Yin/Yang expression of CCN family members: Transforming growth factor beta 1, via ALK5/FAK/MEK, induces CCN1 and CCN2, yet suppresses CCN3, expression in human dermal fibroblasts

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Abstract

The role of the microenvironment in driving connective tissue disease is being increasingly appreciated. Matricellular proteins of the CCN family are signaling modifiers that are secreted by cells into the extracellular matrix microenvironment where they have profound, context-dependent effects on organ development, homeostasis and disease. Indeed, CCN proteins are emergent targets for therapeutic intervention. Recent evidence suggests that, in vivo, CCN3 has effects opposing CCN2. Moreover, when CCN3 expression is high, CCN2 expression is low. That is, they appear to be regulated in a yin/yang fashion, leading to the hypothesis that the CCN2:CCN3 ratio is important to control tissue homeostasis. To begin to test the hypothesis that alterations in CCN2:CCN3 expression might be important in skin biology in vivo, we evaluated the relative ex vivo effects of the profibrotic protein TGFbeta1 on dermal fibroblasts on protein and RNA expression of CCN3 and CCN2, as well as the related protein CCN1. We also used signal transduction inhibitors to begin to identify the signal transduction pathways controlling the ability of fibroblasts to respond to TGFbeta1. As anticipated, CCN1 and CCN2 protein and mRNA were induced by TGFbeta1 in human dermal fibroblasts. This induction was blocked by TAK1, FAK, YAP1 and MEK inhibition. Conversely, TGFbeta1 suppressed CCN3 mRNA expression in a fashion insensitive to FAK, MEK, TAK1 or YAP1 inhibition. Unexpectedly, CCN3 protein was not detected in human dermal fibroblasts basally. These data suggest that, in dermal fibroblasts, the profibrotic protein TGFbeta1 has a divergent effect on CCN3 relative to CCN2 and CCN1, both at the mRNA and protein level. Given that the major source in skin in vivo of CCN proteins are fibroblasts, our data are consistent that alterations in CCN2/CCN1: CCN3 ratios in response to profibrotic agents such as TGFbeta1 may play a role in connective tissue pathologies including fibrosis.
Introduction

Fibrosis, as a pathology, is characterized by excessive deposition of extracellular matrix, comprised principally of type I collagen, resulting in scar tissue that ultimately culminates in organ dysfunction and death. Collectively, fibrosis and fibrosis-associated disorders account for ~45% of the health care costs and deaths in the Western world [1]. As a feature of end-stage disease, the contribution of fibrosis to human disease would be expected to rise due to an increasingly aging population. Fibrotic conditions of the skin include: hypertrophic scars that occur in response to burns or wounding, keloids, or scleroderma, in which skin (and internal organs) progressively scars resulting in dermatological effects such as itching, skin tightness and reduced mobility [2,3].

The effector cell of fibrosis is the fibroblast, which responds to profibrotic cytokines such as TGFbeta by increasing production, contraction, adhesion and remodeling of the surrounding extracellular matrix [2, 4]. Initially it was believed, owing to its profound in vitro and in vivo effects and its potent upregulation in connective tissue disease, that targeting TGFbeta and its canonical signaling pathways would have profound palliative effects on fibrotic conditions. However, it is now widely appreciated due to its established pleiotropic effects, to not be an appropriate therapeutic target due to lack of efficacy relative to observed side effects [4,5]. This problem was surmised a priori, leading to the search in the early 1990s for downstream effectors or cofactors of TGFbeta that may have more selective profibrotic effects [6]. Indeed, parallel studies examining: (1) non-canonical TGFbeta signaling; (2) the mechanobiology of the profibrotic effector cell, the myofibroblast; and (3) collagen structure conclusively established that an enhanced, autocrine pro-adhesive signaling pathway was essential to promote and sustain fibrosis [7–11].

The convergence of these approaches, namely those involving the identification of possible cofactors/downstream mediators of TGFbeta and of an autocrine pro-adhesive signaling loop in promoting and sustaining fibrosis, have supported the hypothesis that targeting the cellular microenvironment may be an appropriate therapeutic approach [2, 12, 13]. In particular, the CCN family of secreted pro-adhesive matricellular proteins are of interest [14, 15]. CCN2 (formerly called CTGF), which is induced in fibroblasts by the potent profibrotic cytokine TGFbeta, was hypothesized as being a mediator of fibrosis as early as the mid-1990s [6, 16, 17]. Indeed, conditional knockout strategies have shown CCN2 expression by fibroblasts is required for fibrosis in a variety of mouse models [15, 18–21]. Conversely, CCN2 is not required for cutaneous tissue repair [22], emphasizing its selective profibrotic action and its potential utility as a specific anti-fibrotic target. Significantly, an anti-CCN2 antibody strategy (FG-3019) is currently entering a Phase III trial for idiopathic pulmonary fibrosis [23].

