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TRANSFECTION OF CYP4B1 INTO THE CORNEA INCREASES ANGIOGENIC ACTIVITY OF THE LIMBAL VESSELS

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Abstract

Injury to the ocular surface induces the production of the corneal epithelial-derived 12-hydroxyeicosatetrienoic acid (12-HETrE), which exhibits stereospecific potent inflammatory and angiogenic properties and is formed by a cytochrome P450 enzyme, CYP4B1. We have cloned the rabbit corneal CYP4B1 into the expression plasmid pIRES2-EGFP and examined the effect of CYP4B1 overexpression on corneal inflammation *in vivo* and limbal vessel sprouting *ex vivo*. Cultured rabbit corneal epithelial (RCE) cells transfected with pIRES2-EGFP-CYP4B1 metabolized arachidonic acid to 12-HETrE at a rate 5 times higher than that of pIRES2-EGFP-transfected cells (3.53±0.08 vs. 0.62±0.10 nmol/h/10^6 cells; mean±SE, n=6, $p<0.05$), indicating a functional expression of the CYP4B1. Injection of either plasmid into the rabbit cornea resulted in EGFP fluorescence in the corneal epithelium. However, corneal neovascularization, as measured by the length of penetrating blood vessels, was significantly greater in the corneas of eyes transfected with the pIRES2-CYP4B1 as compared with pIRES2-EGFP. Corneal limbal explants from eyes transfected with pIRES2-CYP4B1 demonstrated a marked angiogenic activity (46±10 vs. 12±3 mm capillary length, n=6, $p<0.05$), which correlated with increased levels of 12-HETrE, the CYP4B1-derived angiogenic 12-hydroxyeicosanoid (0.93±0.18 vs. 0.15±0.02 pmol/explant, n=6, $p<0.05$), and was inhibited (76±5 %) by the CYP inhibitor 17-ODYA. The results further implicate the corneal CYP4B1 as a component of the inflammatory and angiogenic cascade initiated by injury to the ocular surface and raise the possibility of a new therapeutic target for preventing corneal neovascularization, namely, the CYP4B1-12-HETrE system.

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Introduction

Angiogenesis or neovascularization is regarded as a component of the inflammatory reparative response of a given tissue to injury. While neovascularization has some positive effects such as facilitating healing by enabling transport of immunologic humeral and cellular factors for repairing the tissues, persistence of blood vessels within the cornea is detrimental in that it degrades corneal transparency. Neovascularization of the normally avascular cornea is seen in many pathological conditions, which include infection, mechanical and chemical injury, chronic exposure to hypoxia and following corneal transplantation. Corneal neovascularization, whathever the cause, leads to decreased vision, recurrent corneal erosion and incompetent barrier function, thus presenting a serious clinical problem for which treatment is lacking. The molecular mechanisms that control corneal neovascularization are not fully understood. Since inflammation precedes the corneal neovascularization seen following injury, many studies have concentrated on the role of humeral and corneal-derived inflammatory mediators in the regulation of this process. Among these mediators are various arachidonic acid-derived eicosanoids of the cyclooxygenase, lipoxygenase and cytochrome P450 monooxygenase (CYP) pathways.

Previous studies have identified CYP-dependent arachidonic acid metabolism in the corneal epithelium and established it as a primary corneal epithelial inflammatory pathway in rabbit models of ocular surface inflammation (Laniado Schwartzman et al., 1997b). The corneal epithelial CYP metabolizes arachidonic acid to two major 12-hydroxyeicosanoids, 12-HETE and 12-HETrE, which exhibit stereospecific potent inflammatory and angiogenic properties. That these two metabolites are critical tissue-derived mediators of ocular surface inflammation is strongly supported by studies from our laboratory demonstrating that: 1) their synthesis and primarily that of the bioactive R enantiomers is increased following hypoxic and chemical injury

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in vitro and *in* vivo (Conners et al., 1995b; Conners et al., 1997; Vafeas et al., 1998); 2) their levels positively correlate with the *in situ* inflammatory response; 3) inhibition of their synthesis attenuates ocular surface inflammation *in vivo,* suggesting a potential cause-effect relationship (Conners et al., 1995a; Laniado Schwartzman et al., 1997a); and 4) their biological activities, in particular those of 12(R)-HETrE, *in vitro* and *in vivo,* are characteristic of potent inflammatory mediators (including vasodilation, neutrophil chemotaxis, and angiogenesis). The importance of these metabolites to human pathophysiology is underscored by the demonstration that these two metabolites are present in human tears, and more significantly, their levels are much higher in tears from subjects with ocular inflammation (Mieyal et al., 2001).

We have previously isolated a 1.82 kb CYP cDNA from hypoxia-treated rabbit corneal epithelium with a 98% sequence homology to the lung CYP4B1 (Mastyugin et al., 1999; Mastyugin et al., 2004). The expression of the CYP4B1 in rabbit corneal organ cultures was induced by hypoxia and by known chemical inducers of the CYP4 gene family such as clofibrate and phenobarbital and its increased expression was associated with increased production of 12-HETE/12-HETrE (Mastyugin et al., 1999). Further studies demonstrating that antibodies against CYP4B1 inhibited hypoxia-induced 12-HETE and 12-HETrE synthesis (Mastyugin et al., 1999) provided substantial evidence that, in the corneal epithelium, CYP4B1 is involved in the hypoxia-induced 12-HETE and 12-HETrE synthesis and ocular surface inflammation. Indeed, increased expression of CYP4B1 mRNA in the corneal epithelium during hypoxic injury *in vivo* corresponded well with the progression of the anterior surface inflammatory response, including corneal thickness and inflammatory score, as well as with the synthesis of the inflammatory and angiogenic eicosanoid, 12-HETrE (Mastyugin et al., 2001).

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In this study, we cloned the rabbit corneal CYP4B1 into a bicistronic expression plasmid and examined the effect of CYP4B1 overexpression on corneal inflammation *in vivo* and limbal vessel sprouting *in vitro*. The results demonstrated that the pIRES-EGFP-4B1 construct effectively expressed the CYP4B1 in cultured rabbit corneal epithelial cells as evidenced by increased arachidonate metabolism to 12-HETrE. Moreover, CYP4B1 transfection of the corneal epithelium *in vivo* yielded an inflammatory response in the ocular surface and stimulated angiogenic activity of limbal explants that was mediated by increased 12-HETrE production.

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Methods

Plasmids. The full-length (1.82 kb) corneal CYP4B1 cDNA (Mastyugin et al., 2004) was cloned into the MCS cloning site of the pIRES2-EGFP plasmid (Clontech Laboratories, Inc. Palo Alto, CA) via AccI and XmaI restriction sites. CYP4B1 and EGFP were translated separately from a single bicistronic mRNA. The cloned plasmid was termed pIRES2-EGFP-4B1 and the pIRES2-EGFP plasmid was used as the control.

Transfection of plasmids in cultured cells. Immortalized rabbit corneal epithelial (RCE) cells were a generous gift from Dr. K. Araki, Department of Ophthalmology, University of Osaka, Japan (Araki et al., 1993). Cells were grown to 70-80% confluence in T-75 fibronectin-coated flasks containing DMEM/F12 supplemented with 15% (v/v) heat-inactivated fetal bovine serum (FBS); 48 h prior to transfection, the medium was substituted with DMEM/F12 containing 0.5% FBS. Cells were incubated with a mixture containing 6 µl of the LipofectAMINE^{TM} Reagent (Invitrogen) with and without 1 µg plasmid DNA for 6 h in DMEM medium deprived of FBS at 37^0C. The medium was then replaced with DMEM/F12 supplemented with 15% (v/v) FBS. To assess transfection efficiency, cells were cultured in 4-well slides (SonicSeal®, Nalgene Nunc International, Rochester, NY) at a density of 20,000 cells/well and transfected with pIRES-EGFP or pIRES-EGFP-4B1 (800 ng/well) as described above. The medium was removed 24h later; cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. Cells were then washed with PBS and EGFP fluorescence was detected with an Olympus Fluoview FV300 confocal laser-scanning microscope using a X63/1.4 objective.

