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Jessica Weant

David D Eveleth

Amuthakannan Subramaniam

Jennifer Jenkins-Eveleth

Michael Blaber

See next page for additional authors

Authors

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Regenerative responses of rabbit corneal endothelial cells to stimulation by fibroblast growth factor 1 (FGF1) derivatives, TTHX1001 and TTHX1114

Jessica Weant[a], David D. Eveleth[a], Amuthakannan Subramaniam[a], Jennifer Jenkins-Eveleth[a], Michael Blaber[b], Ling Li[c], David M. Ornitz[c], Asaf Alimardanov[d], Trevor Broadt[e], Hui Dong[e], Vinay Vyas[e], Xiaoyi Yang[e] and Ralph A. Bradshaw[a,f]

[a]Trefoil Therapeutics, Inc, San Diego, CA, USA; [b]Department of Biomedical Sciences, College of Medicine, Florida State University, Tallahassee, USA; [c]Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO, USA; [d]Therapeutics Development Branch, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD, USA; [e]Biopharmaceutical Development Program, Advanced Technology Research Facility, Frederick National Laboratory for Cancer Research (FNLCR), Leidos Biomedical Research Inc, Frederick, MD, USA; [f]Department of Physiology and Biophysics, University of California, Irvine, Irvine, CA, USA

ABSTRACT

Utilising rabbit corneal endothelial cells (CEC) in three different paradigms, two human FGF1 derivatives (TTHX1001 and TTHX1114), engineered to exhibit greater stability, were tested as proliferative agents. Primary CECs and mouse NIH 3T3 cells treated with the two FGF1 derivatives showed equivalent EC_{50} ranges (3.3–24 vs.1.9–16. ng/mL) and, in organ culture, chemically lesioned corneas regained half of the lost endothelial layer in three days after treatment with the FGF1 derivatives as compared to controls. *In vivo*, following cryolesioning, the CEC monolayer, as judged by specular microscopy, regenerated 10–11 days faster when treated with TTHX1001. Over two weeks, all treated eyes showed clearing of opacity about twice that of untreated controls. In all three rabbit models, both FGF1 derivatives were effective in inducing CEC proliferation over control conditions, supporting the prediction that these stabilised FGF1 derivatives can potentially regenerate corneal endothelial deficits in humans.

Abbreviations: FGF1: fibroblast growth factor 1; CECs: corneal endothelial cells; FGFR: fibroblast growth factor receptor; BME: β-mercaptoethanol; miL-3: murine recombinant interleukin-3; pRCEC: primary rabbit corneal endothelial cell; FNC: fibronectin, collagen and albumin; FBS: foetal bovine serum; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PES: phenazine ethosulfate; EdU: 5-ethynyl-2′-deoxyuridine; H(B)SA: human (bovine) serum albumin

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Cornea; endothelium; fibroblast growth factor1; primary tissue culture; opacity clearing; regeneration

Introduction

Tissue growth in higher organisms is produced by increases in cell size (hypertrophy) and cell number (hyperplasticity). The stimuli that control and produce these responses are basically external to the cell and include interactions with extracellular matrix, other cells, and soluble factors. Importantly, the ability of cells to respond to these external signals, and the nature of these responses, is dependent on a variety of conditions, e.g. cell cycle status, differentiative state, etc. such that the activation of a pathway in one cell type might lead to proliferation while in another, produce apoptotic cell death. One major element in the regulation of growth events is the polypeptide growth factors which are, for the most part, soluble ligands that are exported from their cells of origin and then interact with target cells through cell surface receptors (Bradshaw and Dennis 2011; Heldin et al. 2014). There are several types of growth factor families based on the kind of receptor they utilise. One of these, the fibroblast growth factor receptor (FGFR) family, is composed of seven members coded by four unique genes (Ornitz et al. 1996; Zhang et al. 2006; Ornitz and Marie 2015) and the associated ligand family of fibroblast growth factors (FGFs) contains 22 members that are subdivided into seven groups.

FGF1 and FGF2, the first members of this family to be identified (Gospodarowicz 1975; Thomas et al. 1980; Esch et al. 1985; Gimenez-Gallego et al. 1985) constitute one group and are distinct from the other

CONTACT Ralph A. Bradshaw rablab@uci.edu Department of Physiology and Biophysics, University of California, Irvine, CA 92697, USA

FGFs in that they lack signal peptides that direct their export from cells via the endoplasmic reticulum/Golgi pathway (Esch et al. 1985; Gimenez-Gallego et al. 1985; Jaye et al. 1986). A structural consequence of this is that the cysteine residues of FGF1 and 2 remain in the reduced state and are subject to oxidation or other modifications which result in inactivation once they leave the reducing environment of the cytoplasm. However, they are protected to a considerable degree by interactions with heparin and heparan sulphate proteoglycans, which function as co-factors for receptor activation (Ornitz 2000; Monsonego-Ornan et al. 2002; Cochran, Li, and Ferro 2009) The biological consequence of FGF1 and FGF2 being released in the reduced state is rapid loss of activity in oxidising environments, consistent with the biological role of these FGFs in acute wound responses and signalling over short spans of time and distance.

Because of the ability of FGF1 to stimulate full signal transduction from all seven FGFRs, the only member of the entire family able to do so (Ornitz et al. 1996; Zhang et al. 2006), FGF1 derivatives that have been modified to increase their stability and to eliminate their potential inactivation due to thiol oxidation are attractive candidates for pharmaceutical applications in germane regenerative situations (Xia et al. 2012, 2016). One such target is the endothelial cell monolayer on the posterior side of the cornea. While there is a slow loss of these cells during the normal ageing process (Gipson 2013), certain dystrophic conditions lead to more rapid losses and, left untreated, such patients eventually lose the ability to pump fluid out of the cornea leading to edoema, corneal opacity and loss of vision (Vedana, Villarreal, and Jun 2016). Presently, the only therapeutic option for such patients is the transplantation of healthy endothelial cells from a normal donor, and tens of thousands of such surgeries are performed every year (Ortega et al. 1991; Brych et al. 2001; Afshari et al. 2006; Van Meter 2014).

To explore the feasibility of using suitable FGF1 derivatives to treat corneal endothelial dystrophies pharmaceutically, several engineered FGF1s were evaluated, from which TTHX1001 and TTHX1114 were chosen. Both derivatives are characterised by three mutations and exhibit increased stability and lowered susceptibility to oxidation and proteolysis (Xia et al. 2012, 2016). The applicability of these FGF1 derivatives for regenerating CECs in three rabbit paradigms was tested.

