Retinitis pigmentosa (RP) is a heterogeneous group of inherited retinal degenerations, with more than 60 genes at play (the Retinal Information Network (RetNet)). All cases of RP are characterized by photoreceptor apoptosis, leading to gradual loss of vision and eventual blindness [1,2]. RP is the most common inherited retinal disorder worldwide, with an incidence of approximately 1/4,000, and affects more than 2 million people [3]. RP is, however, still incurable.

Of major importance in RP and other retinal diseases are neurotrophic factors, which stimulate the survival and differentiation of neurons. They comprise neurotrophins, neurokines, the fibroblast growth factor family, the transforming growth factor family, and the insulin-like growth factor family [4]. Several studies have examined steroid hormone signaling in retinal disease. Progesterone has been shown to protect retinal cells from apoptosis in a rat model of retinal ischemia [5], while other studies show no protection afforded by progesterone in light damage models of retinal degeneration [6,7]. The relationship between progesterone and neurotrophic factor signaling has also been disparate, having been shown to negatively and positively modulate brain-derived neurotrophic factor (BDNF) [8].

In contrast to those studies, our group has described the neuroprotective effects of the synthetic progestin, norgestrel, in a light-induced and an inherited model of RP [9]. Norgestrel prevented photoreceptor cell death and partially restored retinal function via a mechanism that included upregulation of the neurotrophic factor basic fibroblast growth factor (bFGF). The objective of the present study was to investigate whether the protection provided by norgestrel is likely to be mediated by other neurotrophins.

Correspondence to: Thomas Cotter, Cell Development and Disease Laboratory, Biochemistry Department, Biosciences Institute, University College Cork, Cork, Ireland; Phone: +353 21 4901321, FAX: +353 21 4901382; email: t.cotter@ucc.ie
METHODS

Cell culture: The 661W photoreceptor cell line was provided by Dr. Muayyad Al-Ubaidi (Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK). Cells were routinely grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum (both from Sigma, Arklow, Ireland) and 1% penicillin/streptomycin, at 37 °C in a humidified atmosphere with 5% CO₂. At 24 h post seeding, the cell medium was changed, and the cells were treated with 20 μM norgestrol or dimethyl sulfoxide (DMSO) vehicle control for 30 min to 6 h.

Mice: C57BL/6 or homozygous rd10/rd10 mice (B6.CXB1-Pde6brd10/J) were maintained and handled in accordance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. Mice were euthanized by cervical dislocation. All procedures were approved by the Health Products Regulatory Authority (HPRA), Ireland.

Norgestrel diet: At pup postnatal day (P) 10, the rd10 mothers were switched from their normal chow to either a control (LabDiet 5053 control diet) or norgestrel diet (LabDiet 5053, custom diet containing D(-)-norgestrel; Sigma #N2260). The norgestrel chow contains 0.05% norgestrel (500 ppm), which equates to a daily intake of approximately 80 mg/kg, assuming a 30 g mouse consumes around 5 g of food/day. The pups of dams that consumed the norgestrel diets received norgestrol through the dams’ milk from P10 until weaning at P20. Supplementation of a mouse maternal diet with neuroprotective agents has previously been shown to provide neuroprotection in pups [16].

Retinal explant culture: C57BL/6 mice from P30 to P40 were culled and decapitated. The eyes were enucleated under aseptic conditions. The anterior segment, vitreous body, and sclera were removed, and the retina was mounted on Millicell-CM3 multiwell plate (Millipore, Billerica, MA), photoreceptor-side down. Explants were cultured without RPE in a sphere with 5% CO₂, 1.8 mM KH₂PO₄, and blocked and permeabilized in 5% donkey serum with 0.1% Triton X-100. Cells were fixed on coverslips in 4% PFA for 20 min followed by blocking and permeabilization with 5% donkey serum with 0.1% Triton X-100. Subsequently, sections or cells were incubated with primary antibody solutions overnight at 4 °C: anti-LIF 1/400 (Santa-Cruz Cat# SC20087) and anti-glutamine synthetase (GS) 1/100 (Millipore Cat# MAB302). Antibody binding was detected by using conjugated secondary antibodies (Alexa Fluor 594 or Alexa Fluor 488; Invitrogen, Carlsbad, CA) at a dilution of 1/500 for 1 h at room temperature. Sections incubated with secondary antibody only confirmed positive immunolabeling with primary antibodies (data not shown). Hoechst 33,342 (1 μg/ml; Sigma-Aldrich) was used to counterstain the nuclei. Sections were mounted with Mowiol mounting medium (Sigma) and viewed under a fluorescence microscope (Leica DM LB2; Leica).

