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Procollagen C-proteinase Enhancer 1 (PCPE-1) Functions as an Anti-angiogenic Factor and Enhances Epithelial Recovery in Injured Cornea

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Abstract

Procollagen C-proteinase enhancer 1 (PCPE-1) has been characterized as a protein capable of enhancing the activity of bone morphogenetic protein 1/tolloid-like proteinases (BTPs) in the biosynthetic processing of C-propeptides from procollagens I–III. This processing step is thought necessary to the formation of collagen I–III monomers capable of forming fibrils. Thus, PCPE-1 is predicted to play an important role in scarring, as scar tissue is predominantly composed of fibrillar collagen. Corneal scarring is of great clinical importance, as it leads to loss of visual acuity and, in severe cases, blindness. Here, we have investigated a possible role for PCPE-1 in corneal scarring. Although differences in corneal opacity associated with scarring following injury of *Pcolce*^{−/−} and WT mice using full-thickness excision or alkali burn models of corneal injury were not grossly apparent, differences in procollagen I processing levels between *Pcolce*^{−/−} and WT primary corneal keratocytes were consistent with a role for PCPE-1 in corneal collagen deposition. An unexpected finding was that neoangiogenesis, which follows alkali burn cornea injury, was strikingly increased in *Pcolce*^{−/−} cornea, compared to WT. A series of aortic ring assays confirmed the anti-angiogenic effects of PCPE-1. Another unexpected finding was of abnormalities of epithelial basement membrane and of re-epithelialization following *Pcolce*^{−/−} corneal injury. Thus, PCPE-1 appears to be of importance as an anti-angiogenic factor and in re-epithelialization following injury in cornea, and perhaps in other tissues as well.

Keywords

angiogenesis; collagen; cornea; epithelium; extracellular matrix; PCOLCE1; BMP1

Introduction

Procollagen C-proteinase enhancer 1 (PCPE-1) is a secreted 55-kDa glycoprotein that can enhance the activity by which bone morphogenetic protein 1/tolloid-like proteinases (BTPs) cleave procollagen C-propeptides, thereby producing mature types I–III collagen monomers capable of forming fibrils (Adar, et al., 1986, Steiglit, et al., 2002, Steiglit, et al., 2006).

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Consistent with a role in collagen biosynthetic maturation, increased PCPE-1 expression is reported to accompany fibrosis onset in animal models of hepatic cirrhosis (Hassoun, et al., 2016, Ippolito, et al., 2016, Ogata, et al., 1997), cardiac fibrosis (Kessler-Icekson, et al., 2006, Yu, et al., 2013), and skeletal muscle fibrosis (Hassoun, et al., 2016); and in human dermal scarring (Wong, et al., 2014). Differences in PCPE-1 expression levels in quiescent versus proliferating smooth muscle cells (Kanaki, et al., 2000), and the induced anchorage-independent growth of cultured fibroblasts in which the PCPE-1 gene has been disrupted (Masuda, et al., 1998), have also suggested a possible role for PCPE-1 in cell proliferation control. It was also recently reported that PCPE-1 can bind the anti-angiogenic factor endostatin and can itself inhibit angiogenesis in an *in vitro* assay (Salza, et al., 2014). In addition, PCPE-1 may be necessary for lumen formation in a model of *in vitro* angiogenesis, although the latter effect may be secondary to PCPE-1's role in collagen biosynthesis and consequent effects on extracellular matrix (ECM) stiffness (Newman, et al., 2011).

A related gene product, PCPE-2, shares only 43% amino acid sequence identity with PCPE-1, but has an identical domain structure (Xu, et al., 2000) and can enhance BTP procollagen C-proteinase activity (Steiglitz, et al., 2002). Consistent with this activity, mice null for the PCPE-2 gene, *Pcolce2*, show reduced myocardial collagen deposition in an *in vivo* model of pressure overload hypertrophy (Baicu, et al., 2012). Interestingly, *Pcolce2*^{-/-} mice also show a deficit in conversion of pro-apoA-I to mature apoA-I, and thus, a deficit in HDL biogenesis, presumably because PCPE-2 can enhance the biosynthetic cleavage of pro-apoA-I by BTPs (Francone, et al., 2011).

We previously demonstrated that ablation of the murine PCPE-1 gene, *Pcolce*, results in viable mice with bones that are abnormally massive and have altered geometries, to compensate for inferior material properties (Steiglitz, et al., 2006). Collagen fibrils in both *Pcolce*^{-/-} bone and tendon were found to have abnormal morphologies that included irregular, scalloped profiles and abnormal branching (Steiglitz, et al., 2006). Interestingly, *Pcolce*^{-/-} bone and tendon collagen fibril morphological abnormalities are more pronounced than those of *Bmp1*^{-/-}/*Tll1*^{-/-} doubly null embryos, in which the BTPs BMP1 and tollid-like 1 (TLL1) have both been ablated (Muir, et al., 2014, Pappano, et al., 2003, Steiglitz, et al., 2006). This is despite the fact that retained procollagen 1 C-propeptides are readily detected in the fibrils of *Bmp1*^{-/-}/*Tll1*^{-/-} tissues, but not in the collagen fibrils of *Pcolce*^{-/-} tissues, indicating that procollagen C-proteinase activity is more compromised in the former than in the latter (Muir, et al., 2014, Pappano, et al., 2003, Steiglitz, et al., 2006). Thus, we previously suggested (Steiglitz, et al., 2006) that the profound and varied effects of PCPE-1 ablation on *Pcolce*^{-/-} tissues cannot be explained by mere diminution of collagen biosynthetic processing. The latter suggestion further suggests additional PCPE-1 *in vivo* roles.

To obtain further insights into PCPE-1 *in vivo* roles, we have begun examining characteristics of *Pcolce*^{-/-} soft tissues. Evidence of PCPE-1 corneal expression (Kessler, et al., 1990), the facility for observation and experimental manipulation of this tissue, and the profound effects that corneal scarring, associated with abnormal collagen deposition, has on vision led to the current study on effects of PCPE-1 ablation on responses to corneal injury.

