Regulation and Function of Trefoil Factor Family 3 Expression in the Biliary Tree

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Microarray analysis identified trefoil factor family 3 (TFF3) as a gene expressed in biliary epithelial cells (BECs), regulated by interleukin (IL)-6, and potentially involved in biliary pathophysiology. We therefore studied the regulation and function of BEC TFF3, *in vitro* and *in vivo* in IL-6^{+/+} and IL-6^{-/-} mice subjected to chronic bile duct ligation for 12 weeks. *In vitro* studies showed that IL-6 wild-type (IL-6^{+/+}) BECs expressed higher TFF3 mRNA and protein levels than IL-6-deficient (IL-6^{-/-}) BECs. BEC TFF3 expression is dependent primarily on signal transducer and activator of transcription (STAT3) signaling, but the reciprocal negative regulation known to exist between the intracellular IL-6/gp130 signaling pathways, STAT3 and mitogen-activated protein kinase (MAPK), importantly contributes to BEC TFF3 expression. Specifically blocking STAT3 activity with a dominant-negative molecule or treatment with a growth factor such as hepatocyte growth factor, which increases MAPK signaling, decreases BEC TFF3 expression. In contrast, specifically blocking MAPK activity with PD98059 significantly increased BEC TFF3 expression. Higher BEC TFF3 levels in IL-6^{+/+} BECs were associated with significantly better migration than IL-6^{-/-} BECs in a wound-healing assay and defective IL-6^{-/-} BEC migration was reversed with exogenous TFF3. *In vivo*, hepatic TFF3 mRNA and protein expression was limited to BECs and dependent significantly on STAT3 signaling, but was influenced by other factors present after bile duct ligation. Com-

parable results were obtained in normal and diseased human tissue samples. In conclusion the regulation and function of BEC TFF3 expression is similar to the colon. BEC TFF3 expression depends primarily on gp130/STAT3 and contributes to BEC migration and wound healing. Therefore, use of recombinant IL-6 or TFF3 peptides should exert a therapeutic role in preventing biliary strictures in liver allografts. (Am J Pathol 2004, 165:1907–1920)

The contribution of epithelial cells to wound healing has been extensively studied for barriers such as the skin and colon.¹ However, we are unaware of studies in the literature devoted to understanding these processes in the biliary tree, despite the fact that biliary epithelial cells (BECs) must protect the underlying stroma and other liver cells from the extremely toxic actions of bile salts.

Ulceration and ineffective restoration of the epithelial lining of the biliary tree contributes to many biliary tract diseases, such as primary sclerosing cholangitis, extrahepatic biliary atresia, and preservation injury-related biliary tract strictures in liver allografts.² Defects of the biliary epithelial lining lead to sludge formation, fibrosis of the bile duct wall, and luminal narrowing. Subsequent intrahepatic stagnation of toxic bile salts damages the liver and eventually leads to cirrhosis.³ If the molecular mechanisms of BEC repair reactions were better understood, some biliary diseases, such as preservation injury-related strictures,² might be avoided altogether if the donor could be pretreated to either prevent the damage and/or promote effective BEC repair.

Interleukin (IL)-6 is produced normally at low levels by the BECs, perhaps stimulated by the bile salts,⁴ and secreted into the bile.⁵ Virtually any bile duct insult, such as obstruction,^{6–8} infection,^{8,9} or immunological damage^{5,10,11} triggers sharp increases in IL-6 mRNA and protein production by the BECs and peribiliary hemalymphoid cells.¹² This leads to subsequent autocrine,

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paracrine, and juxtacrine gp130 signaling in BECs. Biliary insults such as bile duct ligation (BDL) also trigger production of other cytokines and growth factors, such as tumor necrosis factor (TNF)- α , hepatocyte growth factor (HGF), transforming growth factor (TGF)- β that are produced primarily by peribiliary stromal and hemato lymphoid cells, and not by BECs, but contribute to BEC pathophysiology *in vivo*.^{6,13}

A previous study from our lab showed that interleukin-6-deficient (IL-6^{-/-}) mice develop decompensated biliary cirrhosis at a higher rate than wild-type (IL-6^{+/+}) controls¹⁴ after BDL because of impaired biliary tree integrity.¹⁴ However, the molecular mechanisms responsible for impaired biliary tree integrity remain undefined.

The goal of this study was to identify possible molecular mechanisms that could contribute to impaired biliary tree integrity in IL-6^{-/-} mice after BDL.¹⁴ A microarray analysis identified the trefoil factor family (TFF)^{15,16} of peptides as candidate genes expressed in BECs and regulated by IL-6 signaling that could potentially contribute to biliary tree integrity. TFF are mucin-associated proteins that increase the viscosity of mucins and help protect epithelial linings from insults. TFF also contributes to restitution of epithelial barriers in the gastrointestinal tract by promoting epithelial cell migration in wound healing assays.¹⁷

Initial analyses were performed *in vitro*, using complete serum-free medium (C-SFM), a medium that stimulates BEC IL-6 production^{13,18} and magnifies the differences between IL-6^{+/+} and IL-6^{-/-} BECs related primarily to BEC IL-6 production. Isolating the BECs in culture also removes potential confounding paracrine influences of other growth factors and cytokines that are produced by peribiliary stromal and hematopoietic cells after BDL *in vivo*.^{6,19,20} Examples include TNF- α , HGF, and TGF- β , which are not produced or differentially regulated by BECs under this condition (data not shown), but can significantly decrease TFF3 expression.²¹ Finally however, the dynamic regulation of TFF3 mRNA and protein expression was studied in relationship to STAT3 and MAPK signaling, *in vivo*, using a chronic BDL model in IL-6^{+/+} and IL-6^{-/-} mice. The purpose of the *in vitro* studies was to determine the relative contribution of IL-6 signaling pathways to BEC TFF3 expression, *in vivo*, under real disease conditions.

Results show that BEC TFF3 mRNA and protein expression are primarily dependent on STAT3 signaling, which is deficient in IL-6^{-/-} mice. However, the reciprocal negative regulation known to exist between the STAT3 and MAPK signaling pathways also influences BEC TFF3 expression. In addition, BEC TFF3 contributes to restitution of the BEC lining by enhancing cell migration and wound healing.

Materials and Methods

Mice

Male C57BL/6 mice (8 to 12 weeks old), the dominant parental strain for the IL-6^{+/+} and IL-6^{-/-} mice, were the

source of bile ducts for the primary mouse BEC cultures. The IL-6^{-/-}-deficient mice were created by replacing a 2.1-kb fragment of the IL-6 gene, containing the proximal promoter region and the first three exons with MC1-Neo poly(A)⁺ cassette, as reported by Poli and colleagues.²² The original genetic background of the mice was ~75% C57BL/6, 20% 129SV, and 5% mixed F1. For these experiments, the mice were bred and maintained in the animal facility at the University of Pittsburgh on a standard diet of Purina Isopro 3000 (Purina, Richmond, IN) that contains 5% fat.

Isolation of Mouse BECs, Culture Conditions, and BEC Transfection

Primary cultures of mouse BECs were prepared as previously reported.¹⁸ For all experiments, except where noted, BECs were cultured on collagen gels in complete serum-free medium (C-SFM), which stimulates IL-6 production by IL-6^{+/+}, but not by IL-6^{-/-} BECs.¹⁸ C-SFM consisted of Dulbecco's modified Eagle's medium/F12 medium (Sigma, St. Louis, MO) supplemented with 5.4 g/L D-glucose, 50 μ g/ml gentamicin, antibiotic-anti-mycotic (100 U/ml penicillin G sodium, 100 μ g/ml streptomycin sulfate, 250 ng/ml amphotericin B), 10 mmol/L HEPES, 2.5 mg/ml bovine serum albumin (Sigma), insulin-transferrin-selenium-X (10 mg/L insulin, 5.5 mg/L transferrin, 6.7 ng/L sodium selenite), 0.1 mmol/L minimal essential media nonessential amino acid solution, 2 mmol/L L-glutamine, 32 ng/ml thyroxine (Sigma), 10 ng/ml prostaglandin E1 (Sigma), 40 ng/ml hydrocortisone (Sigma), 10 μ mol/L forskolin (Sigma), and 50 μ g/ml trypsin inhibitor (Sigma). Simple, serum-free medium contains Dulbecco's modified Eagle's medium/F12 supplemented with 5.4 g/L D-glucose, 50 μ g/ml gentamicin, antibiotic-anti-mycotic (100 U/ml penicillin G sodium, 100 μ g/ml streptomycin sulfate, 250 ng/ml amphotericin B), 10 mmol/L HEPES, and 2.5 mg/ml bovine serum albumin.