RNA isolation, cDNA synthesis, and real-time PCR: tRNA isolation from 661W cells and retinal explants was performed using an RNeasy Midi Kit (Qiagen, Manchester, UK.) according to the manufacturer’s instructions, including DNase treatment to digest residual genomic DNA. Cells were lysed by vortexing and explants by homogenization using a pellet pestle cordless motor (Sigma). RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR (RT–PCR) was performed using murine QuantiTect Primer Assays (Qiagen) and SYBR Green JumpStart Taq ReadyMix (Sigma) in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Life Technologies, Paisley, UK). The RT-PCR profile consisted of 40 cycles of 30 s at 95 °C, 60 s at 60 °C and 30 s at 70 °C. The mRNA values were normalized to the geometric mean of three endogenous reference genes: β actin, Gapdh, and Hprt. Relative changes in gene expression were quantified using the comparative Ct (DΔCt) method as described by Livak and Schmittgen [17].

Western blotting: Protein samples were isolated along with tRNA from the RNeasy Kit (midi) from Qiagen. After the ethanol centrifugation step, the flow-through, which contains total protein, was retained. Protein was then precipitated by adding the flow-through to 4X volume acetone at −20 °C overnight. Samples were centrifuged at 3577 ×g for 15 min at 4 °C. The acetone was decanted and pellets were allowed to air dry before being re-suspended in 50 μl protein rehydration buffer (7 M Urea, 2 M thiourea, 4% CHAPS (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) (w/v), 1 M Tris pH 8.8) containing Complete, EDTA-free Protease Inhibitor Cocktail Tablets (Roche; Dublin, Ireland). The total protein concentration was determined with Bradford assay,
using bovine serum albumin (BSA) as the standard. Equivalent amounts of protein were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in 4X Protein Sample Loading Buffer (LI-COR Biosciences UK Ltd, Cambridge UK) and then transferred onto nitrocellulose membranes (Schleicher & Schuell, Whatman, Dassel, Germany). Membranes were blocked with 5% (w/v) BSA in Tris-buffered saline/0.1% Tween-20 (TBST) for 1 h at RT and then incubated at 4 °C overnight with primary antibody: anti-LIF (1:1,000; Santa Cruz Cat# SC20087) and anti-tubulin (1:10,000; Sigma Cat# T5168). Membranes were washed three times for 5 min in TBST before the appropriate Alexa Fluor fluorescent secondary antibodies diluted 1:10,000 in 5% BSA/TBST solution were added. Blots were scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences) for fluorescent detection of the secondary antibodies. Fluorescence signal intensity was quantified using Image Studio Lite software (LI-COR Biosciences).

siRNA transfection: Cells were transfected with specific siRNA (25 nM) targeted against murine Lif (Flexi-Tube siRNA, Qiagen) or a non-targeting scrambled control (AllStars Negative Control siRNA, Qiagen). Transfections were performed using HiPerFect transfection reagent (Qiagen) according to the manufacturer’s protocol.