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Arachidonic acid metabolism. 24 h post transfection, pIRES2-EGFP-, pIRES2-EGFP-4B1- or vehicle-transfected cells were washed with PBS twice and assessed for arachidonic acid metabolism as previously described (Conners et al., 1997). Briefly, cells (10^6) were incubated with ^{14}C-arachidonic acid (1 µCi) and NADPH (I mM) in the presence and absence of indomethacin (5 µM), NDGA (15 µM) or 17-ODYA (5 µM) for 30-120 min at 37^{o}C. The incubation medium was extracted with ethyl acetate and radiolabeled metabolites were separated by HPLC. To assess endogenous production of 12-HETrE, cells were incubated with the calcium ionophore A23187 (5 µM). 12-HETrE extracted from the incubation medium was quantified by negative chemical ionization (NCI)-gas chromatography-mass spectrometry (GC-MS) as described below.

Transfection of CYP4B1 into rabbit cornea in vivo. All animal studies adhered to the ARVO statement for the Use of Animals in Ophthalmic and Vision research. Male New Zealand rabbits (2.2-2.5 kg) were anesthetized with Ketamine (50 mg/kg) and Xylazine (20 mg/kg) intramuscularly. Plasmids (2 µg/4 µl saline) or the vehicle control (saline) were administered to the right eye using a 30G1/2 needle inserted repeatedly into the limbus over 360^o. The inflammatory/neovascular response was monitored by slit lamp microscopy for up to 7 days after transfection. Quantitative analysis of corneal neovascularization was performed using Image Pro-Express Software (Cyber Media). Rabbits were sacrificed at different time points and corneas including the limbus were removed, dissected into four pieces and processed for immunofluorescence measurement, angiogenic activity and 12-HETrE production.

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Immunostaining. Corneas were washed twice with PBS and fixed in 4% paraformaldehyde-PBS, pH 7.4 for 4 h at room temperature. Fixed corneas ware washed with PBS. Corneas were then subjected to three successive 1 h incubations in solutions containing 5, 10 and 30% sucrose-PBS, pH 7.4. Each cornea was sectioned transversely (16 μm thick) and then processed for immunohistochemistry. Sections were washed with PBS three times at room temperature for 5 min and blocked with 1% non-fat milk-PBS- 0.2% Triton X-100 (blocking solution) at room temperature for 30 min. Primary antibody (anti-EGFP, mouse monoclonal 3E6, Molecular Probes, Eugene, OR) was added (1:200 dilution) and sections were further incubated for 48 h at 4^0C. The sections were washed five times with PBS for 1 min each wash and incubated with 1:500 dilution of the secondary antibody (fluorescein goat anti-mouse IgG (H+L) conjugate, Molecular Probes, Eugene, OR) for 3 h at room temperature. Negative controls included sections incubated with non-immune serum instead of primary or secondary antibody. The sections were washed and mounted in SlowFade medium (Molecular Probes, Eugene, OR) and analyzed by confocal (Olympus Fluoview, FV300) and fluorescence (Nikon epifluorescence) microscopy.

Limbal neovessel formation. The angiogenic activity of limbal vessels was examined *ex vivo* using corneal-limbal explants as previously described with some modifications (Jiang et al., 2004). 48 h post transfection, corneas were excised with a limbal ring washed 4 times and cut into 4 pieces. Each piece was placed onto fibronectin-coated 24-well plates. Matrigel GFR (250 μl; BD Biosciences, Bedford, MA) was added into each well on top of the corneal-limbal explant and allowed to solidify for 30 min, after which 500 μl EGM-2 (Cambrex, Walkersville, MA) was added with and without 17-ODYA (10 μM). In some experiments, 3 nmols of 12(R)-HETrE were

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added to explants cultured in the presence of 17-ODYA. Culture medium was collected every 2 days for measurements of 12-HETrE. The angiogenic response of the limbus was determined over time (at day 3, 5 and 7) by measuring the length of the neovessel sprouts and counting the branching points using the Image Pro-Express Software. To determine whether neovessel sprouts were composed of endothelial cells, corneal-limbal explants were placed in glass-bottom culture dishes and cultured as described above. At day 4, the lectin *Ulex europeus* (Sigma, MO), which specifically binds to endothelial cells, was added to the medium in a final concentration of 10 µg/ml and incubated with the explants for 1 h at 37^{o}C as described (Brodsky et al., 2003). Cultures were washed 3 times with MCDB 131 medium (GIBC-BRL, Grand Island, NY) and endothelial cell fluorescence was examined by confocal microscopy.

Measurements of 12-HETrE. The amount of 12-HETrE in culture medium was quantified by negative chemical ionization-gas chromatography-mass spectrometry (NCI-GC-MS) as previously described (Mieyal et al., 2000). Briefly, 10 pg of [12-$^{2}H_3$]-12(R)-HETrE was added to each sample as the internal standard and 12-HETE and 12-HETrE were extracted with ethyl acetate, isolated by reverse phase-HPLC and derivatized to the pentafluorobenzyl ester and trimethylsilyl ether. NCI-GC/MS was performed on a HP6890 mass spectrometer (Hewlett-Packard, Palo Alto, CA) interfaced with a capillary gas chromatographic column (HP-5MS, 30mX0.25mmX0.25µm, Agilent). Single ions with m/z 393 corresponding to the derivatized 12-HETrE, m/z391 for 12-HETE and other mono-HETEs, and m/z 396 for the derivatized [$^{2}H_3$]-12(R)-HETrE internal standard (Figure 2) were selected for monitoring. Total 12-HETrE in each sample was determined by comparison of the ratio of ion intensities (393:396) vs. standard

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curves of derivatized 12-HETrE/[$^{2}H_{3}$]-12(R)-HETrE molar ratios obtained from NCI-GC/MS analysis.

Data analysis. Student's t-test was used to evaluate the significance of differences between groups (control vs. treatment). A p value less than 0.05 was considered significant. All data are presented as mean±SE.

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Results

CYP4B1-transfected RCE cells produce 12-HETrE

Transfection of rabbit corneal epithelial cells (RCE) with the pIRES2-EGFP-4B1 yielded a marked expression of EGFP within 24h (Figure 1). Similar results were obtained for the control plasmid pIRES2-EGFP (Figure 1). Due to the lack of CYP4B1 antibody, we examined whether cells transfected with the CYP4B1 cDNA carry out oxidation of arachidonic acid to 12-HETE and 12-HETrE, an enzymatic activity ascribed to this enzyme based on *in vitro* and *in vivo* studies (Vafeas et al., 1998; Mastyugin et al., 1999). Figure 2 depicts HPLC patterns of radiolabeled metabolites recovered in the medium of RCE cells incubated with ^{14}C-labeled arachidonic acid. RCE cells transfected with the CYP4B1 cDNA metabolized arachidonic acid to a product that had the HPLC elution profile of authentic 12-HETrE. 12-HETrE was formed at a rate of 3.53±0.08 nmol/h/10^6 cells (mean±SE, n=5) in cells transfected with the CYP4B1 as compared to a rate of 0.62±0.10 nmol/h/10^6 cells (mean±SE, n=6, $p<0.001$) in cells transfected with the control plasmid, pIERS2-EGFP. The production of 12-HETrE was not affected by cyclooxygenase or lipoxygenase inhibitors, indomethacin and NDGA, respectively. Omission of NADPH from the incubation medium decreased formation of 12-HETrE by 57±4% (mean±SE, n=4, $p<0.05$). Addition of 17-ODYA, an inhibitor of CYP-derived arachidonic acid metabolism, inhibited the production of 12-HETrE by 70% (1.07±0.34 nmol/h/10^6 cells, mean±SE, n=3, $p<0.002$), as previously described (Vafeas et al., 1998). An additional broad polar peak appears in incubations of pIERS2-EGFP-4B1-transfected cells that may contain arachidonate-derived metabolites. The identity of these metabolites is unknown and extensive studies are needed to identify their origin and structure. However, their levels increased as a function of time and they

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may constitute previously described polar metabolites of 12-HETE and 12-HETrE (Nishimura et al., 1991).

