferritoid mRNA remains unchanged.

As qRT-PCR measures the steady state levels of an mRNA, for ferritoid we do not know for certain whether the increases observed during normal development – or in the organ cultures in response to T3 or serum – reflect increased rates of transcription, increased mRNA stability, or both. However, during normal development it is likely that increased transcription is involved, as the levels of ferritoid mRNA go from essentially background in the pre-ferritoid stage CE, to ones that rapidly increase 13 fold at the time ferritoid protein becomes detectable (Beazley et al., 2008). In the organ cultures it is also likely that increased transcription and/or mRNA stability are responsible for the increased content of ferritoid mRNA in the T3 or serum-containing medium, as compared to the serum-free cultures. For the serum-free cultures the other alternative for their having comparatively low levels of ferritoid mRNA is increased

mRNA degradation. However, a general increase in mRNA degradation is ruled out by the observation that as that levels of ferritin mRNA in serum-free medium are the same as in serum or T3-supplemented media, and while this does not eliminate a specific alteration in the degradation of the closely-related ferritoid mRNA, it makes this less likely.

All of these results are consistent with thyroxine being a major factor in the regulation of ferritoid and ferritin synthesis. However, it is unlikely that thyroxine is the only factor involved, as in the organ cultures its stimulation on the synthesis of ferritin protein, and ferritoid mRNA and protein is only partial – as compared to that effected by serum.

We considered that another potential regulatory factor may be iron, as in other cell types it has long been known that iron is involved in the translational regulation of ferritin (reviewed in Pantopoulos, 2004), and we have previously shown that the initiation of synthesis of both ferritoid and ferritin can be reversibly inhibited by removing iron from the culture medium with the iron chelator deferoxamine (Beazley et al., 2008). However, when we attempted to initiate the synthesis of ferritoid and ferritin by increasing the concentration of iron in the serum-free medium, no effect was observed (data not shown). Therefore, other factor(s) that are involved in the initiation of ferritin and ferritoid synthesis remains to be determined. Potential factors include those growth factors involved in the development and maintenance of the CE, such as epidermal growth factor (EGF), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), and transforming growth factor-beta (TGF- β) (reviewed in Kinoshita et al., 2001).

Materials and Methods

Chorioallantoic membrane (CAM) graft

Fertile eggs (White Leghorn) were obtained from Hyline (Elizabethtown, PA), incubated at 38°C, and staged by the criteria of Hamburger and Hamilton (1951). Host embryos were prepared as described previously (Zak and Linsenmayer, 1985a; Zak and Linsenmayer, 1985b). Briefly, at embryonic day 3 (E3), the air sacs at the blunt end of the eggs were punctured with an 18 gauge needle and a window was cut through the eggshells over the embryos. The eggshell fragments were then removed, the windows were covered with Parafilm, and the eggs were returned to the humidified 38°C incubator until donor corneas were ready for explantation onto the CAM.

Donor embryos were removed into a Petri dish of sterile Hank's balanced saline solution (Invitrogen) and staged according to Hamburger and Hamilton (1951). Whole corneas were explanted to the CAM as follows: corneas were dissected from adjacent tissues and transferred with the aid of a small spatula to a site on a host CAM which had been lightly abraded with a mini scalpel blade to facilitate engraftment of the donor corneas. The mini scalpel blade was gently scraped 3-4 times across a small area of the CAM, just enough to cause small blood spots to appear. Excess fluid was removed with a sterile Pasteur pipet to ensure good contact between the cornea and the CAM. The window was then resealed with Parafilm and the host returned to the incubator for up to 6 days.

Corneal organ culture

Corneas were dissected from day 8 embryos (Stage 34; Hamburger and Hamilton, 1951) and cultured epithelial side up for 48 hours in various media conditions. Serum-free media consisted of DMEM:F-12 (1:1) (Invitrogen) with 5 μ g/mL insulin (Sigma), 5ng/mL hEGF (Invitrogen), and 100U/mL penicillin/100 μ g/mL streptomycin (Invitrogen). This medium was supplemented either of 10% fetal calf serum (Hyclone) (serum-containing medium), or triiodo-L-thyronine (T3) (Sigma) (T3-supplemented medium). Cytotoxicity was assayed by lactate

dehydrogenase activity in the culture medium [using an LDH-Cytotoxicity Kit II (BioVision) following the manufacturer's protocol]. Proliferation was assayed by 5-Bromo-2'-deoxy-uridine (BrdU) (10 μ M, Roche) labeling of the cultures for the final 24 hours of the 48 hour culture period, with labeled cells visualized as described below.

