Overexpression of Human HMW FGF-2 but Not LMW FGF-2 Reduces the Cytotoxic Effect of Lentiviral Gene Transfer in Human Corneal Endothelial Cells

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Purpose. Recently, insertion of immuno-modulatory or anti-apoptotic genes into corneal endothelial cells (HCECs) came into focus. Basic FGF-2 occurs in one secreted (low molecular weight, LMW, 18 kD) and four nuclear (high molecular weight, HMW, 22–34 kD) isoforms. HMW isoforms are known differentiation and survival factors, while LMW FGF-2 is a known mitogen. The effect of FGF-2 overexpression of each of the five known isoforms on HCEC cell survival after lentiviral gene transfer in different culture media was investigated.

Methods. Cells were transduced with lentiviral vectors encoding for each of the five FGF-2 isoforms. Transduction efficiency and expression of individual FGF-2 isoforms was assessed by marker gene transfer and western blotting. Primary HCECs were cultured and transduced in four different media previously described for HCEC cultivation or corneal organ cultivation. Cytotoxic effect of virus infection and a possible rescue effect of FGF-2 overexpression were determined by resazurin conversion assay.

Results. Transduction with FGF-2 encoding lentiviral vectors resulted in overexpression of the respective isoform in all tested cell populations. Western blotting after total cell lysis proved nuclear localization of transgenic HMW isoforms. Overexpression of HMW FGF-2—especially 34 kD FGF-2—reduced lentiviral cytotoxicity, while overexpression of LMW FGF-2 aggravated viral cytotoxicity.

Conclusions. Cytotoxicity of lentiviral gene transfer in corneal endothelial cells may be reduced by using bicistronic vectors that encode for the target gene and the 34-kD isoform of human FGF-2. Such cotransduction of a survival factor may increase cell survival after gene transfer, thereby improving gene therapeutic approaches. (*Invest Ophthalmol Vis Sci.* 2012;53:3207–3214) DOI:10.1167/iovs.12-9423

Human corneal endothelial cells (HCECs) were shown to express 18-kD FGF-2 and the FGF-2 receptors FGFR-1 and FGFR-2.[1,2] FGF-2 mainly reacts on mesodermal and neuroectodermal cells including corneal endothelial cells, where it can trigger re-entry into the cell cycle.[3–7] As a culture medium supplement, FGF-2 was shown to sustain corneal endothelial cell survival and proliferation in serum-reduced and serum-free culture media.[1,2,4,5,8–13] Furthermore, after cell transplantation of corneal endothelial cells on denuded donor corneas, addition of FGF-2 to the organ culture medium supported a differentiated morphology of the transplanted cells, although it did not promote cell proliferation in situ.[14,15]

To date, five isoforms of FGF-2 are known. The 18-kD low molecular weight (LMW) isoform of FGF-2 is cytosolic and can be secreted by an unconventional mechanism independent of ER/Golgi processing,[16,17] while the four high molecular weight (HMW) isoforms of FGF-2, with sizes between 22–34 kD, localize to the nucleus due to a nuclear localization sequence (NLS)-like signal.[18] All five known isoforms are encoded by a single mRNA and share the sequence of the 18-kD isoform, with the HMW isoforms being N-terminal elongations of the LMW isoform.[18,19] In normal rabbit corneal endothelial cells, coexpression of the 18-kD and 24-kD isoforms was observed during proliferation, with expression of the 24-kD isoform being reduced after reaching confluence.[20] Selective or induced overexpression of the HMW isoforms can promote cell survival under starvation conditions, and some transformed cell lines like SK-Hep2 or HeLa express some of these isoforms constitutively.[18,21–23]

Lentiviruses are very efficient tools to transduce HCECs, but it could recently be shown that lentiviral gene transfer is cytotoxic to HCECs depending on vector design.[24] Therefore, this study sought to determine if overexpression of LMW or either of the HMW isoforms of human FGF-2 can minimize lentiviral cytotoxicity and increase cell survival after lentiviral gene transfer. Examining the effect of FGF-2 overexpression under different nutrient conditions was another point of interest, because it could also be shown that the choice of culture medium influences lentiviral cytotoxicity in HCECs.[24] Here the native expression and overexpression of the five FGF-2 isoforms after lentiviral gene transfer was investigated in normal HCECs in vitro under conventional or stress conditions (enriched or nutrient-poor culture media).

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METHODS

Cell Culture

All cell culture experiments adhered to the tenets of the World Medical Association (WMA) Declaration of Helsinki. All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. HCECs were isolated from organ-cultured corneas (donor age 49.3 ± 28.7 years, ranging from 2–78 years) that were unsuitable for keratoplasty due to unclear serological results using the method of Engelmann et al.[13] Cells were cultured in medium $F99_{HCEC}$ (F99 supplemented with 5% FCS [Gibco Invitrogen, Karlsruhe, Germany]); 20 μg/mL ascorbic acid (Sigma Aldrich, St. Louis, MO); 10 ng/mL human recombinant FGF-2 (Gibco Invitrogen); 20 μg/mL human recombinant insulin (Sigma Aldrich); 2.5 μg/mL amphotericin B and 50 μg/mL gentamycin (Biochrom AG, Berlin, Germany) in a well of a 24- or 12-well plate pre-coated with a mixture of 10 μg/mL laminin (Gibco Invitrogen) and 10 mg/mL chondroitin sulphate (Sigma Aldrich) solubilized in F99. From passage P1 on, the cells were cultured in pre-coated T25 tissue culture flasks. Primary (i.e., untransduced) HCECs of passages P2 to P8 after isolation from donor corneas were used for experiments. The immortalized human corneal endothelial cell population HCEC-12[25] was cultured in Medium $F99_{HCEC}$, and its clonal daughter cell lines HCEC-B4G12 and HCEC-H9C1[26] were cultured under serum-free conditions in medium Human Endothelial-SFM (Gibco Invitrogen) without antibiotics. The human embryonic kidney cell line 293T[27] and the human fibrosarcoma cell line HT1080[28] were cultured in Dulbecco's modified Eagle's medium (Gibco Invitrogen) supplemented with 10% fetal calf serum and penicillin/streptomycin (Gibco Invitrogen) as antimicrobial agents in T75 or T175 tissue culture flasks. All cells were detached using trypsin/EDTA (0.05%/0.02%; Gibco Invitrogen) and collected by centrifugation at 100 × g for 5 minutes. Trypsin was either quenched with serum-containing growth medium, or was quenched using protease inhibitor cocktail (cOmplete; Roche Applied Science, Mannheim, Germany) for serum-free cultured cells. Cells were then resuspended in their respective medium and seeded into new flasks or multiwell plates depending on the purpose of use.