Materials and methods

FGF1 derivatives

TTHX1001, containing K12V, C117V, and P134V substitutions was expressed as the N-Phe, 140 amino acid form of human FGF1 as described (Xia et al. 2012). Some preparations also had an N-terminal extension containing a His-tag sequence for purification purposes (Brych et al. 2001). TTHX1114, which is characterised by C16S, A66C and C117V mutations, where an intrachain disulphide bond is also formed between C83 and the substituted cysteine at 66, was prepared as the N-Phe 140 residue structure as above and as the N-Met-FGF1 (141-amino acid form) by the Frederick National Laboratory for Cancer Research, Biopharmaceutical Development Program, and supplied to Trefoil through a CRADA collaboration with the NCATS TRND program. Both derivatives are annotated as the 140-residue sequence (Jaye et al. 1986). TTHX1001 has the most sensitive cysteine at 117 (Ortega et al. 1991) replaced while TTHX1114 has no free thiols.

Proliferation assays in tissue culture

The BaF3/FGFR1c assay was performed as previously described (Ornitz et al. 1992; Xia et al. 2016). Briefly, BaF3 murine lymphoid cells were transfected to express the FGFR1c isoform (Ornitz et al. 1992; 1996). Cells were maintained in RPMI 1640 medium (Sigma Chemical, St. Louis MO) supplemented with 10% newborn bovine serum (Sigma Chemical, St. Louis MO), 50 µM β-mercaptoethanol (BME), 0.5 ng/mL murine recombinant interleukin-3 (mIL-3, PeproTech Inc, Rocky Hill NJ), 2 mM L-glutamine, penicillin-streptomycin ("BaF3 culture medium"), and G418 antibiotic (600 µg/mL). FGFR1c expressing BaF3 cells were washed twice in BaF3 "assay medium" ("culture medium" lacking both mIL-3 and BME) and plated at a density of 30,000 cells per well in a 96-well assay plate in assay medium containing heparin sulphate (1 µg/mL) and concentrations of recombinant native FGF1 (#100-17 A, PreproTech Inc.) and TTHX1001 and TTHX1114 mutants ranging from 20 to 5120 pM. The cells were incubated for 36 h and DNA synthetic activity was determined by adding 1 µCi of ^{3}H-thymidine in 50 µL of BaF3 assay medium to each well. Cells were harvested after 4 h by filtration through glass fibre paper. Incorporated ^{3}H-thymidine was counted on a MicroBeta plate scintillation counter (PerkinElmer, Waltham MA).

Primary rabbit corneal endothelial cell (pRCEC) cultures were established by digestion of excised Descemet's membranes from fresh rabbit corneas with Accutase to release the endothelial cells and plating on fibronectin, collagen and albumin (FNC) (Athenaes Cat:# 0407H)) coated tissue culture plastic

using a modified Joyce's medium, Opti-MEM supplemented with 8% foetal bovine serum (FBS), insulin-transferrin-selenium, and Ca + ascorbate. For proliferation assays, second or third passage cultures were seeded onto 96-well plates at cell density (2×10^5 cells/well in 200 µL culture medium) in media containing 8% FBS and incubated at 37 ± 1 °C, 5 ± 1% CO_2 24 h before beginning treatment. On the second day, after removal of culture medium, a serum free modified Joyce's media, Opti-MEM supplemented with insulin-transferrin-selenium, and Ca^{2+} + ascorbate was added into the plate (200 µL/well) to induce quiescence. The plate was incubated at 37 °C, 5% CO_2 for 24 ± 2 h with a subsequent medium change. Diluted samples (20 µL/well) and controls were transferred to the wells containing 200 µL of cells per well and incubated at 37 ± 1 °C, 5 ± 1% CO_2 for 24 ± 2 h. After 5 days of medium changes and dosing of diluted samples and controls, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) was added (20 µL/well) and incubated for four h at 37 ± 1 °C and 5 ± 1 % CO_2 and the sample absorbance at 490 nm was read. The background absorbance readings in the wells with medium were subtracted from the measurements. The data were analysed by fitting to a 4-parameter logistic function (Prism version 6).

The NIH/3T3 cell proliferation assay was performed using the Promega CellTiter96® AQueous One Solution Cell Proliferation Assay with (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine ethosulfate (PES) electron coupling reagent. NIH/3T3 cells were cultured in DMEM supplemented with 10% heated-inactivated FBS and sodium pyruvate. The cells were harvested and seeded into a 96-well plate at cell density (3×10^5 cells/well in 100 µL culture medium) and incubated at 37 ± 1 °C, 5 ± 1% CO_2 overnight. On the second day, after removal of culture medium, the quiescent medium (DMEM + 25% F17) was added into the plate (100 µL/well). and incubated at 37 °C, 5% CO_2 for 6 ± 1 h. The diluted samples (20 µL/well) and controls were transferred to the wells of the 96-well plate containing 100 µL of cells per well and incubated at 37 ± 1 °C, 5 ± 1% CO_2 for 20 ± 1 h. After the 20-h incubation period, Cell Titer96® AQueous One Solution was added (20 µL/well) and incubated for another four h at 37 ± 1 °C and 5 ± 1 % CO_2. The plate was read at 490 nm. The background readings in the wells with medium were subtracted from read outs of the sample wells. The data were analysed using a 4-parameter logistic function (SoftMax Pro from Molecular Device).

The 5-ethynyl-2′-deoxyuridine (EdU) click-it reaction with Alexafluor 488 and subsequent staining with Hoechst 33342 was performed as described previously (Eveleth et al. 2018).

Proliferation assays in organ culture

Corneas from fresh rabbit eyes (Pel-Freez) were dissected leaving about 2 mm of sclera attached. Lesions were produced by a variation of the method of Ljubimov (Saghizadeh et al. 2010). This method uses long chain alcohols such as heptanol or octanol to remove cells without damaging the basement membrane (Hatchell et al. 1983; Chung et al. 1998). Corneas were placed in a hemispherical agar well and excess fluid was removed using paper towel slivers applied at the edge. A 4 mm diameter piece of Whatman #1 filter paper saturated with n-octanol was placed in the centre of the cornea for 30 sec and the cornea then placed in Dulbecco's PBS to rinse. Corneas were cultured in Opti-MEM without serum as described above. At 24 h post lesioning, corneas were randomised to various drug treatment groups. Medium was changed daily. Lesion size was evaluated using Trypan blue staining, which stains areas of the cornea with endothelial damage but is not itself toxic to the endothelial layer (Means et al. 1995; van Dooren, Beekhuis, and Pels 2004) and quantitated as % of corneal area using Image J (Abramoff, Magalhaes, and Ram 2004). The methodology used in this study was designed to preserve the function of the corneal endothelial cells.

Ex vivo organ cultured corneas ($n = 8$ per group) were lesioned as described above and immediately exposed to TTHX1114 (100 pg/mL) for the indicated duration of time followed by rinsing in culture media and incubation for the remainder of the three-day experiment in media without drug.