Assessment of cell viability: The CellTiter96® AQueous One Solution Cell Proliferation Assay (MTS; Promega, Madison, WI) was used to quantitate cell viability. A total of 4×10^4 cells per well were seeded in 96-well plates 6 h before transfection with siRNA. The following day, the cell media were replaced with serum-free media containing either 20 µM norgestrel or vehicle control (DMSO). Un-transfected cells maintained in complete media without treatment provided a positive control. Twenty hours after treatment, the cells were incubated with 20 µl of the MTS solution for 4 h at 37 °C. The quantity of the formazan product was measured with absorbance at 490 nm using a microplate reader (Molecular Device Corporation, SpectraMax Plus 384, Sunnyvale, CA). This is directly proportional to the number of living cells; thus, the absorbance of the formazan formed in the un-transfected, un-stressed control cells was taken as 100% viability.

Statistical analysis: Data are presented as mean values ± standard error of the mean (SEM) and are representative of at least three individual experiments. Data were statistically analysed using ANOVA (Graph Pad, Prism 6, GraphPad Software, Inc., La Jolla, CA) with values of p < 0.05 being considered statistically significant.

RESULTS

Norgestrel increases LIF in vitro: Our previous data showed that administration of norgestrel increases expression of bFGF at the protein level in wt C57BL/6 and rd10 mice, supporting the concept that the neuroprotection afforded by norgestrel is mediated in part through bFGF [9]. There are, of course, several other neurotrophic factors known to provide neuroprotection [4,18,19], which may also contribute to norgestrel’s effects. We have already shown that ciliary neurotrophic factor (CNTF) and BNDF are not such contributors [9]. Here, we screened 661W cells for any changes in mRNA expression of the neurotrophins Lif, epidermal growth factor (Egf), hepatocyte growth factor (Hgf), and nerve growth factor (Ngf) along with bFGF, in response to norgestrel (Figure 1A). At 1 and 3 h post-norgestrel challenge, Lif mRNA was significantly upregulated compared to vehicle control, which cells were treated with for 6 h. When cells were treated with vehicle control at each time point, the upregulation of Lif following norgestrel treatment for 1 h remained significant (Figure 1B). This increase was matched with a significant increase in functional protein (Figure 1C), which was corroborated by immunofluorescence staining (Figure 1D). As expected, bFGF mRNA was also upregulated in response to norgestrel at 3 h (Figure 1A). This delay in comparison to Lif supports the observation by Joly et al. that upregulation of Lif is required for that of bFGF [20].

Norgestrel increases LIF ex vivo: To investigate whether norgestrel increases LIF in an ex vivo model of retinal stress, we employed live retinal explants, a system we have previously demonstrated is a good model to study the retina under stress [21,22]. We first screened explants for any change in neurotrophic factor mRNA expression in response to norgestrel and found Lif to be significantly upregulated at 3 and 6 h post treatment compared to the 24 h vehicle control (Figure 2A). When compared to vehicle control at all time points studied, the increases in Lif mRNA at 3 and 6 h remained significant (Figure 2B). An increase at the protein level was observed 6 h post norgestrel treatment, with a slight increase in LIF in the inner plexiform layer (IPL) and a marked increase in the outer plexiform layer (OPL) and the photoreceptor outer segment layer (OSL; Figure 2C). Costaining with Hoechst dye allowed orientation of the retinal cell layers.