Construction of FGF-2 Expression Vectors

The FGF-2 expression constructs are based on the replication-deficient lentiviral vector p6NST50[29] that contains a woodchuck hepatitis virus (WHV) posttranscriptional regulatory element (WPRE) and an EGFP marker gene coupled to a zeocin resistance gene (EGFP-zeo) downstream of an internal ribosomal entry site (IRES) (Fig. 1). The FGF-2 18-kD isoform gene was cloned from pUbFGF into a multiple cloning site (MCS) upstream of the IRES, producing p6NST50 FGF-2 18 kD. The HMW isoforms of the FGF-2 gene were composed of the 18-kD isoform cloned from pUbFGF linked to the respective expression-optimized upstream sequences (GeneArt, Regensburg, Germany), and likewise inserted upstream of the IRES, producing p6NST50 FGF-2 *x* kD, with *x* standing for 22, 22.5, 24, or 34. Individual cloning strategies and primers are available on request. All PCR-derived fragments are sequenced.

Lentivirus Production and Cell Transduction

Lentiviral vectors based on p6NST50[29] pseudotyped with a modified foamy virus envelope protein (FV Env) were produced as previously described[24] by cotransfection of 293T cells with equal amounts (5 μg DNA) of Env-encoding plasmid (pczPFVenv PE01[29]), Gag/Pol-encoding plasmid (pCD/NL-BH[30]), and viral transfer vector plasmid encoding for only EGFP-zeo (p6NST50) or for EGFP-zeo and one of the FGF-2 isoforms (p6NST50 FGF-2 x kD) using polyethyleneimine (PEI) transfection reagent (Sigma Aldrich). Cell-free viral vector containing 293T-supernatants were stored at −80°C until further use.

Transductions were performed by infection of 5×10^4 cells plated 24 hours (HT1080) or 72 hours (primary HCECs of passages P2 to P8) in advance in 6-well plates. HT1080 were incubated with 2-mL virus-containing 293T supernatants, either undiluted or diluted 1:10, and 1:100 in DMEM + 10% FCS. Primary HCECs were incubated with 2-mL virus-containing 293T supernatants diluted 1:10 in $F99_{HCEC}$. After 6 hours, the medium was changed to the respective fresh, virus-free culture medium. Uninfected cells, cells treated with supernatant from 293T cells transfected with pUC19 and treated as to produce virus (mock) and cells transduced with the EGFP only encoding vector (p6NST50) served as controls. Transduction efficiency of the vectors was determined in HT1080 cells by marker gene transfer assay as previously described.[24]

Western Blotting

Transduced, mock transduced, and uninfected cells were subjected to mild lysis (extraction of cytoplasmic proteins) or total lysis (extraction of whole cell protein) at 72 hours after infection. For mild lysis, cells were lysed in lysis buffer (10 mM Tris/HCl pH 8.0, 140 mM NaCl, 0.025% NaN_3, 1% Triton X-100) and centrifuged at 16,060 × g for 2 minutes to separate cell organelles from the cytoplasmic fraction. Equal volumes of 2x PPPC buffer (100 mM Tris/HCl pH 6.8, 24% glycerol, 8% SDS, 0.02% Brilliant Blue G, 2% 2-mercaptoethanol) were added to the supernatants and samples were boiled for 10 minutes at 95°C. For total lysis, cells were lysed in 2x PPPC and homogenized by passing through polymeric homogenization columns (QiaShredder homogenizer; Qiagen, Hilden, Germany) by a 2-minute centrifugation at 16,060 × g in order to obtain cytoplasmic and cell organelle constituents. Equal volumes H_2O were added and samples were boiled for 10 minutes at 95°C. The protein samples were electrophoresed by tricine SDS-PAGE on 10% polyacrylic acid (PAA) gels (running buffer: anode 0.2 mol/L Tris base, pH 8.9; cathode 0.1 mol/L Tris base, 0.1 mol/L Tricine, 0.1% SDS) or on 4–12% bis-tris gradient gels (NuPAGE 4–12% Bis Tris Gels; Invitrogen) (running buffer: NuPAGE MES SDS Running Buffer; Invitrogen; 50 mM 2-[N-morpholino]ethanesulfonic acid, 50 mmol/L Tris Base, 0.1% SDS, 1 mmol/L EDTA, pH 7.3), and semidry blotted onto Amersham Hybond ECL nitrocellulose membranes (GE Healthcare, Munich, Germany) at 0.9 mA/cm² for 1.5 hours. After protein transfer membranes were blocked in 5% non-fat milk in PBS-T and probed with a monoclonal mouse anti-human FGF-2 antibody (clone 3H3, Calbiochem; Merck Chemicals Ltd., Darmstadt, Germany) at a concentration of 0.5 μg/mL in 5% non-fat milk overnight at 4°C, and a secondary polyclonal goat anti-mouse IgG HRP-conjugated antibody (Dako,

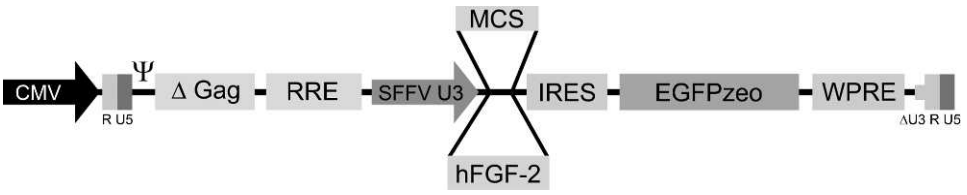

FIGURE 1. Lentiviral vector p6NST50: Sequences for human FGF-2 isoforms were cloned into an MCS downstream of the SFFV U3 promoter. Cap-independent expression of the marker gene EGFP-zeo is driven by an IRES, while the WPRE element regulates transgene expression by stabilizing nuclear export of transcribed RNA. Vector size ranges from 9448 bp (p6NST50 FGF2 18 kD) to 9833 bp (p6NST50 FGF2 34 kD). RRE: Rev responsive element, ΔGag: partially deleted Gag.