In addition to CCN2, CCN1 has context-specific profibrotic effects [24]. Thus, clinically, a more precise strategy might be to target both CCN1 and CCN2 simultaneously. In that regard, another member of the CCN family, CCN3, is reciprocally regulated by CCN2 in a model of diabetes [25,26], in glomerular cell proliferation [27], and chondrocyte differentiation [28]. Moreover, CCN3 protein has antifibrotic effects in a diabetes model [29]. These data have led to the hypothesis that a high CCN2:CCN3 ratio drives fibrosis and that normalizing this ratio by adding CCN3 may have antifibrotic effects [14, 30]. In addition, reciprocal regulation of CCN1 and CCN3 activities has also been previously discussed [31]. However, no studies have simultaneously examined the regulation of CCN1, CCN2, and CCN3, possibly because, until recently, the concept of all three proteins being members of the same family and therefore worthy of studying them simultaneously has not achieved widespread recognition [30, 32].

To begin to address this conceptual deficit, we elected to determine to add TGFbeta1 to human dermal fibroblasts and simultaneously monitor the expression of CCN1, CCN2 and
Moreover, we use chemical signal transduction inhibitors to identify if a common pathway mediates TGFbeta1’s effects on CCN1, CCN2 and CCN3. Our data provide new and valuable insights into the reciprocal regulation of CCN proteins in fibroblasts and into the signaling mechanisms downstream of TGFbeta1 in driving expression of key profibrotic mediators in fibroblasts.

Methods

Cell culture

All cell culture experiments were performed using primary human foreskin fibroblasts obtained from healthy humans (American Type Culture Collection CRL2094) that were previously shown to differentiate into myofibroblasts in the presence of TGFbeta1 or mechanical tension [33–35]. Cells were cultured to passage 8 in high glucose DMEM (Invitrogen) supplemented with 10% FBS and 1% antibiotic-antimycotic solution. Cells were plated on tissue culture plastic at an approximate density of 60000 cells/plate and grown overnight at 37°C in a humidifier containing 5% CO2. At approximately 70% confluence, cells were serum starved by replacing high glucose DMEM with low glucose DMEM containing 0.5% FBS and 1% antibiotic-antimycotic solution. After serum starving overnight, cells were pre-treated with either DMSO or one of the following small molecule inhibitors: SB-431542 (Tocris, 10 μM), PF573228 (Sigma, 10 μM), (5Z)-7-oxozeaenol (Tocris, 400 μM), U0126 (Sigma, 30 μM), or Verteporfin (Sigma, 695 nM). TGFbeta1 (R&D Systems, 4 ng/ml) was added 30 minutes after inhibitors for either 6 hours (for RNA collection) or 24 hours (for protein collection).

Generation of CCN3-overexpressing fibroblasts

Custom lentiviral particles were designed and obtained from Sigma-Aldrich. CCN3 overexpressing cells were generated using a CSTORFV Mission TRC3 Custom Human ORF Lentivirus (pLX317) containing a specifically designed CCN3 expression vector. Transduction control cells were generated using an ORFBFPV Mission TRC3 ORF GFP Lentivirus. For transduction, human foreskin fibroblasts were incubated with viral particles at a moiety of infection of 1.5 supplemented with 5 μg/mL Polybrene for 24 hours. Successfully transduced cells were selected for using puromycin as described by the manufacturer (Sigma-Aldrich). Surviving cells were cultured to passage 8.

Real-time PCR

Total RNA was obtained from treated cells after 6 hour treatments described above. TriZol extraction using phenol-chloroform was used to isolate RNA from cell lysates to be used for real-time PCR. RNA concentrations and integrity were measured via Nanodrop 2000 (Thermo Scientific). A total of 1 μg of RNA from each sample was reverse transcribed using qScript Supermix (QuantaBio), producing cDNA. SYBR green real-time PCR was then performed by combining cDNA (7 ng/well), SYBR master mix (Thermo Scientific) and gene specific primers. Signal changes were detected using a ViiA 7 Real-Time PCR System (Thermo Scientific). The following gene specific primers were purchased from Life Technologies for use in our experiment: CCN1 Fw: 5’-CGGCTCCCTGTGTTTTGGAAT-3’, Rev: 5’-TTGAGCAGCTGGACCATGAA-3’; CCN2 5’-GAGGAGTGGCATTGTGACG-3’, 5’-TCTTCCAGTCCGTA-3’; CCN3 5’-GTGCTACTGCTGGCTGAGGCTAA-3’, 5’-CTGTAAGCTGCAAAGGGTAA GG-3’; EDN1 5’-AGAAACAGTCTTAGCGCTGA-3’, 5’-TGGACTGGGAGTGGGTTTTTC T-3’; ITGA11 5’-CTGTGCGCCAGGTTCACG-3’, 5’-TGTAGCCAAAGAGGCGGTTC-3’; β-actin 5’-CCTCGGCTTTGCGATCC-3’, 5’-CGCGCCGATATCATCAG-3’. Samples
were run in triplicate and expression values were standardized to control values from β-actin primers using the ΔΔCt method. Biological repeats are indicated. GraphPad Prism software was used to perform a one-way ANOVA with Tukey’s post-hoc test to determine statistical significance.