Materials and methods

Animals and corneal wound healing models

C57BL/6 mice 8 – 12 weeks of age were housed in a 12 h light/12 h dark cycle environment, with access to food and water at all times. Equal numbers of male and female mice were used. Corneal wound healing experiments were performed under general and topical anesthesia. After iris dilatation with 1% atropine sulfate (MED-PHARMEX), 1 drop/10 min was administered during the 30 min prior to surgery. Mice also received an intraperitoneal injection of Ketamine 100 mg/kg (MidWest Veterinary Supply) and 10 mg/kg Xylazine (LLOYD laboratories) and a drop of topical anesthesia (proparacaine hydrochloride 0.5%; AKORN) in the left eye. A subcutaneous injection of Meloxicam (1 mg/kg; Norbook laboratories) at the time of surgery was also used. All surgical procedures were carried out on the left eye, and by the same person, to insure consistency among experiments. The 1-mm penetrating keratectomy healing model was performed at the center of the cornea using ophthalmologic microsurgical tools under a stereomicroscope, as described (Galiacy, et al., 2011, Malecaze, et al., 2014, Massoudi, et al., 2012). To prevent infection, an ophthalmic antibiotic ointment (Akorn, active ingredients neomycin, polymyxin, B sulfate and bacitracin zinc) was applied to the left eye (3 times/day) for 4 days post-surgery and a 1% solution of atropine sulfate was also applied 3 times/day for 4 days post-surgery to prevent adhesions between the cornea and iris during healing. Fluorescein eye stain (I-Glo, Jorvet) was applied once per week post surgery to visualize the extent of the wound. The Corneal epithelial abrasion model was conducted after demarcating the central corneal with a 2 mm trephine. The delimited area was then removed with a Beaver Sclerotome Blade (Azmedsurgical). The alkali burn model was performed with a 2 mm diameter Whatman paper disk soaked in 1M NaOH and placed in the center of the cornea for 30 sec. The eye was rinsed immediately after with 10 ml phosphate buffered saline (PBS). To prevent the cornea from drying after epithelial abrasion or alkali burn, an ophthalmic ointment (Akorn) was applied to the injured eye. Post-operatively, all mice from the 3 corneal injury models received acetaminophen-supplemented water (0.5 mg/ml) immediately, and continuing ad libitum, and a subcutaneous injection of Meloxicam (1 mg/kg) at day 1 post-injury to control pain. Mouse corneas were observed at specific time points during healing for opacity and epithelial recovery. Animals were sacrificed at specific time points post injury, the eyes removed, embedded in OCT, immediately frozen and stored at –80°C for cryosectioning, or fixed 24 h in 10% formalin and paraffin embedded, for histological analysis.

All mice were housed and treated in accordance with NIH guidelines, using protocols approved by the Research Animal Resources Center of the University of Wisconsin-Madison.

Clinical observation and histopathology of corneas

Corneal opacity was measured under a dissection microscope as follows: grade 0, completely clear cornea; grade 1, trace opacity that did not interfere with visualization of fine iris detail; grade 2, moderate obscuration of the iris and lens; and grade 3, complete corneal opacification. Opacity grading was performed in a masked manner. Corneal re-epithelialization was evaluated under a dissection microscope by assessing the percentage of

cornea stained (not re-epithelialized) and not stained (re-epithelialized) with fluorescein dye (I-Glo, Jorvet). Quantitation of stained and unstained areas was performed using ImageJ software.

Mouse corneal primary fibroblast (keratocyte) culture

After sacrifice, eyes were removed and placed in ice-cold PBS containing 10 IU/ml penicillin-streptomycin. The cornea was isolated (without including the limbus) and the epithelium and endothelium were mechanically removed. Corneal stroma was then minced, placed on a tissue culture dish and let dry for a few minutes. Growth medium (Dulbecco's modified Eagle's medium, 10 IU/ml penicillin-streptomycin) supplemented with 10% fetal bovine serum was then added and the dish was incubated at 37°C. After 10 days, the corneal stroma explants were removed and the fibroblasts were cultured to confluence. For immunoblotting, cells were serum starved for 16 h, for PCPE-1, or for 4 or 6 h, for collagen $\alpha 1(I)$, in growth medium supplemented with 100 $\mu\text{g/ml}$ ascorbic acid (Sigma) and 100 μM modified Eagle's medium Nonessential Amino Acids (Cellgro). Conditioned media were collected and concentrated on Amicon Centrifugal Filters (Millipore) prior to immunoblotting.

Immunoblotting

Cornea was surgically removed, cleaned of external tissue (e.g. iris), and then ground in extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 0.5% NP40, and 0.5% deoxycholate) containing protease inhibitors (Complete/mini/EDTA-free, Roche). The supernatant was collected after centrifugation for 10 min at 15,682 $\times g$ and 4°C, mixed with 2X Laemmli buffer, and boiled with 2.5 % β -mercaptoethanol. Amounts of sample loaded on a 10% polyacrylamide gel were normalized to have similar amounts of loading control proteins α -tubulin or GAPDH. For keratocyte conditioned media (see above), samples were resolved on 10% or 6% SDS-PAGE gels for detecting PCPE-1 or collagen $\alpha 1(I)$ chains, respectively. Samples loaded were derived from equal volumes of conditioned media and subjected to SDS-PAGE, under reducing conditions, and immunoblotting. Blots were incubated overnight at 4°C with rabbit polyclonal antibodies to PCPE-1 (SAB2104455, Sigma-Aldrich, 1:1500), PCOLCE2 (ab156224, Abcam, 1:500), or the pro $\alpha 1(I)$ chain C-telopeptide or C-propeptide [antibodies LF67 and LF41, respectively kind gifts of Larry Fisher (Fisher, et al., 1995)], 1:5000], or were incubated 1h at room temperature with rabbit anti-GAPDH (G9545, Sigma-Aldrich, 1:10000) or mouse anti- α -Tubulin (clone DM1A, 05–829, Millipore, 1:10000). Secondary antibodies (goat anti-rabbit, 1:10000 and goat anti-mouse, 1:10000, Bio-Rad) were applied for 1h at room temperature.

