Effect of Connective Tissue Growth Factor on Protein Kinase Expression and Activity in Human Corneal Fibroblasts

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PURPOSE. To investigate signal transduction pathways for connective tissue growth factor (CTGF) in human corneal fibroblasts (HCF).

METHODS. Expression of 75 kinases in cultures of serum-starved (HCF) were investigated using protein kinase screens, and changes in levels of phosphorylation of 31 different phosphoproteins were determined at 0, 5, and 15 minutes after treatment with CTGF. Levels of phosphorylation of three signal transducing phosphoproteins (extracellular regulated kinase 1 [ERK1], extracellular regulated kinase 2 [ERK2] [MAPKs], and signal transducer and activator of transcription 3 [STAT3]) were measured at nine time points after exposure to CTGF using Western immunoblots. Inhibition of Ras, MEK1/2 (MAPKK), and ERK1/2, on CTGF-stimulated fibroblast proliferation and collagen gel contraction was assessed using selective inhibitors farnesylthiosalicylic acid, PD-98059, and SB203580, respectively.

RESULTS. Thirty two of the 75 kinases (43%) evaluated by the kinase screen were detected in extracts of quiescent HCF, suggesting these kinases are available to respond acutely to CTGF exposure. Addition of CTGF increased levels of phosphorylation of five phosphoproteins (ERK1 and 2, MEK1/2 [MAPKK], STAT3, and SAPK/JNK), and decreased levels of phosphorylation of 14 phosphoproteins (including protein kinases B and C) after 5 and 15 minutes. Further analysis of ERK1 and 2 and STAT3 phosphorylation showed rapid increases within 1 minute of CTGF exposure that peaked between 5 and 10 minutes then returned to pretreatment levels by 30 minutes. Treatment of HCF with selective inhibitors of Ras, MEK 1/2, and ERK1/2 individually blocked both CTGF induced cell proliferation, and collagen gel contraction.

CONCLUSIONS. Results from protein kinase screens and selective kinase inhibitors demonstrate Ras/MEK/ERK/STAT3 pathway is required for CTGF signaling in HCF. (*Invest Ophthalmol Vis Sci.* 2012;53:8076–8085) DOI:10.1167/iovs.12-10790

Connective tissue growth factor (CTGF) is a 38-kDa secreted, cysteine-rich peptide that was first identified in conditioned media from cultures of human umbilical vein endothelial cells (HUVEC).^{1,2} CTGF belongs to the CCN [CTGF, Cyr61/Cef10, Nov] family of secreted cysteine-rich proteins, which possess growth regulatory functions and are involved in cell differentiation.^{3–5} CTGF increases the production of components of the extracellular matrix such as collagen, integrin, and fibronectin. Furthermore, CTGF has been shown to be significantly up-regulated in a variety of fibrotic disorders such as biliary fibrosis, sclerotic skin fibroblasts, corneal scar tissue, atherosclerotic blood vessels, and inflammatory bowel disease, suggesting that CTGF is involved in promoting pathological fibrosis.^{6–10} Another growth factor that has been implicated in regulating scarring is TGF- β . Important links between CTGF and TGF- β actions were established by experiments, which showed that TGF- β up-regulated the synthesis of CTGF and that CTGF mediated the effects of TGF- β on synthesis of collagen, alpha smooth muscle actin, and proliferation of fibroblast cultures.^{11–14} However, CTGF alone did not induce anchorage-independent growth of fibroblasts, which distinguishes some its actions from those of TGF- β .¹⁵

CTGF was implicated in playing several key roles in corneal scarring and contraction.¹⁶ All three isoforms of TGF- β increased CTGF mRNA and protein expression in human corneal fibroblasts (HCF).^{17,18} CTGF stimulated contraction of relaxed collagen matrix populated by HCF, and furthermore, antisense oligonucleotides to CTGF blocked TGF- β 1-mediated contraction, demonstrating that CTGF alone was sufficient for contraction and that CTGF was necessary for TGF- β -mediated contraction.^{16,18–20} Using stressed collagen matrix, CTGF alone did not stimulate matrix contraction, but its expression was necessary for TGF- β 1-stimulated contraction.¹⁹ Thus, CTGF plays a central role in mediating TGF- β 1-induced contraction of fibroblast-populated collagen lattices. These data suggest that CTGF is important in regulating corneal scarring and contraction. However, there is very little information regarding the acute signaling events in cells treated with CTGF. In one independent long term study, the induction of CTGF gene expression by TGF- β , and the induction of collagen synthesis by CTGF were both shown to be inhibited by elevated levels of intracellular cAMP.²⁰ In telomerase immortalized human cornea stromal fibroblasts, TGF- β induction of CTGF was inhibited by SP600125 which is mitogen-activated protein kinase (MAPK) specific inhibitor that targets stress-activated

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protein kinase (JNK). These data showed that the JNK pathway plays a key role in TGF- β induction of CTGF.²¹

CTGF was reported to cross-link to a cell surface protein of approximately 250 kDa in a chondrosarcoma cell line.²² Another study using bone marrow-derived stromal cells reported that CTGF cross-linked to a 620-kDa cell surface protein that was identified as the low-density lipoprotein receptor-related protein (LRP)/ α -2-macroglobulin receptor.²³ We recently reported CTGF was shown to bind to and exhibit activity by binding to the type 2 insulin-like growth factor receptor (IGF) in corneal fibroblasts.²⁴

There is minimal information about the signaling pathway regulated by CTGF. Some information shows CTGF stimulated phosphorylation of both p42/44 mitogen activated protein kinase (p42/44 MAPK) and p38 MAPK in a human chondrosarcoma-derived chondrocytic cell line, suggesting that these proteins may mediate the actions of CTGF in the induction of chondrocyte differentiation.²⁵ In addition, CTGF stimulation of fibronectin expression was reported to involve the Src, p42/44 MAPK, and protein kinase B pathways in mesangial cells.²⁶ In renal fibroblast activity, the importance of the Ras and Rho GTPase families have also been reported.²⁷ In human corneal epithelial cells, CTGF was shown to induce epithelial migration via the Ras/MEK/ERK signaling pathway.²⁸ In this study, we investigated the expression patterns of protein kinases and the levels of phosphorylated proteins in HCF treated with CTGF to better understand the signal transduction pathways regulated by CTGF.

MATERIALS AND METHODS

Cell Culture

Cultures of HCF were established by outgrowth from corneal explants, obtained in compliance with the tenants of the 2012 Declaration of Helsinki, as described previously.²⁹ Briefly, epithelial and endothelial cells were removed from corneas that were unsuitable for corneal transplantation, the stroma was cut into cubes approximately 1 mm³, and placed in culture medium (equal parts Dulbecco's Modified Eagle Medium [Gibco BRL, Grand Island, NY], Medium 199 [Gibco], Ham's F₁₂ nutrient mixture [Gibco BRL] containing 1 mM NaHCO₃, and buffered with 25 mM HEPES at pH 7.4 [Gibco BRL]). The medium was supplemented with 10% heat-inactivated fetal bovine serum and 1 $\times$ antibiotic-antimycotic (Gibco BRL). HCF grown to confluence were serum starved for 48 hours in order to create quiescent corneal fibroblast. After 48 hours, cells are stimulated with CTGF and were examine for the expression of several different protein kinases and phosphoproteins.

