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ORIGINAL ARTICLE

Transforming growth factor β1 regulates the expression of CCN2 in human keratinocytes via Smad-ERK signalling

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Abstract

Connective tissue growth factor (CCN2/CTGF) and transforming growth factor β1 (TGF-β1) are important regulators of skin wound healing, but controversy remains regarding their expression in epithelial cell lineages. Here, we investigate the expression of CCN2 in keratinocytes during reepithelialisation and its regulation by TGF-β1. CCN2 was detected in the epidermis of healing full-thickness porcine wounds. Human keratinocytes were incubated with or without 10 ng/ml TGF-β1, and signalling pathways were blocked with 10-μM SIS3 or 20-μM PD98059. Semi-quantitative real-time PCR was used to study CCN2 mRNA expression, and western blot was used to measure CCN2, phosphorylated-ERK1/2, ERK1/2, phosphorylated-Smad3 and Smad2/3 proteins. CCN2 was transiently expressed in neoepidermis at the leading edge of the wound in vivo. In vitro, CCN2 expression was induced by TGF-β1 at 2 hours (7·5 ± 1·9-fold mRNA increase and 3·0 ± 0·6-fold protein increase) and 12 hours (5·4 ± 1·9-fold mRNA increase and 3·3 ± 0·6-fold protein increase). Compared with inhibiting the SMAD pathway, inhibiting the mitogen-activated protein kinase (MAPK) pathway was more effective in reducing TGF-β1-induced CCN2 mRNA and protein expression. Inhibition of the MAPK pathway had minimal impact on the activity of the SMAD pathway. CCN2 is expressed in keratinocytes in response to tissue injury or TGF-β1. In addition, TGF-β1 induces CCN2 expression in keratinocytes through the ras/MEK/ERK pathway. A complete understanding of CCN2 expression in keratinocytes is critical to developing novel therapies for wound healing and cutaneous malignancy.

Introduction

The skin protects the body from the external environment (1). The protective barrier is provided by the keratinocytes of the epidermis and by the dermis, which consists of fibroblasts that produce extracellular matrix (2,3). Upon injury, normal wound healing must occur in an organised fashion for tissue integrity to be restored. Cutaneous wound healing is a complex process involving the migration of inflammatory cells to the wound site, deposition of extracellular matrix by fibroblasts and the re-establishment of an intact epithelial barrier. These processes are tightly regulated by a variety of growth factors and cytokines, and abnormalities in any of these processes may result in the clinical phenotype of chronic non-healing wounds or hypertrophic scars (4,5). Although novel therapies have been developed to augment normal wound healing, treatment of aberrant wounds remains a complex endeavour that costs the United States over 25 billion dollars annually (6).

Key Messages

- connective tissue growth factor (CCN2) play an important role in regulation of migration in the healing wound
- this study investigates the effects of transforming growth factor β1 (TGF-β1) on CCN2 expression in the context of the Smad and ERK signalling pathways
- studies were performed in vitro and using a porcine wound healing model

- CCN2 was shown to be expressed in keratinocytes in response to tissue injury or TGF-β1
- TGF-β1-induced CCN2 expression in keratinocytes through the ras/MEK/ERK pathway

The re-establishment of an intact epithelial barrier is an essential feature to a healed wound and is accomplished through the directed migration of keratinocytes in response to molecular mediators. CCN2, previously named connective tissue growth factor, has emerged as a regulator of cell migration. CCN2 is the second member of the CCN family of six cysteine-rich proteins named Cyr61, CCN2, Nov and WISP1, 2 and 3 (7–9). Members of the CCN family of proteins contain four structurally conserved domains that regulate cellular activity by binding molecules such as growth factors, integrins and proteoglycans (10). In the context of normal wound healing, CCN2 is well established as a profibrotic factor that plays a major role in cell proliferation, migration, adhesion, angiogenesis and maintenance of the extracellular matrix (7,11). Expression of CCN2 was initially thought to be confined to cells of mesenchymal origin, but a growing body of evidence indicates that CCN2 is also expressed in epithelial cells (12–17). This is due in part to advances in cancer genomics, which has linked over-expression of CCN2 to multiple carcinomas, particularly metastatic melanoma (18–20).

The potential role of CCN2 in the physiology of certain non-healing wounds, fibrosis and cutaneous malignancy presents a unique opportunity for the development of novel therapeutics. CCN2 and its intracellular pathways in epidermal cells are thus clinically important as they may one day serve as both a biomarker and therapeutic for a variety of pathologies. The main obstacle to using CCN2 as a therapeutic target will be its cell type-specific expression and widespread involvement in matricellular processes. Modulating expression of CCN2 in a single organ may have an unpredictable impact on many cell types as illustrated by the diverse effects of CCN2 on keratinocytes, fibroblasts and melanocytes. For instance, our previous finding that CCN2 promotes the migration of keratinocytes suggests that inhibiting dermal CCN2 expression for anti-fibrotic therapy may prevent reepithelialisation (15). Conversely, up-regulating CCN2 expression with the intent of achieving closure of a non-healing wound may pose a risk of cancer secondary to keratinocytes or melanocytes exhibiting more invasive and migratory behaviours. Thus, while CCN2 holds great promise for the development of novel therapeutics, a better understanding of the cellular events and signalling pathways involved in its expression are needed.

Transforming growth factor β1 (TGF-β1) is one of the most potent inducers of CCN2 (9,21,22). TGF-β1-regulated gene expression is generally mediated through Smad signalling (23). TGF-β1 binds to its primary (type II) receptor, allowing recruitment, transphosphorylation and activation of the signalling (type I) receptor. This receptor complex activates receptor-activated Smads (R-Smads), primarily Smad 2 and Smad 3, by phosphorylating serine residues at the C-terminal. The activated R-Smads form a complex with Smad 4 and subsequently translocate into the nucleus, where they bind to Smad-binding elements (SBE) in promoters of TGF-β1-responsive genes. In addition to Smad signalling, TGF-β1 is known to activate other signalling pathways, including the mitogen-activated protein kinase (MAPK) pathway. Within the MAPK pathway, TGF-β1 directly regulates CCN2 expression through effector proteins ERK, p38 and JNK in a variety of cell types to facilitate cellular activity (24). Regulation of CCN2 by TGF-β1 may be cell type-specific even within a single organ. For instance, TGF-β1 induces CCN2 through the Smad signalling pathway in hepatocytes but has little effect on CCN2 expression in hepatic stellate cells (25). Conversely, the Jak-Stat signalling pathway is intimately involved in TGF-β1 induction of CCN2 in lung endothelial cells (26).

Numerous studies have identified cross-talk between Smad and ERK signalling activated by TGF-β1. ERK induced by TGF-β1 can regulate Smad activity and function by direct phosphorylation of Smads or indirectly by modifying the activity of coactivators/corepressors that mediate Smad-DNA binding (27–29). In addition, Smads have been shown to mediate activation of ERK, adding more complexity to the relationship between the Smad and ERK pathways (27,30). It has been shown that both Smad and ERK signalling cascades are involved in TGF-β1-induced CCN2 expression in mesenchymal cells (31–33). However, in keratinocytes, the potential interactions between Smad and ERK in TGF-β1-induced CCN2 expression remain unexplored. Thus, we sought to investigate the localisation of CCN2 in epidermal wound healing and to characterise TGF-β1-induced CCN2 in the context of the Smad and ERK signalling pathways.

Methods

Ethics statement

The research protocol and consenting procedures were reviewed and approved by the institutional review boards (IRB) of Partners Human Research Committees at the Brigham and Women's Hospital (IRB protocol no. 2010P002947). The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Human skin was obtained from patients undergoing elective plastic surgery. The IRB committee deemed full written consent unnecessary, and oral consent was considered appropriate as no patient identifiers were collected, and the tissue would have otherwise been routinely discarded. Oral consent was obtained after careful review of an informational sheet describing what researchers would do with the otherwise-discarded tissue. After the surgery, medical staff contacted the researchers, and the de-identified tissue was collected. This study was conducted according to the principles expressed in the Declaration of Helsinki.