Immunofluorescence

For cryosections the following primary antibodies and dilutions were used: rat monoclonal IgG1 anti-mouse PCPE-1 (MAB2239; R&D Systems, 1:100); rat monoclonal IgG2 anti-mouse F4/80 (clone BM8, eBioscience, 1:100); rat monoclonal IgG2 anti-mouse Ly6G (clone 1A8, BioLegend, 1:100); rabbit anti- $\alpha 1(I)$ collagen C-telopeptide (LF67, 1:400), rabbit anti-collagen VII NC1 domain (1:1000, a kind gift of Alexander Nyström). The following secondary antibodies and dilutions were used: Alexa Fluor 488, 546 or 594 goat anti-rat IgG; and Alexa Fluor 488 or 546 goat anti-rabbit IgG (Invitrogen Molecular Probes,

1:750). Briefly, for anti-PCPE-1 and anti-collagen I co-immunostaining, cryosections (5 μ m thick) were fixed at 4°C with 4% paraformaldehyde and permeabilized with triton X-100. Samples were then incubated with primary and secondary (2 and 1 h, respectively) antibodies at room temperature. For anti-collagen VII, primary antibody was incubated overnight at 4°C. For anti-F4/80 and anti-Ly6G immunostaining, samples were fixed with acetone and incubated with primary antibody overnight at 4°C. Secondary antibodies were applied for 1 h at room temperature in a dark humidified chamber. Nuclei were counterstained with diamidino-2-phenylindole (DAPI, Sigma) and samples were mounted with Mountant PermaFluor (Thermo Scientific).

For whole mounts, eyes were collected by enucleation, fixed 20 min in 4% paraformaldehyde at 4°C, rinsed 5 min in PBS and placed in cold 0.3% bovine serum albumin/PBS. Corneas were then surgically removed, taking care to include the limbus, with radial cuts to produce a “petal” shape to facilitate mounting. Corneas were then fixed 30 min with -20°C methanol, permeabilized 20 min with 1% Triton/phosphate buffered saline at 4°C, and blocked 1 h with 10% bovine serum albumin in 0.1% Triton/Tris buffered saline at room temperature. Primary antibody (rat anti-mouse CD31, BD Pharmingen; 1:100) was incubated on corneas overnight at 4°C. Corneas were rinsed and the secondary antibody (Alexa Fluor 488 goat anti-rat IgG; 1:200) was applied for 4 h at room temperature. Corneas were rinsed 4 $\times$ 15 min and mounted with PermaFluor (Thermo Scientific).

Immunohistochemistry

Paraffin embedded tissue sections were probed with rabbit monoclonal anti-human α -smooth muscle actin (5264-1, EPITOMICS, 1:8000) and LF67 (1:400). Sections (5 μ m thick) were deparaffinized and antigen retrieval was performed using sodium citrate. Primary antibodies were incubated overnight at 4°C and the secondary antibody, goat anti-rabbit, tagged with horseradish peroxidase (Bio-Rad, 1:1000), was incubated for 1h at room temperature.

Aortic Ring Assay

Aortic ring assays were performed as described (Baker, et al., 2012), with the following exceptions: Aortas were cut into ~0.8 mm sections, embedded in a rat tail collagen I (Millipore) gel, and cultured 8 days, with media changed at days 4 and 6 after embedding. The presence of endothelial cells in the aortic sprouts was confirmed by CD31 staining (not shown). Quantitation of numbers of angiogenic sprouts per ring was by counting under a dissecting microscope (WT, n= 30 rings; *Pcolce*^{-/-}, n= 23 rings). For treatment with recombinant human PCPE-1, a previously described cDNA insert encoding human PCPE-1 fused to a BM40 signal peptide (Steiglitz, et al., 2002), to optimize secretion, was PCR-amplified with primers that gave it a C-terminal His-tag, and was ligated into pcDNA4. The recombinant PCPE-1 was produced by transfection into human embryonic kidney 293 cells, and purified on a HisPur cobalt spin column (Thermo Scientific). For the aortic ring assay, recombinant PCPE-1 was then incorporated into the collagen I gel during its preparation and was also present in culture media at the indicated concentrations.

***In vitro* procollagen cleavage assay**

This assay was conducted essentially as described (Huang, et al., 2009). 300 ng human type I procollagen was incubated with 150 ng recombinant human PCPE-1 in a buffer containing 50 mM Tris-HCl, pH 7.5 and 150 mM NaCl. After 30 min incubation at 37° C, 100 mM CaCl₂ with/without 30 ng of mammalian tolloid (mTLD) was added to a final concentration of 5 mM CaCl₂. Reactions were stopped after 6 h at 37° C by adding SDS sample buffer. Proteins were then separated by SDS-PAGE on a 6% polyacrylamide gel, transferred to nitrocellulose membrane and probed with LF67 antibody (1:5000).

Statistical analysis

Analyses were performed with a 2-tailed Fischer's *t*-test. A *P* value < 0.05 was considered statistically significant. Data are reported as means ± SEM.

Results

PCPE-1, but not PCPE-2, is readily detectable in cornea

Although it has been reported that PCPE-1 is not readily detectable by immunostaining in uninjured mouse cornea (Malecaze, et al., 2014), immunoblotting in the present study readily detected PCPE-1 as a ~55 kDa band in WT mouse cornea, while its absence in *Pcolce*^{-/-} cornea demonstrated the specificity of the assay (Fig. 1a). These results are consistent with a previous report of immunoblotting detection of PCPE-1 in cornea (Kessler, et al., 1990). Due to the possibility of functional redundancy between PCPE-1 and PCPE-2, immunoblotting was performed to determine whether PCPE-2 is found in WT cornea. As can be seen (Fig. 1b), PCPE-2 was not detected in WT cornea, although it could be readily detected as a ~48 kDa band in heart, a tissue previously demonstrated to express PCPE-2 RNA (Steiglitz, et al., 2002). Immunoblotting with anti-PCPE-2 antibodies was also unable to detect PCPE-2 expression in *Pcolce*^{-/-} corneal extracts, indicating that corneal PCPE-2 expression is not induced in a compensatory manner in response to PCPE-1 ablation (data not shown). Immunofluorescence staining found WT corneal PCPE-1 (Fig. 1c) to be localized to stroma, but did not detect PCPE-1 in corneal epithelium (Fig. 1c'). Collagen I appeared to be at grossly similar levels in *Pcolce*^{-/-} and WT corneal stroma (Fig. 1c'', c''') and, in large part, co-localized with PCPE-1 in the latter (Fig. 1d-d''). Consistent with the previously demonstrated ability of recombinant PCPE-1 to enhance *in vitro* processing of procollagen I by BMP1/tolloid-like proteinases (BTPs) (Steiglitz, et al., 2002) and the demonstrated role for endogenous PCPE-1 in enhancing procollagen I biosynthetic processing in mouse embryo fibroblast cultures (Steiglitz, et al., 2006), immunoblotting found procollagen I processing to be reduced in cultures of primary stromal fibroblasts (keratocytes) isolated from *Pcolce*^{-/-} cornea, compared with controls (Fig. 1e'). Immunoblotting also confirmed the presence and absence of PCPE-1 expression in WT and *Pcolce*^{-/-} primary keratocytes, respectively (Fig. 1e).