Protein Kinase and Phosphoprotein Arrays

HCF cultures were grown to complete confluence with the edges of all HCF coming into contact with another HCF in six 75 cm² (fourth passage, 4.8×10^5 cells/well) tissue culture flasks in serum supplemented medium. The cell monolayers represented cells pooled from multiple donors. All cells were used at passage number four. Following serum starvation for 48 hours, cells were rinsed three times with PBS. The cell monolayers were then incubated with 25 ng CTGF per milliliter in serum-free culture medium containing 0.1% BSA to reduce possible nonspecific binding of CTGF that could alter phosphorylation of kinase proteins. Two 75 cm² flasks served as negative controls and were lysed immediately. The remaining four 75 cm² flasks were treated with CTGF protein at two time points (5 and 15 minutes). Following the appropriate duration of stimulation, the solution was removed and the cells were washed with PBS. Subsequently, 400 μ L of cell lysis solution (20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 40 mM β -glycerophosphate, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM

phenylmethylsulfonyl fluoride, 3 mM benzamidine, 5 μ M pepstatin-A, 10 mM leupeptin, and 0.5% Triton-X-100, pH 7.0) was added directly to the appropriate flask. Cell lysate was centrifuged for 30 minutes at 14,000 rpm at 4°C. The supernatants were removed and protein concentrations were measured using a BCA protein assay kit (Pierce, Rockford, IL). The Triton-X-100 soluble supernatant fractions were further processed for analysis of protein kinase levels and protein phosphorylation levels as specified by Kinexus Bioinformatics Corporation Analysis (Vancouver, Canada). Briefly, cell lysates were diluted 4-fold with sample buffer to give final concentrations of 12.5% glycerol, 31 mM Tris-HCl, pH 6.8, 1% SDS, 0.02% bromophenol blue, 1.25% β -mercaptoethanol, and a final protein concentration of 1 μ g/ μ L. Samples were then heated in boiling water for 4 minutes.

The protein extracts from control samples were analyzed using Kinetworks Protein Kinase Screen, KPKS, 1.2 (Kinexus Bioinformatics Corporation). The level of expression of 75 different protein kinases was assessed using 600 μ g of cell lysate protein. In addition to the protein kinase screen, a phosphorylation status screen, (Kinetworks Phospho-Site Screen, KPSS 1.3) was also performed on treated samples using 350 μ g of cell lysate. KPSS 1.3 measured the relative level of phosphorylation of 36 unique sites on 31 different phosphoproteins using antibodies to defined phosphorylated epitopes (see Table 3). Quantification of the immunoreactive bands on both the KPKS and KPSS blots was performed with enhanced chemiluminescence (ECL) detection using a Bio-Rad FluroS Max Imager (Bio-Rad Laboratories, Hercules, CA) and Bio-Rad Quantity One Software (Bio-Rad Laboratories).³⁰ Counts per minute (CPM) levels were computed using average of zero time points. The values presented in Tables 1 and 3 are the mean $\pm$ SE cpm, which was calculated as 50% of the range of the two values from replicate samples ($n = 2$).

Western Blot Analysis of Selected Proteins in an Expanded Time Course

Based on the results of the protein kinase array and the phosphoprotein array, additional Western blot analyses were performed to measure extracellular regulated kinase 1 (ERK1), extracellular regulated kinase 2 (ERK2), and signal transducer and activator of transcription 3 (STAT3). Briefly, cultures of HCF were grown to complete confluence in 12-well culture plates (fourth passage, 2.4×10^4 cells/well), and after serum starvation for 48 hours, were treated with 25 ng CTGF per mL for 0, 1, 2, 3, 4, 5, 10, 15, 30, and 60 minutes then cells were lysed using the lysis solution as described as above. To verify that the induction of protein kinase expression and phosphorylation was due solely to the presence of CTGF, cells were also treated with CTGF in the presence of neutralizing CTGF antibody (goat anti-CTGF provided by Grotendorst laboratory) for 10 minutes. Following cell lysis, samples were centrifuged for 30 minutes at 14,000 rpm at 4°C. Protein samples (13 μ g per well) were separated on a 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA). After blocking, membranes were incubated with the appropriate primary antibody (1:100 mouse anti-pERK $\frac{1}{2}$ [cat # sc-8019; Santa Cruz Biotechnologies, Santa Cruz, CA], 1:100 mouse anti-pSTAT3 [cat # sc-81,492; Santa Cruz Biotechnologies]). Alkaline phosphatase conjugated secondary antibodies were used followed by colorimetric detection with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate. Quantification of bands was performed using Kodak Digital Science software (Kodak, Rochester, NY). Normalization of results was achieved by comparison to levels of β -Actin (1:5000 mouse anti- β -Actin, [Oncogene, San Diego, CA]). Results are expressed as the mean $\pm$ SE, and were analyzed for statistical significance using ANOVA followed by Tukey's HSD post-hoc comparison of means using Statistica software (Statistica, Tulsa, OK).

Inhibition of CTGF Stimulated Cell Proliferation

To assess the effect of CTGF signaling by the MEK1/2 pathway, cell proliferation assays were conducted with specific and potent inhibitors

TABLE 1. Detectable Protein Kinase Expression in Quiescent Human Corneal Fibroblast Cultures

Protein Kinase	Counts Per Minute, Mean $\pm$ SE
Bone marrow X kinase	500 $\pm$ 00
Calmodulin-dependent kinase 1	250 $\pm$ 70
Calmodulin-dependent kinase 4	1640 $\pm$ 640
Cancer Osaka thyroid oncogene (Tpl2)	4530 $\pm$ 100
Casein kinase 2 (a)	280 $\pm$ 70
Casein kinase 2 (b)	340 $\pm$ 10
Casein kinase 2 (c)	2990 $\pm$ 340
Cyclin-dependent kinase 9	300 $\pm$ 30
Death associated protein kinase 1	240 $\pm$ 170
Extracellular regulated kinase 1 (a)	7670 $\pm$ 70
Extracellular regulated kinase 1 (b)	3120 $\pm$ 200
Extracellular regulated kinase 2 (a)	2090 $\pm$ 340
Extracellular regulated kinase 2 (b)	3150 $\pm$ 460
Extracellular regulated kinase 2 (c)	770 $\pm$ 370
Extracellular regulated kinase 3 (a)	70 $\pm$ 10
Extracellular regulated kinase 3 (b)	290 $\pm$ 00
Focal adhesion kinase	800 $\pm$ 40
G protein-coupled receptor kinase 2 (BARK2)	100 $\pm$ 20
Inhibitor NF kB kinase alpha/beta	1260 $\pm$ 400
Janus kinase 1	760 $\pm$ 220
MAP kinase kinase 1 (MKK1)	310 $\pm$ 100
MAP kinase kinase 2 (MKK2)	160 $\pm$ 70
MAP kinase kinase 6 (MEK6)	150 $\pm$ 0
Oncogene Raf 1	2270 $\pm$ 960
P38 Hog MAP kinase	690 $\pm$ 290
Protein kinase A (cAMP-dependent protein kinase)	250 $\pm$ 30
Protein kinase B alpha	100 $\pm$ 20
Protein kinase C Beta1	6930 $\pm$ 2730
Protein kinase C epsilon	160 $\pm$ 160
Protein kinase C mu (a)	300 $\pm$ 300
Protein kinase C mu (b)	70 $\pm$ 70
Protein kinase C zeta	920 $\pm$ 490
Protein kinase G1 (cGMP-dependent protein kinase)	470 $\pm$ 90
Ribosomal S6 kinase 1	340 $\pm$ 130
Ribosomal S6 kinase 2	390 $\pm$ 20
Stress activated protein kinase (JNK) (a)	220 $\pm$ 40
Stress activated protein kinase (JNK) (b)	100 $\pm$ 100
v-mos Moloney murine sarcoma viral oncogene homolog 1	530 $\pm$ 210
v-raf murine sarcoma viral oncogene homolog B1	830 $\pm$ 70
ZIP kinase (death associated protein kinase 3)	350 $\pm$ 170