Endothelial regeneration in vivo: the corneal cryofreeze model

All *in vivo* experiments were carried out at Absorption Systems, San Diego CA. All aspects of animal care and use were approved by their IACUC (protocol #17C281Q1) including the lesioning of both eyes, which was deemed acceptable because the lesions create blurry central vision without impacting peripheral vision and thus allow the animals to adapt and function normally. In the rabbit cryogenic injury model used (Okumura et al. 2011), New Zealand white rabbits were anaesthetised with an

intramuscular injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). One drop of topical proparacaine hydrochloride anaesthetic (0.5%) was placed in each of the animal's eyes prior to the procedure. Additional topical ocular anaesthesia was utilised during the procedure as needed. The eyes were cleaned with betadine and then rinsed with basic salt solution (BSS). Both corneas of each rabbit were frozen using a cryothermia unit (Brymill cryounit) (either 2 or 3 mm) for 15–30 sec, killing the CECs in the central cornea. The lesioning process was assessed by slit lamp. Test and vehicle control articles were administered via bilateral intracameral injections 1 h after the freeze process. Animals were maintained under anaesthesia between the cornea freezing and test article administration procedures. Additional anaesthesia was administered as needed. A 30-gauge needle was inserted parallel to the surface of the eye directly at or above the limbus at the 11 o'clock position. The needle was advanced into the anterior chamber and the test article or vehicle control (approximately 10 µL per injection) was administered. Immediately after the cryogenic lesion procedure, each animal underwent clinical ophthalmic examinations, fluorescein staining, slit-lamp photography, and specular microscopy to confirm the severity of the corneal lesion. Endothelial cell counts and imaging specular microscopy using a Konan specular microscope and endothelial camera was performed on both eyes of all study animals at baseline (prior to lesion creation/dosing), day 0, 3, 5, 7, 11, and 14 post-lesion/dosing. Clinical ophthalmic examination (slit-lamp biomicroscopy) to measure corneal clearing was performed on both eyes at baseline (prior to creation of the lesion) including indirect ophthalmoscopy, on day 0 and on days 3, 5, 7 (±2) post lesion. Day 14 (±2) examinations were performed immediately prior to euthanasia. Ophthalmic examination scoring utilised a modified McDonald-Shadduck scoring system (McDonald and Shadduck 1977).

Results

Tissue culture reponses

To provide an initial comparison of the survival/proliferative activity of the TTHX1001, TTHX1114 and native FGF1, the three ligands were assayed in the presence of heparin with BaF3 cells modified to express FGFR1c[3] (Figure 1). BaF3-FGFR1c cells show increasing survival/mitogenic response as measured by ^{3}H-thymidine incorporation with increasing wild type FGF1 protein concentrations, with maximum

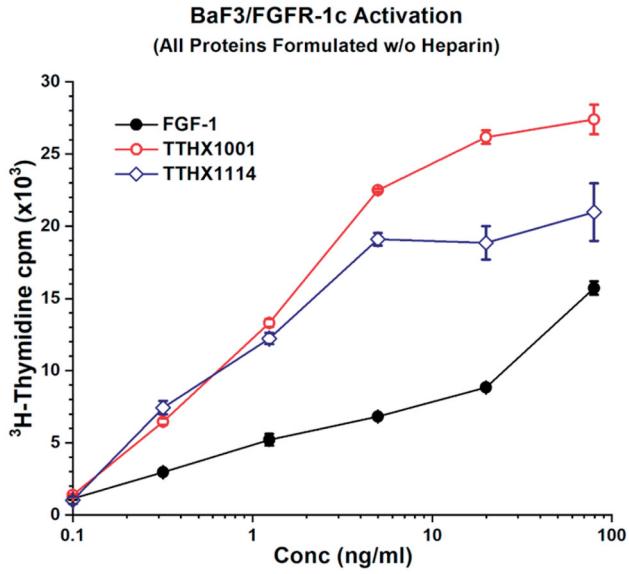

Figure 1. Stimulation of ^{3}H-thymidine incorporation into the murine lymphoid cell line, BaF3, expressing FGFR1c by recombinant native FGF1, TTHX1001 and TTHX1114. DNA synthetic activity was determined by adding 1 µCi of ^{3}H-thymidine in 50 µL and the harvested cells counted on a MicroBeta plate scintillation counter (PerkinElmer, Waltham MA). Black closed circles: native FGF1; open triangle: TTHX1114; open circles: TTHX1001 ($N = 3$ per a group).

stimulation occurring at the highest tested concentration. Both TTHX1001 and TTHX1114 exhibited ~15-fold greater activity compared to wild type FGF1, and the BaF3-FGFR1c cells approached maximum survival/growth stimulation with FGF1 mutant concentrations of 5 ng/mL (320 pM). These findings indicate that in this test (with one FGFR receptor type), both stabilised derivatives are approximately an order of magnitude more potent than native FGF1.

The stimulatory effects of TTHX1001 and TTHX1114 on dissociated cultures of pRCECs was confirmed using the MTT assay (van Meerloo, Kaspers, and Cloos 2011) (Figure 2a). The MTT assay does not assess growth responses directly but rather measures the conversion of the tetrazolium salt to formazan by mitochondrial dehydrogenases, which can be assayed colorimetrically. Although the MTT assay is commonly used to measure changes in cell number in proliferative assays, it can be subject to some variation (Liu and Dalgleish 2009) so the same responses were also monitored by EdU incorporation, which is a measure of DNA synthesis (Figure 2(b)) (Chehrehasa et al. 2009). A similar stimulatory effect of TTHX1114 was observed in pRCECs using EdU incorporation. The MTT and EdU assays showed a similar dose response curve in TTHX1114 dosed cells. Cells that were dosed with 100 ng/mL were shown to have greatest metabolic activity and highest EdU