The MAPK inhibitor, PD98059 (Calbiochem, La Jolla, CA), recombinant TGF- β (R&D Systems, Minneapolis, MN) and recombinant HGF (Toyobo, Shiga, Japan), and recombinant mutein IL-6 (ImClone System, Somerville, NJ) were used to treat the mouse BECs in several experiments. An adenoviral vector coding for a dominant-negative form of STAT3 (AdSTAT3-DN) and a control viral vector (AdGFP) were used to transiently transfect normal mouse BECs.²³ Both the AdSTAT3-DN and the AdGFP express green fluorescent protein (GFP) used to measure viral titer and transfection efficiency. The dose used to transfect the BECs was 25 PFU per cell.

High-Density Oligonucleotide Array Analysis

BECs (8×10^5) from IL-6^{+/+} and IL-6^{-/-} mice were seeded in a 10-cm collagen gel dish in C-SFM supplemented with 50 μ g/ml bovine pituitary extract and 10 ng/ml epidermal growth factor for 7 days until the cultures were ~70% confluent. The medium was removed and the cells were washed twice with phosphate-buffered saline (PBS) and incubated in S-SFM for 24 hours and then

Table 1. Primers for Real-Time PCR

Gene	Genbank no.	Forward primer (5' to 3')	Reverse primer (5' to 3')	Product size
mTFF3	D38410	TGGTCCAAGGGTAGCAAGCAT	CAGCCTTGTGTTGGCTGTGAG	102 bp
mGAPDH	M32599	TGGCAAAGTGGAGATTGTTGCC	AAGATGGTGATGGGCTTCCCG	156 bp

changed to C-SFM for 48 hours, as in previous experiments.¹⁸ Finally, total RNA was extracted from the BECs by TRIzol reagent (Invitrogen, Carlsbad, CA), and purified with Qiagen RNeasy kit (Qiagen, San Diego, CA). Five μ g of total RNA were used in the first strand complementary DNA (cDNA) synthesis with T7-d(T)24 primer [GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)24] by Superscript II (Invitrogen). The second strand cDNA synthesis was performed at 16°C by adding *Escherichia coli* DNA ligase, *E. coli* DNA polymerase I, and RNaseH in the reaction. This was followed by the addition of T4 DNA polymerase to blunt the ends of newly synthesized cDNA. The cDNA was purified through phenol/chloroform and ethanol precipitation. The purified cDNA were then incubated at 37°C for 4 hours in an *in vitro* transcription reaction to produce cRNA labeled with biotin using MEGAscript system (Ambion, Inc., Austin, TX). cRNA (15 to 20 mg) was fragmented by incubating in a buffer containing 200 mmol/L Tris-acetate (pH 8.1), 500 mmol/L KOAc, and 150 mmol/L MgOAc at 95°C for 35 minutes. The fragmented cRNA were then hybridized with a pre-equilibrated Affymetrix chip containing 12,655 cDNA species (U74A ver.2 array; Affymetrix Inc., Santa Clara, CA) at 45°C for 14 to 16 hours. After the hybridization cocktails were removed, the chips were then washed in a fluidic station with low-stringency buffer (6 $\times$ SSPE, 0.01% Tween 20, 0.005% anti-foam) for 10 cycles (two mixes/cycle) and stringent buffer (100 mmol/L MES, 0.1 mol/L NaCl, and 0.01% Tween 20) for four cycles (15 mixes/cycle), and stained with SAPE (strepto-avidin phycoerythrin). This was followed by incubation with biotinylated mouse anti-avidin antibody, and restained with SAPE. The chips were scanned in a HP ChipScanner (Affymetrix Inc.) to detect hybridization signals. Hybridization data were analyzed through Microarray suite 5.0 automatic software and GeneChip Expression Analysis software (Affymetrix Inc.) for comparison analysis of two samples.

SYBR Green Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA pellets were resuspended in RNase-free water, followed by removal of potential contaminating DNA by treatment with DNase I (Invitrogen). Total RNA was used for reverse transcription with an oligo dT and a Superscript II (Invitrogen) to generate first strand cDNA. TFF3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expressions were quantified in duplicate by SYBR Green two-step, real-time RT-PCR on an ABI Prism 7000 sequence detection system (PE Applied Biosystems, Foster City, CA). PCR reaction mixture was

prepared using SYBR Green PCR Master Mix (PE Applied Biosystems). The forward (F) and reverse (R) primers were selected using Primer Express software (PE Applied Biosystems), and are shown in Table 1. The identities of the each PCR fragment were confirmed with DNA sequencing (data not shown). The standard curve was constructed with fivefold serial dilutions of total cDNA from the IL-6^{+/+} BECs. The five points always showed a strong linear relationship ($R^2 > 0.99$) between the threshold cycle (Ct) and the initial copy number of each cDNAs (data not shown). TFF3 mRNA expression was normalized with the level of GAPDH mRNA used as an internal control. Dissociation curve analyses were performed for each reaction to ensure amplification of specific product. To exclude PCR amplification of contaminating genomic DNA, RT-negative controls (samples containing RNA that was not reverse transcribed) were performed.

In Vitro Wound-Healing Assay on Collagen Gel

A primary mouse BEC wound-healing assay on collagen gel (migration assay) was newly established for these studies. Before BEC plating, an 8-mm length of glass micropipette (1 μ l; Drummond Scientific, Broomall, PA) was laid on 48-well plates coated with a collagen gel (see Figure 5A). The micropipette prevented cell attachment to this area. Then primary mouse BECs (6 $\times$ 10⁴ cells/well) were plated and grown to confluence in C-SFM supplemented with bovine pituitary extract and epidermal growth factor for 2 days. The medium was removed and the cells were washed twice with PBS and incubated in C-SFM for 24 hours. To inhibit cell proliferation, mitomycin C (Sigma Chemical Co., St. Louis, MO) was added (1.0 μ g/ml) to C-SFM 2 hours before initiating the wound-healing assay. The wound was created by removing the glass micropipette from the collagen gel, which left an area uncovered by BECs.

The BECs were then left untreated or treated with recombinant human TFF3 (rTFF3) (kind gift from Dr. D.K. Podolsky, Massachusetts General Hospital, Boston, MA) in C-SFM and incubated at 37°C for 12 hours. Photographs were taken at 0 hour and 12 hours, and the relative distance traveled by the BECs between the acellular fronts was determined. Cell counts were also performed at 0 hour and 12 hours to verify that mitomycin C effectively inhibited BEC proliferation.

Operative Procedures and Isolation of the Biliary Tree

IL-6^{-/-} mice and littermate IL-6^{+/+} controls (8- to 14-week-old males) were used for the BDL studies. Accord-

ing to the Institutional Animal Care and Use Committee protocol number 0101176B-1, all mice underwent methoxyflurane anesthesia and were aseptically subjected to double ligation of the common bile duct below the bifurcation and single ligation above the pancreas with dissection between the distal and proximal knots. Animals ($n = 3$ to 5 in each group at each time point) were sacrificed before BDL and 1, 3, 7, and 12 weeks after BDL. Whole liver, including equal amounts of perihilar tissue, was used for RNA and protein isolation. Some of the analyses on whole mouse liver after BDL were conducted on tissues saved from previous experiments,^{7,14} but none of the results have been previously reported.

To determine the effect of IL-6 replacement therapy on IL-6^{-/-} mice, a group of IL-6^{-/-} mice was treated via subcutaneous injection with either IL-6 mutein (ImClone System), a modified version of recombinant human (rh)IL-6 protein,²⁴ or with normal saline as a control. Daily treatment was started at 6 weeks after BDL and continued for 6 weeks, at a dose of 1 μ g/g body weight/day in a 0.04% solution of pyrogen-free normal saline.

Western Blot Analysis

Protein extraction and Western blotting were performed as previously described,²⁵ with minor modifications. Briefly, nuclear and cytosolic proteins were extracted from homogenized mouse whole liver, and aliquots were measured for protein concentration, using the BCA protein assay kit (Pierce, Rockford, IL). Total protein was extracted from cultured BECs using Chaps cell extract buffer (Cell Signaling Technology, Inc., Beverly, MA). For phospho-p44/p42 MAPK and phospho-STAT3 Western blotting, 50 μ g of liver nuclear proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using different concentrations of gels (p44/p42, 12%; STAT3, 8%). The separated proteins were transferred to nitrocellulose membranes that were subsequently incubated with anti-phospho-p44/p42 MAPK antibody (Thr202/Tyr204; Cell Signaling Technology, Inc.). For TFF3 Western blotting, frozen cell pellets and mouse liver tissues were boiled for 1 minute to preferentially extract small proteins. Then proteins were extracted from those samples. Thirty μ g of BEC total protein or 50 μ g of liver cytosolic protein were separated on a 10 to 20% gradient Tris-HCl precast gel (Bio-Rad, Hercules, CA), then blotted onto polyvinylidene difluoride membranes, and incubated with anti-TFF3 antibody (kind gift from Dr. D.K. Podolsky) at the dilution of 1:250 overnight. Recombinant human TFF3 as a positive control is a kind gift from Dr. D.K. Podolsky. All signals were detected using enhanced chemiluminescence reagents (NEN; Life Science Products, Boston, MA).