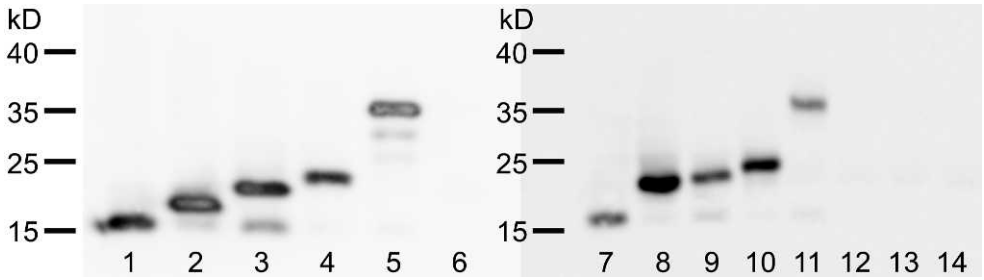

Figure 2. Representative immunoblot analysis of 293T and HT1080 cells: 293T cells (*lanes 1–6*) were transfected to produce lentiviral vectors and subjected to total cell lysis and subsequent immunoblotting for FGF-2. HT1080 cells (*lanes 7–14*) were transduced with lentiviral vectors and likewise analyzed. All five isoforms were overexpressed after transfection/transduction of 293T and HT1080 cells (*lanes 1, 7*: 18 kD; *lanes 2, 8*: 22 kD; *lanes 3, 9*: 22.5 kD; *lanes 4, 10*: 24 kD; *lanes 5, 11*: 34 kD). Controls (*lanes 6, 12*: transfected or transduced with the EGFP only encoding vector, *lane 13*: mock-transduced; *lane 14*: uninfected) gave no detectable signal.

Hamburg, Germany) at a dilution of 1:1000 for 2 hours at room temperature. The blots were developed with luminol for enhanced chemiluminescence using the ECL Plus Western Blotting Detection System (GE Healthcare) after blotting from 10% PAA gels or Immobilon Western HRP Substrate (Millipore, Schwalbach/Ts., Germany) after blotting from 4–12% bis-tris gradient gels, documented using an imaging system (LAS-3000; Fujifilm Europe GmbH, Dusseldorf, Germany) and analyzed with Image Gauge (Fujifilm) and Photoshop (Adobe) software. Blotting was repeated four times with cells from four different donors.

Cytotoxicity Assay

Primary HCECs of passages P2 to P8 were seeded in medium $F99_{HCEC}$ at densities of 1250, 2500, or 5000 cells/well into 96-well plates and allowed to attach and spread for 24 hours. The medium was then changed to test media and cells were infected with virus-containing 293T supernatant at a 1:10 dilution in the respective test medium. Test media were HCECs growth medium $F99_{HCEC}$, its unsupplemented basal medium F99, Human Endothelial-SFM (abbreviated SFM), and MEM (Biochrom AG) supplemented with 2% FCS (hereafter referred to as MEM). Uninfected cells, cells infected with EGFP only encoding vector (p6NST50), or cells treated with a 1:10 dilution of virus-free supernatant from 293T cells after transfection with pUC19 (mock) served as controls. Medium was changed to fresh medium 24 hours after infection and cells were further cultured for up to 9 days with medium changes every other day. Cell survival was determined at 3, 6, and 9 days after infection by resazurin conversion using a cell viability assay (CellTiter-Blue; Promega, Mannheim, Germany) according to the manufacturer's instructions. Resazurin conversion was measured fluorometrically in a plate reader (SPECTRAFluor Plus; Tecan, Crailsheim, Germany) using a 545-/595-nm excitation/emission filter pair at 3 hours after adding the test reagent. Data were recorded and analyzed using Magellan (Tecan) and Microsoft Excel software (Microsoft Deutschland GmbH, Unterschleissheim, Germany). Experiments were performed in quadruplicates (four donors) with two replicates per donor ($n = 8$).

Statistical Analysis

Values are given as mean ± SEM. Data were grouped by culture medium, initial cell number, and duration of cultivation, and each group contains the data points for the different treatments. Independent variables were culture medium ($F99_{HCEC}$, F99, MEM, SFM); initial cell number (1250, 2500, or 5000 cells/well); duration of cultivation (3, 6, or 9 days); and transduction (uninfected, mock-transduced, transduced with p6NST50, or with vectors encoding for one of the five FGF-2 isoforms). Statistical analysis was performed by multifactorial univariate analysis of variance (multi-way ANOVA) followed by post hoc analysis (Bonferroni-test). Significance was accepted at $P < 0.05$. Statistical analysis was performed using SPSS 17.0 (Chicago, IL).

Results

Vector Production and Recombinant Vector Titers

The first experimental setup examined whether transfection of 293T cells for viral vector production led to expression of the transgene, FGF-2. The results indicated that 293T producer cells transfected with p6NST50 did not express FGF-2 or expressed it below the detection threshold, while 293T producer cells transfected with FGF-2 encoding vector plasmids expressed the respective FGF-2 isoforms in abundance (Fig. 2, lanes 1–6). Therefore, it was expected that the viruses that were produced by transfected 293T cells could likewise induce overexpression of the respective FGF-2 isoforms.

In line with the expression analysis in transfected 293T cells, the different FGF-2 isoforms could also be detected in HT1080 cells after transduction with the respective lentiviral vectors. After mild cell lysis, only the LMW isoform was detectable, but not the HMW isoforms (see Supplementary Material and Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9423/-/DCSupplemental). HMW FGF-2 isoforms could only be detected after total cell lysis, indicating that they were located to the nucleus as expected (Fig. 2, lanes 7-14). Like in 293T cells, LMW FGF-2 was coexpressed after transduction with HMW encoding vectors. Transduction efficiencies of lentiviral vector encoding for only EGFP or for EGFP and one of the FGF-2 isoforms were similar, ranging from 1.37×10^6 for p6NST50 FGF-2 34 kD to 3.96×10^6 for p6NST50 (Fig. 3).