To further confirm 12-HETrE production, cells were stimulated with the calcium ionophore A23187 to release endogenous arachidonic acid and 12-HETrE levels were analyzed by NCI-GC/MS. As seen in Figure 3C, a single peak with a mass ion m/z 393 and elution profile identical to that of the 12-HETrE internal standard (Figure 3B) was detected in incubates from cells transfected with pIRES2-EGFP-4B1. Quantitative analysis indicated that 12-HETrE levels in cells transfected with the CYP4B1 were 5-fold higher than cells transfected with the control plasmid, pIRES2-EGFP (Figure 3D). Since CYP4B1 belongs to the CYP4 gene family that generally catalyzes oxidation at the ω-carbon of fatty acids (Rettie et al., 1995; Fisher et al., 1998), 20-HETE levels in RCE were also assessed. Although the production of 20-HETE was higher in cells transfected with pIRES2-EGFP-4B1 compared to cells transfected with the control plasmid, its levels were 7 times lower than 12-HETrE levels (Figure 3D), suggesting that 12-HETrE is the primary arachidonic acid metabolite of the corneal CYP4B1.

Corneal epithelial transfection of CYP4B1 *in vivo* induces corneal neovascularization.

In addition, we examined whether corneal epithelial transfection can be achieved *in vivo* and determined the duration of EGFP expression within the cornea. As described in Methods, the expression vector pIRES2-EGFP was inserted into the limbus over 360^{o} followed by a topical application of the remaining plasmid solution. No significant signs of inflammation or corneal neovascularization were seen by biomicroscopic examination of the treated eyes over the 6-day period of this experiment. Eyes were excised and corneas processed for EGFP detection by fluorescence and immunohistochemistry 3 and 6 days after treatment. The results shown in

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Figure 4 indicate that the plasmid inserted circumferentially into the limbus effectively transduced EGFP expression in the corneal epithelium, which lasted for up to 6 days (Figure 4B and C). A similar EGFP expression pattern was also observed in corneas transfected with pIRES2-EGFP-4B1 (Figure 4 E and F). However, in comparison with eyes transfected with the control plasmid, eyes transfected with the pIRES2-EGFP-4B1 demonstrated increase in limbal vasodilation and, more importantly, corneal neovascularization, as compared to eyes transfected with pIRES2-EGFP (Figure 5A and B). Figure 5C depicts a representative picture of blood vessels penetrating from the limbus into the avascular cornea of eyes transfected with pIRES2-EGFP-4B1. This neovascularization response was hardly detected in eyes transfected with pIRES2-EGFP (not shown). This response was further quantified and the results clearly demonstrated a 4-fold difference in the number of neovessels penetrating the cornea (Figure 5D).

In vivo transfection of pIRES2-EGFP-4B1 increases corneal 12-HETrE production and neovascularization

To the extent that CYP4B1 is an injury-inducible CYP whose catalytic activity includes the oxidation of arachidonic acid to 12-HETrE, a potent angiogenic eicosanoid, increased expression of CYP4B1 protein might result in increased angiogenic response of corneal-limbal explants. Corneal limbal explants were taken from eyes 2 days after transfection with either pIRES2-EGFP-4B1 or pIRES2-EGFP or the vehicle control, and their capacity to produce a capillary-like network when grown on Matrigel was examined over a 7-day-period. Figure 6 shows representative photographs of the angiogenic response in these cultures. Explants from control corneas or corneas transfected with pIRES2-EGFP exhibited only rare (1 out of 5 preparations) sprouts (Figure 6A and B), whereas explants from corneas transfected with pIRES2-EGFP-4B1 showed a prominent angiogenic response

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in all preparations as evidenced by a marked increase in capillary-like tube formation (Figure 6, C and D). The origin of the cells sprouting from the explants was examined using the lectin *Ulex europeus*, which specifically binds to endothelial cells. As seen in Figure 6 (E and F), the majority (75±20%, n=10) of cells forming capillary tube-like networks were stained with *Ulex europeus*, indicating that the sprouting cells were primarily endothelial cells.

Quantitative analysis of the angiogenic response is shown in Figure 7. Capillary-like tube formation as measured by total capillary length, increased by 3-fold in corneal explants from eyes transfected with pIRES2-EGFP-4B1 as compared to explants from pIRES2-EGFP- transfected eyes 5 and 7 days in culture. Explants that were not transfected with plasmids (sham-vehicle transfected) showed similar angiogenic activity as with pIRES2-EGFP-transfected explants. That the increased expression of CYP4B1 contributed to the angiogenic activity was further confirmed using a CYP selective inhibitor. Addition of 17-ODYA (10 μM) to the culture medium of pIRES2-EGFP-4B1-transfected corneas blocked the angiogenic activity by 78, 80 and 70% at days 3, 5 and 7, respectively (Figure 8), suggesting that CYP4B1 activity underlies the increased angiogenic activity. More importantly, the inhibitory effect of 17-ODYA was reversed by the addition of 12-HETrE (Figure 8), further suggesting the involvement of 12-HETrE in mediating the angiogenic response displayed by pIRES2-EGFP-4B1-transfected corneas. Indeed, GC/MS analysis of the culture medium indicated that pIRES2-EGFP-4B1-transfected corneas produced significantly more 12-HETrE than pIRES2-EGFP- or sham- transfected explants (Figure 9).

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Discussion

CYP-derived eicosanoids are recognized as important autocrine and paracrine mediators of cell function. They have been implicated in the regulation of cell proliferation and differentiation, vascular tone, ion transport mechanisms, inflammation and angiogenesis (Kroetz and Zeldin, 2002; Roman, 2002). This diversity of biological activities stems from numerous CYP isoforms that exhibit cell-specific localization and regulation and have the ability to catalyze arachidonic acid oxygenation to an array of structurally different metabolites (Capdevila and Falck, 2001). 12-HETrE is one of these CYP-derived arachidonic acid metabolites; it is produced by the corneal epithelium in response to injury and exhibits powerful stereospecific inflammatory properties including angiogenic activity *in vitro* and *in vivo* (Laniado Schwartzman et al., 1997b). We have previously identified the inducible CYP in the corneal epithelium as CYP4B1 and demonstrated a correlation between its increased expression, 12-HETrE production and the inflammatory response in eyes exposed to hypoxic injury (Mastyugin et al., 1999; Mastyugin et al., 2001). The present study provides strong evidence that CYP4B1 expression in the corneal epithelium is associated with increased angiogenic activity, presumably via the production of 12-HETrE, an activity clearly detected for the expressed CYP4B1 protein *in vitro*.

We cloned the full-length cDNA of the corneal CYP4B1 into a bicistronic vector allowing the simultaneous expression of EGFP and CYP4B1. Such a feature was essential for evaluation of expression efficiency since an antibody against CYP4B1 is not available. Using EGFP as a marker, we were able to demonstrate high transfection efficiency for this bicistronic expression plasmid in RCE cells *in vitro*. The functional expression of CYP4B1 following transfection was determined by measuring the putative catalytic activity of CYP4B1, i.e., oxidation of arachidonic acid to 12-HETrE. This activity was ascribed to the corneal CYP4B1 after numerous correlative studies