Immunohistochemical Staining for TFF3 and Phospho-STAT3

Immunohistochemical localization of TFF3 and phospho-STAT3 protein was performed using formalin-fixed paraffin-embedded whole mouse and human liver tissue sec-

tions with a routine indirect avidin-biotin-immunolabeling procedure, as previously described.⁶ The human tissues used in this study were obtained from archived samples maintained in the department of pathology according to exempt Institutional Review Board protocol number 010259. Tissue sections were first deparaffinized in xylene and rehydrated. The sections were then blocked with Tris-buffered saline (20 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl) containing 10% goat serum for 20 minutes at room temperature, then incubated with anti-TFF3 antibody²⁶ diluted at 1:1000 or with anti-phospho-STAT3 antibody (Tyr705; Cell Signaling Technology, Inc.) diluted at 1:100 at 4°C overnight. Nonimmune isotype-matched immunoglobulin was used in place of the primary antibody in the negative controls.

Statistical Analysis

The values shown for the various tests are the mean $\pm$ SD of three to five representative experiments. The Student's *t*-test was used to compare two groups. One-factor analysis of variance was used to detect significant intergroup differences, in which case individual study groups were compared using Fisher's protected least significant difference test. A *P* value of less than 0.05 was considered to be statistically significant.

Results

Oligonucleotide Array Profile Comparing IL-6^{+/+} and IL-6^{-/-} BECs under Conditions of Stress

In an effort to identify candidate genes that are expressed in BECs, regulated by IL-6, and potentially involved in biliary tree integrity, we first compared the microarray gene expression pattern of IL-6^{+/+} and IL-6^{-/-} mouse BECs *in vitro*. For this analysis, the BECs were cultured in complete serum-free medium (C-SFM) for 48 hours. This medium stimulates IL-6 production by IL-6^{+/+} BECs, but IL-6^{-/-} BECs^{13,18} are not capable of IL-6 production. Therefore, C-SFM accentuates differences between the IL-6^{+/+} and IL-6^{-/-} BECs related to BEC IL-6 production. Because BECs produce IL-6 *in vivo* after biliary tract injury,^{5,6} these culture conditions mimic biliary injury. However, using cell cultures isolates the BECs from the confounding influences of other cytokines and growth factors such as TNF- α , HGF, and TGF- β , which are produced by non-BECs after BDL, but not expressed or differentially regulated in the BECs in C-SFM (data not shown). These factors are produced primarily by peribiliary stromal and hematolymphoid cells after BDL,⁶ and can either have a direct effect on TFF3 expression (eg, TNF- α ²¹), or induce biological effects similar to TFF3, such as HGF-enhanced BEC migration.²⁷

The top 10 up-regulated genes in IL-6^{+/+} compared to IL-6^{-/-} BECs determined by Affymetrix GeneChip are shown in Table 2. The level of TFF3 mRNA expression was significantly higher in IL-6^{+/+} BECs than in IL-6^{-/-} BECs. TFF3 is known to contribute to epithelial integrity and barrier function in the intestine.^{15,26,28} Other mem-

Table 2. Up-Regulated Genes in IL-6^{+/+} BEC Compared with IL-6^{-/-} BECs by Oligonucleotide Array Analysis

No.	Genbank no.	Gene	Expression level*	Fold Change [†]	Function
1	AJ005567	Small proline-rich protein 2I	4864	105.1	Squamous cell differentiation
2	AJ005563	Small proline-rich protein 2E	7177	95.7	Squamous cell differentiation
3	AJ005560	Small proline-rich protein 2B	11,539	86.3	Squamous cell differentiation
4	AF039663	AC133 antigen homolog	3996	80.9	Cell antigen
5	AJ005559	Small proline-rich protein 2A	14,794	67.8	Squamous cell differentiation
6	J03953	Glutathione transferase GT9.3	3374	58.1	Cell metabolism
7	D38410	TFF3 (Trefoil factor 3, intestinal)	3823	56.5	Mucosal cytoprotective function
8	M17327	Leukemia virus modified polytropic provirus DNA	2571	49.8	Endogenous provirus
9	L06047	Glutathione-S-transferase, alpha 1	3408	45.8	Cell metabolism
10	AB003305	Proteasome beta type subunit 5, pseudogene	2044	40.2	Peptide production
	Z21858	TFF1 (pS2)	167	4.4	Mucosal cytoprotective function
	U78770	TFF2 (spasmolytic polypeptide)	236	5	Mucosal cytoprotective function

* mRNA expression level in IL-6^{+/+} BECs.

[†] Calculated by Affymetrix comparison analysis software. Corresponding mRNA expression in IL-6^{-/-} BECs was used as a base line. These results are representative of two separate experiments.

bers of the trefoil factor family, TFF1 and TFF2 mRNA were expressed more strongly in IL-6^{+/+} than IL-6^{-/-} BECs, but at much lower levels than TFF3 in the IL-6^{+/+} BECs (Table 2). The microarray results were confirmed by gel analysis of RT-PCR for TFF1, TFF2, and TFF3 mRNA expression (data not shown).

Quantitative Comparison of TFF3 mRNA and Protein Expression between IL-6^{+/+} and IL-6^{-/-} BECs

We next conducted SYBR Green quantitative real-time RT-PCR for TFF3 mRNA expression in mouse BECs. When the IL-6^{+/+} BECs were maintained in C-SFM, a medium that stimulates endogenous IL-6 production, TFF3 mRNA expression was significantly higher than that of IL-6^{-/-} BECs in similar conditions (Figure 1A). As a control, IL-6^{+/+} BECs and IL-6^{-/-} BECs cultured in S-SFM, a medium that does not stimulate endogenous IL-6 production, showed significantly less TFF3 mRNA expression. These results validated the microarray analysis and other real-time PCR analyses in mice showing that TFF3 is preferentially expressed in the liver.²⁹ Western blotting of BEC protein obtained from experiments conducted in C-SFM, the medium that stimulates BEC IL-6 production, showed that IL-6^{+/+} BECs also express more TFF3 protein than IL-6^{-/-} BECs. In addition, exogenous rIL-6 can increase significantly TFF3 protein expression in IL-6^{-/-} BECs (Figure 1, B and C).

Exogenous rIL-6 Increases TFF3 mRNA and Protein Expression in IL-6^{-/-} BECs

Exogenous recombinant IL-6 (rIL-6) should increase the low levels of TFF3 mRNA expression normally seen in IL-6^{-/-} BECs. As shown in Figure 2A, the addition of 10 to 100 pg/ml of rIL-6 to IL-6^{-/-} BECs resulted in increased TFF3 mRNA expression in a concentration-dependent manner. However, very high levels (1000 pg/ml) of rIL-6, resulted in reversion of TFF3 mRNA expression to

baseline levels, or actually inhibited TFF3 expression. There was also a difference in TFF3 protein expression in IL-6^{-/-} BECs treated with rIL-6, confirmed by Western blotting (Figure 2, B and C). TFF3 protein expression in IL-6^{-/-} BECs treated with rIL-6 (100 pg/ml) was significantly higher than control levels (Figure 1C). Endogenous production of IL-6 by BECs is within the physiological range of 10 to 200 pg/ml, as shown in previous experiments.¹⁸

Neutralizing Anti-IL-6 Antibody Decreases TFF3 mRNA Expression in IL-6^{+/+} BECs

If IL-6 protein released from the IL-6^{+/+} BECs is responsible for the enhanced TFF3 mRNA expression when the cells are kept in C-SFM, treatment of the IL-6^{+/+} BEC cultures with neutralizing anti-IL-6 antibody should reverse this effect. As expected, addition of 10 μ g/ml of neutralizing anti-IL-6 antibody to IL-6^{+/+} BECs successfully blocked the enhanced expression of TFF3 mRNA compared to the same concentration of control IgG (Figure 3).