Western blot

Proteins were harvested using radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 100 mM Tris-HCl pH 7.4, 1% NP40, 0.1% SDS, 5 mM EDTA, 1X protease inhibitor cocktail) from cells after 24 hour treatment. Protein concentrations were approximated using a BCA protein assay kit (Thermo Scientific), according to instructions provided by the distributor. An equal amount of protein (50 μg) was added to each well of an SDS-PAGE polyacrylamide gel (5% stacking, 10% separating). Protein samples were resolved and then transferred to a nitrocellulose membrane. Membranes were blocked in 5% non-fat skim milk in TBST (100mM Tris-HCl, pH 7.4, 0.01% Tween-20) for 1 hour, and then incubated with primary antibody overnight at 4˚C in 5% milk solution. The following antibodies were used: anti-CCN1 (1:1000; abc102; Santa Cruz), anti-CCN2 (1:500; sc14939; Santa Cruz) and anti-beta-actin (1:8000; A1978; Sigma-Aldrich). Anti-CCN3 antibody (dilution 1:2000), was used as described in the paper disclosing the generation of the antibody [36]. Membranes were washed thoroughly in TBST and then incubated with HRP-conjugated secondary antibody for 1 hour. Horseradish peroxidase-conjugated donkey anti-goat (705-036-147), donkey anti-rabbit (711-036-152) and donkey anti-mouse (715-035-150) were obtained from Jackson Immunoresearch Laboratories. Membranes were washed thoroughly and then exposed to SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) for 5 minutes before visualization using X-ray film.

Results

TGFβ1 induces CCN1 and CCN2 yet suppresses CCN3 expression in human dermal fibroblasts via ALK5

To begin to assess if, in human dermal fibroblasts, TGFβ1 affects CCN1, CCN2 and CCN3 expression in a yin/yang fashion, we treated serum-starved cells overnight and then cultured them with or without TGFβ1 for an additional 6 h (for RNA analysis) or 24 h (for protein analysis). RNA and protein were harvested. For our protein analysis, consistent with previous studies, we examined total cell extracts to capture CCN proteins in the process of being secreted through the Golgi. In our initial experiments, as a control, we also treated cells in the presence or absence of ALK5 inhibitor, as ALK5 is the TGFβ type I receptor for fibroblasts [4]. As expected, TGFβ1 induced CCN1 and CCN2 mRNA and protein, consistent with prior studies [16, 37] (Fig 1). Moreover, consistent with our prior studies, ALK5 inhibition blocked TGFβ1 induced CCN1 mRNA and protein in human dermal fibroblasts [38] (Fig 1). Furthermore, TGFβ1 also induced CCN2 mRNA protein in a fashion sensitive to ALK5 inhibition; conversely, TGFβ1 suppressed CCN3 mRNA expression; this was also blocked by addition of ALK5 inhibitor (Fig 1).

However, to our surprise, CCN3 protein was undetectable in human dermal fibroblasts either in the presence or absence of added TGFβ1 (Fig 1). CCN3 was readily detected NCI-H295R cells, a tumor line that expresses CCN3 [33], indicating the validity of our method of detecting CCN3 protein (Fig 1). That is, CCN3 does not appear to be basally expressed by proliferating human dermal fibroblasts (Previous studies have shown that low levels of intracellular CCN3 are produced in fibroblasts in culture; in growing cells the expression of CCN3 protein is quickly downregulated [37]). Collectively however, these data are consistent with
the idea that, at least at the mRNA level, TGFbeta1 has opposing effects on CCN1 and CCN2 as compared to CCN3 in human dermal fibroblasts and that this effect is mediated by ALK5. TGFbeta1 induces CCN1 and CCN2 expression in human dermal fibroblasts via FAK