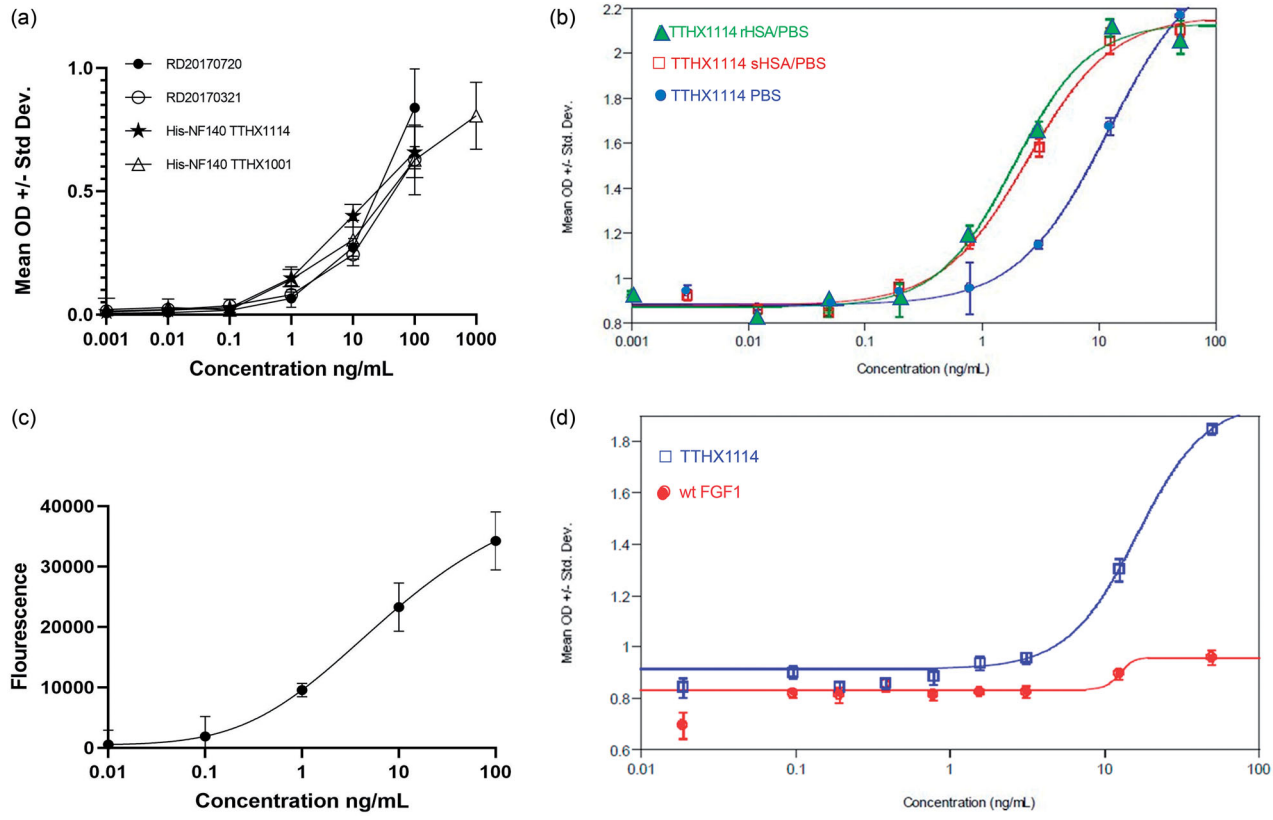

Figure 2. Dose response of rabbit primary corneal endothelial cells (pRCECs) and mouse NIH/3T3 cells to FGF1 derivatives, TTHX1001 and TTHX1114, in tissue culture. Standard deviation was used to plot variances. (a) pRCEC responses to various derivatives as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); black filled in circles: TTHX1114 (NM141), Lot 70720; open circles: TTHX1114 (NM141), Lot 70321; filled in stars: TTHX1114 (His-NF140); and open triangles: TTHX1001 (His-NF140) ($N = 4$ per a group). (b) pRCEC response to TTHX1114 (His-NF140) as measured by 5-ethynyl-2′-deoxyuridine (EdU) incorporation; ($N = 3$ per a group). (c) Dose response of mouse NIH 3T3 cell proliferation by TTHX1114 (NM141) in PBS (closed circles) and in the presence of 0.1% recombinant (closed triangles) or serum-derived (open squares) human serum albumin (HSA) as measured by MTT; ($N = 3$ per a group) (d) Dose response of mouse NIH/3T3 cells to TTHX1114(NM141), Lot 70321 in PBS (open squares) and wt FGF1 in PBS/0.1% bovine serum albumin (closed circles)(no heparin) as measured by MTT ($N = 3$ per a group).

incorporation ratio. Using the same preparation of engineered FGF1 [TTHX1114(His-NF140)], the observed EC_{50} values determined by the two protocols were essentially the same: 4.56 and 3.37 ng/mL (Table 1). The results between the two methods, MTT and EdU, differ by 27% which is well within validated standards for a bioassay assay thus confirming that with pRCECs, the two methods can be used interchangeably. Similar results were observed with the NIH/3T3 cells (data not shown).

Using the observed EC_{50}s as indices, these responses were compared to those of mouse NIH 3T3 cells (Table 1), a well-validated standard for FGF-induced proliferation assays. The EC_{50}s observed for the four FGF1 samples with pRCECs ranged from 3.3 to 24 ng/mL with MTT or EdU. These were comparable to the EC_{50} values for the FGF1 derivatives acting on the NIH/3T3 cells (1.9–16 ng/mL) including wild type FGF1, which yielded a value of 12.6 ng/mL (Figure 2(d)), as determined with MTT. The samples tested in Figure 2(a) included two different preparations of TTHX1114(NM-141), TTHX1114(His-NF-140) and TTHX1001 (His-NF-140) and all were in

Table 1. EC50 responses of primary rabbit corneal endothelial cells and mouse NIH/3T3 cells to TTHX1001 and TTHX1114.

Compound	Drug Lot	Cell Type	Buffer	EC_{50} ng/mL	Assay
TTHX1114(NM141)	RD20170720	pRCEC[a]	PBS	15.07	MTT
TTHX1114(NM141)	RD20170321	pRCEC[a]	PBS	18.8	MTT
TTHX1114(His-NF140)	02012014	pRCEC[a]	A[c]	4.56	MTT
TTHX1001(His-NF140)	12072014	pRCEC[a]	PBS	24.14	MTT
TTHX1114(His-NF140)	02012014	pRCEC[a]	A[c]	3.37	EdU
TTHX1114(NM141)	RD20170720	3T3[b]	PBS	12.08	MTT
TTHX1114(NM141)	RD20170720	3T3[b]	PBS/rHSA[d]	1.87	MTT
TTHX1114(NM141)	RD20170720	3T3[b]	PBS/sHSA[e]	2.44	MTT
TTHX1114(NM141)	RD20170321	3T3[b]	PBS	16.226	MTT
wtFGF1	RD20170321	3T3[b]	PBS	12.61	MTT

[a]Primary rabbit corneal endothelial cells
[b]Mouse NIH/3T3 cells
[c]Buffer A: 50 mM sodium phosphate, 100 mM NaCl, 10 mM ammonium sulphate, 2 mM DTT, and 0.5 mM EDTA (pH 7.5)
[d]PBS with 0.1% recombinant human serum albumin
[e]PBS with 0.1% serum derived human serum albumin.

Table 2. TTHX1001 induced cornea endothelial cell recovery following cryolesioning in the rabbit as measured by specular microscopy over a period of two weeks.

Endothelial cell characteristics							
		Cell density, cells/mm^2		% Hexagonal		Average cell area, um^2	
		TTHX1001	Veh	TTHX1001	Veh	TTHX1001	Veh
Baseline	Animal #1	3083	3250	59	63	325	308
	Animal #2	3332	3268	54	63	300	307
Day 3	Animal #1	2639	nd	38	nd	379	nd
	Animal #2	nd	nd	nd	nd	nd	nd
Day 7	Animal #1	2612	nd	51	nd	385	nd
	Animal #2	2862	nd	40	nd	350	nd
Day 14	Animal #1	3528	3545	54	36	285	283
	Animal #2	3069	2909	53	47	327	344

"nd" refers to No Data due to the inability of the specular microscope to identify discrete CEC borders. This resolves by day 7 in drug treated and day 14 in vehicle treated animals allowing for countable cells.