MAPK Signaling Decreases BEC TFF3 Expression whereas STAT3 Increases TFF3 Expression in Mouse BECs

The IL-6 family of cytokines activates two distinct intracellular signaling pathways, STAT3 and Src-homology tyrosine phosphatase 2 (SHP)-Ras-MAPK pathways, and there is reciprocal negative regulation between them.³⁰ Coordinated and simultaneous activation of the STAT1/3 and SHP2-Ras-ERK pathways appears to transduce activities regulating cellular differentiation and apoptosis (STAT1/3) and mitogenesis (SHP2-Ras-ERK).²⁶ It has been suggested that the two pathways may maintain cellular homeostasis in some dynamic cellular systems, such as the immune system, by balancing positive and negative signals.²⁶

Figure 1. Comparison of TFF3 mRNA and protein expression by IL-6^{+/+} and IL-6^{-/-} mouse BECs in two different media: S-SFM does not stimulate endogenous IL-6 production, whereas C-SFM stimulates endogenous IL-6 production. **A:** TFF3 mRNA expression was significantly higher in IL-6^{+/+} than in IL-6^{-/-} BECs in C-SFM (**, *P* < 0.01). In addition, TFF3 mRNA expression in IL-6^{+/+} BECs was significantly greater in C-SFM than in S-SFM after 48 hours of incubation by real-time PCR (**, *P* < 0.01). **B:** Western blot analysis for TFF3 protein in mouse IL-6^{+/+} and IL-6^{-/-} BECs kept in C-SFM for 72 hours. TFF3 protein band was present at ~7 kd. Recombinant human TFF3 (rTFF3) was used as a positive control. **C:** Intensity of each band in B was quantified by densitometry and statistically analyzed. IL-6^{+/+} BECs showed higher TFF3 protein expression than IL-6^{-/-} BECs in C-SFM (**, *P* < 0.01). Addition of rIL-6 (100 pg/ml) resulted in a significant up-regulation of TFF3 protein expression in IL-6^{-/-} BECs (**, *P* < 0.01 compared to IL-6^{-/-} BECs).

Phenotypes common to gp130-signaling domain mutants and mutants with disrupted gp130 signaling (IL-6^{-/-} or IL-11Rα1^{-/-} mice) such as decreased colonic TFF3 expression and enhanced susceptibility to colitis are likely to result from the altered signaling throughout of the STAT1/3 cascade, somewhat irrespective of that of the SHP2-Ras-ERK pathway.²⁶ Therefore, we first tested the hypothesis that directly blocking STAT3 signaling should decrease the high level of TFF3 mRNA expression in IL-6^{+/+} BECs using an adenoviral vector encoding a dominant-negative form of STAT3 (AdSTAT3-DN). IL-6^{+/+} BECs infected for 2 days with either AdSTAT3-DN or the control vector (AdGFP) were then

Figure 2. The effect of exogenous rIL-6 on TFF3 mRNA and protein expression in IL-6^{-/-} BECs. **A:** The addition of rIL-6 (10 to 100 pg/ml) to IL-6^{-/-} BECs kept in C-SFM for 24 hours up-regulated TFF3 mRNA expression in a concentration-dependent manner (*, *P* < 0.05; **, *P* < 0.01 versus control). **B** and **C:** There was also a significant increase in TFF3 protein expression at 100 pg/ml, as evidenced by densitometry analysis of the Western blots (*, *P* < 0.01 versus controls).

tested for TFF3 mRNA expression. Despite a similar infection ratio of 30 to 40% in both groups as determined by GFP expression (Figure 4B), BECs transfected with AdSTAT3-DN showed significantly less TFF3 mRNA expression than BECs transfected with the control vector (Figure 4B).

However, because strong STAT3 signaling diminishes the MAPK pathway and vice versa,³⁰ we next determined

Figure 3. The effect of neutralizing anti-IL-6 antibody on TFF3 mRNA expression in IL-6^{+/+} BECs. The addition of 10 µg/ml of anti-IL-6 antibody to IL-6^{+/+} BECs kept in C-SFM for 48 hours significantly lowered TFF3 mRNA expression compared to the same concentration of control IgG antibody (*, $P < 0.05$).

the effect of inhibiting BEC MAPK signaling with the expectation that BEC TFF3 mRNA expression would be increased. Indeed, treatment of IL-6^{+/+} BECs, which already express IL-6 and high levels of TFF3 mRNA with PD98059, a specific inhibitor of MAPK kinase 1 (MAPKK1) activation and p44/p42 MAPK activity, significantly up-regulated TFF3 mRNA expression compared to control treatment (Figure 4B). Similarly, the normally low level of TFF3 mRNA expression in IL-6^{-/-} BECs showed significant up-regulation when the BECs were treated with PD98059. However, the levels of TFF3 mRNA expression in IL-6^{-/-} BECs treated with PD98059 were still much lower than the control level of IL-6^{+/+} BECs.

Finally, we tested the effect of other growth factors (eg, HGF) and cytokines (TGF-β) that are produced primarily by periductal stromal and hematolymphoid cells after BDL.⁶ TNF-α has already been shown to decrease TFF3 expression.²¹ These growth factors and cytokines are important in intestinal epithelial wound healing, but are not produced or differentially regulated by BEC cultures in C-SFM.¹³ The results show that HGF and TGF-β significantly decrease BEC TFF3 mRNA and protein expression in a concentration-dependent manner Figure 4, D and E.

The Effect of IL-6 and TFF3 on BEC Restitution in a Wound-Healing Assay

TFF3 contributes to restitution of epithelial defects in the intestines.²⁶ Therefore, we next tested the ability of TFF3 to influence BEC wound healing *in vitro* and compared the ability of IL-6^{+/+} and IL-6^{-/-} BECs to fill an artificially created wound in a BEC monolayer on a collagen gel (Figure 5A; see Materials and Methods). IL-6^{+/+} BECs that show significantly higher TFF3 expression also showed significantly better migration and wound healing than IL-6^{-/-} BECs after 12 hours in C-SFM (Figure 5, B and C). If lower TFF3 expression in IL-6^{-/-} BECs is a reason for impaired cell migration and wound healing, treatment of IL-6^{-/-} BECs with exogenous recombinant

Figure 4. Regulation of TFF3 mRNA expression by the two intracellular signaling pathways, MAPK and STAT3 signaling, *in vitro*. **A:** Transfection with an equal dose of adenoviral vectors coding for a dominant-negative form of STAT3 (AdSTAT3-DN) or the control vector (AdGFP) to IL-6^{+/+} BECs showed similar transfection ratios (30.5% AdGFP versus 43.3% AdSTAT3-DN) by examining GFP expression (top) by fluorescent microscopy and the corresponding phase contrast images (bottom). **B:** IL-6^{+/+} BECs were transfected for 2 days with either AdSTAT3-DN or AdGFP, and then incubated in C-SFM for 24 hours. TFF3 mRNA expression was significantly lower in IL-6^{+/+} BECs transfected with AdSTAT3-DN than with AdGFP (*, $P < 0.05$). **C:** The addition of PD98059 (25 µmol/L), a selective inhibitor of MAPK kinase 1 (MAPKK1), significantly increased TFF3 mRNA expression in IL-6^{+/+} and IL-6^{-/-} BECs kept in C-SFM for 24 hours compared to 0.1% DMSO (vehicle control) (*, $P < 0.05$; **, $P < 0.01$). **D:** BEC treatment with either HGF (0 to 50 ng/ml) or TGF-β (0 to 100 ng/ml) significantly decreased BEC TFF3 mRNA expression (*, $P < 0.04$). **E:** Western blot analysis for TFF3 in IL-6^{+/+} BECs treated with 0 to 50 ng/ml of HGF for 72 hours. Similar to TFF3 mRNA, TFF3 protein was reduced in HGF-treated BECs.

TFF3 (rTFF3) should improve migration and wound healing. As expected, rTFF3 significantly increased the ability of IL-6^{-/-} BECs to repair the wound at a concentration of 1.0 mg/ml for 12 hours in C-SFM (Figure 5D) in the presence of mitomycin C, which successfully inhibited BEC proliferation (data not shown). Therefore, the effect of IL-6 and rTFF3 on wound healing could be confidently attributed to BEC restitution and not to BEC proliferation.

Dynamic Changes of TFF3 Expression after BDL and Impaired Expression in IL-6^{-/-} Mice

We subjected IL-6^{-/-} and IL-6^{+/+} mice (littermate) controls to BDL to determine whether the differences between IL-6^{+/+} and IL-6^{-/-} BEC production of TFF3 had relevance *in vivo*. This model provides an opportunity to determine the net effect of BDL on expression of BEC TFF3 *in vivo* and the relative contribution of IL-6 signaling. Mice from each group were sacrificed before, and 1, 3, 7,

Figure 5. BEC migration in a wound-healing assay conducted on collagen gel substrate *in vitro*. **A:** Before plating the BECs, a glass micropipette was laid on the collagen gel to prevent cell attachment or growth into this area. Then the BECs were plated and grown to a confluent BEC monolayer. The glass micropipette was then removed from the collagen gel creating an artificial wound in the BEC monolayer, which was filled in by BECs migrating from the edge of the wound. **B:** Comparison of IL-6^{+/+} and IL-6^{-/-} BECs kept in C-SFM 12 hours after creating the wound. Photographs were taken immediately after creating the wound (0 hour) and 12 hours later. The leading edge of BECs showed that IL-6^{+/+} BECs migrated significantly more than IL-6^{-/-} BECs. **C:** Quantification of migration distance for IL-6^{+/+} and IL-6^{-/-} BECs on collagen gels kept in C-SFM for 12 hours. IL-6^{+/+} BECs showed significantly better migration than IL-6^{-/-} BECs (*, $P < 0.05$). **D:** The effect of recombinant TFF3 (rTFF3) on IL-6^{-/-} BEC migration kept in C-SFM for 12 hours on collagen gel. The addition of rTFF3 at concentrations of 1.0 mg/ml significantly enhanced the migration distance of IL-6^{-/-} BECs (*, $P < 0.05$ versus control).

and 12 weeks after BDL.^{6,7,14} A group of IL-6^{-/-} mice was treated with exogenous rIL-6 or normal saline as a control to determine the effect of IL-6 replacement therapy on IL-6^{-/-} mice. This daily treatment was started at 6 weeks after BDL and continued for 1 or 6 weeks.