of the MEK1/2 pathway. These included an inhibitor of Ras, (farnesylthiosalicylic acid [FTS]; Biomol Int, Plymouth Meeting, PA), a MEK inhibitor (PD-98059, 2'-amino-3'-methoxyflavone; Fisher Scientific, Pittsburgh, PA).³¹ and an ERK1/2 inhibitor (SB203580; University of Florida College of Medicine, Gainesville, FL) Briefly, 40 wells of a 96-well plate were seeded with HCF (fourth passage, 3000 cells/well) in medium containing 10% serum for 24 hours then placed in serum free medium for 48 hours. The medium was removed, wells were washed with PBS and replaced with serum free culture medium containing 0.1% BSA and the following five different additions: (1) serum free medium, (2) 10% fetal bovine serum, (3) CTGF (50 ng/mL), (4) CTGF (50 ng/mL) and selective inhibitor, and (5) selective inhibitor. CTGF was added at 50 ng/mL to maximize the stimulation of cell proliferation and gel contraction. After 48 hours of further culturing, 20 μ L of

CellTiter 96 Aqueous One Solution Reagent (MTS; Promega, Madison, WI) was added and the absorbance at 490 nm (and/or 450 nm) was recorded after 2 hours. The level of reduced MTS product (formazan) measured at designated wavelength is a measure of the metabolic oxidative activity of cells and reflects the number of viable cells in the culture well. Results are expressed as the mean percentage change from serum free medium $\pm$ SE of eight replicates for each condition, and average values were analyzed for statistical significance using ANOVA followed by Tukey's HSD post-hoc comparison of means using Statistica software (Statistica).

Inhibition of CTGF-Induced Collagen Gel Contraction

Fibroblast-populated collagen gels (FPCG) were prepared by mixing concentrated DMEM (Sigma-Aldrich) with type 1 rat tail collagen (5 mg/mL; Sigma-Aldrich). The pH of the mixture was neutralized with 0.1 M NaOH before adding concentrated DMEM containing HCF, to give a final cell density of 5×10^5 /mL of gel mixture. Following polymerization, the gels were detached from the culture well using a pipette tip and were cultured in the respective test medium. Test medium categories were similar to those for the proliferation assay: (1) serum free medium, (2) CTGF (50 ng/mL), (3) CTGF (50 ng/mL) and selective inhibitor, and (4) selective inhibitor. The same selective inhibitors were chosen for both the cell proliferation and gel contraction assays. FPCG contraction was measured following 72 hours by taking digital photographs from a fixed distance. Areas were calculated in pixels with image analysis software (Image Tool; University of Texas Health Sciences Center San Antonio, San Antonio, TX). Triplicate samples per treatment were tested. Each experiment was repeated at least three times with fibroblasts from different donors. Results are expressed as the mean percentage change from serum free medium $\pm$ SE of triplicates for each condition, and average values were analyzed for statistical significance as aforementioned.

RESULTS

Protein Kinase Expression Analysis

The signal transduction pathway(s) for CTGF are not established in cells, especially in HCF. It is reasonable to hypothesize that acute CTGF signaling will involve phosphorylation of cellular proteins by protein kinases. However, there is no extensive survey that identifies protein kinases that are expressed in quiescent corneal fibroblasts, and, thus, would theoretically be available to participate in CTGF signal transduction. Therefore, we utilized a Western blot protein array to identify kinases that are expressed in cultures of quiescent corneal fibroblasts.

As shown in Table 1, a total of 32 protein kinases were detected in extracts of quiescent HCF cultures (representative blots shown in Fig. 1). This represents 43% of the total 75 protein kinases that can be detected by the KPKS 1.2 array. Six kinases were detected by two or more antibodies, generating a total of 40 possible unique antigenic sites on 32 different kinase proteins listed in Table 1. Kinases detected by more than one antibody were designated with lowercase letters. The kinase array measured the relative levels of kinase proteins in cultures of quiescent HCF, regardless of phosphorylation status of the kinases. Thus, these 32 kinases are expressed in detectable levels in quiescent HCF, and would theoretically be available to participate acutely in CTGF signaling pathways.

Table 2 lists the 43 kinases were undetectable by the kinase protein Western blot array. Four of the kinases were undetectable by two or more antibodies directed against different antigenic sites, which provides high confidence that these four kinases were not present at detectable levels. Three

TABLE 2. Protein Kinases Undetectable in Quiescent Human Corneal Fibroblast Cultures

Protein Kinases	
3-phosphoinositide dependent protein kinase 1 (PKB kinase)	Mammalian sterile 20-like 1
Bruton agammaglobulinemia tyrosine kinase	MAP kinase interacting kinase 2
Calmodulin-dependent kinase kinase	MAP kinase kinase 4 (MEK4)
Casein kinase 1 delta	Oncogene Lyn
Casein kinase 1 epsilon	Oncogene SRC
c-SRC tyrosine kinase	p21 activated kinase 1 (PAK alpha)
Cyclin-dependent kinase 1 (cdc2)	p21 activated kinase 3 (PAK beta)
Cyclin-dependent kinase 2	Protein kinase B alpha (b)
Cyclin-dependent kinase 4	Protein kinase C delta
Cyclin-dependent kinase 5	Protein kinase C epsilon
Cyclin-dependent kinase 6	Protein kinase C gamma
Cyclin-dependent kinase 7	Protein kinase C lambda
DNA-activated protein kinase	Protein kinase C theta
dsRNA dependent kinase	Protein kinase C zeta (b)
Elongation factor-2 kinase (eEF2k)	Protein tyrosine kinase 2
Fyn oncogene related to SRC (a)	RhoA kinase
Fyn oncogene related to SRC (b)	Ribosomal S6 kinase 1 (a)
Germinal centre kinase	Ribosomal S6 kinase 2 (b)
Glycogen synthase kinase 3 alpha	S6 kinase p70 (a)
Glycogen synthase kinase 3 beta	S6 kinase p70 (b)
Hematopoietic progenitor kinase 1	S6 kinase p70 (c)
Inhibitor of kappa light polypeptide gene enhancer in B-cells (IKKbeta)	Spleen tyrosine kinase
Janus kinase 2	Yamaguchi sarcoma viral oncogene homolog 1
Kinase suppressor of Ras 1	Zeta-chain (TCR) associated protein kinase
Lymphocyte-specific protein tyrosine kinase	ZIP kinase (death associated protein kinase 3) (a)

kinases that were probed by more than one antibody were detected by at least one antibody, but not by all the antibodies. Therefore, we considered these three kinases to be undetectable. Since the 43 kinases were not detectable by the Western blot array, they theoretically would not be available to participate acutely in CTGF signaling pathways.