All animal procedures were approved by the Harvard Medical Area Standing Committee on Animals and conformed with regulations related to animal use and other federal statutes (protocol number 693). All animals were handled in strict accordance with good animal practice, and the study was conducted adhering to the institution's guidelines for animal husbandry.

Reagents

Recombinant human TGF-β1 was purchased from Calbiochem (Gibbstown, NJ, USA). MEK 1 inhibitor PD98050 was obtained from Cell Signaling Technology (Danvers, MA); Smad 3 inhibitor SIS3 was from Millipore (Bedford, MA); and actinomycin D was from Sigma (St. Louis, MO). Polyclonal antibodies against CCN2 (L-20) came from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit-anti-phospho-p44/42 (phosphorylated ERK), rabbit anti-p44/42 (ERK), rabbit-anti-phospho-Smad3 (Ser 423/425), rabbit-anti-Smad 2/3 and rabbit-anti-lamin A/C were from Cell Signaling Technology. HRP-conjugated mouse-anti-GAPDH was purchased from Abcam (Cambridge, MA). Biotinylated secondary antibodies (rabbit-anti-goat and donkey-anti-rabbit) were from Vector Laboratories (Burlingame, CA). Alexa-fluor-488 donkey-anti-goat IgG and Alexa-fluor-488 donkey-anti-rabbit IgG were from Invitrogen (Carlsbad, CA). Reagents used for positive controls included rat thoracic aorta myoblast whole cell lysate (A10) from Santa Cruz Biotechnology. Dispase, trypsin and ethylenediaminetetraacetic acid (EDTA) were obtained from Invitrogen. EpiLife keratinocyte medium and keratinocyte supplement were from GIBCO (Grand Island, NY). Dulbecco's Modified Eagle's Medium (DMEM) was from Sigma Aldrich (Steinheim, Germany). High pure RNA isolation kits were purchased from Roche Diagnostics (Indianapolis, IN). Quantitative real-time Polymerase Chain Reaction (qRT-PCR) kits were obtained from Applied Biosystems (Foster City, CA). FAM™Taqman®MGB probes and Taqman® Universal PCR Master Mix as well as TaqMan probes and primers for 18S RNA were purchased from Applied Biosystems. Bicinchoninic Acid (BCA) protein assay reagent kit as well as all western blot reagents was purchased from Bio-Rad (Hercules, CA). Chemiluminescence detection kit was from GE Health Care (Westborough, MA). NE-PER™ nuclear and cytoplasmic extraction reagents were purchased from Thermo Scientific (Rockford, IL).

Porcine wound model

The in vivo wound model has been extensively described (34,35). Briefly, full-thickness wounds measuring 1·5 × 1·5 cm were created on the dorsum of three pigs and covered with sterile dry gauze. The pigs were returned to the pen and monitored during recovery from anaesthesia. Biopsies were taken on postoperative days 6, 10, 14, 18 and 22 with a 0·5-cm margin from the tattoo marking the wound border, with a total of three samples per pig per biopsy day ($n = 9$). Tissue samples were embedded in an optimal cutting temperature compound (Tissue-Tek O.C.T., Sakura, Fintec, CA) and snap-frozen in liquid nitrogen.

Immunohistochemistry and immunofluorescence staining

Cryosections (6 μm) were incubated at 4°C overnight with primary antibody diluted 1:200. Slides were subsequently incubated with biotinylated rabbit anti-goat followed by incubation with avidin-biotin-peroxidase complex. Aminoethylcarbazole was used for detection, and sections were counterstained with haematoxylin.

For immunofluorescence detection of CCN2 and phosphorylated Smad 3, keratinocytes were cultured on collagen I-coated cover slips in a serum-free medium for 24 hours. As indicated by the figure legends, some chambers were pre-treated with 20-μM MEK inhibitor PD98059 for 60 minutes prior to the addition of 10 ng/ml TGF-β1 for 24 hours. Keratinocytes cultured without the addition of inhibitors or growth factors served as negative controls. Following treatment, cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature. After 5 minutes permeabilisation with 0·1% Triton X-100, non-specific binding sites were blocked using 1% BSA in PBS. CCN2 was detected with primary antibodies diluted 1:300, followed by incubation with Alexa Fluor 488 donkey anti-goat IgG, diluted 1:500. Phosphorylated Smad 3 was detected with primary antibodies diluted 1:500, followed by incubation with Alexa Fluor 568 donkey anti-goat IgG, diluted 1:500. Slides were mounted with aqueous mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (ProLong Gold, Invitrogen) and assessed by fluorescence microscopy.

Cell culture

Primary human keratinocytes were isolated from the epidermis as previously described (34). Briefly, skin was incubated at 4°C with 1 U/ml dispase in DMEM overnight, and the epidermis was mechanically separated from the underlying dermis. The epithelial sheets were treated with 0·05% trypsin and 0·01% EDTA for 10 minutes. Keratinocytes were cultured in EpiLife keratinocyte medium supplemented with bovine insulin (5 μg/ml), hydrocortisone (0·5 μg/ml), human recombinant epidermal growth factor (0·1 μg/ml), 0·4% bovine pituitary extract and 60 μM calcium chloride. Cells were expanded using serial passage, and cells from passages 1–3 were used for the experiments.

RNA preparation

Primary human keratinocytes were grown in serum-free conditions as described above. Keratinocytes from days 1–7 were harvested each day for RNA isolation. When indicated, keratinocytes were treated with 1, 5 or 10 ng/ml TGF-β1 for 2 or 12 hours. For inhibition studies, keratinocytes were pre-treated with 20-μM MEK 1 inhibitor PD98050, 10 μM Smad 3 inhibitor SIS3 and/or 5 μg/ml actinomycin D for 1 hour prior to incubation with 10 ng/ml TGF-β1, as indicated in the figure legends. Cells were harvested with 0·05% trypsin and 0·01% EDTA, washed with PBS, and pelleted via centrifugation at 200 *g* for 7 minutes at 4°C. The high pure RNA isolation kit was used to extract total RNA from the cells according to kit protocol.

Quantitative real-time PCR

As per the thermal cycling protocol, cycling consisted of 2 minutes at 95°C and 10 minutes at 95°C, followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute. The

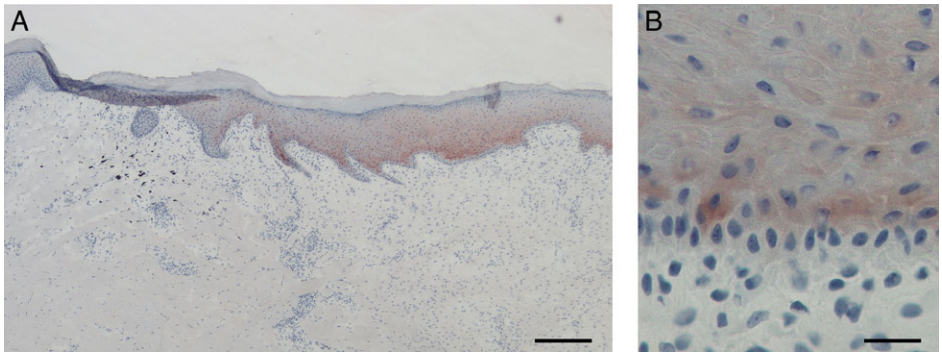

Figure 1 Epidermal connective tissue growth factor (CCN2) expression in vivo. (A) CCN2 protein expression in vivo was detected by immunohistochemistry 10 days after wounding. CCN2 was transiently expressed in the leading edge of the neoepidermis. Red colour represents AEC-positive immunoreactivity, and the blue colour is the haematoxylin counter-stain. (B) Magnified image of the dermal–epidermal junction showing that CCN2 expression was confined to the suprabasal keratinocytes. Scale bar equals 200 μm in (A) and 25 μm in (B).