Before BDL, TFF3 mRNA expression was significantly higher in the normal livers of IL-6^{+/+} compared to IL-6^{-/-} mice (Figure 6A). At this time point, both STAT3 and MAPK signaling was present in the IL-6^{+/+}, but MAPK-predominant signaling was observed in the IL-6^{-/-} mice (Figure 7A). After BDL, TFF3 mRNA expression in IL-6^{+/+} mice livers was down-regulated and remained at low levels until 3 weeks (data not shown), consistent with the up-regulation of TNF- α , HGF, and TGF- β during this time, as reported in previous studies.^{6,19,20} However, between 7 and 12 weeks after BDL, TFF3 mRNA levels in the IL-6^{+/+} mice livers returned to near baseline levels (Figure 6A). In contrast, IL-6^{-/-} mice livers showed a slight, but statistically significant up-regulation of TFF3 mRNA between 3 to 7 weeks compared to baseline levels in the IL-6^{-/-} mice. The slightly increased TFF3 mRNA levels in the IL-6^{-/-} mice persisted until 12 weeks after BDL, but these levels were still significantly lower than IL-6^{+/+} livers 12 weeks after BDL (Figure 6A). The low baseline levels of TFF3 mRNA in the IL-6^{-/-} mice livers at 12 weeks after BDL could be prevented by daily treatment with rIL-6 treatment for 6 weeks (Figure 6A).

Figure 6. Quantification of TFF3 mRNA and protein expression in mouse whole liver before and after BDL. **A:** Time course of TFF3 mRNA expression by IL-6^{+/+} and IL-6^{-/-} whole liver before and after BDL. IL-6^{-/-} mice were treated daily with either saline (controls) or rIL-6 starting at 6 weeks after BDL and continuing for 1 or 6 weeks. TFF3 mRNA expression was significantly higher in the normal whole liver of IL-6^{+/+} mice than that of IL-6^{-/-} mice before BDL (**, $P < 0.01$). By 12 weeks after BDL, IL-6^{-/-} livers expressed significantly lower TFF3 mRNA than in IL-6^{+/+} mice liver or IL-6^{-/-} mouse liver treated with exogenous rIL-6 (**, $P < 0.01$ versus the other two). **B:** TFF3 protein expression in mouse whole liver at 12 weeks after BDL by Western blot analysis. Cytosolic protein was extracted from IL-6^{+/+} and IL-6^{-/-} mouse liver treated with control saline or rIL-6 for 6 weeks. Three different mouse liver samples were used for each condition. rTFF3 was used as a positive control. The IL-6^{-/-} mice treated with rIL-6 were obtained 24 hours after the last IL-6 dose. **C:** TFF3 protein levels were quantified by densitometry and statistically analyzed. TFF3 protein expression in IL-6^{+/+} mice liver was significantly higher than that in IL-6^{-/-} mouse liver treated with control saline or rIL-6 (*, $P < 0.05$). IL-6 treatment significantly increased TFF3 levels in the IL-6^{-/-} liver above that of saline-treated IL-6^{-/-} livers (x, $P < 0.05$).

There was also a significant difference in TFF3 protein expression in IL-6^{+/+} and IL-6^{-/-} livers at 12 weeks after BDL, confirmed by Western blotting (Figure 6B). TFF3 protein expression in 12-week IL-6^{+/+} livers was significantly higher than that in saline-treated IL-6^{-/-} livers (Figure 6C). Treatment of the IL-6^{-/-} mice for 6 weeks with rIL-6 also significantly increased TFF3 protein levels compared to saline-treated IL-6^{-/-} mice, but the level of TFF3 protein expression did not return to the levels seen in IL-6^{+/+} mice at 12 weeks after BDL (Figure 6C).

TFF3 mRNA and Protein Expression Correlates Primarily with STAT3 Phosphorylation

We next measured the level of nuclear phospho-STAT3 (p-STAT3) and phospho-p44/p42 MAPK (p-p44/p42) expression in mouse whole liver by Western blotting to determine whether there was a relationship between TFF3 expression and p-STAT3 or p-p44/p42 expression

Figure 7. Activation of STAT3 and MAPK signaling in mouse liver before and after BDL. **A:** Western blotting for phospho-STAT3 (p-STAT3) and phospho-p44/p42 MAPK (p-p44/p42) were used to study the association between gp130 downstream signaling (MAPK and STAT3) and TFF3 expression in IL-6^{+/+} and IL-6^{-/-} mice whole livers before (normal) and 12 weeks after BDL. Three different mouse liver samples were used for each time point. Nuclear p-STAT3 expression is greater in IL-6^{+/+} than IL-6^{-/-} mice before and at 12 weeks after BDL, confirming impaired STAT3 signaling in the IL-6^{-/-} mice. In contrast, there was no clear difference between IL-6^{+/+} and IL-6^{-/-} mice in nuclear p-p44/p42 expression. **B** and **C:** p-STAT3 protein levels (**B**) and p-p44/p42 protein levels (**C**) were quantified by densitometry (*, $P < 0.05$; **, $P < 0.01$).

in vivo. In normal mice before BDL, nuclear p-STAT3 expression was significantly higher in IL-6^{+/+} than IL-6^{-/-} livers (Figure 7, A and B), but pMAPK was similarly detectable in both groups. By 12 weeks after BDL, nuclear p-STAT3 protein in IL-6^{+/+} livers was slightly below baseline values, but still significantly higher than that in IL-6^{-/-} livers. In addition, the slightly increased TFF3 expression was associated with slightly increased pSTAT3 in IL-6^{-/-} mice. These findings are consistent with previous studies showing defective STAT3 signaling is primarily responsible for the decreased TFF3 expression and susceptibility to colitis in IL-6^{-/-} mice and gp130 mutants.^{26,30–32} There were no differences in the level of p-p44/p42 levels between the IL-6^{+/+} and IL-6^{-/-} mouse livers before or 12 weeks after BDL. However, the

values at 12 weeks were higher than those at 1 week and IL-6^{-/-} mice tended to show higher p-p44/p42 levels at both time points (Figure 7, A and C). Thus, it appears that BEC TFF3 mRNA and protein expression is dependent primarily on STAT3 activation and signaling (Figure 6, A and B; Figure 7, A and B), but other factors such as HGF or TNF- α ²¹ can obviously influence levels *in vivo*, depending on the conditions.

Immunohistochemical Staining for TFF3 and Phospho-STAT3 Protein Expression in Mouse Bile Duct-Ligated Liver

Because TFF3 is a mucin-associated peptide and the above studies show that STAT3 signaling importantly contributes to TFF3 expression, we expected TFF3 protein to localize to mucin-secreting BECs that also expressed nuclear p-STAT3. Indeed, immunohistochemical staining of mouse liver at 12 weeks after BDL, showed TFF3 protein expression primarily in the large bile ducts (Figure 8A) and peribiliary glands (Figure 8A, inset) in the IL-6^{+/+} mice. In contrast, TFF3 protein was more weakly present in the large bile ducts (Figure 8B) and peribiliary glands of IL-6^{-/-} mouse livers. Some flattened BECs covering small mucosal defects with minimal mucin expression also expressed TFF3, sometimes more strongly than the nearby cuboidal or columnar BECs (Figure 8B, inset). In addition, nuclear p-STAT3 and TFF3 protein co-localized to the same areas of the large bile ducts and peribiliary glands (Figure 8C) in IL-6^{+/+} mouse liver at 12 weeks after BDL. However, there were also mucin-producing TFF3⁺ BECs that were negative for nuclear pSTAT3.