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(Conners et al., 1995a; Vafeas et al., 1998) as well as studies using inhibitors of CYP-derived arachidonic acid metabolism (Stoltz et al., 1994; Mastyugin et al., 1999). The results described here are the first to indicate that CYP4B1 catalyzes the oxidation of arachidonic acid to 12-HETrE. Thus, cultured cells originating from the corneal epithelium, the site of synthesis of 12-HETrE, have little capacity to produce 12-HETrE. It is well known that CYP expression and activity rapidly decline upon culture (Paine, 1990; Lin et al., 1995) and, in as much as 12-HETrE synthesis is a CYP-derived reaction, it is not surprising that cultured RCE cells exhibited low levels of 12-HETrE production. Transfection of RCE cells with plasmid containing the CYP4B1 cDNA resulted in higher capacity to produce 12-HETrE. In cells transfected with pIRES2-EGFP-4B1, the levels of 12-HETrE produced following addition of exogenous arachidonic acid or stimulation of endogenous arachidonic acid release, were 5-fold higher than those in cells transfected with the control plasmid pIRES2-EGFP. Moreover, the production of 12-HETrE in these cells was inhibited by 17-ODYA and was not affected by either indomethacin or NDGA, further establishing 12-HETrE as a CYP-derived eicosanoid in corneal epithelial cells. Oxidation of arachidonic acid to 12-HETrE may be a unique feature of the corneal CYP4B1 since the lung CYP4B1 has been identified as a short fatty acid hydroxylase with little affinity for arachidonic acid (Fisher et al., 1998). The mechanism of CYP reactions with olefins typically involves the formation of an epoxide. Thus, the initial step of oxygenation would be the formation of an 11,12-EET intermediate. Alternatively, CYP can directly oxidize arachidonic acid to 12-HETE via a lipoxygenase-like reaction without an intermediate epoxide with the bioactive R enantiomer as the predominant stereoisomer (Capdevila et al., 1986; Keeney et al., 1998). The formation of 12(R)-HETrE would require additional rearrangement of 11,12-EET, either through direct epoxide rearrangement to an isomeric 12-keto-eicosatrienoic acid or through oxidation of 12(R)-HETE to 12-keto-HETE, an intermediate that has been identified in

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incubates of corneal microsomes with arachidonic acid and NADPH (Nishimura et al., 1991; Yamamoto et al., 1994).

The finding that CYP4B1 transfected RCE cells produced 12-HETrE was seminal to our attempt of linking 12-HETrE production to inflammation and neovascularization. Using the same expression plasmid, we showed that local transfection of pIRES2-EGFP-4B1 by injecting directly into the limbus, resulted in a significant transgene expression within the corneal epithelium, with little expression in the surrounding tissues. Thus, this method of transfection directed the CYP4B1 transgene into the site of its expression within the ocular surface. Moreover, EGFP immunofluorescence was detected over a period of 6 days. A similar method of plasmid transfection was used in mice demonstrating a transgene expression within the corneal tissues that was maximal by 24 h and was evident up to 5 days (Stechschulte et al., 2001; Moore et al., 2002). A corneal neovascular response ensued after transfection of CYP4B1 that was not apparent in the ocular surface of eyes transfected with the control plasmid not containing the CYP4B1 cDNA. While a cause-effect relationship was not established in this study, the difference in this response between experimental and control plasmids suggested that CYP4B1 drives the neovascular response, presumably via production of angiogenic eicosanoids such as 12(R)-HETrE.

Indeed, one of the main characteristics of 12(R)-HETrE is its powerful angiogenic activity (Masferrer et al., 1991; Mezentsev et al., 2002). This activity is believed to be mediated via its binding to a specific, high affinity, binding site on limbal microvessel endothelial cells (Stoltz and Laniado Schwartzman, 1997) and its ability to elicit cellular transduction pathways (NFκB and p42/44 MAPK) that culminate with the induction of angiogenic proteins such as VEGF and IL-8 (Laniado-Schwartzman et al., 1995; Stoltz et al., 1996; Mezentsev et al., 2002). Thus, 12(R)-HETrE is a corneal epithelial-derived angiogenic factor whose synthesis is induced

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in response to injury and which in a paracrine manner acts on the limbal vessels to activate endothelial cells via a specific receptor/binding site into the angiogenic phenotype.

To provide a link between CYP4B1, 12-HETrE and the angiogenic response, measurements were done in a setting where sufficient materials could be obtained for quantitative analysis. Thus, we proceeded with experiments using limbal-corneal explants from transfected eyes and evaluated their angiogenic activity as correlated to 12-HETrE producing capacity to establish a cause-effect relationship. The results demonstrated that explants from eyes transfected with the CYP4B1 containing plasmid exhibited a much higher angiogenic activity than those from eyes transfected with the control plasmid. The increased angiogenic activity was accompanied with an increase in the levels of the angiogenic 12-HETrE. These results indicate that increased expression and activity of the CYP4B1 contributed to the increased angiogenic capacity. The fact that this angiogenic activity was attenuated in the presence of the CYP metabolic inhibitor, 17-ODYA, and that addition of 12-HETrE reversed the inhibitory action of 17-ODYA, further implicates CYP4B1 and its catalytic product, 12-HETrE, in the angiogenic process of the ocular surface.

In summary, this study is the first to demonstrate that the corneal CYP4B1 catalytic activity includes the synthesis of 12-HETrE. The results strongly implicate the corneal CYP4B1 as a component of the inflammatory cascade initiated by injury of the ocular surface. Recent interest in inhibiting neovascularization has focused on VEGF (Witmer et al., 2003). Our previous studies showing that VEGF mediates the angiogenic activity of 12-HETrE (Mezentsev et al., 2002) and that injury to the cornea is associated with a concerted induction of CYP4B1 and VEGF (Mastyugin et al., 2001), together with the results presented here, raise the possibility of a new approach to the problem of preventing corneal neovascularization that targets the CYP4B1-

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12-HETrE system. To this end, we have shown the presence of the cytochrome P450 system in human corneas (Abraham et al., 1987) and documented the presence of 12-HETrE in human tears (Mieyal et al., 2001). More importantly, ocular surface inflammation of diverse etiologies was associated with increased 12-HETrE tear levels. 12-HETrE's levels were especially high in contact lens-related inflammation in which all patients had slept with their lenses in place and had an acute ocular surface inflammation on waking. Hypoxia, which is believed to be a major factor in contact lens-induced inflammation, has been shown to induce CYP4B1 expression and stimulate 12(R)-HETrE synthesis (Mastyugin et al., 1999). Herpes simplex keratitis and corneal foreign body, both injuries to the corneal epithelium, also had marked elevations of 12-HETrE. The significant increase of 12-HETrE in the tear film of inflamed human eyes supports a positive correlation between 12-HETrE production and ocular surface inflammation that is consistent with the relationship previously established in rabbit models and with its hypothesized role as a *paracrine* mediator of inflammation.

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Footnotes

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Legends for Figures

Figure 1. EGFP fluorescence in RCE cells transfected with the pIRES2-EGFP-4B1 construct. **A)** Control vehicle (lipofectamine)-transfected cells; **B)** pIRES2-EGFP-transfected cells; **(C)** pIRES2-EGFP-4B1-transfected cells. Photographs were taken at a magnification of X40.

Figure 2: HPLC elution profiles of arachidonic acid metabolites in transfected RCE cells. Cells transfected with pIRES2-EGFP or pIRES2-EGFP-CYP4B1 were incubated with ^{14}C-arachidonic acid (1 µCi, 20 nmols) and radiolabeled metabolites extracted and separated by HPLC as described in Methods. (A) 20-HETE standard; (B) 12-HETE standard; (C) 12-HETrE standard; (D) pIRES2-EGFP-4B1-transfected cells; and (E) pIRES2-EGFP-transfected cells.

Figure 3: GC/MS analysis of 12-HETrE produced by pIRES2-EGFP or pIRES2-EGFP-4B1-transfected RCE cells following incubation with A23187 (5 µM). Representative SIM-GC/MS chromatograms showing the elution profile of mono-HETEs at m/z 391 (A), the internal standard $^{2}H_{3}$-12-HETrE at m/z 396 (B) and the cell-produced 12-HETrE at m/z 393 (C). (D) GC/MS quantitative analysis; results are mean±SE, n=3, *$p<0.05$.

Figure 4. EGFP immunofluorescence in corneal sections from control untreated eyes (A, D); eyes 3 and 6 days after transfection of pIRES2-EGFP (B and C, respectively); and eyes 3 and 6 days after transfection of pIRES2-EGFP-4B1 (E and F, respectively). Pictures were taken at magnification of 20X. Strong fluorescence intensity is seen in the corneal

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epithelium layers with moderate intensity in the stroma and weak intensity in the endothelium.