WT and *Pcolce*^{-/-} mice show similar levels of corneal opacity, but *Pcolce*^{-/-} corneas have delayed wound closure following penetrating keratectomy

PCPE-1 levels have been shown to be induced in mouse corneal wound beds after penetrating keratectomy (full-thickness corneal excision), and to be elevated in human corneas scarred by trauma or herpetic keratitis (Malecaze, et al., 2014). Those observations imply a role for PCPE-1 in forming and/or maintaining corneal scarring after injury, as does its role in enhancing biosynthetic processing of fibrillar collagens, the primary protein constituents of scar tissue. To assess such a role, we compared scarring in *Pcolce*^{-/-} and WT mouse corneas subsequent to penetrating keratectomy. At day 14, at which time point the degree of opacity is most pronounced in this model, differences in corneal opacity/scarring between mice of the two genotypes were not grossly discernable (Fig. 2a, a', b). This result was surprising, but may suggest that the differences in levels of procollagen I biosynthetic processing in WT and *Pcolce*^{-/-} keratocyte cultures (Fig. 1e') affect opacity at times post-keratectomy later than that analyzed here. The result may also reflect the semiquantitative nature of our assessment of opacity, and other possible contributors to corneal opacity following injury (see Discussion). However, despite gross similarities in degree of opacity, fluorescein applied to the majority of *Pcolce*^{-/-} (Fig. 2c') (6 of 9), but not WT (Fig. 2c) (2 of 10), corneas could still penetrate the epithelium to underlying stroma at day 14 post keratectomy (Fig. 2c), indicating deficits in *Pcolce*^{-/-} corneal wound closure.

***Pcolce*^{-/-} mice show delayed epithelial repair after corneal epithelial abrasion**

The increased numbers of *Pcolce*^{-/-} fluorescein-positive corneas following penetrating keratectomy (Fig. 2c') suggested a role for PCPE-1 in corneal wound closure. To investigate this more closely, we employed a corneal epithelial abrasion model, in which only the corneal epithelium is damaged, to investigate whether the deficit in wound closure suggested by fluorescein persistence following full-thickness corneal excision involved delayed epithelial recovery after wounding. Results in the abrasion model showed significantly increased fluorescein persistence in *Pcolce*^{-/-} corneas following epithelial abrasion (Fig. 2d–d''', e), consistent with delayed corneal epithelial recovery. In addition, there was a secondary complication of blood in the aqueous humor of the anterior chamber (red), and evidence of edematous inflammation (yellow) in *Pcolce*^{-/-} eyes at day 5 post-abrasion (Fig. 2d''').

WT and *Pcolce*^{-/-} corneas appear to have grossly similar levels of collagen content and myofibroblasts post-penetrating keratectomy

To further examine possible deficits in collagen deposition due to PCPE-1 loss, WT and *Pcolce*^{-/-} (KO) corneas were stained with hematoxylin/eosin, for general morphology, and with Masson's trichrome, for collagen content. As can be seen, WT and *Pcolce*^{-/-} cornea seemed grossly similar in morphology and collagen content, both prior to (Fig. 3a–3a''') and 14 days post keratectomy (Fig. 3b–b'''). Immunostaining for the $\alpha 1(I)$ collagen chain C-telopeptide also suggested grossly similar levels of collagen I content in WT and *Pcolce*^{-/-} uninjured (Fig. 3c and c') and 14 days post keratectomy (Fig. 3d, d') cornea. Immunostaining for the procollagen I pro $\alpha 1(I)$ C-propeptide was negative in both WT and *Pcolce*^{-/-} uninjured cornea (Fig. 3c'', c'''). This lack of C-propeptide signal in uninjured KO

cornea suggests the absence of a large buildup of procollagen I and pC α 1(I) processing intermediates with uncleaved C-propeptides in this tissue. The similar levels of C-propeptide staining observed in WT and *Pcolce*^{-/-} cornea 14 days post keratectomy (Fig. 3d'', d''') is probably indicative of similar levels of procollagen I, newly synthesized as part of the healing process. Staining for α -smooth muscle actin, a marker for differentiation of keratocytes into myofibroblasts (predominantly responsible for collagen deposition during corneal scarring) also showed similar numbers of myofibroblasts in WT (Fig. 3d''') and *Pcolce*^{-/-} cornea (Fig. 3d'''). Quantification demonstrated no significant difference between Masson's trichrome (Fig. 3e) or anti-SMA (Fig. 3f) staining in WT and *Pcolce*^{-/-} cornea 14 days post-keratectomy.

***Pcolce*^{-/-} corneas have delayed epithelial recovery, but no decrease in opacity, following alkali burn**

The penetrating keratectomy model did not show decreased *Pcolce*^{-/-} corneal opacity, but, along with the epithelial abrasion model, did show evidence of delayed *Pcolce*^{-/-} corneal epithelial wound closure, following injury (above). To bolster these observations, we employed a third corneal injury model, the alkali burn model, to compare degrees of corneal opacity and epithelial wound closure in *Pcolce*^{-/-} and WT mice. In addition to loss of epithelial integrity, subsequent tissue scarring/fibrosis also often occurs in alkali-burned cornea (Brodovsky, et al., 2000). Consistent with results of the penetrating keratectomy and epithelial abrasion models, the alkali burn model showed no difference in corneal opacity (Fig. 4a, a', b), but did show delayed epithelial wound closure, in *Pcolce*^{-/-} cornea (Fig. 4c-c''', d). At 5 days post alkali burn the area in WT cornea that was fluorescein stained (Fig. 4c''') was much less than in *Pcolce*^{-/-} cornea (Fig. 4c'''), in which increased fluorescein persistence showed greatly delayed epithelial closure. At 5 days post alkali burn *Pcolce*^{-/-} cornea also showed secondary complications that included aberrant neovascularization (see below).