Prior data from our group and others has suggested that an autocrine proadhesive signaling loop operating through focal adhesion kinase (FAK) sustains fibrosis, and FAK appears to mediate TGFbeta's profibrotic effects [11, 39, 40]. To extend our current data, we then assessed whether addition of a FAK inhibitor could affect the ability of TGFbeta1 to modulate mRNA expression of CCN1, CCN2, and CCN3 in human dermal fibroblasts. We conducted experiments similar to those described above, however we cultured cells in the presence or absence of TGFbeta1 and the presence or absence of the FAK inhibitor PF573228. We found that PF573228 blocked TGFbeta1-induced CCN1 and CCN2 mRNA and protein expression in human dermal fibroblasts (Fig 2). TGFbeta1-suppressed CCN3 expression was insensitive to PF573228 (Fig 2). Given the profibrotic roles of CCN2 and CCN1, these results emphasize the critical, central role of adhesive signaling operating through FAK in mediating fibrogenic responses in response to TGFbeta1.
TGFbeta1 induces CCN1 and CCN2 yet suppresses CCN3 expression in human dermal fibroblasts via MEK

Prior data from our group has indicated that TGFbeta-induced CCN2 expression occurs via MEK/ERK [41]. To assess if the ability of TGFbeta1 to induce CCN1 mRNA and protein expression and suppress CCN3 mRNA expression depended on MEK, we repeated our studies in the presence or absence of the MEK inhibitor U0126. As anticipated, consistent with our prior reports, TGFbeta1-induced CCN2 mRNA and protein expression in a manner that was impaired by U0126 [41] (Fig 3). Similarly, addition of U0126 significantly impaired the ability of TGFbeta1 to induce CCN1 mRNA and protein expression in human dermal fibroblasts (Fig 3). Finally, addition of U0126 did not significantly impaired the ability of TGFbeta1 to suppress CCN3 mRNA expression in human dermal fibroblasts, although U0126 reduced baseline CCN3 mRNA expression (Fig 3). These data emphasize the central role of MEK in mediating the fibrogenic responses of TGFbeta1.

TGFbeta1 induces CCN1 and CCN2 in human dermal fibroblasts via TAK1

Mitogen-activated protein kinase kinase kinase 7 (MAP3K7), also known as TAK1 (TGFbeta-activated kinase 1), once activated, is an upstream activator of MKK/JNK and p38 by the phosphorylation and activation of MAP kinase kinases such as MAP2K1/MEK1, MAP2K3/MKK3, MAP2K6/MKK6 and MAP2K7/MKK7. TAK1 deletion blocks TGFbeta-induced alpha-
smooth muscle actin expression in mouse embryonic fibroblasts, and, in gingival fibroblasts, the TAK1 inhibitor (5Z)-7-Oxozeaenol blocks the ability of TGFbeta1 to induce CCN2 expression [42,43]. To extend these studies, we assessed the ability of (5Z)-7-Oxozeaenol to block the effect of TGFbeta1 on CCN1, CCN2 and CCN3 expression in human dermal fibroblasts.

We found that, when applied to human dermal fibroblasts, (5Z)-7-Oxozeaenol treatment impaired TGFbeta1-induced CCN1 and CCN2 mRNA and protein expression; however, TGFbeta1-suppressed CCN3 mRNA expression was not significantly affected by (5Z)-7-Oxozeaenol (Fig 4). These data suggest that the mechanism by which TGFbeta1 induces CCN2 and CCN1 is divergent from that mediating TGFbeta1-suppressed CCN3 mRNA expression.

TGFbeta1 induces CCN1 and CCN2 in human dermal fibroblasts via YAP1

Previously, we showed that CCN2 expression was promoted by the mechanosensitive oncogene YAP1 [41]. Subsequently, the literature has used both CCN1 and CCN2 as stereotypical genes induced by the hippo/YAP/TAZ pathway [44]. Given these observations and the potential importance of mechanotransduction in mediating pathological fibrosis, we ascertained if the selective YAP1 inhibitor verteporfin [45] could impair the effect of TGFbeta1 on CCN1, CCN2 and CCN3 expression in human dermal fibroblasts.

In human dermal fibroblasts, exposure to verteporfin impaired the ability of human dermal fibroblasts to respond to TGFbeta1 by increasing CCN1 and CCN2 mRNA and protein expression (Fig 5). However, YAP1 inhibition did not significantly affect the ability of
TGFbeta1 to suppress CCN3 mRNA expression and, in fact, appeared to enhance the suppressive effect of TGFbeta in CCN3 mRNA expression (p = 0.06) (Fig 5). These data are consistent with the notions that verteporfin might be used to suppress the fibrogenic effects of TGFbeta1 on fibroblasts; and that the mechanism underlying the ability of TGFbeta1 to induce CCN2 and CCN1 differs from that through which TGFbeta1 suppresses CCN3 mRNA expression.