Figure 5: Corneal neovascularization in transfected eyes. Representative photographs (X16) taken by slit lamp microscope of eyes 3 days after transfection with pIRES2-EGFP (A) and pIRES2-EGFP-4B1 (B). A representative area of corneal neovascularization in pIRES2-EGFP-4B1 transfected eyes at X40 (C). (D) Quantitative analysis of corneal neovascularization. The length (mm) of penetrating blood vessels was normalized to corneal area (1 mm^2); results are mean±SE, n=6, *$p<0.01$.

Figure 6: Sprouting of neovessels in corneal limbal explants from rabbit eyes 2 days after transfection with pIRES2-EGFP or pIRES2-EGFP-4B1. Phase contrast photographs were taken 3 days after explants were placed in culture. **(A)** Explant from a nontransfected, saline-injected eye**; (B),** explant from a pIRES2-EGFP-transfected eye; (**C and D**), explants from a pIRES2-EGFP-4B1-transfected eye. (**E** and **F),** representative phase contrast and fluorescence photographs, respectively, of sprouting neovessels treated with the lectin *Ulex europeus* (10X).

Figure 7: Angiogenic activity of limbal-corneal explants. Angiogenic activity of explants from saline-treated eyes and eyes transfected with pIRES2-EGFP and pIRES2-EGFP-4B1 was measured as the total length of the capillary-like tube network. Results are the mean±SE; n=5; *$p<0.05$ from sham or pIRES2-EGFP-transfected corneas.

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Figure 8: Effect of 17-ODYA and 12-HETrE on the angiogenic activity of limbal-corneal explants from pIRES2-EGFP-4B1-transfected eyes. Explants were cultured in the absence and presence of 17-ODYA (10 μM) with and without addition of 3 nmols of 12-HETrE. Results are the mean±SE; n=5; *$p<0.05$ from control.

Figure 9: Production of 12-HETrE by corneal-limbal explants at day 5 and 7 in culture. Culture medium was collected and 12-HETrE was extracted and subjected to GC/MS analysis. Results are expressed as pg 12-HETrE per sample (equivalent to one quarter of the cornea) and are mean±SE, n=4, *$p<0.05$ from sham or pIRES2-EGFP-transfected corneas.