***Pcolce*^{-/-} corneas have excess infiltration by Ly6G⁺ cells due to deficits in epithelial healing, not increased PMN recruitment**

Although a controlled acute inflammatory response is necessary for proper wound healing (Li, et al., 2006b), an excessive and chronic inflammatory response can result in delayed wound healing that can be associated with damage to corneal ultrastructure (Comaish and Lawless, 2002, Pfister, et al., 1998, Sotozono, et al., 1999). In the corneal epithelial abrasion and alkali burn models, influx of inflammatory cells that include macrophages and polymorphonuclear neutrophils (PMNs) is involved in wound healing (Ishizaki, et al., 1993, Li, et al., 2006a). To determine if the deficits in wound healing observed for *Pcolce*^{-/-} cornea might be associated with abnormalities in the inflammatory response, we compared levels of F4/80⁺ cells (macrophages) and Ly6G⁺ cells (neutrophil PMNs) within *Pcolce*^{-/-} and WT mouse corneas at day 5 after epithelial abrasion or alkali burn. Although the results showed no difference in levels of F4/80⁺ cells infiltrated into WT and *Pcolce*^{-/-} cornea (Fig. 5a, a', b, c, c', d), *Pcolce*^{-/-} corneas had significantly increased numbers of Ly6G⁺ cells, compared to WT (Fig. 5a'', a''', b', c'', c''', d'), at day 5 post-epithelial abrasion (Figs. 5a-b'), or post-alkali burn (Fig. 5c-d').

The corneal epithelial abrasion model used here is known to involve two waves of neutrophil infiltration from the limbus to the cornea, the first wave occurring between 12 and 18 h, and the second between 30 and 36 h post-abrasion (Li, et al., 2006a). To determine whether differences in Ly6G⁺ cell numbers infiltrated into cornea at day 5 post abrasion were due to increased recruitment at 12 – 18 or 30 – 36 h post abrasion, or whether these differences were due to delayed wound closure, we examined Ly6G⁺ cell numbers in WT and *Pcolce*^{-/-} corneas at 12 and 30 h post abrasion (Fig. 5e–f'). Absence of significant differences between Ly6G⁺ cell numbers in *Pcolce*^{-/-} and WT corneas during either the first or second wave of infiltration at 12 h (Fig. 5e, e', f) or 30 h (Fig. 5e'', e''', f') post abrasion supports the conclusion that the large differences at 5 days post abrasion (Fig. 5b', d') are not due to differences in recruitment, but are instead due to delayed wound closure/epithelial healing in *Pcolce*^{-/-} cornea.

***Pcolce*^{-/-} mice have disruptions of collagen VII deposition at the interface between the epithelial basement membrane and stroma after corneal injury**

To begin examining the integrity of *Pcolce*^{-/-} corneal epithelium post injury, we stained for collagen VII, which constitutes the anchoring fibrils that secure the epithelial basement membrane to underlying stroma. Disruption of this protein is involved in corneal epithelial disorders, such as traumatic recurrent corneal erosion (Chen, et al., 2006), which is characterized by repeated periods of breakdown of the corneal epithelium caused by impaired epithelial adhesion to underlying stroma (Kanski, 1999). Although examination by immunofluorescence found no obvious collagen VII abnormalities in uninjured *Pcolce*^{-/-} corneas (Fig. 6a'), there were disruptions in collagen VII staining along sections of injured *Pcolce*^{-/-} corneas at day 14 after penetrating keratectomy (Fig. 6b'), and at day 5 post corneal epithelial abrasion (Fig. 6c') or alkali burn (Fig. 6d'). This phenotype was most pronounced in day 5 post-alkali burn cornea, in which some areas were devoid of collagen VII staining (Fig. 6d''). No disruptions in collagen VII staining were seen in WT cornea under any of the above conditions (Fig. 6a, b, c, d). In contrast to collagen VII staining results, immunofluorescent staining detected no differences between WT and *Pcolce*^{-/-} cornea in staining for laminin 332, a protein constituent of anchoring filaments (a major and intrinsic component of the epithelial basement membrane) subsequent to injury by penetrating keratectomy, epithelial abrasion, or alkali burn (data not shown).

PCPE-1 expression is upregulated in mouse cornea in response to injury, with early upregulation at the epithelial-stromal junction

To gain insights into how PCPE-1 might influence corneal wound healing, we next evaluated PCPE-1 expression and distribution in response to injury of WT cornea. At day 14 post penetrating keratectomy, as in uninjured cornea controls (Fig. 7a), PCPE-1 was present throughout the corneal stroma, but was at particularly high levels surrounding stromal fibroblasts (keratocytes) in the swollen wound itself (Fig. 7b). This suggested induced stromal PCPE-1 synthesis/deposition within the wound, in response to injury. In the alkali burn model, unlike uninjured normal control cornea (Fig. 7c), there seemed to be a relative decrease of PCPE-1 signal at the center of the injured stromal area (Fig. 7d), perhaps reflecting a recovery lag due to the severe nature of the injury. However, in cornea

juxtaposed to the wound there was intense PCPE-1 signal at the epithelial-stromal junction (Fig. 7e).

In the corneal epithelial abrasion model, we assessed the presence of PCPE-1 from days 1 to 5, post abrasion. Results showed strong and rapid increase of PCPE-1 signal at the epithelial-stromal junction at day 1 post abrasion, after which signal intensity at the epithelial-stromal junction decreased and became patchy, and PCPE-1 signal within the rest of the stroma appeared to increase (Fig. 7f–j).

Corneal neoangiogenesis is strikingly increased in the absence of PCPE-1, post alkali burn

An alkali burn of cornea is followed by neovascularization of this normally avascular tissue, as well as by scarring. Thus, because of recent *in vitro* evidence that PCPE-1 can bind the anti-angiogenic factor endostatin, and can itself inhibit angiogenesis in an *in vitro* assay (Salza, et al., 2014), corneas of WT and *Pcolce*^{-/-} mice were examined for degree of neovascularization 5 days post alkali burn. Interestingly, a striking difference in neovascularization following the alkali burn was found between *Pcolce*^{-/-} and WT corneas, with a marked increase in intracorneal hemangiogenesis in *Pcolce*^{-/-} cornea compared with WT, as detected by light microscopy (Fig. 8a, b) and staining for CD31 (PECAM-1) (Fig. 8c, d), and as quantified for the latter (Fig. 8e).