Phosphoprotein Expression Analysis

Forty unique sites on 32 different phosphoproteins were probed by the phosphoprotein arrays. As shown in Table 3, of those 40 unique phosphorylation sites, 22 unique sites on 19 different phosphoproteins were detected on at least one

TABLE 3. Phosphoprotein Levels in Human Corneal Fibroblasts Altered by Treatment with CTGF

Phosphoprotein (Phosphorylated Amino Acid)	Control (No CTGF) Mean $\pm$ SE cpm	5 Mins + CTGF Mean $\pm$ SE cpm (% Change)	15 Mins + CTGF Mean $\pm$ SE cpm (% Change)
Adducin alpha (S724)	1550 $\pm$ 90	1030 $\pm$ 300 (–33%)	680 $\pm$ 120 (–56%)
Adducin gamma (S662)	1670 $\pm$ 160	960 $\pm$ 110 (–43%)	710 $\pm$ 330 (–58%)
Extracellular regulated kinase 1 (T202/Y204)	5760 $\pm$ 1830	11,320 $\pm$ 200 (+96%)	8970 $\pm$ 880 (+56%)
Extracellular regulated kinase 2 (T185/Y187)	12,380 $\pm$ 2460	19,760 $\pm$ 220 (+60%)	15,700 $\pm$ 2240 (+27%)
Glycogen synthase kinase 3 alpha (S21)	260 $\pm$ 30	200 $\pm$ 20 (–21%)	160 $\pm$ 20 (–39%)
Glycogen synthase kinase 3 alpha (Y279)	1040 $\pm$ 90	1170 $\pm$ 520 (+12%)	1510 $\pm$ 40 (+45%)
Glycogen synthase kinase 3 beta (S9)	230 $\pm$ 40	170 $\pm$ 20 (–19%)	100 $\pm$ 0 (–56%)
Glycogen synthase kinase 3 beta (Y216)	2650 $\pm$ 1100	2640 $\pm$ 90 (–0.5%)	1770 $\pm$ 590 (–33%)
MAP kinase kinase 1/2 (S217/S221)	1820 $\pm$ 180	3480 $\pm$ 1260 (+91%)	1100 $\pm$ 230 (–39%)
MAP kinase kinase 3/6 (MEK3/6) (S189/S207)	360 $\pm$ 80	350 $\pm$ 100 (–4%)	300 $\pm$ 70 (–17%)
Oncogene Raf 1 (a) (S259)	1220 $\pm$ 120	570 $\pm$ 70 (–53%)	1120 $\pm$ 20 (–8%)
Oncogene SRC (Y529/Y418)	14,020 $\pm$ 2750	9730 $\pm$ 1350 (–31%)	10,880 $\pm$ 2470 (–22%)
p38 alpha MAP kinase (Y180/Y182)	2370 $\pm$ 10	2000 $\pm$ 130 (–15%)	1240 $\pm$ 520 (–48%)
Protein kinase B alpha (Akt1) (S473)	3860 $\pm$ 310	3290 $\pm$ 220 (–15%)	2930 $\pm$ 740 (–24%)
Protein kinase B alpha (Akt1) (T308)	1680 $\pm$ 70	1160 $\pm$ 160 (–31%)	1210 $\pm$ 10 (–28%)
Protein kinase C alpha (S657)	14,550 $\pm$ 4040	12,720 $\pm$ 1340 (–13%)	14,140 $\pm$ 2200 (–3%)
Protein kinase C alpha/beta (T368)	7650 $\pm$ 1490	5850 $\pm$ 320 (–23%)	6690 $\pm$ 1350 (–13%)
Protein kinase C epsilon (S719)	1670 $\pm$ 200	1440 $\pm$ 60 (–13%)	1940 $\pm$ 10 (+16%)
S6 kinase p70 (b) (T389)	1120 $\pm$ 30	740 $\pm$ 60 (–34%)	920 $\pm$ 130 (–18%)
Signal transducer and activator of transcription1 (Y701)	370 $\pm$ 0	230 $\pm$ 60 (–36%)	520 $\pm$ 70 (+42%)
Signal transducer and activator of transcription 3 (S727)	4530 $\pm$ 1760	6520 $\pm$ 100 (+44%)	8940 $\pm$ 720 (+97%)
Stress-activated protein kinase (JNK) (T183/Y185)	260 $\pm$ 40	600 $\pm$ 70 (+130%)	400 $\pm$ 10 (+52%)

FIGURE 1. Representative protein kinase array blots. The *left* and *right* panels are representative protein blots of 48 hour serum-starved corneal fibroblast cultures exposed to 25 ng/mL CTGF analyzed for 90 unique antigenic sites on a total of 75 different kinases using the KPKS 1.2 array. Quantitative data from the kinase array blots are summarized in Tables 1 and 2. Thirty two of the 75 kinase proteins were detectable in quiescent cultures of HCF and are theoretically available to participate acutely in CTGF signaling pathways.

of the three time points (0, 5, or 15 minutes) after CTGF exposure (representative blots shown in Fig. 2). The levels of phosphorylation of five unique sites on five different phosphoproteins increased above control levels (time 0) at both 5 and 15 minutes of exposure to CTGF. The levels of phosphorylation of 14 unique sites on 14 different phosphoproteins decreased below control levels (time 0) at both 5 and 15 minutes of exposure to CTGF. The levels of phosphorylation of three unique sites on three different phosphoproteins either increased or decreased compared with control levels (time 0) at either 5 and 15 minutes of exposure to CTGF.

Some notable proteins that were phosphorylated include: ERK 1 and 2, which are serine/threonine kinases of the MAPK superfamily, mitogen-activated protein kinase kinase 1/2 (MAP kinase kinase 1/2), STAT3, and JNK. Levels of phosphorylation of ERK1, ERK2, and STAT 3 were chosen for further study at 10 time points extending to 60 minutes of CTGF treatment.

Immunoblot Analysis of Selected Phosphorylated Proteins

Western blot analyses of three selected phosphoproteins (ERK1, ERK2, and STAT 3) were examined using an extended time course of continuous treatment of corneal fibroblasts with CTGF (Figs. 3, 4). As seen in Panel 4A, phosphorylation of ERK1 rapidly increases to a maximum at 4, 5, and 10 minutes of stimulation with CTGF ($P < 0.01$) then begins declining at 15 minutes and returns to prestimulated levels at 30 and 60 minutes of exposure to CTGF. Similarly, as seen in panel 4B, phosphorylation of ERK2 increased to maximum levels at 4 and 5 minutes ($P < 0.01$) then progressively decreased at 10 and 15 minutes and reached prestimulated levels at 30 and 60 minutes of exposure to CTGF. The time course of phosphorylation for STAT3 was somewhat different than for ERK1 and ERK2, in that phosphorylation of STAT3 reached a sharp peak at 5 minutes ($P < 0.01$), and rapidly decreased at 10 minutes to prestimulation level and remained low at 15, 30, and 60 minutes. Thus, CTGF produced a shorter pulse of phosphorylated STAT3 protein in corneal fibroblasts compared with the

FIGURE 2. Representative phosphoprotein array blots. Representative blots of phosphorylated proteins of 48 hours serum-starved HCF cultures are shown at 0 (*left* panel), 5 (*middle* panel), and 15 minutes (*right* panel) following addition of CTGF (25 ng/mL). Quantitative data from the phosphoprotein array blots are summarized in Table 3. CTGF produced changes in 25 of 40 unique phosphorylation sites of 32 different phosphoproteins at 5 or 15 minutes of exposure.