PBS except TTHX1114(His-NF-140), which was in 50 mM sodium phosphate, 100 mM NaCl, 10 mM ammonium sulphate, 2 mM DTT, and 0.5 mM EDTA (pH 7.5) (Buffer A). This same buffer was used for the TTHX1114 (His-NF-140) measurement using EdU (Figure 2(b)). The importance of buffer composition is also underscored by the 3T3 cell assays in which the excipient human serum albumin (0.1%) was added as derived from both recombinant (r) and serum(s) sources (Figure 2(c)). Cells dosed with RD20170720 in PBS had an EC_{50} of 12.08 ng/mL and cells dosed with RD20170720 in PBS with either rHSA or sHSA had EC_{50}s of approximately 2 ng/mL. This clearly shows that the presence of HSA impacted the EC_{50} values measured by about an order of magnitude. This difference in EC_{50} between those cells dosed with TTHX114 in PBS versus PBS plus HSA can largely be attributed to adsorption of TTHX1114 to surfaces. When a carrier protein such as HSA is added to the formulation, the surface of the plastic is "blocked" decreasing the absorption of protein to the surface and thereby greatly lowering the overall concentration of protein in solution.

Response to FGF1 derivatives by endothelial lesions introduced in organ culture

Since the engineered FGF1s potently stimulate the proliferation of corneal endothelial cells in culture, the ability of TTHX1001 and TTHX1114 to accelerate the recovery and regeneration of the corneal endothelial layer *in situ* in rabbit organ culture was tested. A modification of the corneal organ culture and lesioning process developed in the Ljubimov laboratory was used (Saghizadeh et al. 2010) whereby lesions are introduced using filter paper discs saturated in n-octanol.

This model was also used to determine if the acceleration of healing by TTHX1114(NF140) required constant exposure to drug as measured in Figure 3 or if a brief exposure was sufficient. Corneas lesioned and then treated with TTHX1114(NF140) for 60 min followed by incubation in organ culture for up to 72 h demonstrated that acceleration of healing was not different from corneas incubated in the continuous presence of drug for 72 h (Figure 4). Corneas incubated for 3 days without drug healed 58% of the lesion area while corneas incubated for 1 h followed by the remaining 3 days without drug, 24 h with drug followed by the remaining 3 days without drug, or 3 days with drug healed 85%, 83% and 86% (respectively). A one-way ANOVA was performed to compare the mean results between the groups. The results ($p < 0.01$) demonstrate that there is a significant difference between the means of the groups. Using Tukey's multiple comparisons test there is a significant difference between drug treated and untreated ($p < 0.01$) but no significant difference between drug treated groups ($p \geq 0.98$). This indicates that one-hour exposure to drug is equivalent to 3 days of continuous exposure.

Repair of corneal endothelial damage (cryogenic injury) in vivo

In the rabbit *in vivo* corneal cryogenic injury model, TTHX1001 and TTHX1114, when administered as a single intracameral injection following cryogenic lesioning, accelerated the healing of the damaged corneal endothelium as judged by specular microscopy (Figure 5) and measurements of corneal opacity (Figure 6). As shown in Figure 5, the ordered hexagonal array, characteristic of the intact corneal endothelium, appeared in the lesioned eye following injection of 100 ng of TTHX1001 in just 3 days. In the treated eye, both the density of cells and the hexagonality of the cell packing returned to normal in the treated eye at 7 days, with the average cell areas not returning to normal until 14 days (xzc). In the

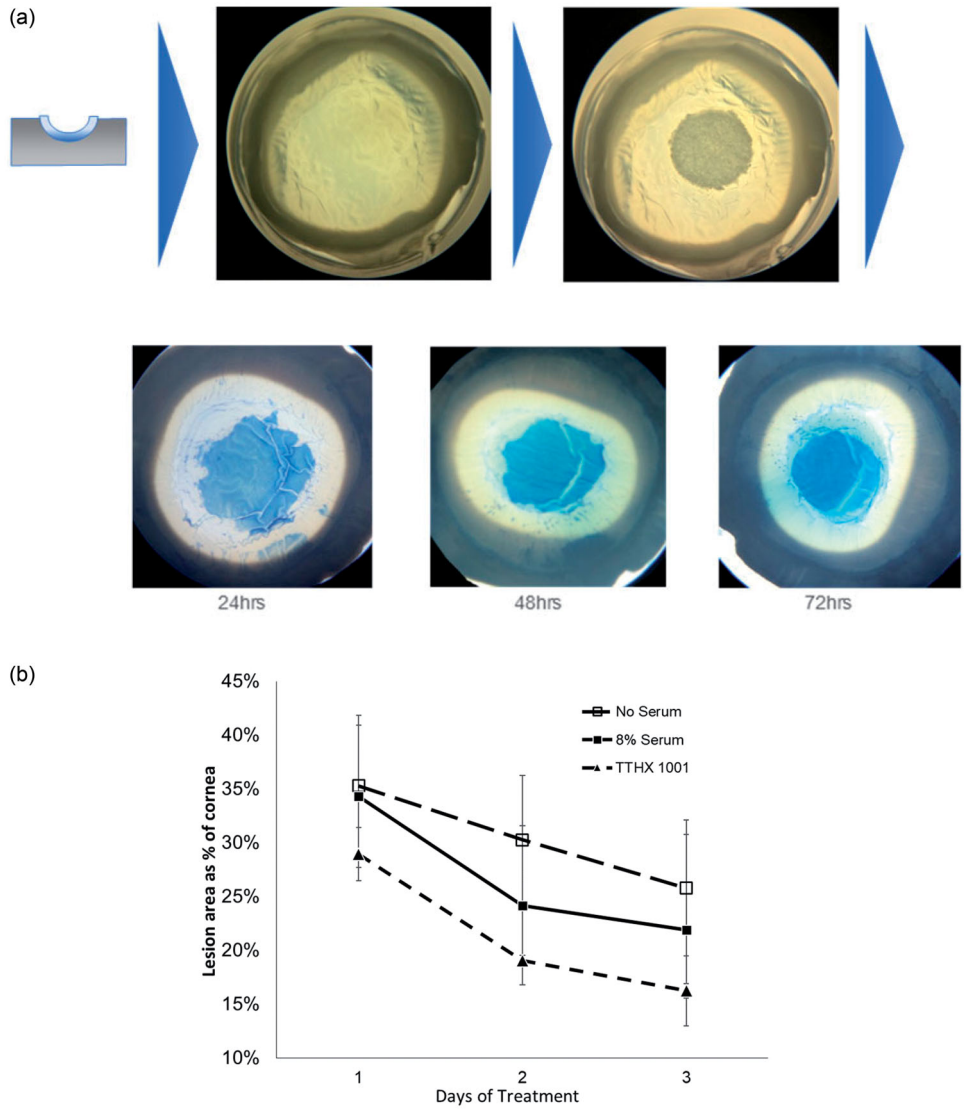

Figure 3. CEC recovery in the corneal organ culture model following chemical lesioning. (a) Top row, left: Schematic representation of the semi spherical agar well used to hold the cornea, posterior side up; middle: untreated cornea *in situ* in agar well; right: cornea after exposure to 4 mm filter paper disc saturated in n-octanol inserted into the centre; Bottom row, left to right: trypan blue stained corneas at 24, 48, and 72 h post-lesioning. (b) Progression of corneal wound healing for the same time period in serum free medium (open square with a solid line), medium with 8% serum (black filled square with long dash lines), and medium with TTHX1001 (100 ng/mL) ($N = 5$ per group) (black filled triangle with medium dash lines). $^{*}p < 0.05$.

vehicle treated eye, cell numbers and areas returned to normal at 14 days but the % hexagonality remained low. Unlike humans, rabbits will regenerate damaged endothelium in about two weeks without any treatment (Figure 5), so the rate of healing by TTHX1001 observed here is 5 times faster than the natural process.