Norgestrel potentiates increases in LIF during photoreceptor degeneration: Endogenous LIF has been shown to increase during photoreceptor degeneration as part of an intraretinal prosurvival cascade [20,23-29]. We looked at the expression of Lif at the mRNA level in rd10 mice at P15 and P20 undergoing retinal degeneration. Compared to the age-matched wild-type C57BL/6 controls, Lif was markedly
Figure 1. Norgestrel increases LIF in 661W cells. A: 661W cells were screened for relative changes in mRNA expression of the neurotrophic factors leukemia inhibitory factor (Lif), epidermal growth factor (Egf), hepatocyte growth factor (Hgf), and nerve growth factor (Ngf), and basic fibroblast growth factor (bFGF) following 20 µM norgestrel treatment for the times shown or vehicle control (ctrl) for 6 h. B: Relative expression of Lif in 661W cells treated with 20 µM norgestrel or vehicle control for the times indicated. For A and B, relative expression was analyzed with real-time (RT) PCR, with the fold change compared to the geometric mean of the three endogenous reference genes. C: Western blot analyses confirm an increase in LIF at the protein level following treatment with 20 µM norgestrel or vehicle control. Densitometry was calculated from three individual experiments and graphed as a percentage of the control. A representative blot is shown. Tubulin acted as the loading control. D: Immunofluorescent staining also shows an increase in LIF immunoreactivity (green) in 661W cells following norgestrel treatment for the times indicated or vehicle control for 6 h. Nuclei were stained with Hoechst dye. Scale bar represents 50 µm. Error bars denote standard error of the mean (SEM) from three independent experiments. **p<0.01, ***p<0.001, ****p<0.0001 (ANOVA).
upregulated (Figure 3A). Interestingly, this increase was lower (approximately 40%) at P20, which coincided with the loss of photoreceptor cells in the outer nuclear layer (ONL), which begins at P16 [30]. To investigate whether norgestrel brings about a further increase in LIF in vivo, immunofluorescence staining was performed on retinal sections from rd10 pups that received either a norgestrel or control diet as described in the Methods section. We have previously shown that norgestrel protects photoreceptors in the rd10 model by reducing apoptosis [9]. At P20, there is preservation of the ONL in the mice that consumed norgestrel daily compared to the control diet (Figure 3C). As photoreceptor loss does not

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**Figure 2.** Norgestrel increases LIF in retinal explant cultures. A: Retinal explants from C57BL/6 were screened for changes in relative mRNA expression of the neurotrophic factors leukemia inhibitory factor (LIF), epidermal growth factor (Egf), hepatocyte growth factor (Hgf), and nerve growth factor (Ngf), and basic fibroblast growth factor (bFGF) in response to treatment with 20 µM norgestrel for the times indicated, or vehicle control (ctrl) for 24 h. B: Relative expression of Lif in C57BL/6 retinal explants treated with 20 µM norgestrel or vehicle control for the times indicated. For A and B, relative expression was analyzed with real-time (RT) PCR, with the fold change compared to the geometric mean of three endogenous reference genes. C: Immunofluorescent staining detects an increase in LIF (green) at the protein level in explants treated with norgestrel for 6 h compared to vehicle control. Sections were counter-stained with Hoechst dye for orientation of the retinal layers. Outer segment layer = OSL; outer nuclear layer = ONL; outer plexiform layer = OPL; inner nuclear layer = INL; inner plexiform layer = IPL. Scale bar represents 50 µm. Error bars denote standard error of the mean (SEM) from three independent experiments. *p<0.05, **p<0.01, ***p<0.001 (ANOVA).
begin until approximately P16 in the rd10 model, no difference in ONL thickness is observed at P15 (Figure 3B). Before photoreceptor loss (P15) and during photoreceptor loss (P20), norgestrel increased LIF levels in the IPL, OPL, and OSL. GS was used to costain for Müller glial cells given that a subset of these cells has been shown to produce LIF during retinal degeneration [20]. Colocalization was observed at several Müller glia end feet in the IPL and extensions in the OPL.

Reduced LIF expression abolishes the norgestrel-mediated protection of stressed photoreceptor cells in vitro: Cone cell death in RP is secondary to that of rods and is the main reason for sight loss. Although the molecular reasons for cone cell death remain unclear, it is known that loss of rod-derived trophic support plays a major role [31]. In vitro removal of trophic support by means of serum starvation has been shown to induce cell death of 661W photoreceptor cells [32-34].