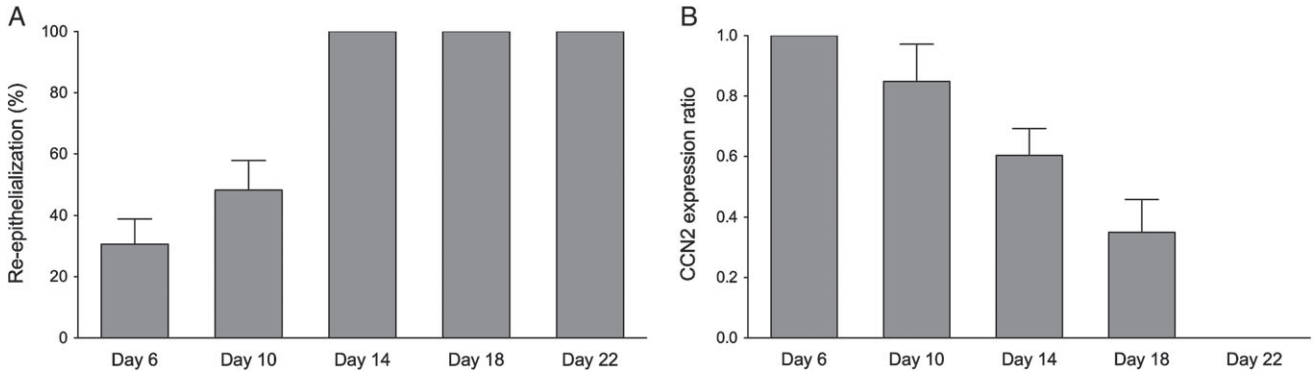

Figure 2 Connective tissue growth factor (CCN2) is transiently expressed during reepithelialisation. (A) The full-thickness porcine wounds were fully reepithelialised by day 14. (B) The linear ratio of CCN2-expressing neoepidermis to total epidermis showed a transient expression of CCN2 during reepithelialisation. Graphs show mean ± SD from four independent experiments, $n = 8$.

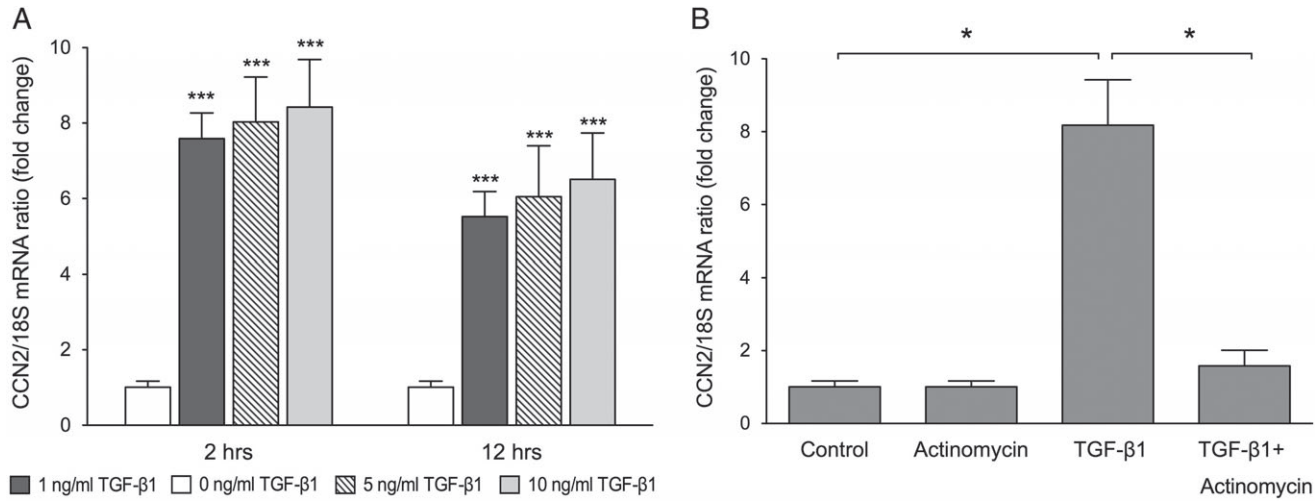

Figure 3 Transforming growth factor β1 (TGF-β1) induces connective tissue growth factor (CCN2) mRNA expression in keratinocytes. (A) Keratinocytes were incubated with or without the addition of 1, 5 or 10 ng/ml TGF-β1 for 2 and 12 hours, and CCN2 mRNA expression was analysed by quantitative real-time PCR. Graphs show mean ± SD from three independent experiments, $n = 6$. (B) To verify that the increased expression of CCN2 by TGF-β1 is because of active transcription, keratinocytes were pre-treated with 5 μg/ml actinomycin D for 60 minutes prior to the addition of TGF-β1. CCN2 mRNA expression was analysed by quantitative real-time PCR after 24 hours incubation. Graphs show mean ± SD from three independent experiments, $n = 6$. *P <0·05 vs. 0 ng/ml TGF-β1 (control), ***P < 0·001 vs. control.

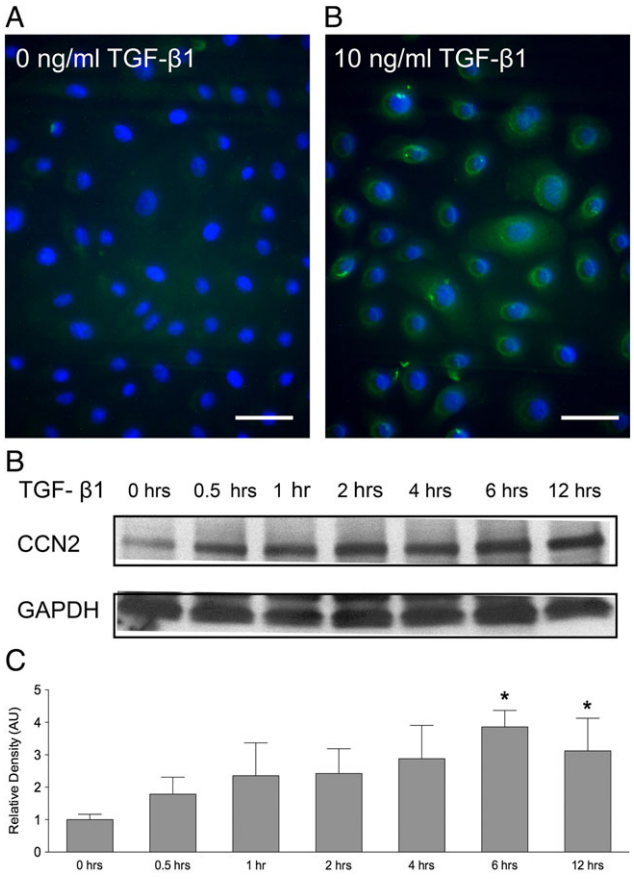

Figure 4 Transforming growth factor β1 (TGF-β1) increases connective tissue growth factor (CCN2) protein production in keratinocytes. CCN2 protein production in vitro was detected by immunohistochemistry. Green staining indicates CCN2, and blue staining indicates nuclei. (A) After 24 hours' culture in a serum-free control culture media, keratinocytes expressed low levels of CCN2. (B) The addition of 10 ng/ml TGF-β1 increased CCN2 protein production. The experiments were repeated three times with similar results. Scale bar equals 20 μm. (C) TGF-β1-induced CCN2 protein production was detected with western blot. An increased production of CCN2 was observed after the addition of TGF-β1, and significantly increased levels of CCN2 were detected after 6 and 12 hours. One representative western blot out of three independently performed experiments is shown. (D) The relative density of the bands was normalised to GAPDH. Graphs show mean ± SD from three independent experiments, $n = 6$. $^*P < 0{\cdot}05$ versus untreated control.

cycle threshold was set within the linear phase of the target gene amplification and above the non-template control background in order to calculate the cycle number at which the transcription was detected as denoted by Ct. The instrument spectral compensations by the 7900 HT SDS software version 2.1 (Applied Biosystems) was used to collect data. Samples were run in triplicate, and expression values were normalised with respect to the housekeeping gene 18S RNA employing the ΔΔCt method (36).