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Figure 1

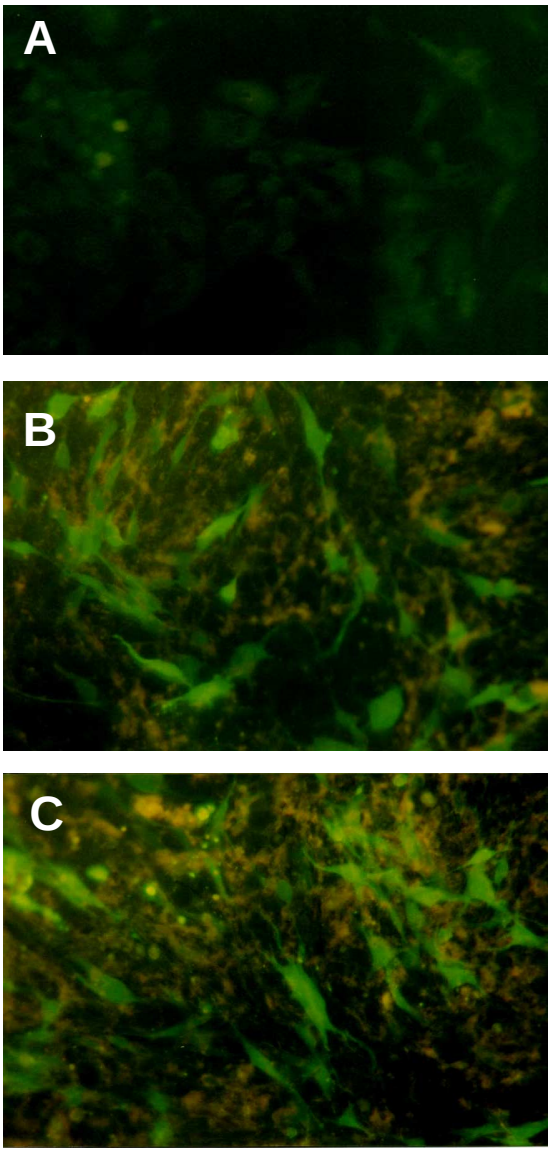

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Figure 2

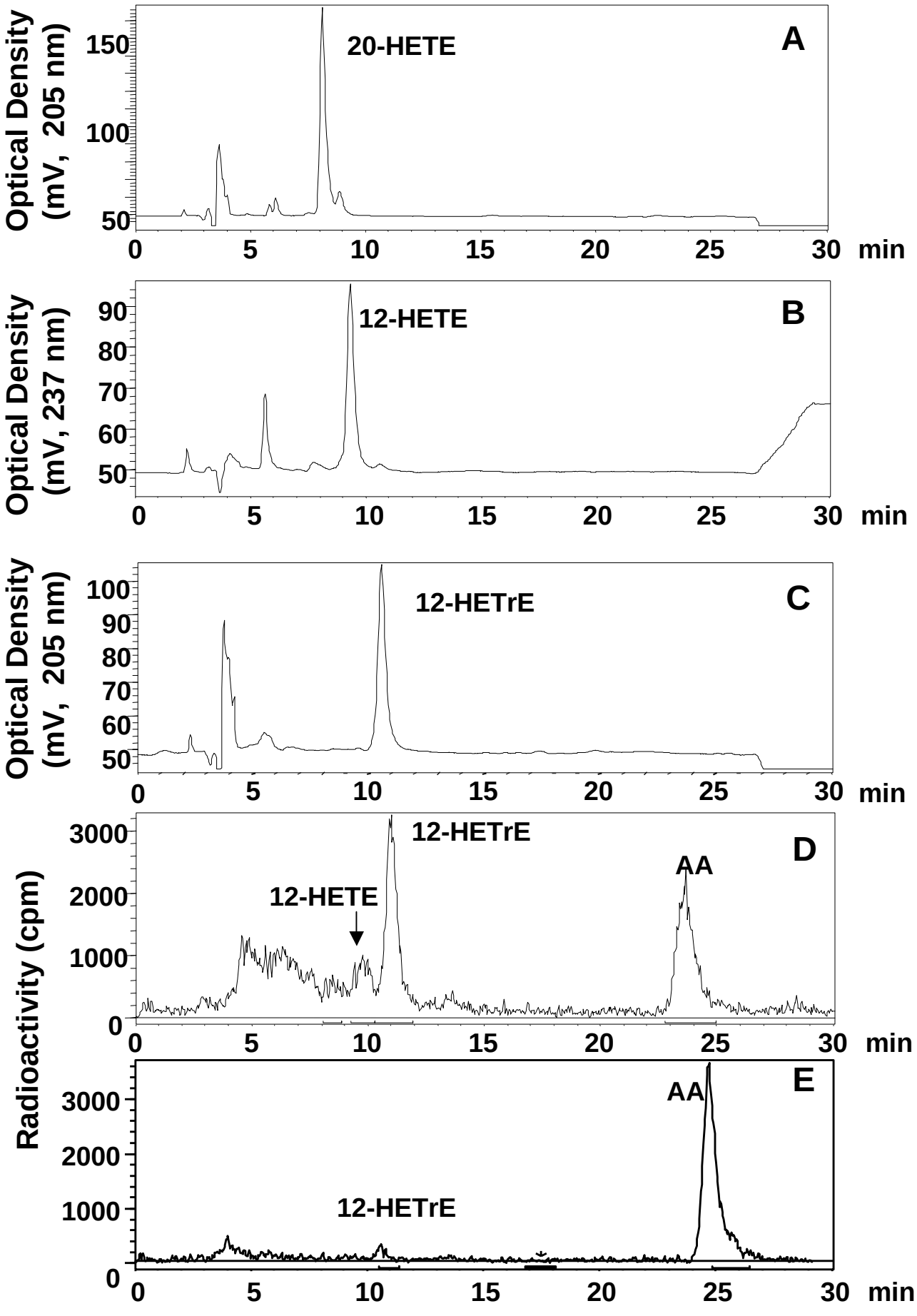

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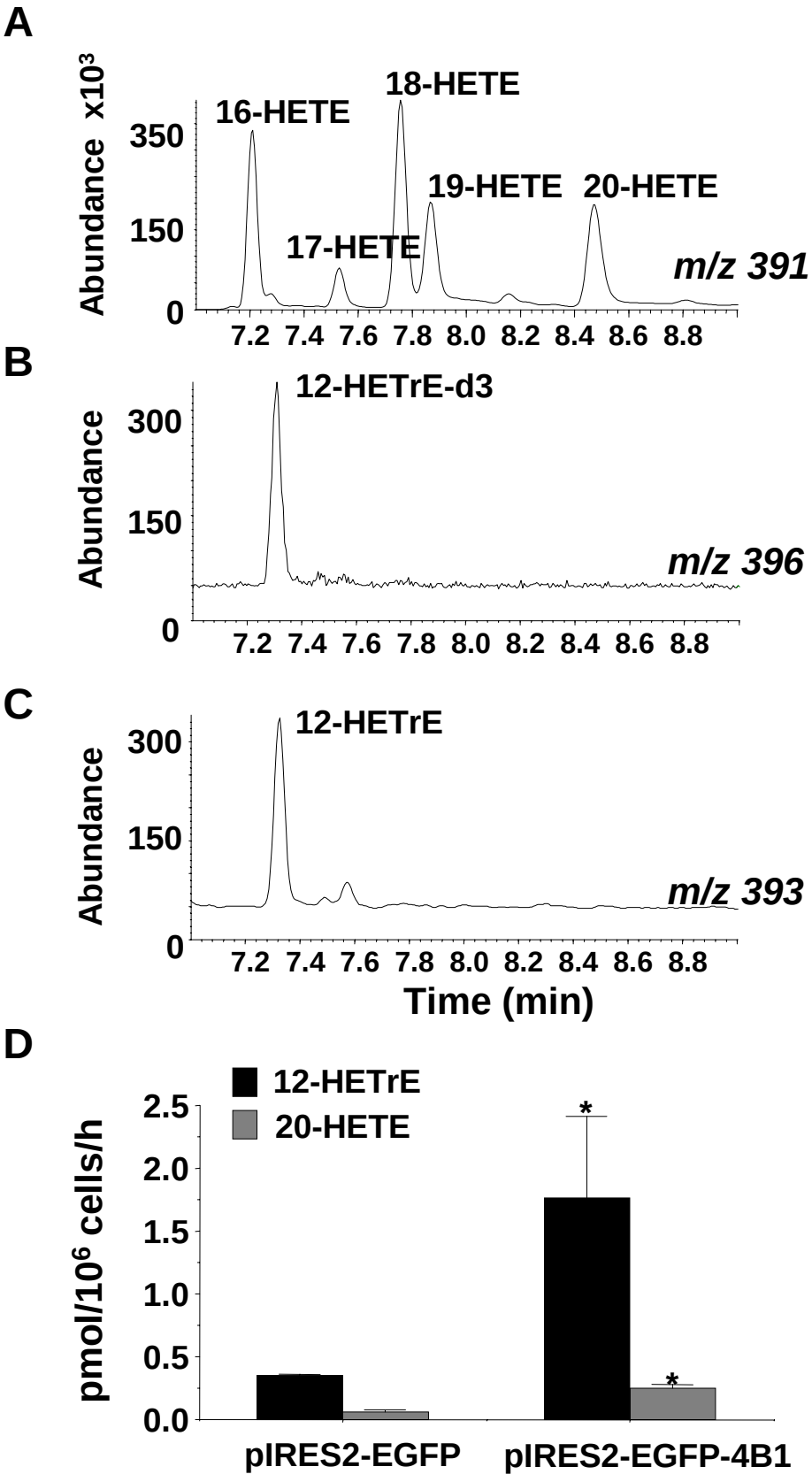
Figure 3



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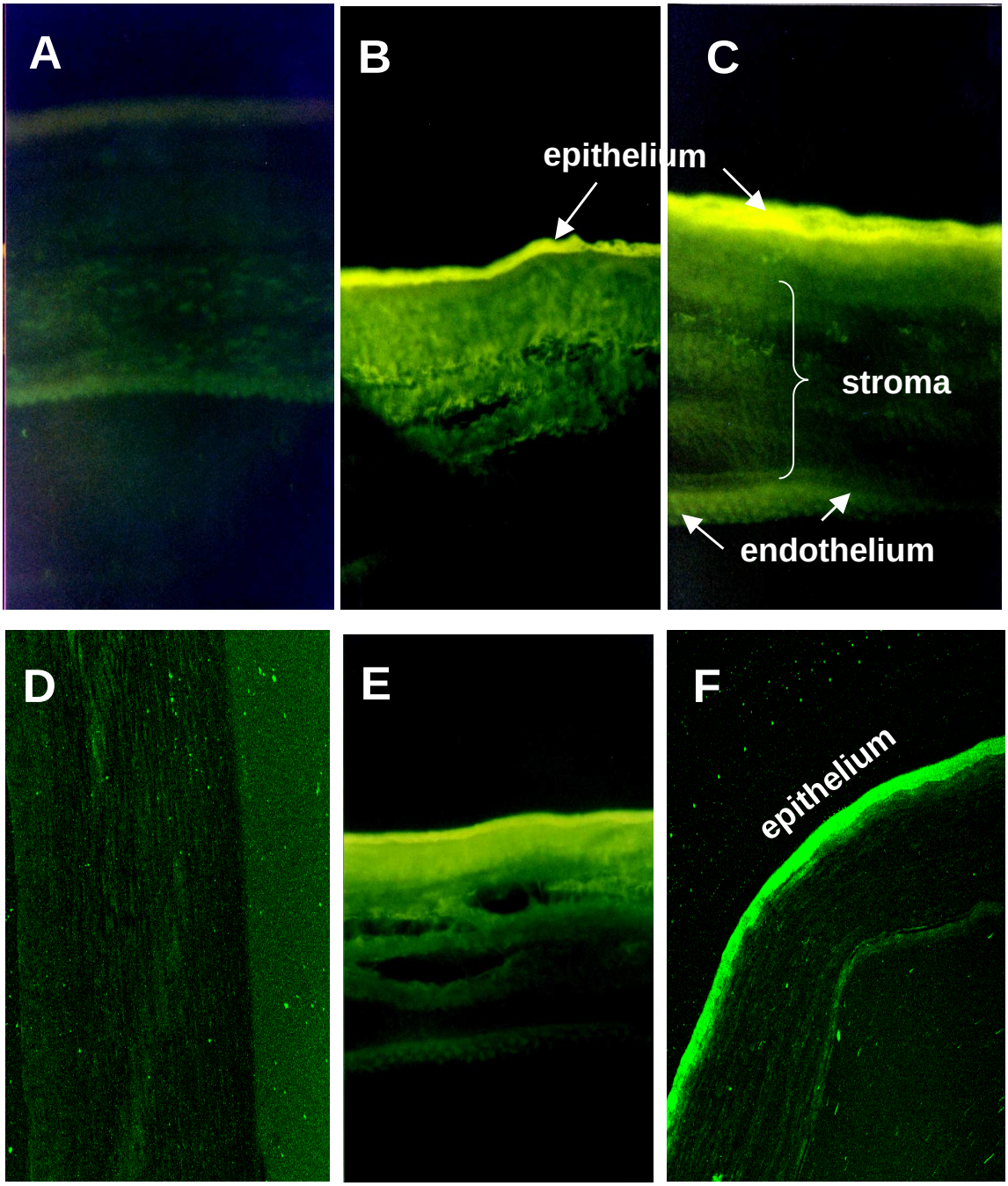