The *ex vivo* aortic ring model has been extensively used to study mechanisms of angiogenesis (Nicosia, et al., 2011). As we previously showed that PCPE-1 RNA is expressed in aorta (Steiglitz, et al., 2002), we compared *ex vivo* angiogenesis in aortic rings from *Pcolce*^{-/-} and WT mice, to gauge the extent to which endogenous PCPE-1 might affect sprouting of angiogenic outgrowths in this model system. As can be seen (Fig. 9a, a', b), sprouting of angiogenic outgrowths was considerably more extensive for *Pcolce*^{-/-} than for WT aortic rings. As another test of the ability of PCPE-1 to affect angiogenesis in the aortic ring assay, recombinant PCPE-1, which was shown to be functional in an *in vitro* assay of enhancement of C-propeptide cleavage from procollagen I (Fig. 9c), was added to *Pcolce*^{-/-} aortic ring cultures. This assay showed PCPE-1 to have a dose-dependent effect in inhibiting angiogenic sprouting (Fig. 9d, d', e).

Discussion

Here, we show by immunoblotting and immunofluorescence that PCPE-1 is a readily detected constituent of normal, uninjured corneal stroma in mouse, and thus available for the earliest responses to corneal injury and healing. Immunoblotting did not detect PCPE-2 in WT or *Pcolce*^{-/-} cornea. Thus, PCPE-2 is unlikely to be available in WT cornea to complement PCPE-1 function, nor is it up-regulated to compensate for loss of PCPE-1 activity. We were unable to detect PCPE-1 in corneal epithelium by immunofluorescence, despite the report of detected PCPE-1 expression in mouse corneal epithelium via quantitative PCR (Malecaze, et al., 2014).

The presence of PCPE-1 in corneal stroma, which has a fibrillar collagen ECM, is consistent with its previously demonstrated role in fibrillar collagen biosynthesis (Adar, et al., 1986,

Steiglitz, et al., 2002, Steiglitz, et al., 2006). Indeed, we found wild type keratocytes to produce readily detectable PCPE-1, and found keratocytes isolated from *Pcolce*^{-/-} corneas to display a decrease in the extent of procollagen I processing. However, this difference in procollagen processing did not correspond to readily discernable differences in corneal opacity or collagen content following keratectomy, or with a readily discernable difference in opacity in WT and *Pcolce*^{-/-} corneas in the alkali burn model. Nevertheless, measurements of collagen content, although showing an absence of large differences in WT or *Pcolce*^{-/-} cornea, were not quantitative. In addition, despite the association of corneal post injury opacity with scarring/collagen deposition, other possible contributors to opacity include edema, light scattering by activated myofibroblastic keratocytes and infiltrated inflammatory cells, and by disorganized extracellular matrix (Malecaze, et al., 2014). Since we don't know the extent to which each of these factors affects the opacity observed here in injured *Pcolce*^{-/-} and control corneas, it is at present difficult to gauge the extent of the role that PCPE-1 plays in scarring following corneal injury.

In addition to scarring, alkali burning causes epithelial erosion and, in contrast with the lack of difference in corneal opacity, a marked and significant delay was observed in re-epithelialization (epithelial recovery) of *Pcolce*^{-/-} corneas compared with WT controls. The latter result in the alkali burn model was consistent with results from the full-thickness corneal excision and epithelial abrasion models, as delayed *Pcolce*^{-/-} re-epithelialization/corneal epithelial recovery was found in all three models. A marked increase in Ly6G⁺ neutrophil numbers in *Pcolce*^{-/-}, compared with WT, cornea 5 days post-epithelial abrasion corroborated the conclusion of delayed re-epithelialization in injured *Pcolce*^{-/-} cornea. Absence of significant differences between Ly6G⁺ neutrophil numbers in *Pcolce*^{-/-} and WT corneas during the first or second wave of infiltration at 12 or 30 h post-abrasion, was consistent with the interpretation that earlier differences in neutrophil numbers resulted from differences in *Pcolce*^{-/-} corneal re-epithelialization, rather than from differences in neutrophil recruitment mechanisms.

Although the role of PCPE-1 in re-epithelialization remains to be determined, the detection of disruptions in the deposition of collagen VII, the major protein constituent of the anchoring fibrils that anchor basement membranes to stroma, suggests some abnormalities of basement membrane structure/function. However, there was an absence of obvious differences between WT and *Pcolce*^{-/-} corneas in distribution of the intrinsic basement membrane protein laminin 332, subsequent to injury by penetrating keratectomy, epithelial abrasion, or alkali burn. Thus, morphological differences between *Pcolce*^{-/-} and WT basement membranes during re-epithelialization may be subtle. Although BTPs have been implicated in the biosynthetic processing and deposition of collagen VII (Rattenholl, et al., 2002), it has been shown that PCPE-1 does not enhance proteolytic processing of procollagen VII *in vitro* (Moali, et al., 2005), while more recent studies have indicated that the *in vivo* processing and deposition of collagen VII are not likely affected by BTPs, at least in skin (Muir, et al., 2016). Thus, the disruption of collagen VII deposition in *Pcolce*^{-/-} cornea seems unlikely to reflect an enhancing role for PCPE-1 in the biosynthetic processing of collagen VII by such proteinases. Rather, the effects of PCPE-1 ablation on collagen VII deposition seem more likely to be secondary to loss of some other PCPE-1 role at the epidermal-stromal junction. Interestingly, the early burst of up-regulation of PCPE-1

expression/deposition that we observed specifically at the epidermal-stromal junction in areas of corneal injury, following epidermal abrasion or alkali burn, is consistent with PCPE-1 role(s) at the corneal basement membrane zone.

Although corneal alkali burn is usually followed by neovascularization of this normally avascular tissue, we found a striking increase in the neovascularization of *Pcolce*^{-/-} cornea, compared to WT, following this type of injury. This observation is of particular interest in the context of a recent report that PCPE-1 can interact with the anti-angiogenic factor endostatin via the PCPE-1 NTR domain (Salza, et al., 2014). The same study also reported that a protein fragment comprising the two PCPE-1 CUB domains, and to a lesser degree full-length PCPE-1, can inhibit angiogenesis in a commercial *in vitro* assay kit that involves tube formation by human umbilical vein endothelial cells (Salza, et al., 2014). In the present report, we show a large increase in intracorneal hemangiogenesis in alkali-damaged *Pcolce*^{-/-} cornea compared to alkali-damaged WT cornea, as detected by antibody to CD31 (PECAM-1). This result is consistent with an *in vivo* anti-angiogenic role for PCPE-1, at least in this tissue. Consistent with the possibility that PCPE-1 may have more general anti-angiogenic properties, our use of the *ex vivo* aortic ring model, widely used to study mechanisms of angiogenesis (Nicosia, et al., 2011), showed sprouting of angiogenic outgrowths to be considerably more extensive for *Pcolce*^{-/-} than for WT aortic rings. Moreover, recombinant PCPE-1 was shown to have a dose-dependent effect in inhibiting growth of angiogenic sprouts.