Treatment with TTHX1114 also improved clearing of corneal opacity from involvement of the entire thickness of the stroma (3+) following injury to some loss of transparency with only the epithelium and/or involvement of the anterior half of the stroma (1+) by Day 14 (Figure 6). In contrast, corneal clearing in contralateral control eyes that received only vehicle progressed to some loss of transparency with cloudiness that extended past the anterior half of the stroma (2+) by Day 14. There was significant acceleration of improvement in opacity compared to contralateral vehicle control eyes at Day 5 for the 1,000 ng/eye and 5,000 ng/eye doses of TTHX1114 and at Day 7 and 14 for all doses. There was no clear differentiation of doses between 100 and 5,000 ng/eye, suggesting that all doses are at the top of the dose response curve. Both TTHX1001 and TTHX1114 were equally effective in the corneal clearing effects.

Discussion

While FGF1 is well known to stimulate proliferation of a wide variety of target cells because of its ability

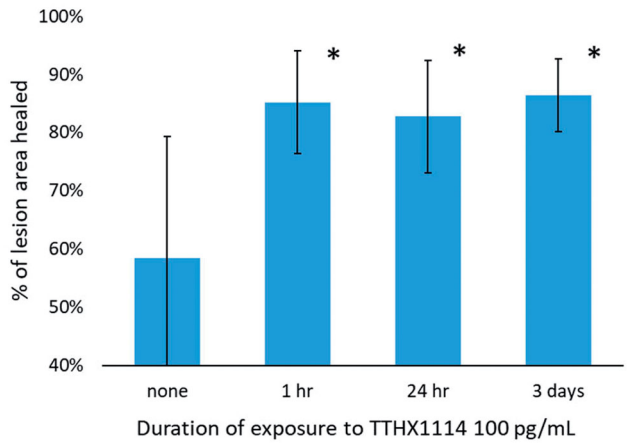

Figure 4. Effect of brief vs sustained treatment of chemically lesioned rabbit corneas. Corneas lesioned as in Figure 3 were exposed to vehicle only for three days, 100 pg/mL of TTHX1114 only for the first h followed by vehicle the remainder of the 3 days, 100 pg/mL of TTHX1114 only for the first 24 h followed by vehicle the remainder of the 3 days, or continuous exposure to drug for 3 days. The area of the endothelial lesion was quantitated using Image J. $^{*}p < 0.01$ vs no drug. Tukey's multiple comparisons test was performed and found a significant difference between the drug treated and vehicle groups. There is no significant difference between the drug treated groups ($N = 6$ per a group).

to activate all seven FGFRs at pM affinity (Ornitz et al. 1996; Zhang et al. 2006), its practical utility is severely compromised by low thermodynamic stability, rapid inactivation at physiologic temperature, susceptibility to proteolysis, and the need for heparin as a stabilising excipient and co-factor (Copeland et al. 1991). However, structural analyses (Zhu et al. 1991; Blaber, DiSalvo, and Thomas 1996; Ogura et al. 1999) have provided a clearer insight into the bases of these pharmaceutically undesirable properties and computational modelling has led to the production of a number of engineered human FGF1 variants that address these limitations to varying degrees (Brych et al. 2001; Xia et al. 2012, 2016). Two of these, TTHX1001 and TTHX1114, each characterised by three amino acid substitutions, were chosen for evaluation in these rabbit CEC proliferation/regeneration studies, because they were less prone to cysteine oxidation, had stabilised conformations and reduced susceptibility to proteolysis, and improved potency. TTHX1001 (K12V, C117V, P134V) has two stabilising mutations and a substitution of the most susceptible cysteine residue (Ortega et al. 1991) and TTHX1114 (C16S, A66C,