FIGURE 3. Representative immunoblots for ERK1, ERK2, and STAT3. Representative immunoblots of phosphorylated ERK1, ERK2, and STAT3 proteins are shown for extracts of HCF cultures at 0, 1, 2, 3, 4, 5, 10, 15, 30, and 60 minutes following treatment with CTGF. Levels of the phosphorylated proteins were measured by densitometry and normalized to the level of beta-actin protein.

longer duration of phosphorylated ERK1 and ERK2. Interestingly, STAT3 activation by other growth factors^{32–35} and its role in oncogenic cell proliferation regulation and differentiation is well documented.^{36–40}

Effect of Selective Kinase Inhibition on CTGF Stimulated Cell Proliferation

Since previous studies have indicated that Ras and MEK 1/2 functions upstream of ERK1, ERK2, and STAT3^{41–43} and CTGF exposure increased levels of phosphorylated MEK 1/2 increased approximately 90% at five minutes (see Table 3), it was reasonable to hypothesize that CTGF may signal, in part, by activating the MEK 1/2 pathway. To investigate this possibility, the effect of the selective Ras inhibitor, FTS, the MEK 1/2 inhibitor, PD-98059, and the ERK1/2 inhibitor SB203580 were evaluated on CTGF-stimulated proliferation of HCF. As shown in the subsets of Figure 5, the addition of 10% serum increased the level of reduced MTS (a surrogate measure of cell number) approximately 3-fold compared with serum-free medium ($P < 0.01$). Addition of CTGF (50 ng/mL) increased MTS approximately 1.5-fold ($P < 0.01$) over serum-free medium, which confirms the mitogenic effect of CTGF on HCF.¹⁸ This effect was once again seen as CTGF induced a 15% increase in proliferation in subsequent assays. Addition of FTS (5 μ M; Fig. 5A), PD-98059 (40 μ M; Fig. 5B), and/or SB203580 (22.2 μ g/mL; Fig. 5C) to CTGF, reduced MTS production to a level that was not significantly different than for cells in serum-free medium, indicating that FTS, PD 98059, and SB203580 effectively blocked the CTGF-stimulated cell proliferation ($P < 0.001$, $P < 0.01$, $P < 0.001$, respectively). Furthermore, addition of inhibitors alone to serum-free medium did not statistically reduce the level of MTS compared with serum-free medium, indicating that at effective concentrations, there were no significant nonspecific toxic effects on HCF.

Effect of Selective Kinase Inhibition on CTGF-Induced Collagen Matrix Contraction

In order to investigate kinase signaling necessary for CTGF induced collagen matrix contraction, the same selective inhibitors used in the cell proliferation assay were also tested in the FPCG setting. As shown in Figures 6A to 6C, serum-free medium resulted in a 46% to 49% gel contraction, while CTGF (50 ng/mL) induced 64% to 71% contraction ($P < 0.001$) of the collagen gel. This approximate 1.5-fold increase, confirms its ability to stimulate lattice contraction. Following addition of the selective inhibitors, this effect was significantly blocked, and resulted in a percentage of gel contraction that was not significantly different from serum-free medium. Addition of FTS (5 μ M) resulted in only 43% contraction (Fig. 6A), PD-98059 (100 μ M) in 59% contraction (Fig. 6B), and SB203580 (22.2 μ g/mL) in 47% reduction (Fig. 6C). CTGF induced contraction results were within $\pm 1.6\%$ SE. All other results were within $\pm 2\%$ SE. As seen in the cell proliferation results, addition of the inhibitors alone did not result in significant differences in contraction from serum free medium, indicating that the inhibitors themselves did not have toxic effects.

DISCUSSION

Extensive data indicates that both TGF- β and CTGF are important growth factors that regulating scar formation in many tissues, including the cornea.^{6,7,44} However, little is known about the signal transduction pathways utilized by CTGF to regulate cellular functions in corneal cells. Results of experiments presented here indicate that the MEK1/2 (MAPKK), ERK1, ERK2, STAT3, and SAPK/JNK pathway is rapidly activated after addition of CTGF to HCF cultures. More specifically, the expanded time course experiments (Figs. 3, 4A–C) showed that CTGF rapidly induced phosphorylation of

FIGURE 4. Immunoblot analyses for ERK1, ERK2, and STAT3. Levels of phosphorylated ERK1(A), ERK2 (B), and STAT3 (C) were measured in cultures of HCF at 0, 1, 2, 3, 4, 5, 10, 15, 30, and 60 minutes of treatment with CTGF using Western blots. Levels of phosphorylated ERK1, ERK2, and STAT3 rapidly increased to a peak between 4 and 10 minutes of exposure to CTGF then progressively decreased to baseline level by 30 minutes. Values are the mean $\pm$ SE of triplicate samples, * indicates different from time 0 with $P < 0.01$.

FIGURE 5. Affect of selective inhibitors CTGF-stimulated proliferation of corneal fibroblasts. (A) Quiescent cultures of HCF were incubated with serum-free medium alone or supplemented with 10% fetal calf serum, CTGF (25 ng/mL), CTGF (25 ng/mL) + FTS (5 μ M), or FTS (5 μ M) alone for 48 hours then cell number was measured by a MTS assay. CTGF increased the number of corneal fibroblasts 15% compared with serum-free medium ($\dagger P < 0.001$). Addition of FTS to CTGF blocked CTGF-stimulated cell proliferation (serum-free versus CTGF + FTS). FTS alone was not toxic to corneal fibroblasts (FTS versus serum-free medium was not significant). Values are the mean percentage change $\pm$ SE of eight replicates for each culture condition. (B) Quiescent cultures of HCF were incubated with serum-free medium alone or supplemented with 10% fetal calf serum, CTGF (25 ng/mL), CTGF (25 ng/mL) + PD-98059 (40 μ M), or PD-98059 (40 μ M) alone for 48 hours then cell number was measured by a MTS assay. CTGF increased the number of corneal fibroblasts 55% compared with serum-free medium ($\dagger P < 0.01$). Addition of PD-98059 to CTGF blocked CTGF-stimulated cell proliferation (serum-free versus CTGF + PD-98059, $P = 0.73$). PD-98059 alone was not toxic to corneal fibroblasts (PD-98059 versus serum-free medium, $P = 0.63$). Values are the mean percentage change from SFM $\pm$ SE of eight replicates for each culture condition. (C) Quiescent cultures of HCF were incubated with serum-free medium alone or supplemented with 10% fetal calf serum, CTGF (25 ng/mL), CTGF (25 ng/mL) + SB203580 (22.2 ng/mL), or SB203580 (22.2 ng/mL) alone for 48 hours then cell number was measured by a MTS assay. CTGF increased the number of corneal fibroblasts 15% compared with serum-free medium ($\dagger P < 0.001$). Addition of SB203580 to CTGF blocked CTGF-stimulated cell proliferation (serum-free versus CTGF + SB203580). SB203580 alone was not toxic to corneal fibroblasts (SB203580 versus serum-free medium was not significant). Values are the mean percentage change $\pm$ SE of eight replicates for each culture condition.

three key signal transducing proteins, ERK1, ERK2, and STAT3 within 1 minute of CTGF exposure, and phosphorylation peaked between 5 and 10 minutes, and returned to pretreatment levels by 30 minutes. The very rapid increase in phosphorylation of ERK1, ERK2, and STAT3 supports the concept that these phosphoproteins mediate the primary signaling events of CTGF rather than being influenced by later secondary signaling pathways.