We previously showed PCPE-1 RNA to be expressed within developing vascular tissues (Steiglitz, et al., 2002), while others have shown that PCPE-1 expression may be involved in inhibiting the growth properties of vascular smooth muscle cells (Kanaki, et al., 2000). In fact, it has been suggested that PCPE-1 may be involved in a more general control of cell growth, as disruption of the PCPE-1 gene *Pcolce* can induce the anchorage-independent growth of cultured fibroblasts (Masuda, et al., 1998). It has also been shown that PCPE-1 may be necessary for lumen formation in a model of *in vitro* angiogenesis, although this effect may be secondary to the role of PCPE-1 in collagen biosynthesis (Newman, et al., 2011).

The molecular/cellular mechanisms, or combination of mechanisms, by which PCPE-1 affects corneal neovascularization after injury remains to be determined. An interesting question in this regard is whether PCPE-1 might, as in its role in collagen biosynthesis, act in anti-angiogenic activities in conjunction with BTPs. This possibility is suggested by previous findings that BTPs are capable of generating protein fragments with potent anti-angiogenic activity from some substrates (Ge, et al., 2007, Gonzalez, et al., 2005), and that BMP1 mRNA is among transcripts induced to highest levels in activated, compared with resting, endothelia (St Croix, et al., 2000).

PCPE-1 has previously been suggested as a therapeutic target for treatment of corneal scarring (Malecaze, et al., 2014). Data presented here suggests the possibility that PCPE-1 may be of particular therapeutic use in prevention of undesirable angiogenesis in injured cornea, and perhaps in other tissues and pathologies as well.

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Fig. 1.

PCPE-1, but not PCPE-2, is readily detectable in mouse cornea. a Immunoblots are shown of extracts of wild type (WT), *Pcolce*^{-/-}, or *Pcolce2*^{-/-} cornea probed with antibodies to PCPE-1, or, b of extracts of WT or *Pcolce2*^{-/-} cornea and heart probed with antibodies to PCPE-2. Blots were also probed with antibodies to a, α -tubulin or, b, GAPDH as loading controls. An asterisk marks the position of an artifactual band detected by the anti-PCPE-2 antibody in all samples. c-c''', Immunofluorescence staining (red) of whole cornea shows PCPE-1 (c and c') and the α 1(I) collagen chain, visualized using the LF67 antibody that recognizes the C-telopeptide of this collagen chain (c'' and c'''), in stroma (s), but not epithelium (ep) (the latter visualized by DAPI staining) of WT cornea. Collagen I (c''') but not PCPE-1 (c') is detected in *Pcolce*^{-/-} (KO) stroma. Immunofluorescence staining for PCPE-1 (d, red) and type I collagen (d', green) in wild type cornea shows the two to be co-localized (d'', yellow) in stroma, and that neither is found in epithelium. For c-c''' and d-d'', scale bars are 20 μ m. e and e', Immunoblotting of conditioned media samples of primary WT and *Pcolce*^{-/-} KO corneal fibroblasts (keratocytes) shows that WT keratocytes express readily detectable PCPE-1 (e, an asterisk marks the position of an artifactual band detected by the anti-PCPE-1 antibody in both the WT and KO media samples), and that type I procollagen is processed more efficiently by WT than by *Pcolce*^{-/-} cultures, as detected by antibody specific to the α 1(I) C-telopeptide (e'). e', Differences in levels of WT and *Pcolce*^{-/-} processing intermediates pCa1(I) and pNa1(I) were more apparent after 4 h, and differences in levels of mature α 1(I) chains were more apparent after 6 h of culturing in serum-free medium. pCa1(I), a processing intermediate that retains the C-propeptide but from which the N-propeptide has been removed; pNa1(I), a processing intermediate that retains the N-propeptide but from which the C-propeptide has been removed.

Fig. 2.

Evaluation of opacity and wound closure at day 14 after corneal full thickness excision, and of epithelial recovery at day 14 after central abrasion. Representative examples of corneal opacity of WT (a) and *Pcolce*^{-/-} (a') eyes, and b quantitation, show no significant difference in the degree of WT and *Pcolce*^{-/-} (KO) corneal opacity at day 14 post-injury. Increased persistence of fluorescein dye is consistent with deficits in *Pcolce*^{-/-} (c') wound closure compared to WT (c) (6 of 9 *Pcolce*^{-/-}, 67%, but only 2 of 10 WT, 20%, eyes remained fluorescein positive at day 14 post-injury). Numbers of mice subjected to corneal full thickness excision were WT, n = 10; and *Pcolce*^{-/-}, n = 9 KO). d-d''' Representative examples of fluorescein persistence in the stroma of WT (d, d'', and d''') and *Pcolce*^{-/-} (d', d''', and d''') mice at different time points subsequent to epithelial abrasion. At 5 days WT (d''') is seen to be fluorescein negative (black), whereas *Pcolce*^{-/-} (d''') is fluorescein positive, with secondary complications that include blood in the aqueous humor of the anterior chamber (red), and evidence of edematous inflammation (yellow). e a graph shows the progression of corneal epithelial wound closure of the KO mice at different healing intervals, as ascertained via ability of fluorescein to penetrate through the epithelium into the underlying stroma. (n = 8 each for WT and *Pcolce*^{-/-} mice). Corneal reepithelialization was quantified by assessing the percentage of cornea stained (not re-epithelialized) and not stained (re-epithelialized) with fluorescein. *, *P* < 0.05; **, *P* < 0.01. NS, not statistically significant. Scale bars: 1 mm.

Fig. 3.

WT and *Pcolce*^{-/-} corneas appear to have grossly similar levels of collagen content and myofibroblasts post-penetrating keratectomy. Sections of a-a''' and c-c''', uninjured; or b-b''' and d-d''', 14 days post-keratectomy WT (a, a'', b, b'', c, c'', d, d'', and d''') and *Pcolce*^{-/-} (KO) (a', a''', b', b''', c', c''', d', d''', and d''') cornea were examined. In a, a', b, and b', sections were stained with hematoxylin and eosin, and in a'', a''', b'', and b''', sections were stained with Masson's trichrome (bottom panels). e The intensity of Masson's trichrome staining for collagen is quantified in a graph (WT n=4, KO n=5). Sections of WT and *Pcolce*^{-/-} uninjured cornea (c-c''') or cornea 14 days post-keratectomy (d-d''') were immunostained with anti-α1(I) telopeptide (c, c', d, and d') or anti-proα1(I) C-propeptide (c'', c''', d'', and d''') antibodies. Sections in d'''' and d''''' were stained with anti-α-smooth muscle actin (SMA) antibodies. f The intensity of anti-SMA staining is quantified in a graph (WT n=4, KO n=5). Scale bars: 50 μm.