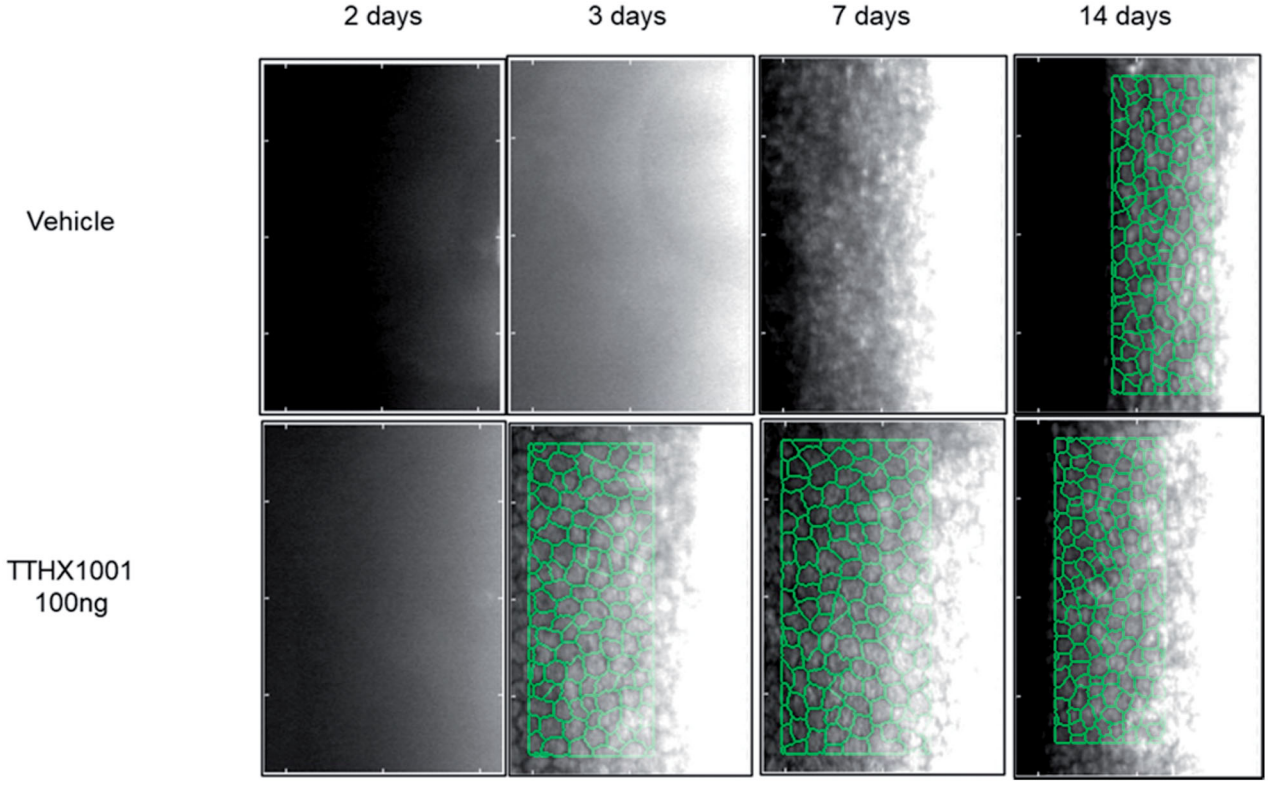

Figure 5. TTHX1001 induced cornea endothelial cell recovery following cryolesioning in the rabbit as measured by specular microscopy over a period of two weeks. The green lines are the outlines of the CECs as determined by the specular microscope; the absence of green lines indicates that the specular microscope could not identify a pattern of CEC borders. For cell counts, hexagonality and cell areas see Table 2.

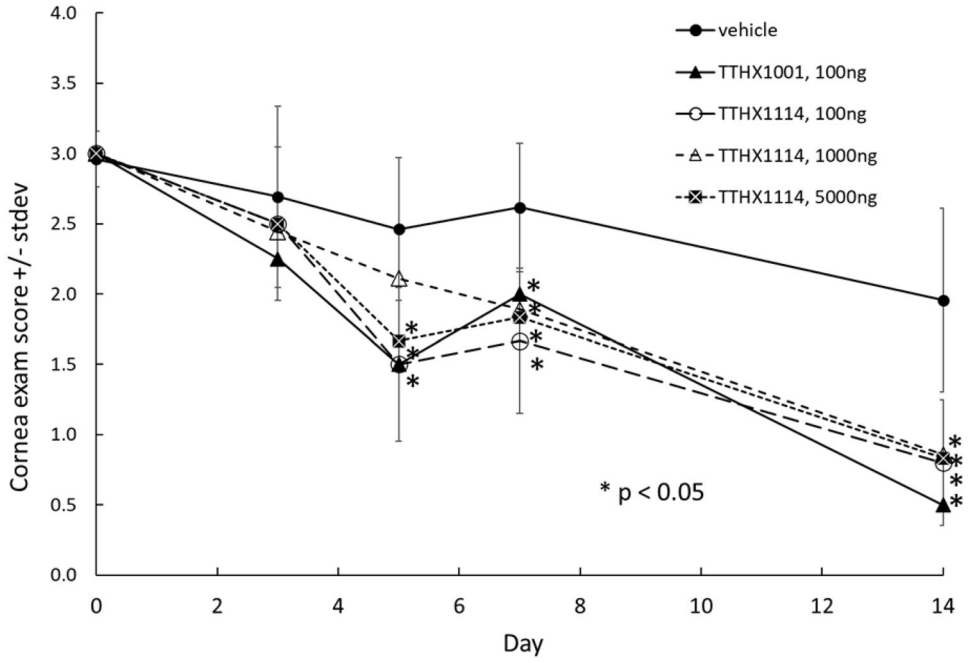

Figure 6. Cornea clearing by TTHX agents in the *in vivo* rabbit cryolesion model. New Zealand white rabbits were subjected to corneal cryolesioning in both eyes with one eye treated with a single intracameral injection of drug and one with vehicle (25 μL) within 2 h of lesioning. Corneas were scored at 3-, 5-, 7-, and 14-days post injury. Differences between means tested using Mann-Whitney. All comparisons to vehicle $p < 0.05$ at days 5, 7, and 14, Scoring: 0 = normal 1= some loss of transparency; only epithelium and anterior stroma involved 2 = involvement of entire thickness of stroma; underlying structures barely visible 3 = involvement of entire thickness of stroma; underlying structures cannot be seen black filled in circles with solid lines: vehicle ($N = 25$); black filled in triangles with solid lines: TTHX1001(NF140), 100 ng ($N = 4$); open circles with long dash lines: TTHX1114 (NM141), 100 ng ($N = 6$); open triangles with medium dash lines: TTHX1114 (NM141), 1000 ng ($N = 6$); filled in squares with a white X and short dash lines: TTHX1114 (NM141), 5000 ng ($N = 6$).

C117V) lacks any thiol groups (A66C-C83 are oxidised to form an internal disulphide bond). These derivatives are essentially fully active in the absence of heparin although they can still bind it. When they are bound to heparin, they are only minimally activated compared to untreated controls in contrast to native FGF1, which requires heparin for significant activity.

The recombinant FGF1 derivatives used in these studies were expressed in bacterial hosts because FGF1 requires no co/post-translational modifications. Two strategies were employed. On the one hand, both TTHX1001 and TTHX1114 were expressed in a construct that contained an N-terminal extension with an enterokinase sensitive sequence immediately adjacent to the N-terminal Phe. Processing of this precursor with enterokinase yielded the 140 amino acid form of FGF1 with an N-terminal Phe. In a few of the experiments reported, the peptide was not removed, and these derivatives thus contained the His-Tag sequence used for purification (Hochuli et al. 1988). The other route employed used a construct in which the initiator f-Met codon was placed immediately upstream from the Phe codon. Bacterial vectors can remove the formyl group but, as with all other organisms, cannot cleave the Met-Phe bond (Bradshaw, Brickey, and Walker 1998), yielding sequences of 141 amino acids with N-terminal Met residues. As judged by the stimulation of cultured pRCECs and mouse NIH 3T3 cells, the presence of the His-tag or the extra Met residue had no significant effect on TTHX1114 function.

Comparison of the responses of rabbit primary CECs and mouse NIH/3T3 cells, considered to be a mitogenic standard for cell proliferation assays, with both derivatives and native FGF1 (with and without heparin) indicated that the derivatives performed as well or better than native FGF1 (with heparin). In a direct comparison assayed with BaF3-FGFR1c cells (Figure 1), both derivatives showed higher levels of activity at equivalent concentrations. On average the EC_{50} value for both CECs and NIH/3T3 cells for TTHX1001 and TTHX1114 was around 10–15 ng/mL. Interestingly, the buffer in which these derivatives were assessed did appear to make a significant difference in the EC_{50} values observed, with PBS clearly being inferior to 50 mM sodium phosphate, 100 mM NaCl, 10 mM ammonium sulphate, 2 mM dithiothreitol, and 0.5 mM EDTA (pH 7.5) (Table 1). Similarly,

the presence of the excipient, human serum albumin, also lowered the EC_{50} by about an order of magnitude. This effect may be related to preventing absorption of the drug on glass surfaces.