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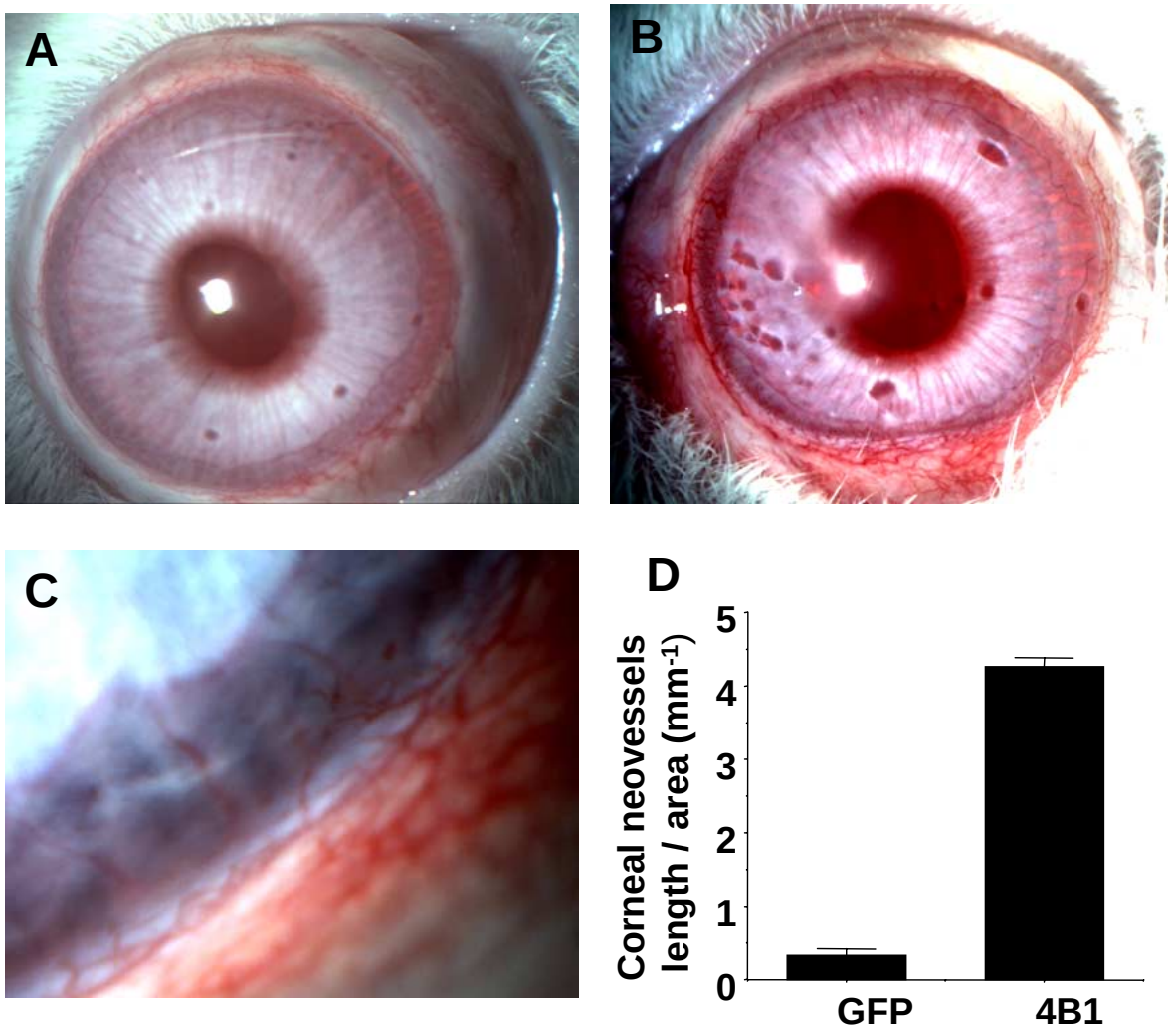

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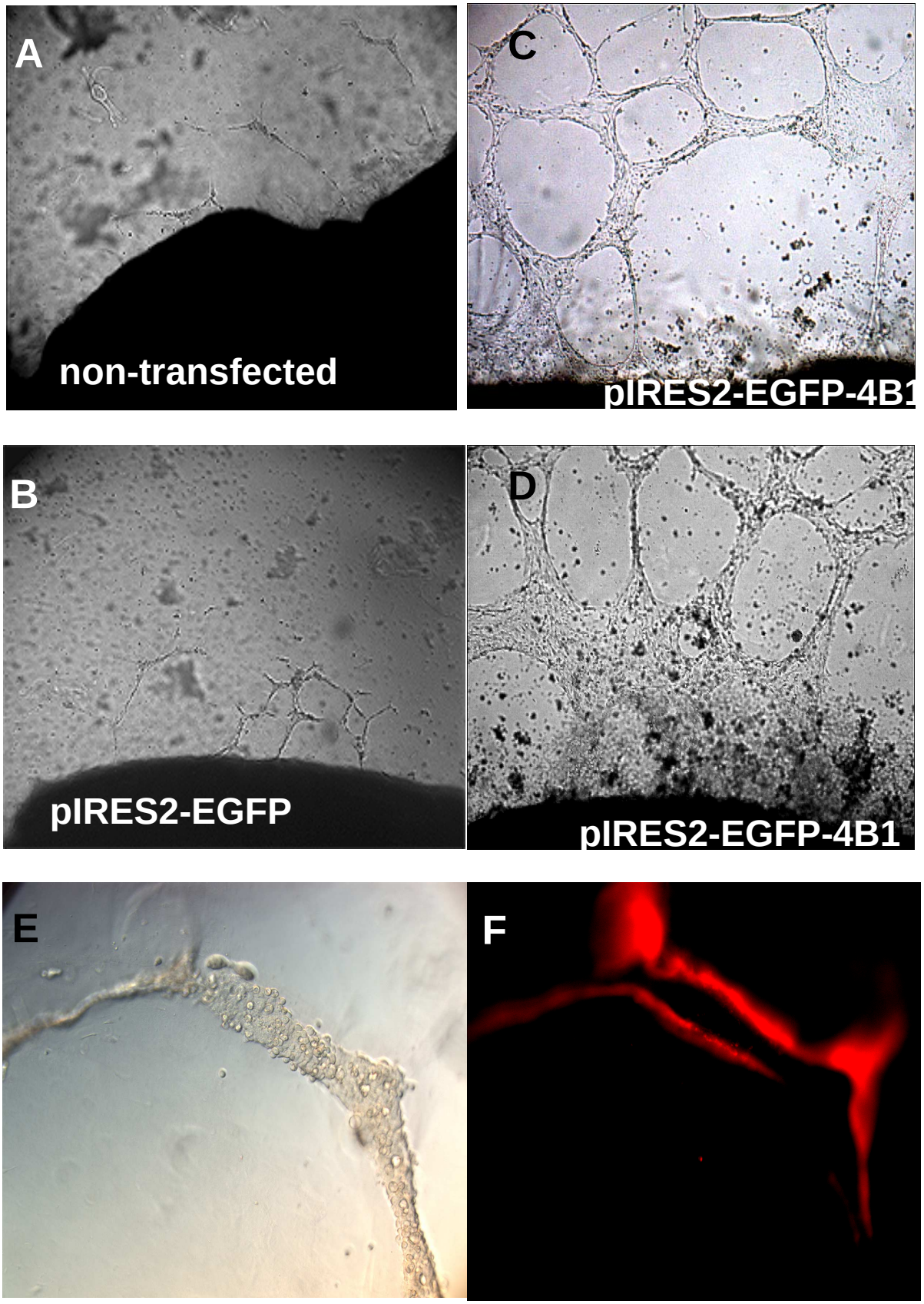