Immunohistochemical Staining for TFF3 and Phospho-STAT3 Protein Expression in Normal and Diseased Human Liver Tissues

Similar to the mouse, TFF3 mRNA expression was much higher than TFF1 and TFF2 in the human liver by real-time PCR (data not shown). In the normal human biliary tree ($n = 3$), TFF3 and nuclear p-STAT3 protein was limited to the medium and large bile ducts and peribiliary mucinous glands (Figure 9; A to C). However, the levels of mucin production and TFF3 expression appeared to be much higher in the human than in the mouse. Patients with biliary obstruction ($n = 3$) (Figure 9; D to F) showed increased TFF3 and nuclear p-STAT3 expression, as estimated by immunohistochemical assessment, in the large intrahepatic and extrahepatic bile ducts, especially in areas of peribiliary mucinous glandular hyperplasia.^{33–36} The mucinous peribiliary glands and elongated cells that were stretching to cover ulcers also showed strong staining. Similar to previous studies,^{37,38} patients with primary biliary cirrhosis ($n = 3$) showed induction or neo-expression of BEC TFF3 only in medium-sized ducts showing inflammatory damage or florid duct lesions (Figure 9; G to I). Co-localization of TFF3 to BECs also expressing nuclear p-STAT3 was particularly evident in

Figure 8. Immunohistochemical staining for TFF3 protein in mouse liver at 12 weeks after BDL showed expression was exclusively limited to the large bile ducts and peribiliary glands (**inset**) in IL-6^{+/+} mouse liver (**A**), but weakly present in the large bile ducts in IL-6^{-/-} (**B**) mouse liver. Note that TFF3 was expressed by columnar mucin-secreting cells and by an elongated (**B, inset**) BEC stretching to cover mucosal ulcers or erosions (**C**). Serial sections were used to co-localize nuclear p-STAT3 and TFF3 protein to the same areas of the large bile ducts and peribiliary glands in IL-6^{+/+} mouse liver at 12 weeks after BDL. The **small rectangular area** highlighted on the low-power or **bottom** image is shown at higher magnification in the **insets** in the **top** portion of the photomicrograph. Note the nuclear p-STAT3 staining in the BECs and peribiliary glands (**top right inset**) in the same areas that are expressing TFF3 (**top left inset**).

PBC livers with florid duct lesions, which were simultaneously positive for TFF3 and nuclear p-STAT3, whereas uninvolved ducts of the same size were negative for both.

Discussion

Several recent studies using knock-in mutations to investigate the effect of IL-6/gp130 signaling in the gastrointestinal tract show that this cytokine-receptor signaling system significantly contributes to mucosal barrier func-

tion.^{26,39} Mice harboring mutations that selectively block all gp130-mediated STAT activity (gp130 Δ^{STAT}), but preserve gp130-mediated MAPK signaling show decreased colonic TFF3 expression, increased sensitivity to sodium dextran sulfate-induced colitis, and impaired mucosal wound healing.^{26,39} In contrast, mice with a mutation selectively abrogating only SHP-2-Ras-ERK (MAPK) signaling (gp130^{757F}) were resistant to colitis, but developed gastric adenomas as a consequence of unopposed STAT3 signaling.^{26,39} A conclusion of these detailed

Figure 9. In normal human liver (A–C), TFF3 protein expression localized mainly to the large bile ducts (A) and mucinous peribiliary glands (B). Nuclear p-STAT3 protein was expressed in the same locations (C). In obstructive cholangiopathy (D–F), TFF3 protein expression increased in the large- and medium-sized bile ducts, especially in areas of peribiliary mucinous gland hyperplasia (D, E), which also expressed nuclear p-STAT3 (F). The association between STAT3 signaling and TFF3 expression was most evident in bile ducts damaged by primary biliary cirrhosis (G–I). TFF3 is not normally expressed in medium-sized intrahepatic bile ducts, but when these ducts are immunologically damaged in primary biliary cirrhosis, they express nuclear p-STAT3 (I) and TFF3 (G, H) that is then secreted into the bile. Previous studies have shown that damaged bile ducts in primary biliary cirrhosis show increased IL-6 production.⁵

studies in the colon is that phenotypes common to gp130-signaling domain mutants and mutants with disrupted gp130 signaling such as in IL-6^{-/-} or IL-11R α 1^{-/-} mice are likely to result from the altered signaling throughput of the STAT1/3 cascade, irrespective of that of the SHP2-Ras-ERK pathway. However, given the reciprocal negative regulation between STAT3 and MAPK signaling, simultaneous and balanced activation of the two gp130-mediated signaling cascades is not only essential for mediating tissue-specific activities of IL-6 family cytokines, but has the potential for fine tuning the balance between negative and positive signals (differentiation and anti-apoptosis versus proliferation) during biological responses to these cytokines.²⁶

A critical linkage between IL-6/gp130/STAT3 signaling and the TFF is supported by striking similarities in the gastrointestinal pathology observed in the gp130 mutants described above and mice deficient for TFF3.^{15,26,39–41} Previous studies show that IL-6^{-/-} mice, as used in this study, most closely mimic the gp130 Δ ^{STAT} mutants: impaired STAT3 signaling and an increased susceptibility to colitis and impaired barrier function.²⁶

TFF contain one or more trefoil motifs composed of six cysteine residues. These proteins influence the rheological properties of mucus gels and contribute to optimal protection of the intestinal mucosa from injury.^{15,16,28,42} Trefoil peptides also enhance intestinal epithelial restitution primarily by stimulating epithelial cell spreading and migration, which accelerates mucosal epithelial recovery after injury.^{40,43} Each of the TFF proteins is differentially regulated in different parts of the gastrointestinal tract: ^{26,39} TFF1 and TFF2 are expressed primarily in the stomach^{44,45} and TFF3 predominates in the small and large intestines.⁴¹ In the liver, this and previous studies have shown that TFF1 and TFF3 are constitutively expressed in the large bile ducts of mouse and human livers.^{29,37,46,47}

A major conclusion of this study is that STAT3 signaling is crucial for BEC TFF3 expression, but the reciprocal negative regulation known to exist between the STAT3 and MAPK signaling pathways²⁶ also importantly influences BEC TFF3 expression. *In vitro*, where isolated BECs can be studied without influences of confounding factors, BEC TFF3 mRNAs and protein expression is significantly decreased by specifically inhibiting STAT3

signaling and increased by inhibiting MAPK signaling, which removes STAT3 inhibition. *In vivo* the most direct correlation was between STAT3 signaling or activation and TFF3 mRNA expression. A similar correlation was seen between STAT3 signaling and TFF3 protein expression, but the differences were not as dramatic, which suggests that posttranslational modification also importantly contributes to BEC TFF3 protein expression. Regardless, TFF3 expression was highest in the IL-6^{+/+} mice before and at 12 weeks after BDL. Increased TFF3 mRNA expression was seen in the IL-6^{-/-} mice only at 12 weeks after BDL when there was also increased STAT3 signaling compared to baseline.

The decrease of hepatic TFF3 expression observed within the first several weeks after BDL is an apparent discrepancy between *in vitro* and *in vivo* results that deserves comment. First, there are a number of growth factors and cytokines, such as TNF- α , TGF- β , and HGF that contribute to BEC pathophysiology after BDL,^{6,48-50} but are not produced primarily by BECs or differentially regulated *in vitro* under the conditions used in this study. However, we and others have shown that TNF- α ,²¹ TGF- β , and HGF can down-regulate BEC TFF3 expression. Thus, additional factors present *in vivo*, but not *in vitro*, significantly influence BEC TFF3 expression. The combination of *in vitro* experiments and *in vivo* experiments enabled us to limit confounding influences and focus more narrowly on IL-6-related pathways, and then subsequently investigate the relative contribution of IL-6-signaling pathways to BEC TFF3 expression under real disease conditions *in vivo*.

During chronic injury or later stages of BDL (7 to 12 weeks after BDL) in IL-6^{+/+} mice, TFF3 mRNA and nuclear pSTAT3 protein expression recover to baseline levels coincident with the periductal mucinous gland hyperplasia, as noted in both the IL-6^{+/+} mice and humans. In contrast, TFF3 levels in the IL-6^{-/-} mice remained depressed, despite the persistent injury associated with permanent BDL. More importantly, the biliary integrity is impaired in untreated IL-6^{-/-} mice, but completely reversed by administration of exogenous IL-6,¹⁴ coincident with an increase of biliary tract TFF3 expression. However, TFF3 protein levels in the rIL-6-treated IL-6^{-/-} mice did not reach levels seen in the IL-6^{+/+} mice, which might be caused by inhibition of STAT3 signaling by the MAPK-predominant activation seen in the IL-6^{-/-} mice at 12 weeks after BDL. Thus, we cannot conclude that deficient BEC TFF3 expression is solely responsible for impaired biliary tree integrity in the IL-6^{-/-} mice, but our findings provide compelling evidence that BEC TFF3 is an important contributor. It would have been ideal to show that replenishing TFF3 alone could reverse the defect in the IL-6^{-/-} mice after long-term BDL, but access to the ligated mouse biliary tree for treatment with rTFF3 was not possible.