Another important action of CTGF revealed by the phosphoprotein screen was the rapid dephosphorylation of 14 of 32 phosphoproteins (14%) at both 5 and 15 minutes, including protein kinases B and C. This suggests that CTGF may also influence the activities of phosphatases that have important roles in gene regulation. This is an important area that remains to be explored in future experiments.

An important signal transduction protein that is immediately upstream of ERK1 and ERK2 is MEK 1/2 (MAP kinase kinase). As shown in Table 3, MEK1/2 is present in relatively high levels in quiescent fibroblasts, and addition of CTGF increased levels of MEK1/2 phosphorylation approximately 90% after 5 minutes, followed by reduced phosphorylation at 15 minutes. Since these data suggest that MEK1/2 is also in the CTGF signal transduction pathway, we investigated the effect of selectively blocking MEK1/2 activity, on CTGF-induced proliferation of corneal fibroblasts with PD-98059.^{45–47} MEK activity and efficacy has been shown in other systems to be

dependent on other key members of the MEK signaling pathway including the upstream protein Ras, as well as the downstream members ERK1/2.⁴⁸ Thus, we chose to investigate the inhibition of these members using FTS,⁴⁹ and SB203580 respectively. As shown in Figure 5B, selective inhibition of MEK1/2 reduced CTGF-stimulated cell proliferation to a level that was not significantly different from levels of cell proliferation in serum-free medium. Moreover, these selective kinase inhibitors reduced CTGF-stimulated cell proliferation to nonsignificant levels (Figs. 5A, 5C). These results indicate that the kinase activities of Ras, MEK1/2, and ERK1/2 are necessary for CTGF-stimulated HCF proliferation.

Although the dependence of the MEK pathway was confirmed by our results for fibroblast proliferation, the role of these members in other important CTGF-induced biological effects is unknown. For this reason, we also chose to investigate the inhibition of these same members of the MEK pathway in the setting of fibroblast-mediated collagen matrix contraction.⁵⁰ As seen in Figure 6, selective inhibition of Ras, MEK1/2 or ERK1/2 activity significantly ($P < 0.001$) reduced CTGF-stimulated collagen gel contraction by approximately 15%. Thus, Ras, MEK1/2, and ERK1/2 are necessary for CTGF-induced collagen matrix contraction.

It is presumed that the initial step in CTGF signaling is the specific binding of CTGF to a high affinity membrane receptor protein. We recently reported that CTGF specifically bound to

FIGURE 6. Affect of selective inhibitors on CTGF-stimulated corneal fibroblast mediated collagen gel contraction. (A) FPCG were cultured in CTGF (50 ng/mL), CTGF (50 ng/mL) + FTS (5 μ M), or FTS (5 μ M) alone. FPCG contraction was measured at 72 hours with image analysis. CTGF induced 64% matrix contraction (* P < 0.001), compared with that induced by SFM of 46%. Addition of FTS to CTGF (SFM + CTGF + FTS) blocked CTGF-stimulated gel contraction (43% contraction, $\ddagger P$ < 0.001). FTS alone (SFM + FTS) was not toxic and induced the same percentage contraction as SFM (46%). Values represent mean percentage contraction $\pm$ SE of triplicate samples per treatment condition. (B) FPCG were cultured in CTGF (50 ng/mL), CTGF (50 ng/mL) + PD 98059 (100 μ M), or PD 98059 (100 μ M) alone. FPCG contraction was measured at 72 hours with image analysis. CTGF induced 71% matrix contraction (* P < 0.001), compared with that induced by SFM of 49%. Addition of PD-98059 to CTGF (SFM + CTGF + PD 98059) significantly blocked CTGF-stimulated gel contraction (59% contraction, $\ddagger P$ < 0.001). FTS alone (SFM + FTS) was not toxic and induced a similar percentage contraction as SFM (55%). Values represent mean percentage contraction $\pm$ SE of triplicate samples per treatment condition. (C) FPCG were cultured in CTGF (50 ng/mL), CTGF (50 ng/mL) + SB203580 (22.2 μ g/mL), or SB203580 (22.2 μ g/mL) alone. FPCG contraction was measured at 72 hours with image analysis. CTGF induced 64% matrix contraction (* P < 0.001), compared with that induced by SFM of 46%. Addition of SB203580 to CTGF (SFM + CTGF + SB203580) blocked CTGF-stimulated gel contraction (47% contraction, $\ddagger P$ < 0.001). SB203580 alone (SFM + SB203580) was not toxic and induced the same percentage contraction as SFM (46%). Values represent mean percentage contraction $\pm$ SE of triplicate samples per treatment condition.

FIGURE 7. Model of CTGF signal transduction in corneal fibroblasts. CTGF stimulation of HCF proliferation is presumably initiated by binding to a membrane receptor protein, which activates a signaling cascade. Addition of CTGF rapidly increases phosphorylation of ERK1 and 2 and STAT3 via upstream activation of MEK1/2 and Ras.

the mannose-6 phosphate/insulin-like growth 2 receptor (IGF-2-R) in corneal fibroblasts, and fibroblasts lacking the IGF-2-R failed to bind and respond to CTGF, indicating the IGF-2-R meets the basic definition of a CTGF receptor protein that mediates biological effects of CTGF²⁴ Other membrane associated proteins have been reported to specifically bind CTGF. Affinity cross-linking of ¹²⁵I-CTGF to a murine bone marrow stromal cell line (BMS2) labeled a protein of 620 kDa that was identified as the low-density lipoprotein receptor-related protein (LRP)²³ and cross-linking of ¹²⁵I-CTGF to a human chondrosarcoma-derived chondrocytic cell line (HCS-2/8) identified a membrane associated protein of approximately 280 kDa.²² However, neither the 620-kDa protein (LRP) nor the 280-kDa protein were shown to be required for generating a biological response to CTGF, so their role as a classical receptor protein for CTGF remains to be determined.

The biological effects of TGF- β and CTGF on fibroblasts have considerable overlap but they are not identical. For example, TGF- β induces CTGF synthesis in corneal fibroblasts, and CTGF mediates TGF- β 's induction of type 1 collagen synthesis.^{18,51} However, unlike TGF- β , CTGF is unable to cause anchorage-independent growth of fibroblasts.¹⁵ TGF- β signaling pathways are relatively well understood. The induction of CTGF in dermal fibroblasts by TGF- β does not occur in a single pathway, but requires a balance between SMAD induction, Ras/MEK/ERK and JNK/MAPK pathways.^{21,52-54} Analogous to the biological effects of both growth factors, the signaling of TGF- β and CTGF is also likely not identical, although overlap such as use of MEK/ERK pathways exists.