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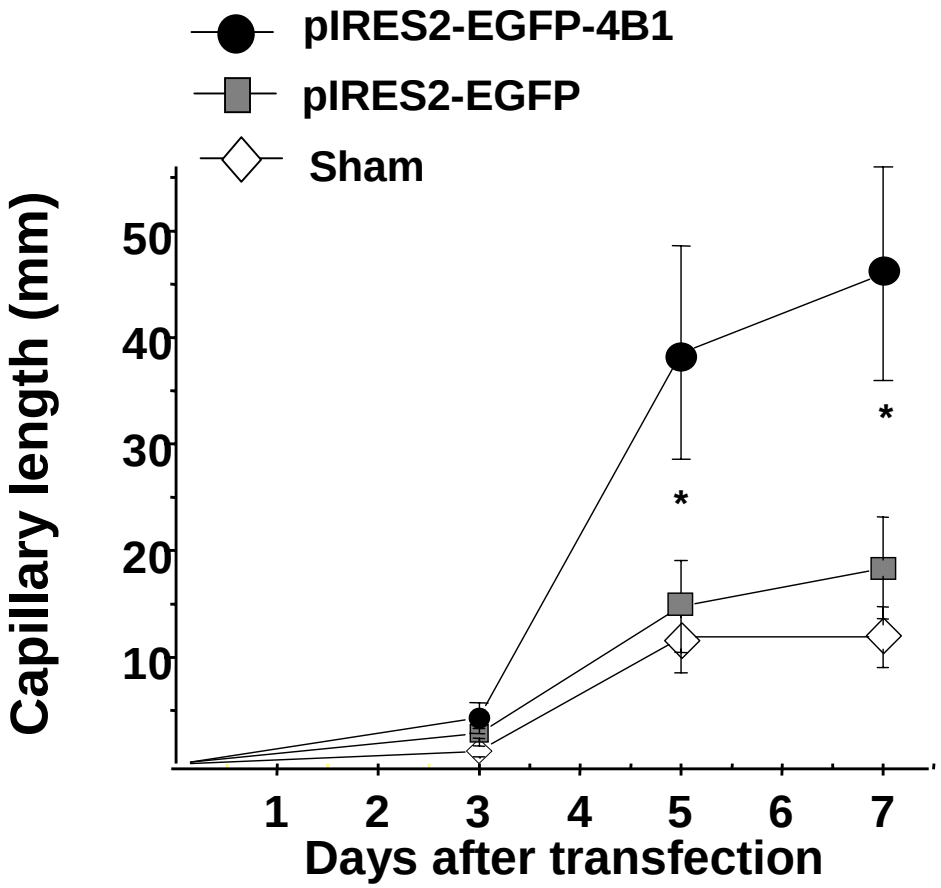

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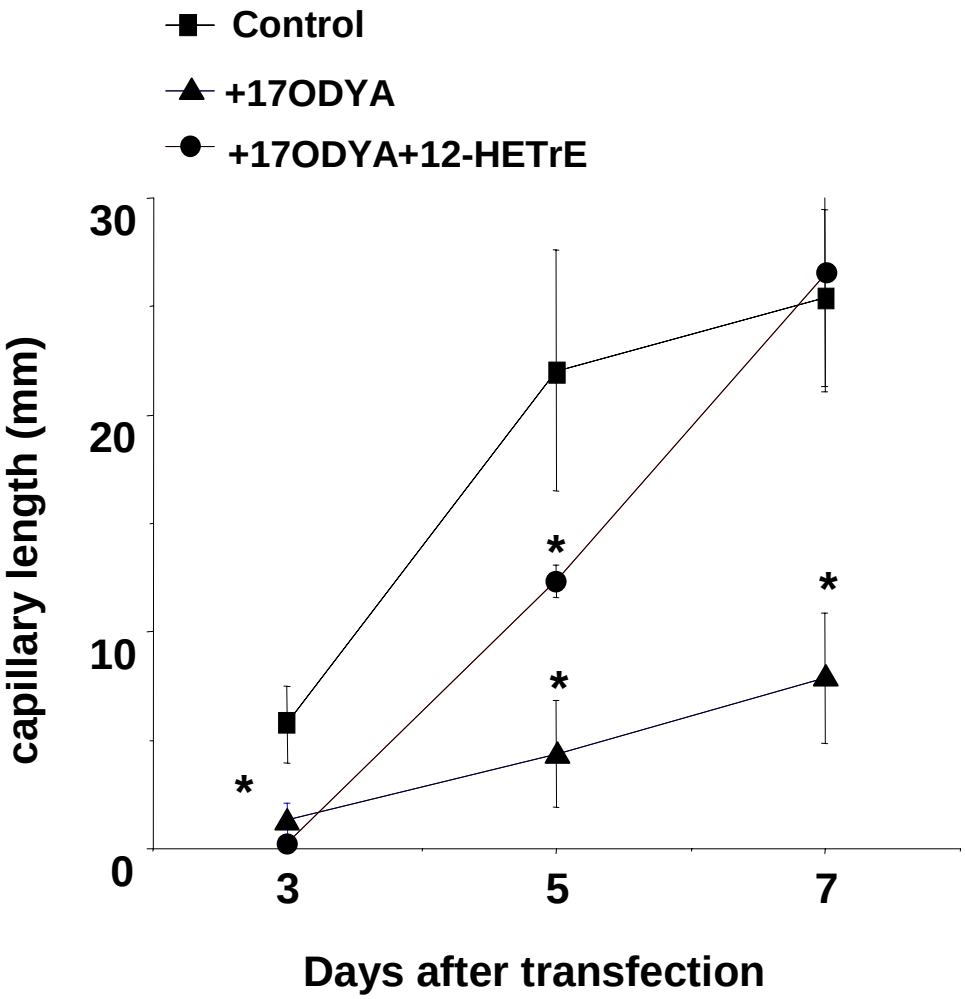

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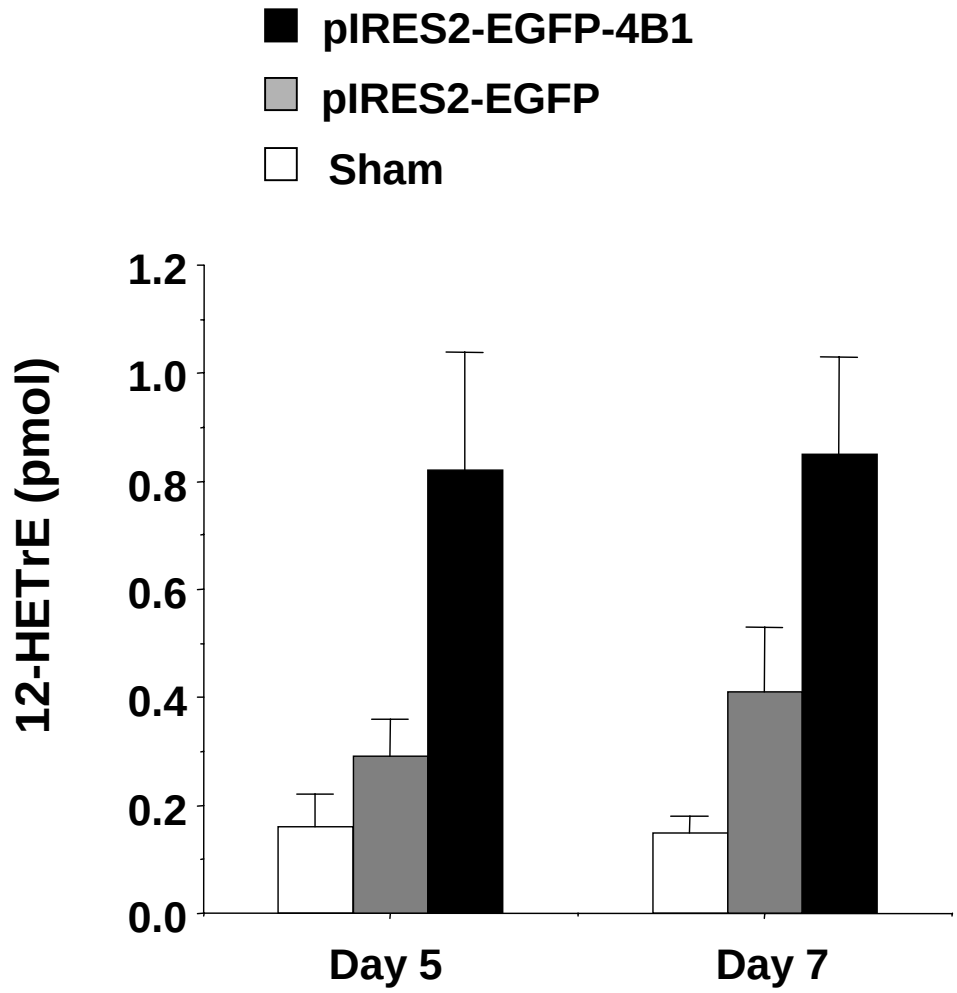

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