Overexpression of CCN3 suppresses TGFbeta1-induced CCN2 protein expression

CCN3 has been proposed to be a potential anti-fibrotic treatment [14]. To examine the possible mechanism underlying such an activity we generated human dermal fibroblasts overexpressing CCN3. In these cells, TGFbeta1 was able to induce CCN1 protein expression; however, the ability of TGFbeta1 to induce CCN2 protein was significantly impaired (Fig 6A and 6B). Unsurprisingly, as: (a) in transduced fibroblasts CCN3 was overexpressed under the control of the non-TGFbeta-responsive CMV promoter; and (b) loss of CCN2 does not impair the ability of TGFbeta to induce expression of fibrogenic mRNA in cultured dermal fibroblasts [18], overexpression of CCN3 was not (a) suppressed by the addition of TGFbeta (b) able to impair the ability of TGFbeta to induce the mRNA expression of the fibrogenic markers endothelin-1 (EDN1) or integrin alpha 11 (ITGA11) [46–48] (Fig 6C).
Discussion

The connective tissue microenvironment is being increasingly appreciated in playing a central role in disease [2,49]. Specifically, the CCN family of matricellular proteins show altered expression patterns in connective tissue disease and are emerging targets for therapeutic intervention [50]. CCN proteins share a similar structure, and have limited in vitro effects, making the development of relevant cell-based bioassays extremely difficult; consequently, is is necessary to study the functional role of CCN proteins in vivo. [15, 30] Of these, CCN2 (CTGF) is the most studied; indeed, anti-CCN2 antibodies are in clinical development [23]. Other CCN family members may have different functional roles in vivo, and indeed, may have opposing effects [30]. Of the other CCN proteins, CCN1 and CCN3 are the most studied; data published thus far suggest that, in vivo, CCN1 may have context-dependent profibrotic effects, whereas CCN3 may be antifibrotic [25–29]. However, until now, no study has simultaneously compared the expression of CCN1, CCN2 and CCN3 in response to the profibrotic protein TGFbeta1 in cultured dermal fibroblasts. Our data suggest that CCN1 and CCN2 are induced by TGFbeta1 via a similar pathway; conversely CCN3 mRNA is reduced by TGFbeta1 through a pathway that is divergent; that is, not involving MEK1, YAP1 or TAK1. These data are consistent with the general hypothesis that CCN1 and CCN2 are expressed in a yin/yang fashion in a way opposite to CCN3 and with a hypothesis that, in vivo, restoring a CCN1/2:CCN3 balance may be of therapeutic value [14, 30]. Similar to previous data showing that CCN2-deficient dermal fibroblasts retained TGFbeta1-responsiveness [18], overexpressing CCN3 in
Fig 6. CCN3 overexpression impairs TGFβ1-induced CCN2 protein expression. Human dermal fibroblasts were transduced with lentiviral particles containing a CCN3 expression vector, while transduction-control cells were transduced with lentiviral particles containing a control expression vector. (A) CCN3 overexpression was confirmed via qPCR and Western blot analysis. CCN3-transduced cells show significantly more CCN3 mRNA and protein expression than transduction-control cells. Statistical differences were determined...
human dermal fibroblasts had no appreciable effect on the ability of TGFbeta to induce mRNA expression of endothelin-1 or integrin alpha 11. It should be reiterated that loss or blockade of CCN2 expression severely impairs fibrogenesis including myofibroblast differentiation in vivo in a fashion that does not appear to involve canonical TGFbeta signaling [15, 18–21, 51,52]. Since CCN proteins have limited ex vivo effects and instead act to integrate signaling emanating from multiple sources [30], direct testing of the potential antifibrotic role of CCN3 requires the use of animal models and is therefore beyond the scope of our current report.

Our studies investigating the mechanism of how non-canonical TGFbeta signaling activates CCN1 and CCN2 expression support the notion that FAK, ERK, TAK1 and YAP1 promote fibrogenic responses. The involvement of adhesive signaling via FAK and ERK [2, 40, 53] in promoting TGFbeta signaling and fibrosis is consistent with prior reports in other systems. YAP1 is known to activate genes in response to mechanotransduction; however, relatively few reports have examined the effect of verteporfin in blocking TGFbeta’s fibrogenic responses. Indeed, only two other reports have examined this question. Specifically, verteporfin was shown to reduce TGFbeta responses in NRK renal cells and in conjunctival fibroblasts [54, 55]. This result is of potential long-term clinical application as verteporfin is in clinical use for macular degeneration [56].

Collectively, our data provide new and valuable insights into the coordinated and opposite regulation of the key CCN family members CCN1, CCN2 and CCN3 in human dermal fibroblasts and are consistent with the long-term hypothesis that alterations in CCN1/CCN2:CCN3 ratio in response to fibrogenic stimuli may be important in driving fibrogenic responses. Our results showing that overexpression of CCN3 in fibroblasts reduces the ability of TGFbeta to induce CCN2 protein expression are consistent with that notion. Our results are also consistent with the notion that TGFbeta induces fibrogenic responses in fibroblasts via non-canonical proadhesive/mechanotransductive pathways and that targeting this pathway, e.g. by verteporfin, may be of value.