We focused on elucidating the signal transduction pathway used by CTGF in corneal fibroblasts since these cells play major roles in corneal scarring. In this study, we have shown that two independent, well established biological effects responsible by CTGF, specifically, fibroblast proliferation, and fibroblast mediated collagen lattice contraction, are clearly dependent upon activation of members of the Ras/MEK/ERK pathway in HCF. It is clear that CTGF signals through Ras, and eventually causes the phosphorylation of MAPKK (MEK), causing a downstream phosphorylation cascade of proteins of members of this pathway including ERK 1 and ERK 2 (Fig. 7). Activating this signal transduction cascade ultimately leads to changes in patterns of gene expression, like the induction of alpha smooth muscle actin that is the hallmark of fibroblast transformation into myofibroblasts and contraction of scar tissue.¹⁹

Controlling corneal scarring is an important clinical goal because of the large number of corneal wounds caused by trauma, surgery, and bacterial or viral infections. Ideally, antiscarring therapies would target specific genes that regulate scarring processes rather than using nonspecific, antiproliferative cancer drugs (mitomycin C and 5-fluorouracil) that can have serious side effects.⁵⁵ Current data indicate that CTGF plays key roles in regulating important aspects of ocular scarring, and results of this study provide the first insight into CTGF's signaling pathways in HCF. This suggests that small molecule kinase inhibitors of the CTGF signaling pathway might be used as local (topical) treatments to reduce corneal scarring without serious side effects.

References

- Bradham DM, Igarashi A, Potter RL, Grotendorst GR. Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. *J Cell Biol.* 1991;114:1285-1294.
- Ryseck RP, Macdonald-Bravo H, Mattei MG, Bravo R. Structure, mapping, and expression of fisp-12, a growth factor-inducible gene encoding a secreted cysteine-rich protein. *Cell Growth Differ.* 1991;2:225-233.
- Hashimoto K, Shindo-Okada N, Tani M, et al. Expression of the Elm1 gene, a novel gene of the CCN (connective tissue growth factor, Cyr61/Cef10, and neuroblastoma overexpressed gene) family, suppresses in vivo tumor growth and metastasis of K-1735 murine melanoma cells. *J Exp Med.* 1998;187:289-296.
- Hurvitz JR, Suwairi WM, VanHul W, El-Shanti H, Superti-Furga A, et al. Mutations in the CCN gene family member WISP3 cause progressive pseudorheumatoid dysplasia. *Nat Genet.* 1999;23:94-98.
- Albrecht C, von Der Kammer H, Mayhaus M, Klaudiny J, Schweizer M, Nitsch RM. Muscarinic acetylcholine receptors induce the expression of the immediate early growth regulatory gene CYR61. *J Biol Chem.* 2000;275:28929-28936.
- Chen MM, Lam A, Abraham JA, Schreiner GF, Joly AH. CTGF expression is induced by TGF-beta in cardiac fibroblasts and cardiac myocytes: a potential role in heart fibrosis. *J Mol Cell Cardiol.* 2000;32:1805-1819.
- Igarashi A, Nashiro K, Kikuchi K, et al. Significant correlation between connective tissue growth factor gene expression and skin sclerosis in tissue sections from patients with systemic sclerosis. *J Invest Dermatol.* 1995;105:280-284.
- Dammeier J, Brauchle M, Falk W, Grotendorst GR, Werner S. Connective tissue growth factor: a novel regulator of mucosal repair and fibrosis in inflammatory bowel disease? *Int J Biochem Cell Biol.* 1998;30:909-922.
- Wunderlich K, Senn BC, Reiser P, Pech M, Flammer J, Meyer P. Connective tissue growth factor in retrocorneal membranes and corneal scars. *Ophthalmologica.* 2000;214:341-346.
- Ito Y, Aten J, Bende RJ, et al. Expression of connective tissue growth factor in human renal fibrosis. *Kidney Int.* 1998;53:853-861.
- Pelton RW, Moses HL. The beta-type transforming growth factor-mediators of cell regulation in the lung. *Am Rev Respir Dis.* 1990;142:S31-S35.
- Igarashi A, Okochi H, Bradham DM, Grotendorst GR. Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. *Mol Biol Cell.* 1993;4:637-645.
- Kikuchi K, Kadono T, Ihn H, et al. Growth regulation in scleroderma fibroblasts: increased response to transforming growth factor-beta 1. *J Invest Dermatol.* 1995;105:128-132.
- Grotendorst GR, Duncan MR. Individual domains of connective tissue growth factor regulate fibroblast proliferation and myofibroblast differentiation. *FASEB J.* 2005;19:729-738.
- Kothapalli D, Frazier KS, Welby A, Segarini PR, Grotendorst GR. Transforming growth factor beta induces anchorage-independent growth of NRK fibroblasts via a connective tissue growth factor-dependent signaling pathway. *Cell Growth Differ.* 1997;8:61-68.
- Daniels JT, Schultz GS, Blalock TD, et al. Mediation of transforming growth factor-beta(1)-stimulated matrix contraction by fibroblasts: a role for connective tissue growth factor in contractile scarring. *Am J Pathol.* 2003;163:2043-2052.
- Tamatani T, Kobayashi H, Tezuka K, et al. Establishment of the enzyme-linked immunosorbent assay for connective tissue growth factor (CTGF) and its detection in the sera of biliary atresia. *Biochem Biophys Res Commun.* 1998;251:748-752.
- Blalock TD, Duncan MR, Varela JC, et al. Connective tissue growth factor expression and action in human corneal fibroblast cultures and rat corneas after photorefractive keratectomy. *Invest Ophthalmol Vis Sci.* 2003;44:1879-1887.
- Garrett Q, Khaw PT, Blalock TD, Schultz GS, Grotendorst GR, Daniels JT. Involvement of CTGF in TGF-beta1-Stimulation of Myofibroblast Differentiation and Collagen Matrix Contraction in the Presence of Mechanical Stress. *Invest Ophthalmol Vis Sci.* 2004;45:1109-1116.
- Duncan MR, Frazier KS, Abramson S, et al. Connective tissue growth factor mediates transforming growth factor beta-induced collagen synthesis: down-regulation by cAMP. *FASEB J.* 1999;13:1774-1786.
- Shi L, Chang Y, Yang Y, Zhang Y, Yu FS, Wu X. Activation of JNK signaling mediates connective tissue growth factor expression and scar formation in corneal wound healing. *PLoS ONE.* 2012;7:e32128.
- Nishida T, Nakanishi T, Shimo T, et al. Demonstration of receptors specific for connective tissue growth factor on a human chondrocytic cell line (HCS-2/8). *Biochem Biophys Res Commun.* 1998;247:905-909.
- Segarini P, Nesbitt D, Li D, Hayes L, Yates III J, Carmichael D. The low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor is a receptor for connective tissue growth factor. *J Biol Chem.* 2001;276:40659-40667.
- Blalock TD, Gibson DJ, Duncan MR, Tuli SS, Grotendorst GR, Schultz GS. A connective tissue growth factor signaling receptor in corneal fibroblasts. *Invest Ophthalmol Vis Sci.* 2012;53:3387-3394.
- Yosimichi G, Nakanishi T, Nishida T, Hattori T, Takano-Yamamoto T, Takigawa M. CTGF/Hcs24 induces chondrocyte differentiation through a p38 mitogen-activated protein kinase (p38MAPK), and proliferation through a p44/42 MAPK/extracellular-signal regulated kinase (ERK). *Eur J Biochem.* 2001;268:6058-6065.
- Crean JK, Finlay D, Murphy M, et al. The role of p42/44 MAPK and protein kinase B in connective tissue growth factor induced extracellular matrix protein production, cell migra-