Western blot

Keratinocytes were grown to 80% confluence in serum-free conditions. Cells were collected with an ice-cold plastic cell scraper at 10 minutes, 30 minutes, 60 minutes or 0–12 hours after adding 10 ng/ml TGF-β1. In some treatment groups, keratinocytes were pre-treated with 10-μM Smad 3 inhibitor SIS3 and/or 20-μM MEK inhibitor PD98059 for 1 hour prior to the addition of TGF-β1. Total cell lysate was separated into cellular and nuclear proteins using the NE-PER™ nuclear and cytoplasmic extraction reagents according to manufacturer's instructions (37). Cytosolic proteins were first extracted by disrupting cell membranes, followed by centrifugation. Intact nuclei were washed with cold PBS and then lysed with high-salt NE-PER™ buffer. Total protein was obtained by lysing cells with radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitor cocktails. Protein concentration was determined using the BCA protein assay reagent kit according to the manufacturer's instructions, and the cell lysates were stored at −80°C. Protein from the cell lysate (40 μg) was boiled in sample buffer and electrophoresed through a 4–20% gradient Tris-glycine ready gel and subsequently transferred to nitrocellulose membranes by immunoblotting. After 1 hour blocking in 5% BSA at room temperature, membranes were incubated with 1:1000 dilutions of goat-anti-CCN2, horseradish peroxidase–conjugated mouse-anti-GAPDH, rabbit-anti-phospho-p44/42 (phospho ERK), rabbit anti-p44/42 (ERK), rabbit-anti-phospho-Smad 3 (Ser 423/425) or rabbit-anti-Smad 2/3 at 4°C overnight, followed by incubation with HRP-conjugated rabbit-anti-goat or goat-anti-rabbit secondary antibodies (dilution 1:5000) for 1 hour at room temperature. Bands were visualised using an enhanced chemiluminescence detection system with dyed molecular-weight markers. Densitometric analysis of the bands was carried out with ImageJ Software (IH-Image – http://rsbweb.nih.gov/ij). The relative phosphorylated-ERK and phosphorylated-Smad 3 values were obtained from normalisation against the total ERK 1/2 or total Smad 2/3 values, respectively, or normalisation of CCN2 against GAPDH values.

Statistical analysis

Values are given as mean ± SD. Student's *t*-test was used when comparing two groups. Statistical comparison of more than two groups was performed using one-way analysis of variance (ANOVA) with Dunn's post hoc test. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad, LaJolla, CA), and *P* values of <0·05 were considered significant.

Results

CCN2 expression in vivo and in vitro

We have previously shown that CCN2 is expressed by the in-growing neoepidermis during wound healing (15). Full-thickness porcine wounds were allowed to heal, and the temporospatial expression of CCN2 was studied over a period of 22 days. The in-growing neoepidermis stained positive for CCN2 (Figure 1A). The expression of CCN2 was confined to the suprabasal layers of keratinocytes (Figure 1B). To assess the percentage of the wounds expressing CCN2, the ratio of CCN2-expressing neoepidermis to negative neoepidermis

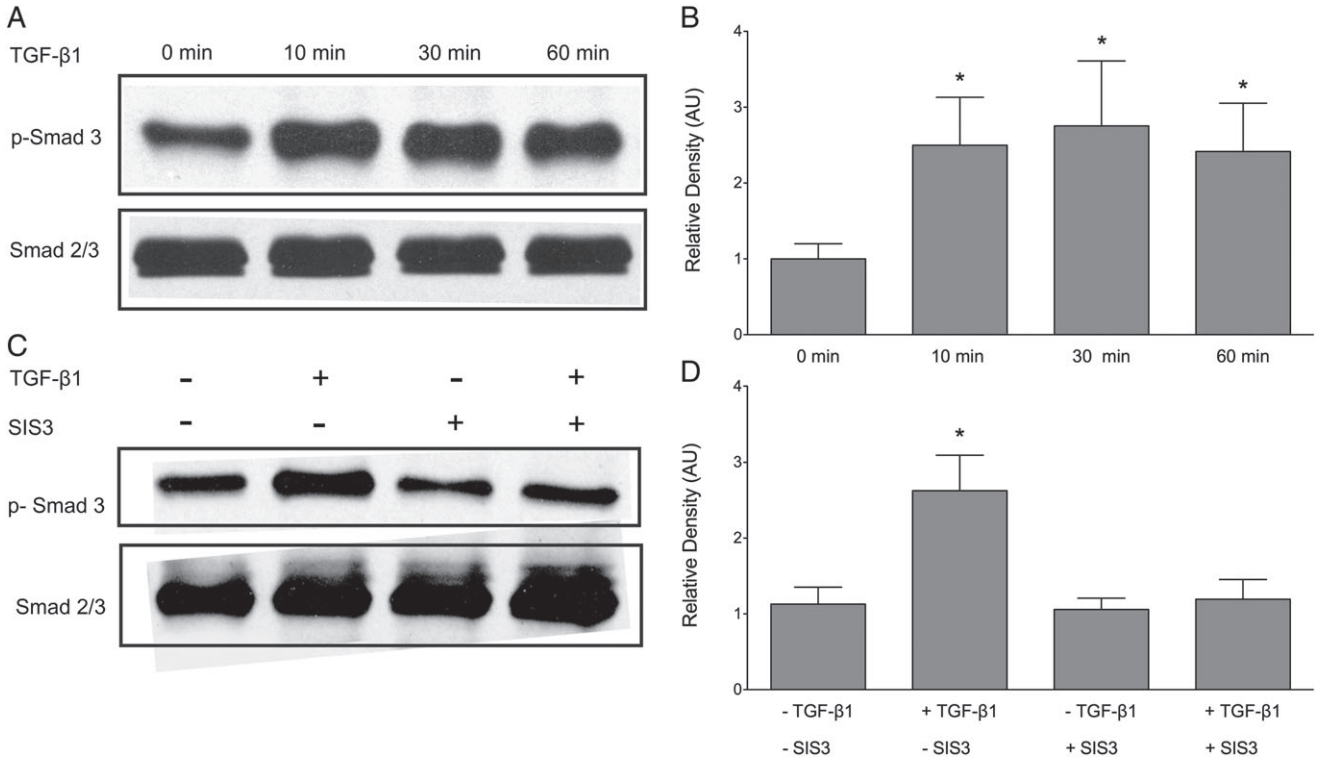

Figure 5 Transforming growth factor β1 (TGF-β1) activates SMAD signalling in keratinocytes. Keratinocytes were incubated with 10 ng/ml TGF-β1 for 0–60 minutes, and phosphorylated-Smad 3 was measured. (A) TGF-β1 induced the phosphorylation of Smad 3. Maximum phosphorylation was seen after 30 minutes. (B) The relative density of the bands was normalised to total Smad. (C) Pre-treatment with 10-μM Smad 3 inhibitor (SIS3) for 60 minutes blocked TGF-β1-induced Smad 3 phosphorylation. (D) The relative density of the bands was normalised to total Smad. One representative western blot out of three independently performed experiments is shown. Graphs show mean ± SD from three independent experiments, $n = 6$. * $P < 0{\cdot}05$ versus untreated control (0 minutes).

was calculated. On day 6, the wounds were 29 ± 7% reepithelialised, and the entire neoepidermis stained positive for CCN2 (Figure 2A and B). On day 10, the wounds were 51 ± 6% reepithelialised, and CCN2 expression started to diminish around the original border. On day 14, the wounds were fully resurfaced, and CCN2 was expressed in the central parts of the wounds in a 0·6:1 ratio. The central parts of the wounds remained positive throughout day 18, and there was no detectable CCN2 expression 22 days after wounding, indicating that CCN2 protein expression in epidermis is transient.