Healing of a mucosal epithelial lining requires two processes: restitution, or spreading and migration of epithelial cells to cover the defect; and regeneration, or proliferation of epithelial cells to replace lost cells.^{1,17} Because it is difficult to directly observe BEC wound healing in the biliary tree under dynamic conditions *in vivo*, we relied on a wound healing assay *in vitro*. *In vitro* IL-6^{+/+} BECs

consistently showed significantly better migration and wound healing than IL-6^{-/-} BECs. In addition, treatment of IL-6^{-/-} BECs with rTFF3 improved migration and wound healing by promoting BEC restitution, but not by BEC proliferation. Impaired skin and colon wound healing has already been convincingly demonstrated in IL-6^{-/-} and STAT3-deficient mice.^{26,51,52}

Clear parallels can be seen between the normal mouse and normal human liver and mouse liver after BDL and human diseases such as biliary obstruction and primary biliary cirrhosis. However, there are also some clear-cut differences. TFF1 and TFF3 levels appear to be up-regulated in human livers after bile duct obstruction, primary sclerosing cholangitis, and in primary biliary cirrhosis as determined by semiquantitative analysis of immunohistochemically stained sections of human liver tissue.^{29,37,46,47} In this study, TFF3 mRNA and protein levels at 12 weeks after BDL, as determined in whole liver, are similar to those seen in normal livers.

There are several possible explanations for the apparent difference between the mouse and human. First, regulation of TFF3 differs between organs in humans and mice.²⁹ Second, it is difficult to compare methodologies (real-time PCR and Western blot) in the current studies versus immunohistochemistry in different types of samples (needle biopsies, wedge biopsies, and resection specimens), in the human analyses. In our experience with both humans and mice, TFF3 expression is constitutive primarily in large bile ducts and peribiliary gland and is expressed in medium-sized bile ducts only after injury. Without routine sampling of the large bile ducts and controlling for the number or percentage of mucin-secreting cells and peribiliary glands, it is difficult to quantify TFF3 expression by immunohistochemistry. Third, mucinous hyperplasia of peribiliary glands appears more prominent in human than in mouse liver after BDL (unpublished observation).

Regardless of the above caveats, we think that it is reasonable to conclude that: 1) TFF3 is the major trefoil peptide expressed in the mouse and human biliary tree; 2) IL-6-mediated gp130 signaling through STAT3 and reciprocal negative regulation via MAPK signaling significantly influence TFF3 mRNA and protein expression in BECs lining the large bile ducts and mucinous peribiliary glands;^{33,34} and 3) the combination of mucins and trefoil family peptides in the biliary tree contribute to mucosal protection and TFF3 contributes to healing of epithelial ulcers by augmenting BEC restitution.^{53,54} Overall, the results are quite similar to observations regarding TFF3 in the colon.²⁶

Understanding the molecular mechanisms of repair reactions in the biliary tree has significant practical implications, particularly for liver allografts. Biliary tract strictures that occur as a consequence of preservation-injury-related denudation of the biliary epithelium affect up to 15% of liver allografts.² Studies are underway to determine whether it is possible to either prevent or lessen the impact of preservation injury to the biliary tract using pretreatment of the donor with either IL-6 or rTFF3.¹⁷ In addition, TFF expression is tightly linked with inflamma-

tion and gastric carcinogenesis. Similar associations would be expected for the biliary tree.

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References

- Jacinto A, Martinez-Arias A, Martin P: Mechanisms of epithelial fusion and repair. *Nat Cell Biol* 2001, 3:E117-E123
- Kukan M, Haddad PS: Role of hepatocytes and bile duct cells in preservation-reperfusion injury of liver grafts. *Liver Transpl* 2001, 7:381-400
- Demetris AJ, Sakamoto T, Liu Z, Yokomuro S, Ezure T, Murase N, Blakolmer K: The ductular reaction in liver disease emphasis on a type I response. *Normal and Malignant Liver Cell Growth*. Edited by WE Fleig. Dordrecht, Kluwer Academic Publishers, 1999, pp 141-155
- Lamireau T, Zoltowska M, Levy E, Yousef I, Rosenbaum J, Tuchweber B, Desmouliere A: Effects of bile acids on biliary epithelial cells: proliferation, cytotoxicity, and cytokine secretion. *Life Sci* 2003, 72: 1401-1411
- Yasoshima M, Kono N, Sugawara H, Katayanagi K, Harada K, Nakanuma Y: Increased expression of interleukin-6 and tumor necrosis factor-alpha in pathologic biliary epithelial cells: in situ and culture study. *Lab Invest* 1998, 78:89-100
- Liu Z, Sakamoto T, Ezure T, Yokomuro S, Murase N, Michalopoulos G, Demetris AJ: Interleukin-6, hepatocyte growth factor, and their receptors in biliary epithelial cells during a type I ductular reaction in mice: interactions between the periductal inflammatory and stromal cells and the biliary epithelium. *Hepatology* 1998, 28:1260-1268
- Liu Z, Sakamoto T, Yokomuro S, Ezure T, Subbotin V, Murase N, Contrucci S, Demetris AJ: Acute obstructive cholangiopathy in interleukin-6 deficient mice: compensation by leukemia inhibitory factor (LIF) suggests importance of gp-130 signaling in the ductular reaction. *Liver* 2000, 20:114-124
- Rosen HR, Winkle PJ, Kendall BJ, Diehl DL: Biliary interleukin-6 and tumor necrosis factor-alpha in patients undergoing endoscopic retrograde cholangiopancreatography. *Dig Dis Sci* 1997, 42:1290-1294
- Scotte M, Daveau M, Hiron M, Delers F, Lemeland JF, Teniere P, Lebreton JP: Interleukin-6 (IL-6) and acute-phase proteins in rats with biliary sepsis. *Eur Cytokine Netw* 1991, 2:177-182
- Kimmings AN, van Deventer SJ, Obertop H, Rauws EA, Huibregtse K, Gouma DJ: Endotoxin, cytokines, and endotoxin binding proteins in obstructive jaundice and after preoperative biliary drainage. *Gut* 2000, 46:725-731
- Akiyama T, Hasegawa T, Sejima T, Sahara H, Seto K, Saito H, Takashima S: Serum and bile interleukin 6 after percutaneous transhepatic cholangio-drainage. *Hepatogastroenterology* 1998, 45:665-671
- Demetris AJ, Lunz JG, Subbotin V, Wu T, Nozaki I, Contrucci S, Yin X: Participation of cytokines and growth factors in biliary epithelial proliferation and mitogenic-inhibition during ductular reactions. *The Pathophysiology of the Biliary Epithelia*. Edited by G Alpini, D Alvaro, M Marzoni, NF LaRusso, G LeSage. Landes Bioscience, Georgetown, TX, 2003, pp 167-191
- Yokomuro S, Tsuji H, Lunz JG, Sakamoto T, Ezure T, Murase N, Demetris AJ: Growth control of human biliary epithelial cells by interleukin 6, hepatocyte growth factor, transforming growth factor beta1, and activin A: comparison of a cholangiocarcinoma cell line with primary cultures of non-neoplastic biliary epithelial cells. *Hepatology* 2000, 32:26-35
- Ezure T, Sakamoto T, Tsuji H, Lunz JG, Murase N, Fung JJ, Demetris AJ: The development and compensation of biliary cirrhosis in interleukin-6-deficient mice. *Am J Pathol* 2000, 156:1627-1639
- Mashimo H, Wu DC, Podolsky DK, Fishman MC: Impaired defense of intestinal mucosa in mice lacking intestinal trefoil factor. *Science* 1996, 274:262-265
- Kindon H, Pothoulakis C, Thim L, Lynch-Devaney K, Podolsky DK: Trefoil peptide protection of intestinal epithelial barrier function: cooperative interaction with mucin glycoprotein. *Gastroenterology* 1995, 109:516-523
- Taupin D, Podolsky DK: Trefoil factors: initiators of mucosal healing. *Nat Rev Mol Cell Biol* 2003, 4:721-732
- Yokomuro S, Lunz JG, Sakamoto T, Ezure T, Murase N, Demetris AJ: The effect of interleukin-6 (IL-6)/gp130 signalling on biliary epithelial cell growth, in vitro. *Cytokine* 2000, 12:727-730
- O'Neil S, Hunt J, Filkins J, Gamelli R: Obstructive jaundice in rats results in exaggerated hepatic production of tumor necrosis factor-alpha and systemic and tissue tumor necrosis factor-alpha levels after endotoxin. *Surgery* 1997, 122:281-287
- Beierle EA, Vauthey JN, Moldawer LL, Copeland III EM: Hepatic tumor necrosis factor-alpha production and distant organ dysfunction in a murine model of obstructive jaundice. *Am J Surg* 1996, 171:202-206
- Loncar MB, Al-azze ED, Sommer PS, Marinovic M, Schmehl K, Kruschewski M, Blin N, Stohwasser R, Gott P, Kayademir T: Tumour necrosis factor alpha and nuclear factor kappaB inhibit transcription of human TFF3 encoding a gastrointestinal healing peptide. *Gut* 2003, 52:1297-1303
- Poli V, Balena R, Fattori E, Markatos A, Yamamoto M, Tanaka H, Ciliberto G, Rodan GA, Costantini F: Interleukin-6 deficient mice are protected from bone loss caused by estrogen depletion. *EMBO J* 1994, 13:1189-1196
- Park JI, Strock CJ, Ball DW, Nelkin BD: The Ras/Raf/MEK/extracellular signal-regulated kinase pathway induces autocrine-paracrine growth inhibition via the leukemia inhibitory factor/JAK/STAT pathway. *Mol Cell Biol* 2003, 23:543-554
- Skelly SM, Tackney C, Hicklin D, Tamkins T, Goldstein N, Waksal H, Dagan S: High-level expression of a biologically active human interleukin-6 mutein. *J Biotechnol* 1994, 34:79-86
- Ni R, Nishikawa Y, Carr BI: Cell growth inhibition by a novel vitamin K is associated with induction of protein tyrosine phosphorylation. *J Biol Chem* 1998, 273:9906-9911
- Tebbutt NC, Giraud AS, Inglese M, Jenkins B, Waring P, Clay FJ, Malki S, Alderman BM, Grail D, Hollande F, Heath JK, Ernst M: Reciprocal regulation of gastrointestinal homeostasis by SHP2 and STAT-mediated trefoil gene activation in gp130 mutant mice. *Nat Med* 2002, 8:1089-1097
- Strain AJ, Wallace L, Joplin R, Daikuhara Y, Ishii T, Kelly DA, Neuberger JM: Characterization of biliary epithelial cells isolated from needle biopsies of human liver in the presence of hepatocyte growth factor. *Am J Pathol* 1995, 146:537-545
- Tomasetto C, Masson R, Linares JL, Wendling C, Lefebvre O, Che-nard MP, Rio MC: pS2/TFF1 interacts directly with the VWFC cysteine-rich domains of mucins. *Gastroenterology* 2000, 118:70-80
- Hertel SC, Chwieralski CE, Hinz M, Rio MC, Tomasetto C, Hoffmann W: Profiling trefoil factor family (TFF) expression in the mouse: identification of an antisense TFF1-related transcript in the kidney and liver. *Peptides* 2004, 25:755-762
- Ishihara K, Hirano T: Molecular basis of the cell specificity of cytokine action. *Biochim Biophys Acta* 2002, 1592:281-296
- Heinrich PC, Behrmann I, Muller-Newen G, Schaper F, Graeve L: Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem J* 1998, 334:297-314
- Auernhammer CJ, Melmed S: Leukemia-inhibitory factor-neuroimmune modulator of endocrine function. *Endocr Rev* 2000, 21:313-345
- Nakanuma Y, Sasaki M, Terada T, Harada K: Intrahepatic peribiliary glands of humans. II. Pathological spectrum. *J Gastroenterol Hepatol* 1994, 9:80-86
- Nakanuma Y, Katayanagi K, Terada T, Saito K: Intrahepatic peribiliary glands of humans. I. Anatomy, development and presumed functions. *J Gastroenterol Hepatol* 1994, 9:75-79
- Katabi N, Albores-Saavedra J: The extrahepatic bile duct lesions in end-stage primary sclerosing cholangitis. *Am J Surg Pathol* 2003, 27:349-355
- Lee KT, Sheen PC, Liu YE: Histomorphometric evaluation of mucin content in stone-containing intrahepatic bile ducts. *J Formos Med Assoc* 1998, 97:788-790
- Srivatsa G, Giraud AS, Ulaganathan M, Yeomans ND, Dow C, Nicoll AJ: Biliary epithelial trefoil peptide expression is increased in biliary diseases. *Histopathology* 2002, 40:261-268