- tion, and actin cytoskeletal rearrangement in human mesangial cells. *J Biol Chem*. 2002;277:44187-44194.
27. Masterson R, Kelynack K, Hewitson T, Becker G. Effect of inhibition of farnesylation and geranylgeranylation on renal fibrogenesis in vitro. *Nephron Exp Nephrol*. 2006;102:e19-e29.
 28. Secker GA, Shortt AJ, Sampson E, Schwarz QP, Schultz GS, Daniels JT. TGFbeta stimulated re-epithelialisation is regulated by CTGF and Ras/MEK/ERK signalling. *Exp Cell Res*. 2008;314:131-142.
 29. Woost PG, Jumblatt MM, Eiferman RA, Schultz GS. Growth factors and corneal endothelial cells: I. Stimulation of bovine corneal endothelial cell DNA synthesis by defined growth factors. *Cornea*. 1992;11:1-10.
 30. Ehses JA, Pelech SL, Pederson RA, McIntosh CH. Glucose-dependent insulinotropic polypeptide activates the Raf-Mek1/2-ERK1/2 module via a cyclic AMP/cAMP-dependent protein kinase/Rap1-mediated pathway. *J Biol Chem*. 2002;277:37088-37097.
 31. Sharma GD, He J, Bazan HE. p38 and ERK1/2 coordinate cellular migration and proliferation in epithelial wound healing: evidence of cross-talk activation between MAP kinase cascades. *J Biol Chem*. 2003;278:21989-21997.
 32. Ebong S, Yu CR, Carper DA, Chepelinsky AB, Ekwuagu CE. Activation of STAT signaling pathways and induction of suppressors of cytokine signaling (SOCS) proteins in mammalian lens by growth factors. *Invest Ophthalmol Vis Sci*. 2004;45:872-878.
 33. Ohkawara B, Shirakabe K, Hyodo-Miura J, et al. Role of the TAK1-NLK-STAT3 pathway in TGF-beta-mediated mesoderm induction. *Genes Dev*. 2004;18:381-386.
 34. Akita S, Akino K, Fukui M. P-II-02 leukemia inhibitory factor gene and vascular endothelial growth factor protein can module embryonic fibroblastic differentiation via GP130-STAT and MAPK signal transduction pathways. *Wound Repair Regen*. 2004;12:A5.
 35. Schulze-Bergkamen H, Brenner D, Krueger A, et al. Hepatocyte growth factor induces Mcl-1 in primary human hepatocytes and inhibits CD95-mediated apoptosis via Akt. *Hepatology*. 2004;39:645-654.
 36. Lian JP, Word B, Taylor S, Hammons GJ, Lyn-Cook BD. Modulation of the constitutive activated STAT3 transcription factor in pancreatic cancer prevention: effects of indole-3-carbinol (I3C) and genistein. *Anticancer Res*. 2004;24:133-137.
 37. Seki Y, Suzuki N, Imaizumi M, et al. STAT3 and MAPK in human lung cancer tissues and suppression of oncogenic growth by JAB and dominant negative STAT3. *Int J Oncol*. 2004;24:931-934.
 38. Chan KS, Carbajal S, Kiguchi K, Clifford J, Sano S, DiGiovanni J. Epidermal growth factor receptor-mediated activation of Stat3 during multistage skin carcinogenesis. *Cancer Res*. 2004;64:2382-2389.
 39. Bharti AC, Shishodia S, Reuben JM, et al. Nuclear factor-kappaB and STAT3 are constitutively active in CD138+ cells derived from multiple myeloma patients, and suppression of these transcription factors leads to apoptosis. *Blood*. 2004;103:3175-3184.
 40. Kanda N, Seno H, Konda Y, et al. STAT3 is constitutively activated and supports cell survival in association with survival expression in gastric cancer cells. *Oncogene*. 2004;23:4921-4929.
 41. Lewis TS, Shapiro PS, Ahn NG. Signal transduction through MAP kinase cascades. *Adv Cancer Res*. 1998;74:49-139.
 42. Cobb MH. MAP kinase pathways. *Prog Biophys Mol Biol*. 1999;71:479-500.
 43. Wierenga AT, Vogelzang I, Eggen BJ, Vellenga E. Erythropoietin-induced serine 727 phosphorylation of STAT3 in erythroid cells is mediated by a MEK-, ERK-, and MSK1-dependent pathway. *Exp Hematol*. 2003;31:398-405.
 44. Igarashi A, Nashiro K, Kikuchi K, et al. Connective tissue growth factor gene expression in tissue sections from localized scleroderma, keloid, and other fibrotic skin disorders. *J Invest Dermatol*. 1996;106:729-733.
 45. Xiao D, Pearce WJ, Longo LD, Zhang L. ERK-mediated uterine artery contraction: role of thick and thin filament regulatory pathways. *Am J Physiol Heart Circ Physiol*. 2004;286:H1615-H1622.
 46. Allen FD, Asnes CF, Chang P, Elson EL, Lauffenburger DA, Wells A. Epidermal growth factor induces acute matrix contraction and subsequent calpain-modulated relaxation. *Wound Repair Regen*. 2002;10:67-76.
 47. Laine P, Reunanen N, Ravanti L, et al. Activation of extracellular signal-regulated protein kinase1, 2 results in down-regulation of decorin expression in fibroblasts. *Biochem J*. 2000;349:19-25.
 48. Fernandez-Garcia B, Vaque JP, Herreros-Villanueva M, et al. p73 cooperates with Ras in the activation of MAP kinase signaling cascade. *Cell Death Differ*. 2007;14:254-265.
 49. Gana-Weisz M, Halaschek-Wiener J, Jansen B, Elad G, Haklai R, Kloog Y. The Ras inhibitor S-trans, trans-farnesylthiosalicylic acid chemosensitizes human tumor cells without causing resistance. *Clin Cancer Res*. 2002;8:555-565.
 50. Daniels JT, Schultz GS, Blalock TD, et al. Mediation of transforming growth factor-beta(1)-stimulated matrix contracted by fibroblasts: a role for connective tissue growth factor in contractile scarring. *Am J Pathol*. 2003;163(5):2043-2052.
 51. Folger PA, Zekaria D, Grotendorst G, Masur SK. Transforming growth factor-beta-stimulated connective tissue growth factor expression during corneal myofibroblast differentiation. *Invest Ophthalmol Vis Sci*. 2001;42:2534-2541.
 52. Saika S, Okada Y, Miyamoto T, et al. Role of p38 MAP kinase in regulation of cell migration and proliferation in healing corneal epithelium. *Invest Ophthalmol Vis Sci*. 2004;45:100-109.
 53. Leask A, Holmes A, Black CM, Abraham DJ. Connective tissue growth factor gene regulation. Requirements for its induction by transforming growth factor-beta 2 in fibroblasts. *J Biol Chem*. 2003;278:13008-13015.
 54. Hayashida T, Decaestecker M, Schnaper HW. Cross-talk between ERK MAP kinase and Smad signaling pathways enhances TGF-beta-dependent responses in human mesangial cells. *FASEB J*. 2003;17:1576-1578.
 55. Noble ME, Endicott JA, Johnson LN. Protein kinase inhibitors: insights into drug design from structure. *Science*. 2004;303:1800-1805.