TGF-β1 induces CCN2 mRNA expression in keratinocytes

One of the most potent inducers of CCN2 is TGF-β1, which was recently shown to up-regulate the expression of CCN2 in corneal epithelial cells (17). To determine whether TGF-β1 induces CCN2 expression in human keratinocytes, cells were incubated with or without the addition of 1, 5 or 10 ng/ml TGF-β1 for 2 or 12 hours (Figure 3A). All concentrations of TGF-β1 led to an increase in CCN2 mRNA expression at 2 and 12 hours. Incubation with 1 ng/ml of TGF-β1 led to a 7·6 ± 1·9-fold increase in CCN2 mRNA expression at 2 hours ($P < 0{\cdot}001$) and a 5·5 ± 2·2-fold increase in CCN2 mRNA expression at 12 hours ($P < 0{\cdot}001$) compared to the untreated control. Incubation with 5 ng/ml of TGF-β1 led to a 8·1 ± 1·4-fold increase in CCN2 mRNA expression at 2 hours ($P < 0{\cdot}001$) and a 6·1 ± 1·7-fold increase in CCN2 mRNA expression at 12 hours ($P < 0{\cdot}001$) compared to untreated control. Incubation with 10 ng/ml of TGF-β1 led to an 8·4 ± 1·9-fold increase in CCN2 mRNA expression at 2 hours ($P < 0{\cdot}001$) and a 6·7 ± 1·8-fold increase in CCN2 mRNA expression at 12 hours ($P < 0{\cdot}001$) compared to untreated control. As maximal increase of CCN2 expression was observed after the addition of 10 ng/ml TGF-β1, this concentration was chosen for subsequent experiments.

To verify that increased expression of CCN2 by TGF-β1 is because of active transcription and not release of pre-existing CCN2 mRNA, keratinocytes were pre-treated with 5 μg/ml actinomycin D for 60 minutes prior to incubation with 10 ng/ml TGF-β1 for 2 hours (Figure 3B). Actinomycin D treatment blocked TGF-β1-induced CCN2 mRNA expression, suggesting transcriptional regulation of CCN2 by TGF-β1.

TGF-β1 enhances CCN2 protein production in keratinocytes

To determine whether TGF-β1-induced CCN2 mRNA expression was reflected by elevated synthesis of CCN2 protein, keratinocytes were cultured in the presence or absence of 10 ng/ml TGF-β1 for 24 hours, and CCN2 protein was detected by immunofluorescence. The results indicate that CCN2

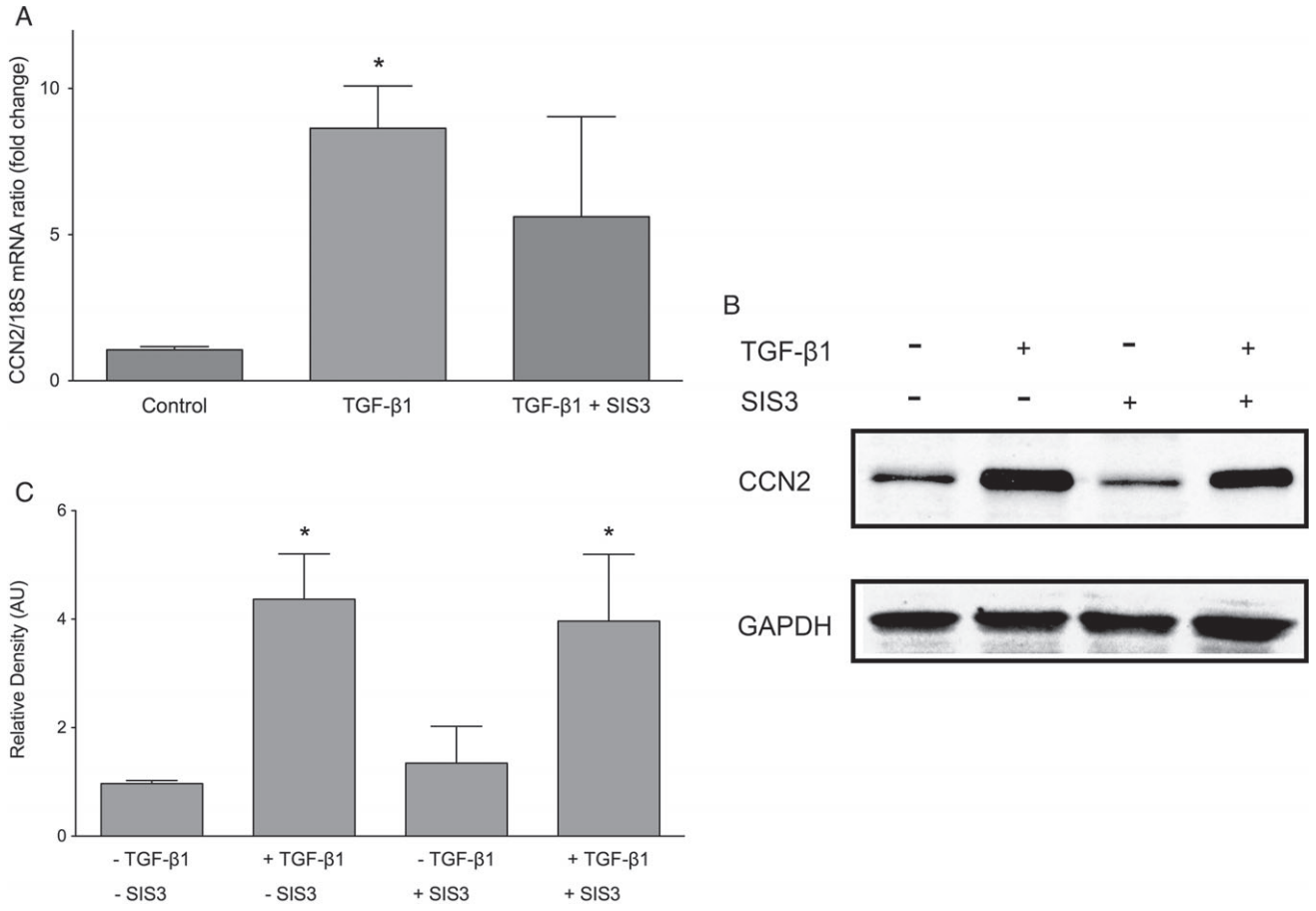

Figure 6 Effect of SIS3 on transforming growth factor β1 (TGF-β1)-induced connective tissue growth factor (CCN2) expression in keratinocytes. Keratinocytes were cultured with or without pre-treatment with 10-μM Smad 3 inhibitor (SIS3) prior to incubation with 10 ng/ml TGF-β1 for 30 minutes. (A) CCN2 mRNA expression was analysed by quantitative real-time PCR. Inhibition of Smad 3 only partially reduced TGF-β1-induced CCN2 mRNA expression. Graphs show mean ± SD from three independent experiments, $n = 9$. $^*P < 0{\cdot}05$ versus untreated controls. (B) Western blot results mirrored the mRNA data. One representative western blot out of three independently performed experiments is shown. (C) The relative density of the bands was normalised to GAPDH. Graphs show mean ± SD from three independent experiments, $n = 6$. $^*P < 0{\cdot}05$ versus untreated control (0 minute).

protein levels were relatively low in the absence of TGF-β1 (Figure 4A). The addition of TGF-β1 led to increased CCN2 production. The expression of CCN2 was primarily perinuclear, as described previously (Figure 4B) (38). Time course analysis of CCN2 expression showed CCN2 expression as early as 30 minutes after TGF-β1 addition (Figure 4C). Maximum levels were reached after 6 hours' stimulation with 10 ng/ml TGF-β1 ($3{\cdot}9 \pm 0{\cdot}5$-fold increase compared to $1{\cdot}0 \pm 0{\cdot}2$-fold with untreated control, $P < 0{\cdot}05$) (Figure 4D).

TGF-β1 activates the SMAD signalling pathway in human keratinocytes

To identify intracellular signalling pathways involved in the regulation of CCN2 by TGF-β1, keratinocytes were incubated with 10 ng/ml TGF-β1 for 0–60 minutes. Immunoblotting was performed on cell lysates, and phosphorylated Smad 3 was measured (Figure 5A). Smad 3 was chosen for this study as it has previously been shown that TGF-β1 induction of CCN2 does not occur in Smad 3 knockout cells (39). Phosphorylation of Smad 3 was detected after 10 minutes and by 30 minutes had reached a maximum (Figure 5A and B). Therefore, the 30-minute post-treatment time point was selected for subsequent analysis of Smad activation. Total Smad 2/3 remained constant at all time points. Next, we tested the effect of Smad 3-specific inhibitor SIS3 on TGF-β1-induced Smad 3 phosphorylation in keratinocytes. SIS3 is a selective inhibitor of TGF-β1-induced Smad 3 phosphorylation and Smad 3-mediated cellular signalling but has no effect on Smad 2 or ERK activation (40). Pre-treatment of keratinocytes with 10 μM SIS3 for 60 minutes blocked TGF-β1-induced Smad phosphorylation at 30 minutes (Figure 5C and D). Therefore, this time point was chosen for all subsequent studies of the involvement of Smad signalling in the induction of CCN2 expression.