38. Kimura Y, Leung PS, Kenny TP, Van De Water J, Nishioka M, Giraud AS, Neuberger J, Benson G, Kaul R, Ansari AA, Coppel RL, Gershwin ME: Differential expression of intestinal trefoil factor in biliary epithelial cells of primary biliary cirrhosis. *Hepatology* 2002, 36:1227–1235
39. Ernst M, Inglese M, Waring P, Campbell IK, Bao S, Clay FJ, Alexander WS, Wicks IP, Tarlinton DM, Novak U, Heath JK, Dunn AR: Defective gp130-mediated signal transducer and activator of transcription (STAT) signaling results in degenerative joint disease, gastrointestinal ulceration, and failure of uterine implantation. *J Exp Med* 2001, 194: 189–203
40. Kanai M, Mullen C, Podolsky DK: Intestinal trefoil factor induces inactivation of extracellular signal-regulated protein kinase in intestinal epithelial cells. *Proc Natl Acad Sci USA* 1998, 95:178–182
41. Podolsky DK, Lynch-Devaney K, Stow JL, Oates P, Murgue B, DeBeaumont M, Sands BE, Mahida YR: Identification of human intestinal trefoil factor. Goblet cell-specific expression of a peptide targeted for apical secretion. *J Biol Chem* 1993, 268:6694–6702
42. Gum Jr JR, Hicks JW, Gillespie AM, Carlson EJ, Komuves L, Karnik S, Hong JC, Epstein CJ, Kim YS: Goblet cell-specific expression mediated by the MUC2 mucin gene promoter in the intestine of transgenic mice. *Am J Physiol* 1999, 276:G666–G676
43. Dignass A, Lynch-Devaney K, Kindon H, Thim L, Podolsky DK: Trefoil peptides promote epithelial migration through a transforming growth factor beta-independent pathway. *J Clin Invest* 1994, 94:376–383
44. Tomasetto C, Rio MC, Gautier C, Wolf C, Hareuveni M, Chambon P, Lathe R: HSP, the domain-duplicated homolog of pS2 protein, is co-expressed with pS2 in stomach but not in breast carcinoma. *EMBO J* 1990, 9:407–414
45. Rio MC, Bellocq JP, Daniel JY, Tomasetto C, Lathe R, Chenard MP, Batzenschlager A, Chambon P: Breast cancer-associated pS2 protein: synthesis and secretion by normal stomach mucosa. *Science* 1988, 241:705–708
46. Sasaki M, Tsuneyama K, Saito T, Kataoka H, Mollenhauer J, Poustka A, Nakanuma Y: Site-characteristic expression and induction of trefoil factor family 1, 2 and 3 and malignant brain tumor-1 in normal and diseased intrahepatic bile ducts relates to biliary pathophysiology. *Liver Int* 2004, 24:29–37
47. Sasaki M, Tsuneyama K, Nakanuma Y: Aberrant expression of trefoil factor family 1 in biliary epithelium in hepatolithiasis and cholangiocarcinoma. *Lab Invest* 2003, 83:1403–1413
48. Aldana PR, Goerke ME, Carr SC, Tracy Jr TF: The expression of regenerative growth factors in chronic liver injury and repair. *J Surg Res* 1994, 57:711–717
49. Saperstein LA, Jirtle RL, Farouk M, Thompson HJ, Chung KS, Meyers WC: Transforming growth factor-beta 1 and mannose 6-phosphate/insulin-like growth factor-II receptor expression during intrahepatic bile duct hyperplasia and biliary fibrosis in the rat. *Hepatology* 1994, 19:412–417
50. Polimeno L, Azzarone A, Zeng QH, Panella C, Subbotin V, Carr B, Bouzahzah B, Francavilla A, Starzl TE: Cell proliferation and onco-gene expression after bile duct ligation in the rat: evidence of a specific growth effect on bile duct cells. *Hepatology* 1995, 21:1070–1078
51. Gallucci RM, Simeonova PP, Matheson JM, Kommineni C, Gurjel JL, Luster MI: Impaired cutaneous wound healing in interleukin-6-deficient and immunosuppressed mice. *FASEB J* 2000, 14: 2525–2531
52. Sano S, Itami S, Takeda K, Tarutani M, Yamaguchi Y, Miura H, Yoshikawa K, Akira S, Takeda J: Keratinocyte-specific ablation of Stat3 exhibits impaired skin remodeling, but does not affect skin morphogenesis. *EMBO J* 1999, 18:4657–4668
53. Vandenhaute B, Buisine MP, Debailleul V, Clement B, Moniaux N, Dieu MC, Degand P, Porchet N, Aubert JP: Mucin gene expression in biliary epithelial cells. *J Hepatol* 1997, 27:1057–1066
54. Zen Y, Harada K, Sasaki M, Tsuneyama K, Katayanagi K, Yamamoto Y, Nakanuma Y: Lipopolysaccharide induces overexpression of MUC2 and MUC5AC in cultured biliary epithelial cells: possible key phenomenon of hepatolithiasis. *Am J Pathol* 2002, 161:1475–1484