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References

1. Borthwick LA, Wynn TA, Fisher AJ. Cytokine mediated tissue fibrosis. Biochim Biophys Acta. 2013 Jul; 1832(7):1049–60. https://doi.org/10.1016/j.bbapain.2012.09.014 PMID: 23046809

2. Schulz JN, Plomann M, Sengle G, Gullberg D, Krieg T, Eckes B. New developments on skin fibrosis—Essential signals emanating from the extracellular matrix for the control of myofibroblasts. Matrix Biol. 2018 Aug; 68–69:522–532. https://doi.org/10.1016/j.matbio.2018.01.029 PMID: 29408279

3. Allanore Y, Simms R, Distler O, Trojanowska M, Pope J, Denton CP, Varga J. Systemic sclerosis. Nat Rev Dis Primers. 2015 Apr 23; 1:15002. https://doi.org/10.1038/nrdp.2015.2 PMID: 27189141

4. Leask A, Abraham DJ. TGF-beta signaling and the fibrotic response. FASEB J. 2004 May; 18(7):816–27 https://doi.org/10.1096/fj.03-1273rev PMID: 15117886

5. Denton CP, Merkel PA, Furst DE, Khanna D, Emery P, Hsu VM, et al. Recombinant human anti-transforming growth factor beta1 antibody therapy in systemic sclerosis: a multicenter, randomized, placebo-controlled phase III trial of CAT-192. Arthritis Rheum. 2007 Jan; 56(1):32–33. https://doi.org/10.1002/art.22289 PMID: 17195236

6. Dammeyer 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 Aug; 30(8):909–22. PMID: 9744082

7. van der Slot AJ, Zuurmond AM, Bardool AF, Wijmenga C, Prujs HE, Sillence DO, et al. Identification of PLOD2 as telopeptide lysyl hydroxylase, an important enzyme in fibrosis. J Biol Chem. 2003 Oct 17; 278(42):40967–72 https://doi.org/10.1074/jbc.M307380200 PMID: 12881513

8. Walraven M, Hinz B. Therapeutic approaches to control tissue repair and fibrosis: Extracellular matrix as a game changer. Matrix Biol. 2018 Oct; 71–72:205–224. https://doi.org/10.1016/j.matbio.2018.02.026 PMID: 29499355

9. Shi-wen X, Paraparam SK, Pala D, Chen Y, Carter DE, Eastwood M, et al. Requirement of transforming growth factor beta-activated kinase 1 for transforming growth factor beta-induced alpha-smooth muscle actin expression and extracellular matrix contraction in fibroblasts. Arthritis Rheum. 2009 Jan; 60(1):234–41. https://doi.org/10.1002/art.24223 PMID: 19116914

10. Liu S, Kapoor M, Denton CP, Abraham DJ, Leask A. Loss of beta1 integrin in mouse fibroblasts results in resistance to skin scleroderm in a mouse model. Arthritis Rheum. 2009 Sep; 60(6):2917–21. https://doi.org/10.1002/art.24801 PMID: 19714619

11. Thannickal VJ, Lee DY, White ES, Cui Z, Larios JM, Chacon R, et al. Myofibroblast differentiation by transforming growth factor-beta1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. J Biol Chem. 2003 Apr 4; 278(14):12384–9. https://doi.org/10.1074/jbc.M20544200 PMID: 12531888

12. Huang C, Ogawa R. Fibroproliferative disorders and their mechanobiology. Connect Tissue Res. 2012; 53(3):187–96. https://doi.org/10.3109/03008207.2011.642035 PMID: 22329637

13. Leask A. Integrin beta1: A Mechanosignaling Sensor Essential for Connective Tissue Deposition by Fibroblasts. Adv Wound Care (New Rochelle). 2013 May; 2(4):160–166.

14. Riser BL, Barnes JL, Varani J. Balanced regulation of the CCN family of matricellular proteins: a novel approach to the prevention and treatment of fibrosis and cancer. J Cell Commun Signal. 2015 Dec; 9(4):327–38. https://doi.org/10.1007/s12079-015-0309-3 PMID: 26698861

15. Leask A. CCN2: a novel, specific and valid target for anti-fibrotic drug intervention. Expert Opin Ther Targets. 2013 Sep; 17(9):1067–71. https://doi.org/10.1517/14728222.2013.812074 PMID: 23848501