Expression of FGF-2 Isoforms in Transduced and Untransduced HCECs

Like in transduced HT1080, western blots performed after mild cell lysis of transduced HCECs gave only signals for the 18-kD isoform of human FGF-2 (see Supplementary Material and Supplementary Fig. S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9423/-/DCSupplemental), while overexpression of transgenic HMW FGF-2 was readily visible in transduced HCECs after total cell lysis (Fig. 4, lanes 1–5). Furthermore, coexpressed LMW FGF-2 was visible after transduction with HMW encoding vectors. Endogenous expression of the 22-/22.5-kD and 24-kD FGF-2 isoforms was also found in cells transduced with the EGFP only encoding vector (Fig. 4, lane 6) as well as in mock transduced (Fig. 4, lane 7) or in uninfected HCECs (Fig. 4, lane 8). These isoforms became more pronounced in cells transduced with FGF-2 ORF–containing vectors when using higher viral vector dilutions of 1:100 and 1:1000 for infection (data not shown).

This study also examined whether the observed expression of certain endogenous HMW isoforms in HCECs was due to

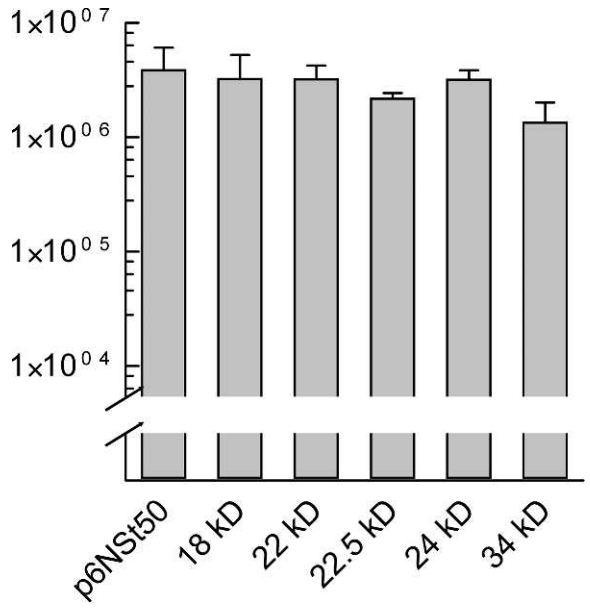

Figure 3. Transduction efficiency of p6NST50-based vectors in HT1080: Cells were transduced with lentiviral vectors encoding for either EGFP alone (p6NST50) or for EGFP and one of the known five isoforms of FGF-2 (18–34 kD). Transduction efficiency was determined by marker gene transfer assay. Titers are given as mean ± SEM. Statistical significance was accepted with $P < 0.05$. Data from $n = 4$ experiments.

cultivation of target cells prior to vector transduction. For this purpose, total cell lysates from cultured untransduced HCECs from two different donors (Fig. 4, lanes 9,10) and three immortalized HCEC populations (Fig. 4, lanes 12–14) were analyzed by immunoblotting after total cell lysis. In addition, Descemet's membranes with corneal endothelium were stripped from two human donor corneas, immediately subjected to total cell lysis, and analyzed likewise (Fig. 4, lane 11). Bands for the 18-kD, 22-/22.5-kD, and 24-kD isoforms were readily visible in untransduced, in vitro cultured primary HCECs (Fig. 4, lanes 9, 10) and in the immortalized HCEC populations (Fig. 4, lanes 12–14). Expression of HMW FGF-2 was not detectable in the stripped Descemet/HCEC lysate. Here, only the 18-kD isoform could be demonstrated (Fig. 4, lane 11). Notably, the 18-kD FGF-2 isoform was differentially detectable in the two uninfected donor cell lysates (Fig. 4, lanes 9, 10), probably because of a differential secretory behavior. A similar but less dramatic difference was also seen in the three immortalized HCEC populations (Fig. 4, lanes 12–14), which were derived from the same donor.

Cytotoxicity of Lentiviral Transduction and Cell Rescue by FGF-2 Expression in Different Culture Media

HCECs were transduced and cultured in four different culture media for 9 days, as described in the Methods section. Generally, metabolic activity of HCECs was lower when cells were cultured in the nutrient-poor media F99 and MEM, and was higher in the enriched media $F99_{HCEC}$ and SFM. As can be seen in Fig. 5, metabolic activity of the cells decreased over the 9-day observation period in media F99 and MEM, but not in media $F99_{HCEC}$ and SFM. In detail, the metabolic activity of uninfected cells (Fig. 5, white bars) cultured in the nutrient-poor media MEM and F99 decreased from day 3 to day 9. In contrast, metabolic activity of uninfected cells cultured in the nutrient-rich medium $F99_{HCEC}$ increased until day 9, and cells cultured in SFM retained a stable metabolic activity. Notably, cells that were transduced with EGFP only encoding vector (p6NST50, Fig. 5, dark grey bars) or HMW FGF-2 isoforms (Fig. 5, light orange to dark red bars) and cultured in MEM benefitted from lentiviral transduction and showed a higher metabolic activity than uninfected cells or cells transduced with 18 kD FGF-2 (Fig. 5, yellow bars). In SFM, neither mock nor lentiviral transduction or overexpression of any of the FGF-2 isoforms seemed to have such a distinct effect on cell metabolic activity than in the other media. Here, overexpression of the 18-kD isoform did not impair cell metabolic activity (Fig. 5, yellow bars in SFM).

In F99 and $F99_{HCEC}$, lentiviral transduction (p6NST50, Fig. 5, dark grey bars) exerted a cytotoxic effect on the cells compared with mock transduced (Fig. 5, light grey bars) or uninfected cells (Fig. 5, white bars). Overexpression of transgenic HMW FGF-2 (22–34 kD, Fig. 5 light orange to dark red bars), especially of the 34-kD isoform, could compensate for the lentiviral cytotoxicity (rescue effect) in $F99_{HCEC}$ and F99 after 9 days. In contrast, overexpression of the transgenic LMW FGF-2 isoform (18 kD, Fig. 5, yellow bars) amplified the cytotoxic effect of lentiviral transduction compared with mock-transduced (Fig. 5, light grey bars) or HMW FGF-2–transduced cells (22–34 kD; Fig. 5, light orange to dark red bars) in $F99_{HCEC}$ and F99 over the 9-day observation period. This effect was similar in MEM, but not in SFM.