Immunofluorescence

Following CAM grafting, explanted corneas were excised from the host CAM and host corneas were dissected from the host embryo. After 48 hours, cultured corneas were removed from culture. Corneas were then washed in PBS, fixed in 4% paraformaldehyde (10-20 minutes on ice), cryoprotected in 8-20% sucrose (30 minutes to overnight at 4°C), and embedded in Tissue Tek OCT. Frozen sections (10 μ m) were cut using a cryostat (Microm), and were mounted on 12-spot slides (ThermoShandon Scientific) coated with BIOBOND (Electron Microscopy Sciences).

Indirect immunofluorescence was performed as described previously (Beazley et al., 2008; Cai et al., 1997). For ferritoid labeling in whole cornea cultures, the blocking solution consisted of BlokHen II (Aves Lab) in PBS; for all other labeling, the blocking solution consisted of 1% BSA and 10% chicken serum in PBS. The primary antibodies were: a monoclonal antibody against chicken ferritin (6D11, undiluted; Zak and Linsenmayer, 1983) and a biotinylated IgY antibody against chicken ferritoid (1:250; Beazley et al., 2008). The secondary antibody for ferritin was either FITC-conjugated goat anti-mouse IgG (Pierce) for double-labeling or TRITC-conjugated goat anti-mouse IgG (Pierce), for single-labeling. To visualize the ferritoid antibody, a TRITC-conjugated streptavidin (Molecular Probes) was used.

To visualize BrdU labeling, slides were pretreated by rinsing with dH₂O and incubation in 2 normal HCl (30 minutes at room temperature). The slides were then treated as described above for indirect immunofluorescence. The primary antibody used was a monoclonal rat antibody against BrdU (1:200, Abcam) and the secondary antibody was TRITC-conjugated goat anti-rat IgG (R&D Systems).

The samples from the CAM grafting experiments were visualized by confocal microscopy using a Leica TCS SP2 laser scanning microscope and detector in 1 μ m optical sections. The samples from the whole cornea cultures were visualized using a Nikon Fluophot microscope, equipped with a SPOT RT real-time CCD camera (Diagnostic Instruments, Inc.).

Isolation of protein from CE and immunoblot

After culture the corneas were washed in PBS and the CE was removed using dispase [4U/mL (Fisher), 20 minutes at 37°C] followed by dissection in PBS. The CE tissue was extracted for 30 minutes with ice cold RIPA Buffer (Boston BioProducts) containing Complete Protease Inhibitor Cocktail (Roche). The samples were spun at 14000 $\times$ g for 10 minutes at 4°C and the supernatant was collected. Protein concentrations were determined by Coomassie Reagent (Pierce) according to the manufacturer's protocol.

For SDS-PAGE, protein samples were incubated at 100°C for 10 minutes in Reducing Buffer (Boston BioProducts) and separated on 12% acrylamide-HCl gels (BioRad). Following electrophoresis, the proteins were transferred onto PDVF membranes (BioRad), and the membranes were blocked with 5% dry milk in PBST (PBS + 0.1% Tween-20) for 1 hour at room temperature. Membranes were then incubated overnight at 4°C in 1.25% dry milk in PBST containing primary antibodies at the following dilutions: anti-ferritin at 1:500 [6D11 (1mg/mL)], anti-ferritoid at 1:10000 (Aves Lab), or anti- α -tubulin at 1:3000 (Ana Spec). The membranes were washed 3 times in PBST for 5 minutes per wash and incubated with the secondary HRP conjugated antibody for 1 hour at RT in PBST at the following dilutions: goat-

anti-mouse at 1:10000 (Sigma), extravidin at 1:10000 (Invitrogen), or sheep-anti-rabbit at 1:5000 (R&D Systems). The membranes were washed 3 times in PBST (5 minutes each) and developed with HyGlo chemiluminescence substrate (Denville). Membranes were washed 4 times in PBST, stripped in Restore (Peirce) for 20 minutes at RT, washed 4 times in PBST and reprobed with anti- α -tubulin (starting at the primary antibody step described above).