TGF-β1-induced CCN2 production in human keratinocytes is in part mediated by Smad 3

To determine whether TGF-β1-induced CCN2 expression in human keratinocytes was mediated through Smad signalling, cells were incubated with 10 μM SIS3 for 60 minutes prior

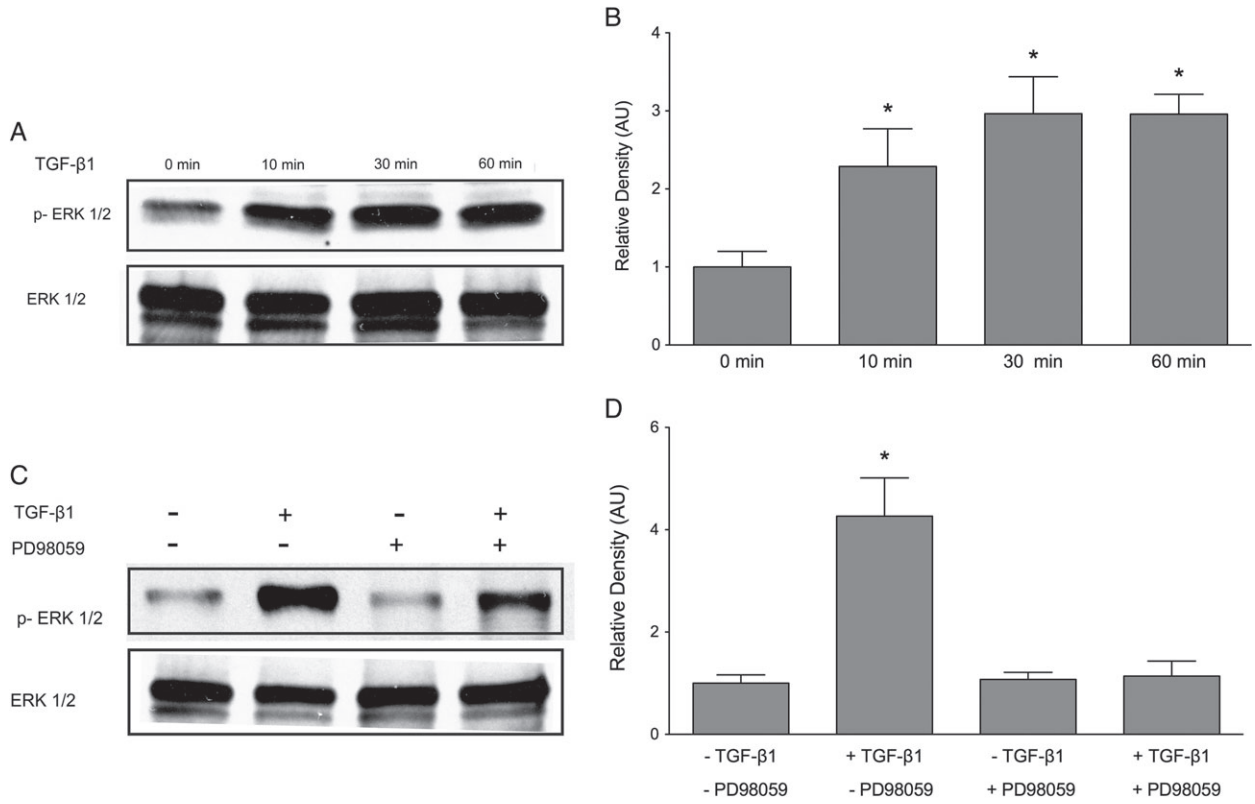

Figure 7 Transforming growth factor β1 (TGF-β1)-induced ERK phosphorylation in keratinocytes. Keratinocytes were incubated with 10 ng/ml TGF-β1 for 0–60 minutes, and phosphorylated ERK was measured. (A) TGF-β1 induced the phosphorylation of ERK as early as 10 minutes of incubation. One representative western blot out of three independently performed experiments is shown. (B) The relative density of the bands was normalised to total ERK. (C) Pre-treatment with MEK inhibitor PD98059 for 60 minutes blocked TGF-β1-induced ERK phosphorylation after 30 minutes' incubation with 10 ng/ml TGF-β1. (D) The relative density of the bands was normalised to total ERK. Graphs show mean ± SD from three independent experiments, $n = 6$.

to the addition of 10 ng/ml TGF-β1 for 30 minutes. Inhibition of Smad 3 only somewhat reduced TGF-β1-induced CCN2 mRNA expression, and this reduction was not statistically significant (Figure 6A). In accordance with the mRNA data, TGF-β1-induced CCN2 protein production is slightly impaired by SIS3 (Figure 6B and C). Taken together, the above findings indicate that TGF-β1-induced CCN2 production in human keratinocytes is only partially mediated by Smad 3, indicating that TGF-β1 might act through alternative pathways.

CCN2 induction by TGF-β1 in human keratinocytes requires ERK activity

Given that pathways other than Smad are known to mediate TGF-β1 signalling, we investigated the contribution of ERK to TGF-β1-induced CCN2. TGF-β1 activates MAPKs, including ERK, p38 and JNK, in a cell type-dependent manner, and ERK has been shown to be required for TGF-β1-induced CCN2 expression in corneal epithelial cells (17). To confirm that TGF-β1 activates the ERK pathway in keratinocytes, immunoblotting was performed to detect phosphorylated ERK. Incubation with 10 ng/ml TGF-β1 induced phosphorylation of ERK measurable after 10 minutes (Figure 7A). At 60 minutes, phosphorylation of ERK in response to TGF-β1 was still evident (Figure 7B).

PD98059 is a specific pharmacological inhibitor targeting MEK-ERK signalling. PD98059 prevents the phosphorylation of MEK, thereby inhibiting ERK (41). In keratinocytes, 60 minutes of pre-treatment with 20-μM PD98059 efficiently prevented TGF-β1-induced ERK activation at 30 minutes (Figure 7C). Therefore, this time point was chosen for all subsequent experiments involving ERK signalling in the induction of CCN2 expression. To determine whether the ERK signalling pathway plays a role in TGF-β1-induced CCN2 expression, keratinocytes were pre-treated with 20-μM PD98059 for 60 minutes prior to incubation with 10 ng/ml TGF-β1 for 30 minutes. Blockage of MEK phosphorylation potently reduced the ability of TGF-β1 to induce CCN2 mRNA and protein expression (Figure 8A–C). Collectively, these data suggest that activation of ERK signalling is necessary for TGF-β1-induced CCN2 expression.