Although the cells survived the 9-day observation period after lentiviral transduction in all media, they showed significantly different metabolic activities. A multifactorial univariate analysis of variance (multi-way ANOVA) revealed that metabolic activity of the cells was significantly influenced by cell seeding density ($P = 4.29 \times 10^{-220}$); culture medium ($P = 1.28 \times 10^{-199}$); transduction (uninfected, mock transduced, transduced with EGFP only encoding vector [p6NST50] or with different FGF-2 isoforms; $P = 1.43 \times 10^{-7}$); and the

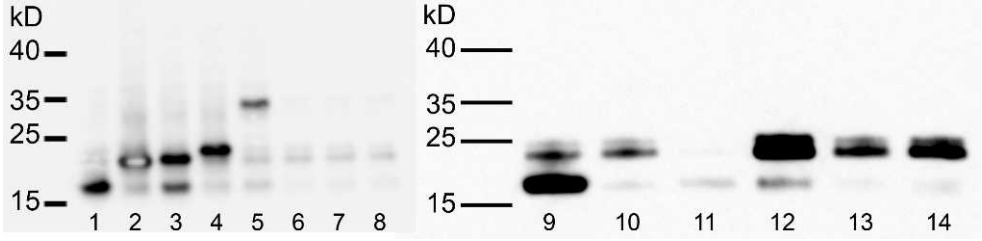

Figure 4. Representative immunoblot analysis of different HCEC populations: Primary HCECs transduced with lentiviral vectors encoding for one of the FGF-2 isoforms showed strong staining for the respective transgene (*lane 1*: 18 kD; *lane 2*: 22 kD; *lane 3*: 22.5 kD; *lane 4*: 24 kD; *lane 5*: 34 kD). Controls showed only a faint staining for 18-, 22-/22.5-, and 24-kD FGF-2 (*lane 6*: EGFP only encoding vector, *lane 7*: mock-transduced, *lane 8*: uninfected). Non-transduced HCECs of two different donors (*lanes 9, 10*) showed bands at 18, 22/22.5, and 24 kD, while stripped Descemet-endothelium-complex (*lane 11*) had only a faint band at 18 kD and an almost undetectable signal at 22/22.5 kD. Three different immortalized HCEC populations (*lane 12*: HCEC-12; *lane 13*: HCEC-B4G12; *lane 14*: HCEC-H9C1) showed mainly protein expression of HMW isoforms.

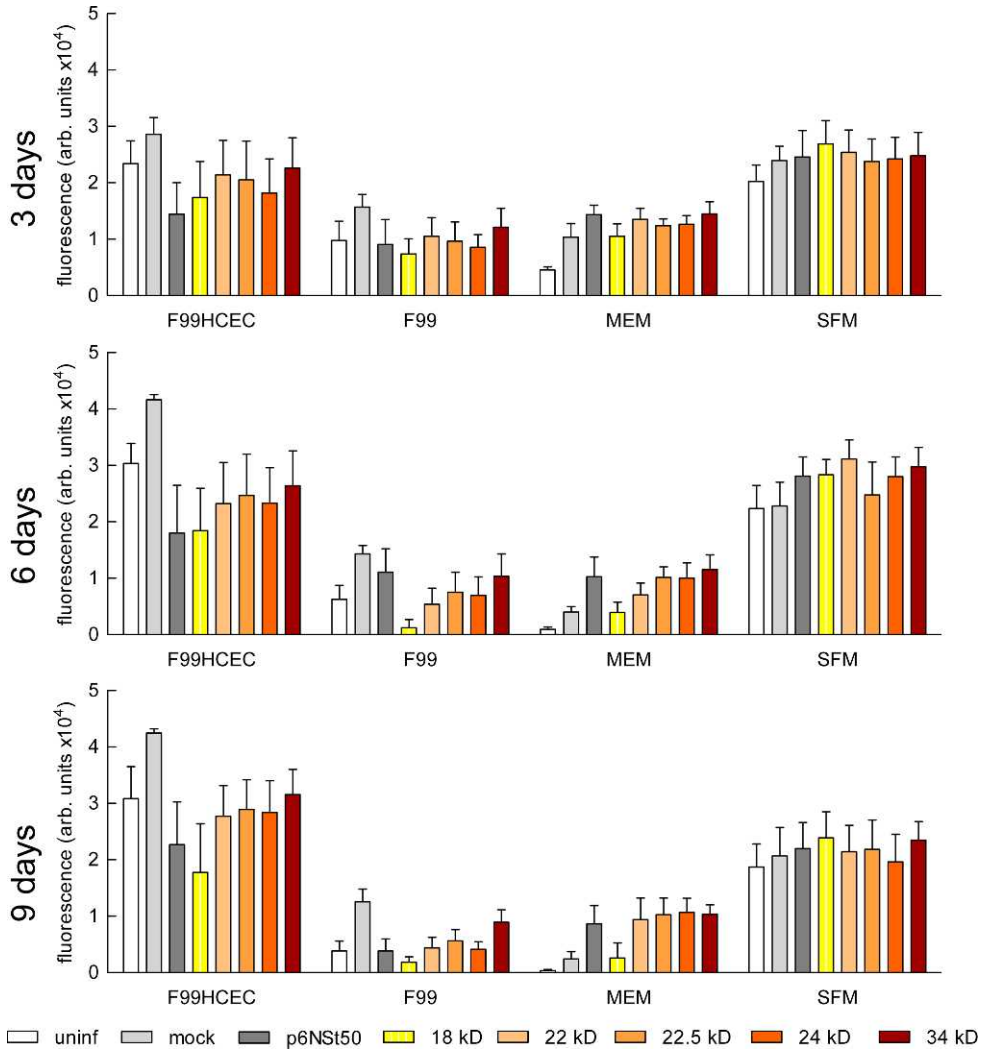

Figure 5. Resazurin conversion by primary HCECs in different culture media. Primary HCECs were seeded at a density of 2500 cells/well and incubated with unsupplemented test medium (uninfected controls, *white bars*), or mock-transduced with virus-free 293T supernatant (mock, *light grey bars*), or transduced with lentiviral vectors encoding for EGFP only (p6NST50, *dark grey bars*) or for one of the FGF-2 isoforms (18–34 kD, *yellow to dark red bars*). Test media were $F99_{HCEC}$, unsupplemented basal medium F99, MEM + 2% FCS, and serum-free SFM. Metabolic activity was determined by resazurin conversion assay after 3, 6, and 9 days of culturing. Mean ± SEM was from $n = 8$ (four donors). Similar results were obtained when seeding the cells at densities of 1250 or 5000 cells/well. Uninf, uninfected.

duration of cultivation after transduction (3–9 days; $P = 1.52 \times 10^{-2}$).