Fig. 4.

Evaluation of opacity and corneal epithelial recovery of *Pcolce*^{-/-} alkali-burned corneas. Representative examples of WT (a) and *Pcolce*^{-/-} (a') corneal opacity 5 days post-alkali burn. b A graph shows the degree of corneal opacity at day 5 post-alkali burn. Absence of PCPE-1 does not seem to affect the degree of corneal opacity subsequent to alkali burn. Representative examples are shown of epithelial closure at days 0 (c and c'), 3 (c'' and c'''), and 5 (c'''' and c''''') post alkali burn, as documented by fluorescein staining. d A time course for post alkali burn corneal epithelial healing, based on fluorescein staining, shows a significant delay in *Pcolce*^{-/-} corneal epithelial recovery. (n = 12 WT and 15 *Pcolce*^{-/-}). **, $P < 0.01$; NS, not statistically significant. Scale bars: 1 mm.

Fig. 5.

Inflammatory response in *Pcolce*^{-/-} corneas after epithelial abrasion and alkali burn. Representative examples (a–a''' and c–c''') and quantification (b and b'; d and d') of immunofluorescent staining are shown for F4/80⁺ cells (macrophages) and Ly6G⁺ cells (neutrophils) that have infiltrated the cornea by day 5 after abrasion (a–a'''; b and b'), or alkali burn (c–c'''; d and d'). There were no statistical differences in numbers of F4/80⁺ cells between WT and *Pcolce*^{-/-} cornea (b and d), but there were much higher numbers of Ly6G⁺ cells within *Pcolce*^{-/-} corneas (b' and d') in both models of cornea injury. For each injury model n = 4. **, *P* < 0.01. Representative examples (e–e''') and quantification (f and f') are shown of immunofluorescent staining to determine numbers of Ly6G⁺ neutrophils recruited to WT and *Pcolce*^{-/-} (KO) cornea at 12 h (f) and 30 h (f') post corneal epithelial abrasion (n = 4 each, WT and *Pcolce*^{-/-} corneas). Scale bars: 50 μm.

Fig. 6.

Evaluation of the distribution of collagen VII in *Pcolce*^{-/-} cornea after injury. Although immunofluorescence staining showed the distribution of collagen VII (red) at the epithelio-stromal junction to be similar in uninjured WT (a) and *Pcolce*^{-/-} (a') corneas, disruptions in collagen VII distribution were observed beneath the epithelium in *Pcolce*^{-/-} (b', arrowheads), but not WT (b) cornea at day 14 post corneal full thickness incision (Ker, keratectomy) b; and in *Pcolce*^{-/-} (c', arrowheads), but not WT (c) cornea in day 5 after mechanical epithelial abrasion (Abr), and was most pronounced in *Pcolce*^{-/-} (d', arrowheads), but not WT (d) cornea day 5 after alkali burn (Alk). For the data from day 5

post alkali burn, an area of *Pcolce*^{-/-} cornea is shown with gaps in staining for collagen VII (d'), and another area is shown that is totally devoid of collagen VII staining (d''). Cell nuclei were stained with DAPI (blue). n = 4 each for WT and *Pcolce*^{-/-} corneas, for each injury model. Scale bars: 20 µm.

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Fig. 7.

Distributions of PCPE-1 in injured mouse cornea. Immunofluorescence staining for PCPE-1 shows PCPE-1 distribution in a normal uninjured mouse control cornea (a) and in the mouse cornea day 14 after full thickness incision (b) (Ker, keratectomy). Increased deposition was observed around keratocytes of the stromal wound bed (arrowheads). Characterization of PCPE-1 deposition in a normal uninjured mouse control cornea (c) and the wound bed of corneas day 5 post alkali burn (d) (Alk), in which decreased signal is seen in some areas (arrowheads). e, PCPE-1 deposition was highly increased at the epithelial-stromal junction juxtaposed to the alkali injury zone (arrowheads). n = 4 each for uninjured corneas, for injured corneas in the incision and alkali burn injury models. A time course is shown for immunofluorescence stained PCPE-1 deposition at the epithelial-stromal junction of unwounded mouse cornea (f) and mouse cornea at days 1 (g), 2 (h), 3 (i), and 5 (j) post

epithelial abrasion. N = 5 for each time point. Stroma, s; epithelium, ep (PCPE-1, red; DAPI, blue). Scale bars: 50 μ m.

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Fig. 8.

Pcolce^{-/-} mice show aberrant neovascularization of cornea following alkali treatment. Gross examination under a dissecting microscope shows increased neovascularization in *Pcolce*^{-/-} corneas (a) compared to wild type (WT) (b). Immunofluorescent staining was performed with antibodies to blood vessel endothelial cell marker CD31 (green) of WT (c) and *Pcolce*^{-/-} (d) corneas. Scale bars: 1 mm. e Immunofluorescence results from b were quantified, as described in Methods, to show the blood vessel areas in WT (n=11) and *Pcolce*^{-/-} (n=20) corneas. *P* value: ***<0.001.

Fig. 9.

PCPE-1 acts as an anti-angiogenic factor in aortic ring assays. Representative dissection microscope photographs (magnification 40x) of wild type (a) (WT, n= 30) and *Pcolce*^{-/-} (a') (KO, n= 23) aortic rings and angiogenic outgrowths. b Quantitation of numbers of angiogenic sprouts per ring. c Immunoblotting shows recombinant PCPE-1 to be functional in the *in vitro* enhancement of the processing of the C-propeptide from the procollagen α 1(I) chain to produce pNa(I) chains that retain N-, but lack C-propeptides. Addition of exogenous PCPE-1 decreases the numbers of angiogenic sprouts per *Pcolce*^{-/-} aortic ring (d'), compared with *Pcolce*^{-/-} aortic rings in the absence of added exogenous PCPE-1 (d). e Quantitation of numbers of angiogenic sprouts per ring. *, $P < 0.05$; ***, $P < 0.001$.