Figure 3. Norgestrel potentiates increases in LIF in degenerating rd10 retinas. A: Relative expression of Lif in P15 and P20 rd10 retinas compared to age-matched wild-type C57BL/6 controls, as measured with real-time (RT) PCR, with fold change compared to the geometric mean of three endogenous reference genes. Error bars denote standard error of the mean (SEM). B and C: Immunostaining of LIF (green) and glutamine synthetase (GS; red) in rd10 retinas at P15 (B) and P20 (C). Increased LIF levels are observed in mice that received the norgestrel diet (lower panels) during retinal degeneration, before photoreceptor loss (P15) and during photoreceptor loss (P20). Colocalization (yellow staining) is observed with a sub-set of Müller glial cells in the OPL and at the Müller glia end feet in the inner plexiform layer (IPL). Scale bars represents 50 µm. Results are representative of n = 3 mice.
This is reversed by treatment with norgestrel (Figure 4A). To investigate whether LIF plays an essential role in norgestrel-mediated cell survival, 661W cells were transfected with specific siRNA targeted against Lif or a non-targeting scrambled control. Cells were then left untreated, or challenged with norgestrel or vehicle control, under serum-free conditions. Following 24 h of serum starvation, cell viability was analyzed with the MTS assay. Knockdown of Lif, which was >50% at the mRNA level (Figure 4D), did not affect cell viability relative to the scrambled control in untreated cells, with both having 40% cell viability (Figure 4B). In non-targeting siRNA transfected cells, norgestrel restored cell viability to 80%, while no change in cell viability was observed in the Lif knockdown cells compared to the vehicle control. Cell viability was taken as a percentage of healthy

Figure 4. LIF is required for norgestrel-mediated cell survival in 661W photoreceptor cells. A: Cells were maintained in complete medium without any treatment, i.e., healthy cells (cells), or were serum starved for 24 h and treated with 20 µM norgestrel (Norg) or vehicle control (ctrl). B: Before 24 h serum starvation, the cells were transfected with siRNA targeted against leukemia inhibitory factor (siLif) or a non-targeting scrambled control (scram). C: Cells were again transfected with siLif or scrambled control followed by serum starvation and treatment with 20 µM norgestrel (Norg) or vehicle control (ctrl). D: Detection of Lif knockdown with real-time (RT) PCR (left) and western blotting (right). Error bars denote standard error of the mean (SEM) from three independent experiments. *p<0.05, **p<0.01 (ANOVA).
cells maintained in complete medium without any treatment (Figure 4A).

**DISCUSSION**

The pleiotropic cytokine LIF is one of more than 50 neurotrophic factors expressed in the central nervous system (CNS) [35]. LIF is involved in the normal development of the retina [36,37], and is a principal prosurvival molecule in retinal degeneration, as one of the main players in promoting photoreceptor survival under noxious conditions. Given that photoreceptors have a high metabolic rate, oxidative stress is a key contributor to the pathology of RP and other retinal degenerative conditions [38-41]. LIF has been shown to be essential for reducing oxidative stress in the retina [42] and in female reproductive tissue [43]. Furthermore, Lif is upregulated in response to endogenous progesterone in the uterus [44], and thus, it is not surprising that we observed increased Lif levels in response to norgestrel.

Progesterone signaling in neurons has gained much interest since the discovery of steroidogenesis in the CNS by Baulieu et al. This group and others have demonstrated the correlation between progesterone and more favorable outcomes after neurologic traumas, such as stroke and traumatic brain injury (TBI) [45,46]. In the retina, de novo synthesis of the progesterone precursor pregnenolone [47] and progesterone receptor expression [48] have been reported, suggesting an important role for this steroid hormone in retinal signaling.

We have previously demonstrated the neuroprotective effects of the synthetic progestin norgestrel in the retina and shown that norgestrel’s actions are mediated, at least in part, by bFGF [9]. The present study expands on this data by identifying another mediator of the norgestrel-induced survival pathway. Of all neurotrophic factors studied in response to norgestrel, Lif exhibited the most marked increase in transcript expression (Figure 1A, 2A), suggesting this cytokine plays a major role in the neuroprotection elicited by this drug. Lif mRNA is significantly upregulated in the 661W photoreceptor cell line and retinal explants with concomitant increases in functional protein. The increase in Lif preceded that of bFGF in 661W cells (Figure 1A); Lif has been shown to be necessary for the upregulation of bFGF during retinal stress [20]. We have previously shown that bFGF is upregulated in retinal explants over time, due simply to the stress of explanting [21], and thus, this may explain why no increase in bFGF was observed in norgestrel-treated explants compared to the control (Figure 2A).