The post hoc test on transduction revealed that transduction with 18-kD FGF-2 (Fig. 5, yellow bars) significantly decreased cell metabolic activity after lentiviral gene transfer compared with mock-transduced cells (Fig. 5, light gray bars) or cells transduced with any of the HMW FGF-2 isoforms (Fig. 5, light orange to dark red bars). It was also observed that transduction with the 34-kD isoform of human FGF-2 (Fig. 5, dark red bars) significantly increased cell metabolic activity compared with uninfected cells (Fig. 5, white bars) or cells transduced with p6NST50 (Fig. 5, dark gray bars) or 18-kD FGF-2 (Fig. 5, yellow bars). Respective *P* values are given in Table.

Discussion

Tissue cultivation of donor corneas for corneal transplantation is accompanied by a loss of endothelial cell density, which continues to a lesser degree after transplantation.[31] Therefore, one aim of our group was to optimize conditions for cell cultivation and cell manipulation, and to investigate mechanisms of cell protection to decrease the observed cell loss. Consequently, methods to positively influence survival of the human corneal endothelium by optimizing culture conditions were tested,[4,12,32–34] and it was shown that corneal endothelial cell viability was markedly improved when cells or whole corneas are cultured in serum-free medium.[12,32] Besides these attempts another group recently reported that transduction with the anti-apoptotic factors Bcl-xL or p35 by lentiviral gene transfer resulted in prolonged endothelial cell survival during organ cultivation under suboptimal conditions (MEM or Optisol).[35] Likewise, it is conceivable that transduction of corneal endothelium with HMW FGF-2, a known survival factor, after preparation of a donor cornea may prolong survival of corneal endothelial cells during organ cultivation. Therefore, it may be speculated that transduction with a survival factor can be a means to protect corneal endothelial cells and ensure their survival during processing from explantation to transplantation.

Table. Statistical Analysis of the Influence of Transduction and FGF-2 Expression on HCEC Metabolic Activity

		P value							
Mean ± SEM	**Transduction**	**Uninf**	**Mock**	**p6NST50**	**18 kD**	**22 kD**	**22.5 kD**	**24 kD**	**34 kD**
14,691 ± 886	uninf		**0.000**	0.699	1.000	**0.007**	**0.001**	**0.003**	**0.000**
20,636 ± 901	mock	**0.000**		**0.000**	**0.000**	**0.002**	**0.014**	**0.004**	1.000
16,435 ± 1069	p6NST50	0.699	**0.000**		1.000	1.000	1.000	1.000	**0.002**
14,576 ± 1170	18 kD	1.000	**0.000**	1.000		**0.026**	**0.005**	**0.015**	**0.000**
17,559 ± 1092	22 kD	**0.007**	**0.002**	1.000	**0.026**		1.000	1.000	0.148
17,929 ± 1120	22.5 kD	**0.001**	**0.014**	1.000	**0.005**	1.000		1.000	0.487
17,683 ± 1075	24 kD	**0.003**	**0.004**	1.000	**0.015**	1.000	1.000		0.224
20,066 ± 1088	34 kD	**0.000**	1.000	**0.002**	**0.000**	0.148	0.487	0.224	

P values from post hoc testing (Bonferroni) after multi-way ANOVA of HCEC metabolic activity determined by resazurin conversion assay and given as fluorescence in arbitrary units. Cells were uninfected, mock-transduced, transduced with EGFP only expressing vector (p6NST50), or with different FGF-2 isoforms (18–34 kD). Statistically significant differences ($P < 0.05$) are shown in bold. Uninf, uninfected.

Lentiviral vectors are powerful and well-proven cell biological research tools to identify mechanisms of cell protection, survival, or immunomodulation, as was recently shown in the works of Fuchsluger et al., Figueiredo et al., and also in this paper.[35–37] The identification of survival mechanisms and survival factors may advance studies of corneal endothelial cell biology, and may also serve as a basis toward developing novel therapeutic approaches. For example, developments in using adenoviral vectors led to the first clinical trials in humans suffering from retinal dystrophies.[38–41] Furthermore, several clinical trials using retro- and lentiviral vectors were initiated during the past two decades, leading to new insights in gene therapy and vector improvement.[42] The development of safe vectors for genetic manipulation and also the development of methods to manipulate cells without modifying their genomic setup is continuously progressing. It is therefore considerable that prospective methods allow for a clinical application of survival or immunomodulatory factors, which were previously identified in the lab with methods such as lentiviral gene transfer in corneal tissue.

Recently it was shown that transduction efficiency of lentiviral gene transfer to human corneal endothelial cells is influenced by culture medium, type of vector, and viral glycoprotein.[24] The type of vector and viral glycoprotein employed also determine the degree of cytotoxicity the viral vector can exert. Viral gene transfer is an effective means of transducing cells with therapeutic genes. In order to protect the cells from viral cytotoxicity, the use of bicistronic vectors that allow coexpression of protective or survival factors may aid cell survival after viral gene transfer. FGF-2 (LMW) is a known mitogen and differentiation factor for human corneal endothelium in vivo and in vitro, as we and others demonstrated.[4,14,15] Therefore, we examined if expression of any of the five known isoforms of human FGF-2 after lentiviral gene transfer can alleviate lentiviral cytotoxicity. We also focused on the effect that expression of any of the five isoforms of FGF-2 after lentiviral transduction might have on HCEC survival when transduction was performed in different culture media that either promote or lower HCEC survival as previously described.[32] It could already be seen that the chosen vector, HIV pseudotyped with a foamy virus envelope glycoprotein, efficiently transduces HCECs in any of the chosen media.[24] It was demonstrated that FGF-2 isoforms were expressed correctly from the expression-optimized constructs and localized correctly in the transduced cells. In contrast to the observed beneficial effect of exogenous administration of 18-kD FGF-2 under serum-reduced conditions, it was found that endogenous overexpression of this isoform can aggravate lentiviral cytotoxicity and can significantly impair cell viability in nutrient-depleted media. This can be concluded from the reduced metabolic activity of cells cultured in serum-free F99 or MEM + 2% FCS after lentiviral transduction with LMW FGF-2, pointing on a cumulative negative effect of suboptimal culture medium and LMW FGF-2 overexpression. A possible explanation may be that LMW FGF-2 acts predominantly as a mitogen rather than a survival factor on in vitro–cultured HCECs. In this case, the nutrient-depleted media cannot sufficiently meet the nutritional requirement of cells that are driven to proliferate by overexpression of 18-kD FGF-2, and the cells eventually die of starvation. When cultured in SFM, which promotes HCEC survival best, this adverse effect of the 18-kD isoform could not be seen.