16. Holmes A, Abraham DJ, Sa S, Shiwen X, Black CM, Leask A. CTGF and SMADs, maintenance of sclerodema phenotype is independent of SMAD signaling. J Biol Chem. 2001 Apr 6; 276(14):10594–601 https://doi.org/10.1074/jbc.M01049200 PMID: 11152469
17. 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 Jun; 4(6):637–45. PMID: 8374172

18. Liu S, Shi-wen X, Abraham DJ, Leask A. CCN2 is required for bleomycin-induced skin fibrosis in mice. Arthritis Rheum. 2011 Jan; 63(1):239–46. https://doi.org/10.1002/art.30074 PMID: 20936632

19. Petrosino JM, Leask A, Accornero F. Genetic manipulation of CCN2/CTGF unveils cell-specific ECM-remodeling effects in injured skeletal muscle. FASEB J. 2018 Sep 14;fj201800622RR.

20. Makino K, Makino T, Stawski L, Lipson KE, Leask A, Trojanowska M. Anti-connective tissue growth factor (CTGF/CCN2) monoclonal antibody attenuates skin fibrosis in models of systemic sclerosis. Arthritis Res Ther. 2017 Jun 13; 19(1):134.

21. Kinashi H, Falke LL, Nguyen TQ, Bovenschen N, Aten J, Leask A, et al. Connective tissue growth factor regulates fibrosis-associated renal lymphangiogenesis. Kidney Int. 2017 Oct; 92(4):850–863 https://doi.org/10.1016/j.kint.2017.03.029 PMID: 28545716

22. Liu S, Thompson K, Leask A. CCN2 expression by fibroblasts is not required for cutaneous tissue repair. Wound Repair Regen. 2014 Jan-Feb; 22(1):119–24. https://doi.org/10.1111/wrr.12131 PMID: 24393160

23. Raghu G, Scholand MB, de Andrade J, Lancaster L, Mageto Y, Goldin J, et al. FG-3019 anti-connective tissue growth factor monoclonal antibody: results of an open-label clinical trial in idiopathic pulmonary fibrosis. Eur Respir J. 2016 May; 47(5):1481–91. https://doi.org/10.1183/13993003.01030-2015 PMID: 26965296

24. Kurundkar AR, Kurundkar D, Rangarajan S, Locy ML, Zhou Y, Liu RM, et al. The matricellular protein CCN1 enhances TGF-beta1/SMAD3-dependent profibrotic signaling in fibroblasts and contributes to fibrogenic responses to lung injury. FASEB J. 2018 Sep 14:fj201800622RR.
36. Kyurkchiev S, Yeger H, Bleau AM, Perbal B. Potential cellular conformations of the CCN3(NOV) protein. Cell Commun Signal. 2004 Sep 10; 2(1):9. https://doi.org/10.1186/1478-811X-2-9 PMID: 15361251

37. Thompson K, Murphy-Mashman H, Leask A. ALK5 inhibition blocks TGF-beta-induced CCN1 expression in human foreskin fibroblasts. J Cell Commun Signal. 2014 Mar; 8(1):59–63. https://doi.org/10.1007/s12079-014-0229-7 PMID: 24567145

38. Scholz G, Martinerie C, Perbal B, Hanafusa H. Transcriptional down regulation of the nov proto-oncogene in fibroblasts transformed by p60v-src. Mol Cell Biol. 1996 Feb; 16(2):481–6. https://doi.org/10.1128/mcb.16.2.481 PMID: 8552074

39. Liu S, Xu SW, Kennedy L, Paia D, Chen Y, Eastwood M, et al. FAK is required for TGFbeta-induced JNK phosphorylation in fibroblasts: implications for acquisition of a matrix-remodeling phenotype. Mol Biol Cell. 2007 Jun; 18(6):2169–78. https://doi.org/10.1091/mbc.E06-12-1121 PMID: 17409352

40. Shi-wen X, Thompson K, Khan K, Murphy-Mashman H, Baron M, et al. Focal adhesion kinase and reactive oxygen species contribute to the persistent fibrotic phenotype of lesional scleroderma fibroblasts. Rheumatology (Oxford). 2012 Dec; 51(12):2146–5

41. Leask A, Holmes A, Black CM, Abraham DJ. Connective tissue growth factor gene regulation. Requirement for its induction by transforming growth factor-beta2 in fibroblasts. J Biol Chem. 2003 Apr 11; 278(15):13008–15 https://doi.org/10.1074/jbc.M210366200 PMID: 12571253

42. Kuk H, Hutchenerreuther J, Murphy-Mashman H, Carter D, Leask A. 5Z-7-Oxozeanol Inhibits the Effects of TGFbeta1 on Human Gingival Fibroblasts. PLoS One. 2015 Apr 30; 10(4):e0123689 https://doi.org/10.1371/journal.pone.0123689 PMID: 25927238