In explant cultures, Lif is increased in the IPL, OPL, and OSL in response to norgestrel. This staining pattern is also observed in degenerating rd10 retinas, before photoreceptor loss (P15) and during photoreceptor loss (P20), suggesting that this cytokine is an important facilitator of norgestrel-mediated retinal protection. Here, containing with a subpopulation of Müller glial cells is evident within the IPL and the OPL (Figure 3A,B). Interestingly, at P20, norgestrel-fed mice showed an increase in GS immunoreactivity (Figure 3C, lower panel). This observation is supported by those of Joly et al., who reported that under conditions of photoreceptor stress, LIF is upregulated in a sub-set of Müller glial cells which brings about a feed-forward mechanism leading to increased Müller cell activity [20]. Moreover, Lif has been shown to be necessary for Müller cell activity following optic nerve crush [49]. GS mops up glutamate, which is produced to noxious levels in the retina following an insult. Accordingly, increases in GS have been shown to be protective against retinal insults [50].

The observed increase in LIF in the OSL and the OPL of the rd10 retinas (Figure 3B,C), together with the fact that rod and cone pedicles reside within the OPL, suggests that photoreceptors may also produce LIF, at least in response to norgestrel, which is certainly the case in vitro (Figure 1). The observation that endogenous LIF expression is decreased in rd10 retinas at P20 compared to P15 (Figure 3A), which coincides with photoreceptor loss, again suggests that this cytokine may be produced by photoreceptor cells. As retinal degeneration progresses, LIF expression would be reduced, which would be detrimental to the retina. Lif has been shown to be an early player in autosomal dominant retinal degeneration, mediating an intraretinal prosurvival cascade, where genetic knockout of Lif resulted in rapid acceleration of photoreceptor loss [20].

To investigate whether LIF is directly linked to norgestrel, 661W cells were serum-starved to mimic the loss of trophic support that occurs during retinal degeneration. Serum starvation induces apoptosis and thus reduces cell viability, which is reversed by norgestrel (Figure 4A). To determine whether LIF is required for this in vitro rescue, Lif expression was reduced using siRNA. With a knockdown of approximately 60% (Figure 4D), norgestrel failed to rescue photoreceptor cells from cell death (Figure 4C); strongly suggesting that LIF is required for norgestrel-mediated photoreceptor survival.

The signaling pathways upstream and downstream of LIF in the retina have been well studied, and it is well documented that this cytokine is a critical mediator of the self-preservation mechanisms employed by the retina when under toxic and degenerative conditions [20,24,27,29,36,51,52]. Here, we showed that the synthetic progestin norgestrel increases levels
of LIF in vitro and in vivo. In the latter, concurrent rescue of the ONL was observed, as previously described [9], strongly suggesting that LIF is involved in norgestrel-mediated retinal protection. Our in vitro studies suggest that norgestrel acts directly to upregulate LIF, as endogenous progesterone does [44]. Our results support previously published findings that LIF is produced by Müller glial cells, and we suggest that it is also produced by photoreceptor cells, at least in response to norgestrel. We recently identified an important receptor for norgestrel, progesterone receptor membrane component 1 (PGRMC1), which we found to be highly expressed in the photoreceptor layer of degenerating rd10 retinas [34].

In conclusion, we identified LIF as an important effector of norgestrel-mediated neuroprotection. We propose that norgestrel acts to maintain increased LIF in degenerating retinas, underscoring the viability of norgestrel as a therapeutic option for treating retinal degenerative conditions, notably RP.

APPENDIX 1. STR ANALYSIS
STR analysis. To access the data, click or select the words “Appendix 1.”

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