According to the current state of knowledge, expression of HMW FGF-2 isoforms triggers differentiation and stress resistance rather than proliferation in various cell types. For example, they can induce resistance against noxious influences, such as heat, oxidative stress, or radiation.[21,43] LMW FGF-2 can induce transformation of normal or non-tumorigenic cells into tumor cells (e.g., of NIH 3T3 fibroblasts). HMW FGF-2 isoforms, on the other hand, can induce growth inhibition and differentiation and are therefore considered primarily as survival and differentiation factors that may act as tumor suppressors.[44] This may explain the increased survival of HCECs after lentiviral transduction with HMW FGF-2 compared with HCECs transduced with LMW FGF-2, as concluded from their higher metabolic activity (e.g., when cultured in MEM).

Taken together, the study results indicate that HMW FGF-2, specifically the 34-kD isoform, supports HCEC survival under stress conditions, such as nutrient depletion and lentiviral infection. Hence, the possible function of HMW FGF-2 as a survival factor that may protect transduced cells from viral cytotoxicity when coexpressed with another gene is an interesting aspect to examine (e.g., in gene therapy approaches such as genetic manipulation of corneal endothelium to reduce immune responses after keratoplasty or in approaches to reduce apoptosis). Since HIV-1 mediated cytopathogenicity also contains apoptosis-inducing elements,[45] anti-apoptotic factors (e.g., Bcl-xL or p35 as described by Fuchsluger et al.[35]) may also support HCEC survival after genetic manipulation when coexpressed from bicistronic vectors. It should also be considered that to date, most studies about genetic manipulation of HCECs focused primarily on the effect of the transgene without factoring in if the experimental conditions are adequately chosen to achieve the aimed goal. Our data show that the transgene as well as experimental conditions like the culture medium can have a major impact on transgene effect, and should be considered in future experiments to avoid masking of effects.