The intact corneal endothelium is a hexagonal array that is characterised by tight junctions, and it is these structures that are detected by the Konan specular microscope (see Figure 5). When lesions are introduced, as was the case in these studies, the wound healing responses observed may be due to the proliferation of new cells and/or migration of already formed cells into the centre of the cornea from the periphery. As the lesion disrupts the contact inhibition that stabilises the monolayer, this may allow arrested proliferation to recur and thus be a contributing factor to the wound healing processes. Transplantation studies with fragments of Descemet's (Baydoun et al. 2018; Birbal et al. 2018) indicate that migration is also a major factor in sealing corneal lesions, being able to cover significant amounts of the disrupted area.

Although human CECs act largely as if they are post-mitotic entities (Van den Bogerd et al. 2018), it is now well established by *in vitro* experiments that they can proliferate and can be expanded for experimentation and transplantation (Toda et al. 2017; Okumura et al. 2018; Parekh et al. 2019; Yam et al. 2019; Frausto et al. 2020; Sie et al. 2020; Numa et al. 2021; Schlöetzer-Schrehardt et al. 2021), that they retain this ability in organ culture (Treffers 1982; Senoo and Joyce 2000; Patel and Bourne 2009; Eveleth et al. 2020) and that CECs from corneas of Fuchs patients retain this ability (Zaniolo et al. 2012). Thus, investigations with human CECs in both tissue and organ culture are feasible and are being pursued (Eveleth et al. 2020). Rabbit CECs, on the other hand, can regenerate themselves (see Figure 5). However, they are susceptible to stimulation, in this case by FGF1 derivatives, and thus provide an animal model for studying these processes, albeit imperfect. The lesions introduced in either the organ culture or *in vivo* experiments are not analogous to the losses in human corneal dystrophies, but they do provide useful systems for assessing the activity of the FGF1 derivatives. The underlying cause(s) for the loss of CECs that characterise Fuchs endothelial cell dystrophy and related disorders are not fully understood (Zhang, McGhee, and Patel 2019). There are clear genetic components to many of the conditions, but these affect many cellular processes and do not as yet provide clear targets.

FGFs are clinically proven regenerative agents for oral mucositis (Yuan and Sonis 2014) and wound healing (Zubair and Ahmad 2019). FGF1 and 2 are endogenous stimulators of CECs that are used as a standard component of CEC culture medium (Lee, Jung, and Heur 2018; Lee et al. 2020) and are both protective (Birbal et al. 2018) and potently mitogenic for CECs (Gospodarowicz, Mescher, and Birdwell 1977; Savion et al. 1982; Baird et al. 1985; Rieck et al. 1995, 2003; Dannowski et al. 2005; Lee, Jung, and Heur 2018). FGFs are known to induce migration (Lee and Kay 2006) and proliferation in bovine (Thalmann-Goetsch, Engelmann, and Bednarz 1997) and human (Dannowski et al. 2005) CECs and to stimulate endothelial wound healing *in vitro* and *in vivo* (Landshman et al. 1987; Schilling-Schon et al. 2000) thus making them candidates for regenerative therapy. However, utility of these proteins as drugs has been hampered by instability (Benington et al. 2020) TTHX1001 and TTHX1114 being stabilised derivatives (Xia et al. 2012, 2016) suggest that they are candidates for a pharmaceutical approach to treating human corneal endothelial dystrophies.

FGF1 and its analogs are not the only agents that may impact the proliferation and migration of CECs. Other growth factors (FGF2, EGF, etc) (Thalmann-Goetsch, Engelmann, and Bednarz 1997) as well as small molecules including p38 MAP kinase inhibitors (Nakahara et al. 2018) and Rho kinase inhibitors (Schlötzer-Schrehardt et al. 2021; Okumura et al. 2016) have been reported to stimulate proliferation and or migration. The Rho kinase inhibitor netarsudil has been clinically tested in humans and does not appear to impact CEC density after 6 months of treatment (Price, Feng, and Price 2021) although it has some effect on corneal thickness in FECD patients (Price and Price 2021). The reported proliferative effects of Rho kinase inhibitors on CECs *in vitro* are not large and the effect of Y-27632 is not reproducible in all laboratories (Meekins et al. 2016), while the proliferative effects of FGF1 *in vitro* are substantial and have been reproduced in several laboratories. In a direct comparison of stimulation of migration *in vitro*, FGF2 stimulated migration more strongly than Y-27632 (Lee and Kay 2006). Currently, a number of investigators are examining the impact of ripasudil on CEC migration in the context of Descemet's stripping only surgery (DSO) as an alternative to transplantation (Macsai and Shiloach 2019; Moloney et al. 2021). While there is not a direct comparison of FGF1 to Rho kinase inhibitors *in vitro* or *in vivo*, comparison of the magnitude of the effects in

separate experiments suggests that FGF1 is at least as effective as Rho kinase inhibitors and may potentially be more so, particularly in stimulating proliferation.

These studies also suggest that the administration of TTHX1114 by intracameral injection may be feasible in humans. The robust response of the rabbit *in vivo* cryogenic injury model to a single injection of TTHX1114 combined with the organ culture data showing that exposure of the corneas to drug for one hour is equivalent to continuous exposure for 3 days support the hypothesis that intracameral injection should be efficacious.

One concern for the use of FGFs for stimulation of CECs is the potential for inducing endothelial-mesenchymal transition (EnMT). FGF2 has been shown to induce some markers of EnMT in CECs although not the classical a-SMA characteristic of EMT (Lee, Jung, and Heur 2018; Lee et al. 2020) and the spectrum of FGF receptors stimulated by FGF1 and FGF2 have significant overlap. However, the transition to a mesenchymal phenotype is accompanied by dramatic changes in cell morphology not seen either in culture or *in vivo* in these experiments. Further, toxicology studies of TTHX1114 administered via the intracameral route have not shown any drug-induced abnormalities in the endothelial layer (D.D. Eveleth, J Weant, S. Pizzuto, manuscript in preparation). TTHX1114 is currently in phase 2 clinical studies for endothelial dysfunction (NCT04520321, NCT04676737).

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Author contributions

J. W., D.D.E., M. B., D. M. O., A. A., and R. A. B. designed the experiments; J. W., D.D.E, A. S., J.J. E., L. L., T. B., H. D., V. V., and X. Y. performed the laboratory work; R.A.B., J. W. and D.D.E. wrote the manuscript. All authors were involved in analysing the data and/or providing editorial comments/corrections to the final article.

Disclosure statement

J. W., D. D. E., A. S., J.J. E., M. B. and R. A. B. are employees and/or hold stock in Trefoil Therapeutics; M. B., D. M. O., and R. A. B. are consultants to Trefoil Therapeutics and are members of its Scientific Advisory Board; L. L., A. A., T. B., H. D., V. V., X. Y. declare no conflict of interests.

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ORCID

David M. Ornitz http://orcid.org/0000-0003-1592-7629

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