RNA isolation and quantitative real-time PCR (qRT-PCR)

Cultured corneas were washed in PBS and the central cornea was isolated using a 1mm trephine. CE tissue was removed, frozen in liquid nitrogen and stored at -80°C. Poly-A+ mRNA was isolated with a Micro-Fast Track Kit 2.0 (Invitrogen) using the manufacturer's protocol; cDNA was generated using iScript (BioRad) and then was diluted to 5ng/ μ L. qRT-PCR was performed using a Stratagene Mx4000 multiplex quantitative PCR instrument system, with SYBR Green reagent (Stratagene), and 10ng of cDNA. In each experiment, all samples were repeated in triplicate. The primers pairs used for ferritin and ferritoid have been described previously (Beazley et al., 2008), and the data was normalized to the ribosomal protein L35a (forward primer: 5'-GGCATAACCACAACACACAG-3', reverse primer: 5'-GCCAGTTATCTACCTTTTCAA-3').

Statistics

Statistical analyses were performed using the one way ANOVA analysis (Microsoft Excel). P values < 0.05 are designated by an asterisk. Error bars represent $\pm$ S.E.M.

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Figure 1. Analysis of ferritoid and ferritin synthesis in corneas explanted to the CAM of older embryos

(A) Schematic diagram of the experimental design. E6 corneas were grafted onto the CAMs of E9 host embryos and both the explanted corneas (explant) and corneas from the host embryos (host) were collected after 1 (+1d) or 3 days (+3d) of incubation. The line at E11 designates when ferritin and ferritoid are normally synthesized in the host embryo (FTN/FTD). (B-C) Immunofluorescence micrographs of explant and host corneas after 1 day (B) or 3 days (C) of incubation. Ferritoid is shown in red and ferritin in green. Nuclei are stained with Hoechst.

Figure 2. Analysis of ferritoid and ferritin synthesis in corneas explanted to the CAM of younger embryos

(A) Schematic diagram of the experimental design. E9 corneas were grafted onto the CAMs of E6 host embryos and both the explanted corneas (explant) and corneas from the host embryos (host) were collected after 2 (+2d), 4 (+4d), and 6 days (+6d) of incubation. The line at E11 designates when the synthesis of ferritin and ferritoid occurs in the host embryo (FTN/FTD). (B-D) Immunofluorescence micrographs of explant and host corneas after 2 days (B), 4 days (C), or 6 days (D) of incubation. Ferritoid is shown in red and ferritin in green. Nuclei are stained with Hoechst.

Figure 3. Effects of serum and T3 on the synthesis of ferritin and ferritoid in corneal organ cultures (immunofluorescence)

(A) Immunofluorescence micrographs for ferritin (FTN) and ferritoid (FTD) in whole corneas cultured in serum-free medium (- Serum), or medium containing either 10% fetal bovine serum (+ Serum) or thyroxine (10 nM T3). Scale bar = 100 μ m. (B) Relative cytotoxicity detected after 48 hours of culture in serum-containing medium (+), serum-free medium (-), or serum-free medium containing T3 (SFM + 10nM T3). (C) Percent of BrdU labeled nuclei in the CE of corneas cultured for 24 hours in serum-containing medium (+), serum-free medium (-), or serum-free medium containing 10nM of T3 (followed by an additional 24 hours in the same media containing Brdu).

Figure 4. Effects of serum and T3 on the synthesis of ferritin and ferritoid in the CE from corneal organ cultures (immunoblot)

(A) Immunoblot for ferritin (FTN) and ferritoid (FTD) in CE tissue lysates from corneas cultured in serum-containing (+) or serum-free (-) medium, or serum-free medium supplemented with T3 (SFM + T3) at concentrations of (1, 10, and 100 nM). (B-C) Quantification of band densities for FTN (B) and FTD (C). Band densities were first normalized to Tubulin (Tub) and then compared to one another with the serum-containing cultures being assigned a value of 100%. Error bars are SEM, and * represents $P < 0.05$.

Figure 5. Effects of serum and T3 on the mRNA for ferritin and ferritoid in the CE from corneal organ cultures

cDNA generated from CE tissue isolated after corneal organ culture in serum-free medium (-serum), serum-containing medium (+ serum), and serum-free medium supplemented with T3 (SFM + 10nM T3) was analyzed by qRT-PCR for ferritin (A) and ferritoid (B). For all conditions n = 3. Error bars are SEM, and * represents P < 0.05.