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References

1. Bednarz J, Weich HA, Rodokanaki-von Schrenck A, Engelmann K. Expression of genes coding growth factors and growth factor receptors in differentiated and dedifferentiated human corneal endothelial cells. *Cornea*. 1995;14:372–381.
2. Wilson SE, Walker JW, Chwang EL, He YG. Hepatocyte growth factor, keratinocyte growth factor, their receptors, fibroblast growth factor receptor-2, and the cells of the cornea. *Invest Ophthalmol Vis Sci*. 1993;34:2544–2561.
3. Gu X, Seong GJ, Lee YG, Kay EP. Fibroblast growth factor 2 uses distinct signaling pathways for cell proliferation and cell shape changes in corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 1996;37:2326–2334.
4. Rieck PW, Gigon M, Jaroszewski J, Pleyer U, Hartmann C. Increased endothelial survival of organ-cultured corneas stored in FGF-2-supplemented serum-free medium. *Invest Ophthalmol Vis Sci*. 2003;44:3826–3832.
5. Engelmann K, Friedl P. Growth of human corneal endothelial cells in a serum-reduced medium. *Cornea*. 1995;14:62–70.
6. Lee HT, Lee JG, Na M, Kay EP. FGF-2 induced by interleukin-1b through the action of phosphatidylinositol 3-kinase mediates endothelial mesenchymal transformation in corneal endothelial cells. *J Biol Chem*. 2004;279:32325–32332.
7. Lee HT, Kay EP. Regulatory role of PI 3-kinase on expression of Cdk4 and p27, nuclear localization of Cdk4, and phosphorylation of p27 in corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 2003;44:1521–1528.
8. Engelmann K, Friedl P. Optimization of culture conditions for human corneal endothelial cells. *In Vitro Cell Dev Biol*. 1989; 25:1065–1072.
9. Engelmann K, Böhnke M. Human corneal endothelial cells in long-term cultures: the influence of conditions for isolation, selective and normal growth and the extracellular matrix on proliferation and morphology. *Chibret Int J Ophthalmol*. 1990;7:3–13.
10. Rieck P, Oliver L, Engelmann K, Fuhrmann G, Hartmann C, Courtois Y. The role of exogenous/endogenous basic fibroblast growth factor (FGF2) and transforming growth factor beta (TGF beta-1) on human corneal endothelial cells proliferation in vitro. *Exp Cell Res*. 1995;220:36–46.
11. Rieck PW, Cholidis S, Hartmann C. Intracellular signaling pathway of FGF-2-modulated corneal endothelial cell migration during wound healing in vitro. *Exp Eye Res*. 2001;73: 639.
12. Hempel B, Bednarz J, Engelmann K. Use of a serum-free medium for long-term storage of human corneas. Influence on endothelial cell density and corneal metabolism. *Graefes Arch Clin Exp Ophthalmol*. 2001;239:801–805.
13. Engelmann K, Bohnke M, Friedl P. Isolation and long-term cultivation of human corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 1988;29:1656–1662.
14. Bohnke M, Eggli P, Engelmann K. Transplantation of cultured adult human or porcine corneal endothelial cells onto human recipients in vitro. Part II: evaluation in the scanning electron microscope. *Cornea*. 1999;18:207–213.
15. Engelmann K, Drexler D, Bohnke M. Transplantation of adult human or porcine corneal endothelial cells onto human recipients in vitro. Part I: cell culturing and transplantation procedure. *Cornea*. 1999;18:199–206.
16. Nickel W. Unconventional secretion: an extracellular trap for export of fibroblast growth factor 2. *Journal of Cell Science*. 2007;120:2295–2299.
17. Temmerman K, Ebert AD, Müller H-M, Sinning I, Tews I, Nickel WA. Direct role for phosphatidylinositol-4, 5-bisphosphate in unconventional secretion of fibroblast growth factor 2. *Traffic*. 2008;9:1204–1217.
18. Delrieu I. The high molecular weight isoforms of basic fibroblast growth factor (FGF-2): an insight into an intracrine mechanism. *FEBS Lett*. 2000;468:6.
19. Arnaud E, Touriol C, Boutonnet C, et al. A new 34-kilodalton isoform of human fibroblast growth factor 2 is cap dependently synthesized by using a non-AUG start codon and behaves as a survival factor. *Mol Cell Biol*. 1999;19:505–514.
20. Gu X, Kay EP. Distribution and putative roles of fibroblast growth factor-2 isoforms in corneal endothelial modulation. *Invest Ophthalmol Vis Sci*. 1998;39:2252–2258.
21. Vagner S, Touriol C, Galy B, et al. Translation of CUG- but not AUG-initiated forms of human fibroblast growth factor 2 is activated in transformed and stressed cells. *J Cell Biol*. 1996; 135:1391–1402.
22. Arese M, Chen Y, Florkiewicz RZ, Gualandris A, Shen B, Rifkin DB. Nuclear activities of basic fibroblast growth factor: potentiation of low-serum growth mediated by natural or chimeric nuclear localization signals. *Mol Biol Cell*. 1999;10: 1429–1444.
23. Bikfalvi A, Klein S, Pintucci G, Quarto N, Mignatti P, Rifkin DB. Differential modulation of cell phenotype by different molecular weight forms of basic fibroblast growth factor: possible intracellular signaling by the high molecular weight forms. *J Cell Biol*. 1995;129:233–243.
24. Valtink M, Stanke N, Knels L, Engelmann K, Funk RH, Lindemann D. Pseudotyping and culture conditions affect efficiency and cytotoxicity of retroviral gene transfer to human corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 2011;52: 6807–6813.
25. Bednarz J, Teifel M, Friedl P, Engelmann K. Immortalization of human corneal endothelial cells using electroporation protocol optimized for human corneal endothelial and human retinal pigment epithelial cells. *Acta Ophthalmol Scand*. 2000; 78:130–136.
26. Valtink M, Gruschwitz R, Funk RH, Engelmann K. Two clonal cell lines of immortalized human corneal endothelial cells show either differentiated or precursor cell characteristics. *Cells Tissues Organs*. 2008;187:286–294.
27. Pear WS, Nolan GP, Scott ML, Baltimore D. Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci USA*. 1993;90:8392–8396.
28. Rasheed S, Nelson-Rees WA, Toth EM, Arnstein P, Gardner MB. Characterization of a newly derived human sarcoma cell line (HT-1080). *Cancer*. 1974;33:1027–1033.
29. Amer DA, Kretzschmar G, Muller N, Stanke N, Lindemann D, Vollmer G. Activation of transgenic estrogen receptor-beta by selected phytoestrogens in a stably transduced rat serotonergic cell line. *J Steroid Biochem Mol Biol*. 2010;120:208–217.
30. Mochizuki H, Schwartz JP, Tanaka K, Brady RO, Reiser J. High-titer human immunodeficiency virus type 1-based vector systems for gene delivery into nondividing cells. *J Virol*. 1998;72:8873–8883.
31. Pels L. Organ culture: the method of choice for preservation of human donor corneas. *British Journal of Ophthalmology*. 1997;81:523–525.
32. Jäckel T, Knels L, Valtink M, Funk RHW, Engelmann K. Serum-free SFM corneal organ culture medium but not conventional MEM organ culture medium protects human corneal endothelial cells from apoptotic and necrotic cell death. *Br J Ophthalmol*. 2011;95:123–130.
33. Rieck PW, von Stockhausen RM, Metzner S, Hartmann C, Courtois Y. Fibroblast growth factor-2 protects endothelial cells from damage after corneal storage at 4 degrees C. *Graefes Arch Clin Exp Ophthalmol*. 2003;241:757–764.

34. Sobottka Ventura AC, Engelmann K, Bohnke M. Fetal calf serum protects cultured porcine corneal endothelial cells from endotoxin-mediated cell damage. *Ophthal Res*. 1999;31:416–425.

35 Fuchsluger TA, Jurkunas U, Kazlauskas A, Dana R. Anti-apoptotic gene therapy prolongs survival of corneal endothelial cells during storage. *Gene Ther*. 2011;18:778–787.

36. Fuchsluger TA, Jurkunas U, Kazlauskas A, Dana R. Corneal endothelial cells are protected from apoptosis by gene therapy. *Hum Gene Ther*. 2011;22:549–558.

37. Figueiredo C, Horn PA, Blasczyk R, Seltsam A. Regulating MHC expression for cellular therapeutics. *Transfusion*. 2007;47:18–27.

38. Ashtari M, Cyckowski LL, Monroe JF, et al. The human visual cortex responds to gene therapy-mediated recovery of retinal function. *J Clin Invest*. 2011;121:2160–2168.

39. den Hollander AI, Black A, Bennett J, Cremers FPM. Lighting a candle in the dark: advances in genetics and gene therapy of recessive retinal dystrophies. *J Clin Invest*. 2010;120:3042–3053.

40. Jacobson SG, Cideciyan AV, Ratnakaram R, et al. Gene therapy for leber congenital amaurosis caused by RPE65 mutations: safety and efficacy in 15 children and adults followed up to 3 years. *Arch Ophthalmol*. 2012;130:9–24.

41. Campochiaro PA. Gene transfer for ocular neovascularization and macular edema. *Gene Ther*. 2012;19:121–126.

42. Maier P, von Kalle C, Laufs S. Retroviral vectors for gene therapy. *Future Microbiol*. 2010;5:1507–1523.

43. Cohen-Jonathan E, Toulas C, Monteil S, et al. Radioresistance induced by the high molecular forms of the basic fibroblast growth factor is associated with an increased G2 delay and a hyperphosphorylation of p34CDC2 in HeLa cells. *Cancer Res*. 1997;57:1364–1370.

44. Quarto N, Fong KD, Longaker MT. Gene profiling of cells expressing different FGF-2 forms. *Gene*. 2005;356:49.

45. Roshal M, Zhu Y, Planelles V. Apoptosis in AIDS. *Apoptosis*. 2001;6:103–116.