Inhibiting ERK does not prevent Smad phosphorylation or nuclear translocation

As both Smad and ERK signalling pathways were found to be involved in TGF-β1-induced CCN2 expression in keratinocytes, we investigated the potential cross-talk between the Smad and ERK signalling pathways. Recent studies have suggested that ERK modifies TGF-β1-induced Smad

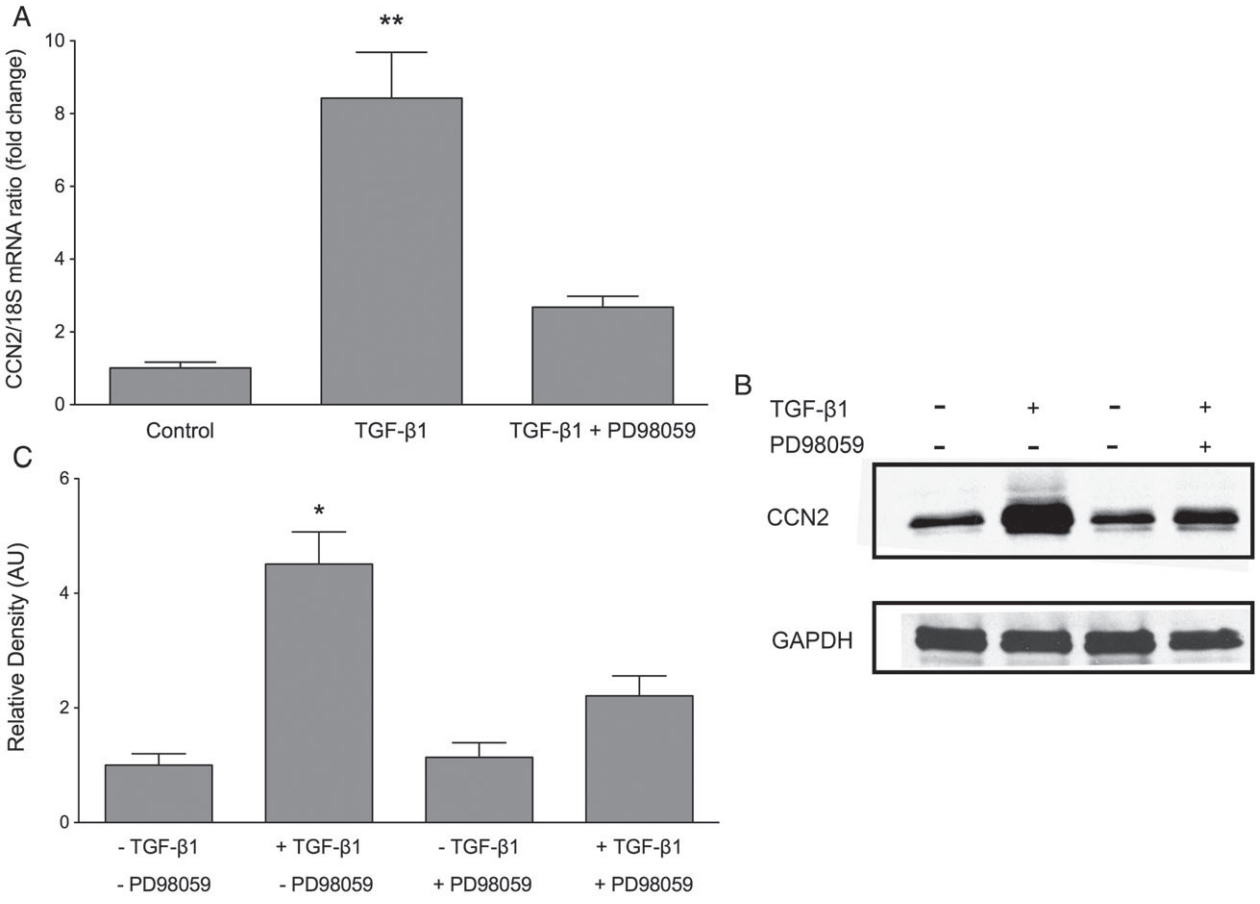

Figure 8 Effect of PD98059 on transforming growth factor β1 (TGF-β1)-induced connective tissue growth factor (CCN2) expression in keratinocytes. Keratinocytes were cultured with or without pre-treatment with MEK 1 inhibitor PD98059 prior to incubation with 10 ng/ml TGF-β1 for 30 minutes. (A) CCN2 mRNA expression was analysed by quantitative real-time PCR. Inhibition of MEK/ERK blocked TGF-β1-induced CCN2 mRNA expression. Data represent the mean ± SD for three separate experiments. Total $n = 9$, $^*P < 0{\cdot}05$ versus untreated controls. (B) Western blotting confirmed the mRNA data. One representative western blot out of three independently performed experiments is shown. (C) The relative density of the bands was normalised to GAPDH. Graphs show mean ± SD from three independent experiments, $n = 6$. $^*P < 0{\cdot}05$ versus untreated control.

signalling by directly phosphorylating Smad 3 (42). To investigate whether ERK is involved in Smad phosphorylation, TGF-β1-induced Smad 3 phosphorylation was studied after inhibition of ERK signalling. Keratinocytes were pre-treated with 20-μM PD98059 for 60 minutes prior to incubation with 10 ng/ml TGF-β1 for 30 minutes. TGF-β1-induced phosphorylation of Smad 3 was not blocked by PD98059 (Figure 9A and B), suggesting that ERK does not mediate TGF-β1-induced Smad 3 phosphorylation in keratinocytes.

Although inhibition of ERK did not reduce TGF-β1-induced Smad 3 phosphorylation, ERK might still regulate Smad signalling by affecting the nuclear translocation of phosphorylated Smad 3 complex. To examine the effect of ERK inhibition on the nuclear translocation of phosphorylated Smad 3, immunofluorescent stainings were conducted to visualise the location of phosphorylated Smad 3 following incubation with TGF-β1. Nuclei were stained with DAPI (blue) to correlate with the nuclear localisation of phosphorylated Smad 3 (red). The fluorescent signal for phosphorylated Smad 3 was undetectable in control keratinocytes cultured in medium (Figure 10A and D). After 30 minutes' incubation with 10 ng/ml TGF-β1, an intense fluorescent signal for phosphorylated Smad 3 was detected in the cell nuclei of keratinocytes (Figure 9B and E). Inhibition of ERK activation by PD98059 has no effect on the nuclear localisation of phosphorylated Smad 3 (Figure 10C and F). In addition to the immunofluorescence studies, nuclear translocation of phosphorylated Smad 3 was measured using western blot. Lamin A/C, a nuclear marker, was used as a control for sample loading and transfer. Keratinocytes were cultured with 10 ng/ml TGF-β1 for 30 minutes with or without PD98059 pre-treatment. Blocking ERK activation with PD98059 did not prevent the nuclear accumulation of phosphorylated Smad (Figure 11A and B). Collectively, these results suggest that inhibiting ERK does not prevent Smad phosphorylation or nuclear translocation.

Discussion

The skin's response to injury is rapid wound closure facilitated by the proliferation and migration of keratinocytes. The directed migration of keratinocytes is particularly important as reepithelialisation yields a cellular interface capable of

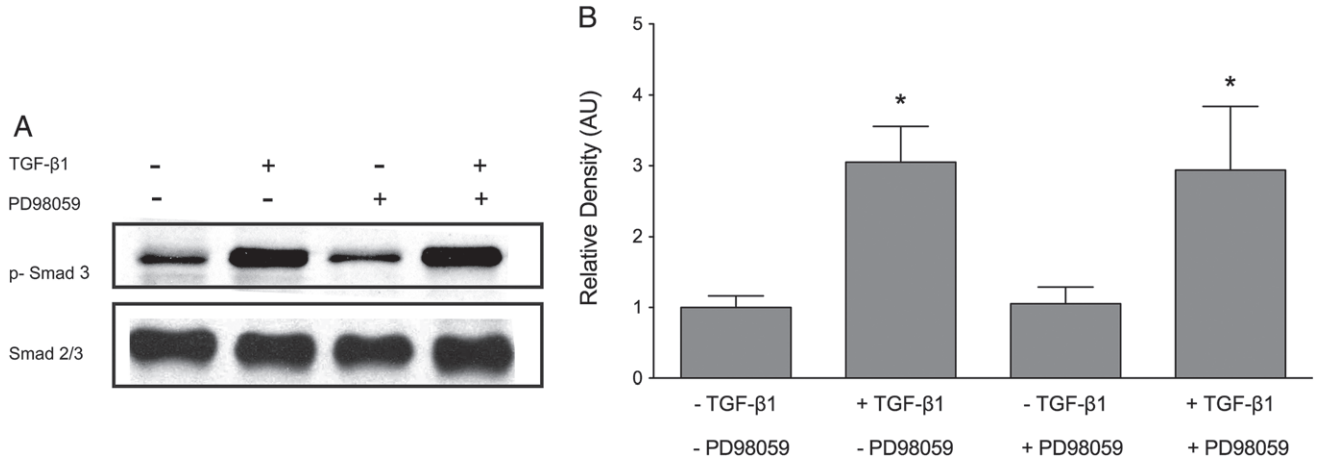

Figure 9 Inhibition of ERK does not prevent transforming growth factor β1 (TGF-β1)-induced Smad phosphorylation. Keratinocytes were cultured with or without pre-treatment with MEK 1 inhibitor PD98059 prior to incubation with 10 ng/ml TGF-β1 for 30 minutes. (A) Blocking ERK did not prevent TGF-β1-induced Smad phosphorylation. (B) The relative density of the bands was normalised to total Smad. One representative western blot out of three independently performed experiments is shown. Graphs show mean ± SD from three independent experiments, $n = 6$. $^*P < 0{\cdot}05$ versus untreated control.