43. Leask A, Holmes A, Black CM, Abraham DJ. Connective tissue growth factor gene regulation. Requirements for its induction by transforming growth factor-beta2 in fibroblasts. J Biol Chem. 2003 Apr 11; 278(15):13008–15 https://doi.org/10.1074/jbc.M210366200 PMID: 12571253

44. Zhou Y, Huang T, Cheng AS, Yu J, Kang W, To KF. The TEAD Family and Its Oncogenic Role in Promoting Tumorigenesis. Int J Mol Sci. 2016 Jan 21; 17(1). pii: E138 https://doi.org/10.3390/ijms17010138 PMID: 26805820

45. Wang C, Zhu X, Feng W, Yu Y, Jeong K, Guo W, et al. Verteporfin inhibits YAP function through up-regulating 14-3-3sigma sequestering YAP in the cytoplasm. Am J Cancer Res. 2015 Dec 15; 6(1):27–37 PMID: 27073720

46. Cipriani P, Di Benedetto P, Ruscitti P, Verzella D, Fischetti M, Zazzeroni F, et al. Macitentan inhibits the transforming growth factor-beta profibrotic action, blocking the signaling mediated by the ETR/TβRI complex in systemic sclerosis dermal fibroblasts. Arthritis Res Ther. 2015 Sep 10; 17:247. https://doi.org/10.1186/s13075-015-0754-7 PMID: 26357964

47. Scholz JN, Plomm M, Sengle G, Gullberg D, Krieg T, Eckes B. New developments on skin fibrosis: evidence of an autocrine endothelin loop operating through the endothelin A and B receptors. Mol Cell Biol. 2006 Jul; 26(14):5518–27. https://doi.org/10.1128/MCB.00625-06 PMID: 16809784

48. Schulz JN, Plomm M, Sengle G, Gullberg D, Krieg T, Eckes B. New developments on skin fibrosis—Essential signals emanating from the extracellular matrix for the control of myofibroblasts. Matrix Biol. 2018 Aug; 68–69:S22–532. https://doi.org/10.1016/j.matbio.2018.01.025 PMID: 29408276

49. Hutchenerreuther J, Leask A. Why target the tumor stroma in melanoma? J Cell Commun Signal. 2018 Mar; 12(1):113–118 https://doi.org/10.1007/s12079-017-0419-1 PMID: 29110248

50. Jun JI, Lau LF. Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets. Nat Rev Drug Discov. 2011 Dec 1; 10(12):945–63. https://doi.org/10.1038/nrd3599 PMID: 22129992

51. Hutchenerreuther J, Vincent K, Norley C, Racanelli M, Gruber SB, Johnson TM, et al. Activation of cancer-associated fibroblasts is required for tumor neovascularization in a murine model of melanoma. Matrix Biol. 2018; 74:52–61. https://doi.org/10.1016/j.matbio.2018.06.003 PMID: 29885461

52. Rebolledo DL, González D, Faundez-Contreras J, Contreras O, Vio CP, Murphy-Ullrich JE, et al. Dener-vation-induced skeletal muscle fibrosis is mediated by CTGF/CCN2 independently of TGF-β. Matrix Biol. 2019 Feb 1. pii: S0907-4504(18)30455-4. https://doi.org/10.1016/j.matbio.2019.01.002 PMID: 30716392

53. Li S, Liu J, Tan J, Li L, Kaltreider MJ, Zhao J, et al. Inhibition of Raf1 ameliorates bleomycin-induced pulmonary fibrosis through attenuation of TGF-beta1 signaling. Am J Physiol Lung Cell Mol Physiol. 2018 Aug 1; 315(2):L241–L247 https://doi.org/10.1152/ajplung.00093.2018 PMID: 29722566

54. Szeto SG, Narimatsu M, Lu M, He X, Sidiqi AM, Tolosa MF, et al. YAP/TAZ Are Mechanoregulators of TGFBeta-Smad Signaling and Renal Fibrogenesis. J Am Soc Nephrol. 2016 Oct; 27(10):3117–3128. https://doi.org/10.1681/ASN.2015050498 PMID: 26961347
55. Futakuchi A, Inoue T, Wei FY, Inoue-Mochita M, Fujimoto T, Tomizawa K, et al. YAP/TAZ Are Essential for TGF-beta2-Mediated Conjunctival Fibrosis. Invest Ophthalmol Vis Sci. 2018 Jun 1; 59(7):3069–3078. https://doi.org/10.1167/iovs.18-24258 PMID: 30025139

56. Gibault F, Corvaisier M, Bailly F, Huet G, Melnyk P, Cotelle P. Non-Photoinduced Biological Properties of Verteporfin. Curr Med Chem. 2016; 23(11):1171–84. PMID: 26980565