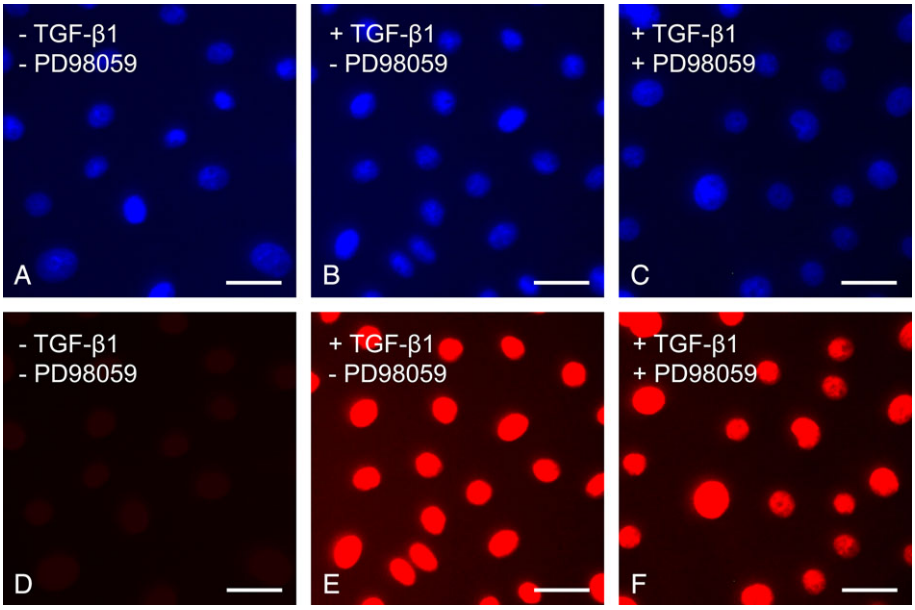

Figure 10 Inhibition of ERK does not prevent Smad nuclear translocation. Nuclear translocation of Smad was detected by immunofluorescence. Phosphorylated Smad 3 was labelled with red fluorescence and cell nuclei with blue fluorescence. (A, C) After 24 hours' culture in a serum-free control culture media, keratinocytes expressed low levels of phosphorylated Smad 3. (B, E) After the addition of 10 ng/ml transforming growth factor β1 (TGF-β1), phosphorylated Smad 3 was detected in the cell nuclei of keratinocytes. (C, F) Inhibition of ERK did not prevent nuclear translocation of phosphorylated Smad 3. The experiments were repeated three times with similar results. Scale bar equals 20 μm in A–F.

maintaining homeostasis in a changing environment. While numerous signalling molecules contribute to reepithelialisation, CCN2 has emerged as a regulator of cell motility in addition to its well-established roles in proliferation, differentiation, angiogenesis and maintenance of the extracellular matrix (17,43–48). The goal of the present study was to examine CCN2 expression in epidermal wound healing and potential involvement of the SMAD and ERK signalling pathways in response to TGF-β1. Using an in vivo porcine model, our study demonstrates that CCN2 is transiently expressed by the in-growing epidermis, strongly implicating a role for CCN2 in epidermal regeneration. We also demonstrate that in keratinocytes, CCN2 mRNA and protein are induced by TGF-β1 signalling through dynamic interactions between the SMAD and ERK signalling pathways.

With regard to wound healing, CCN2 is best known as a profibrotic factor that stimulates the production of matricellular proteins for dermal regeneration. It is currently being explored as a potential target for pro- and anti-fibrotic therapy, with the aims of achieving closure of non-healing wounds and reducing hypertrophic scarring, respectively. Thus, our findings are clinically significant as they confirm a contribution of CCN2 to epidermal wound healing and provide a foundation for two of the major signalling pathways involved. Attempts to alter the wound-healing process by modulating expression of CCN2 in the dermis must also consider possible unfavourable effects on the epidermis.

It has traditionally been thought that CCN2 is a downstream mediator of TGF-β1 in mesenchymal cells but not epithelial cells (9,16,39). For instance, in an early study by Fraizer *et al.*, TGF-β1 injections into the dermis of NIH Swiss mice did not

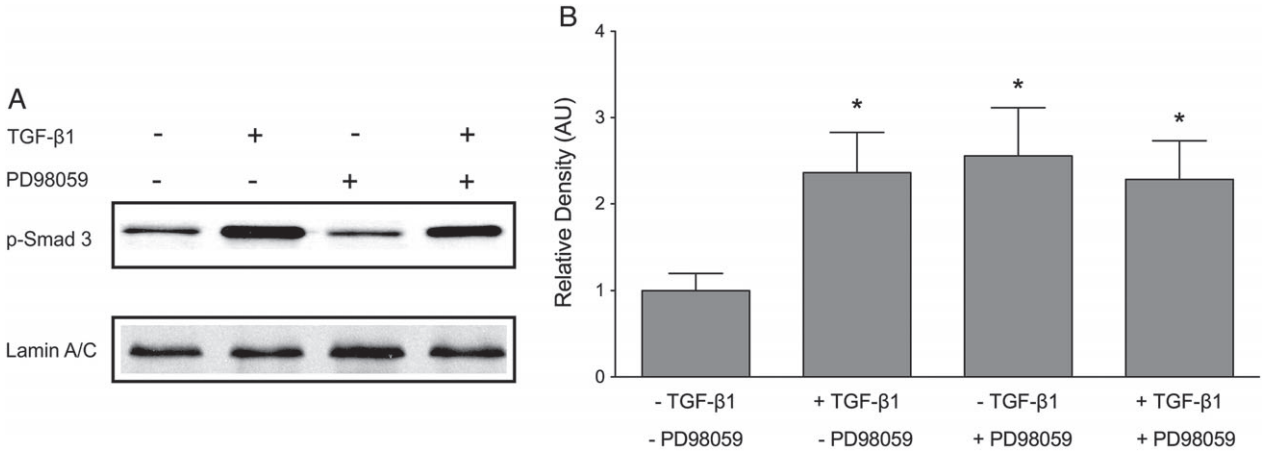

Figure 11 The effect of PD98059 on nuclear phosphorylated Smad 3. Nuclear translocation of phosphorylated Smad 3 was analysed using western blot. Keratinocytes were cultured with or without pre-treatment with 10-μM Smad 3 inhibitor (SIS3) prior to incubation with 10 ng/ml transforming growth factor β1 (TGF-β1) for 30 minutes. Nuclear protein was extracted as described in the Methods section, and lamin A/C was used as a control for sample loading and transfer. (A) TGF-β1 induced the phosphorylation of Smad 3. Inhibition of ERK did not prevent nuclear translocation of phosphorylated Smad 3. (B) The relative density of the bands was normalised to laminin A/C. One representative western blot out of three independently performed experiments is shown. Graphs show mean ± SD from three independent experiments, $n = 6$. $^*P < 0{\cdot}05$ versus untreated control.

result in any detectable CCN2 staining in the epidermis or endothelial cells of large vessels and capillaries (12). However, the authors expressed some doubt regarding the validity of their results, speculating that their injection model might not effectively mirror the wound-healing milieu. Another influential study by Grotendorst *et al.* showed that TGF-β1 induced a 25- to 30-fold increase in CCN2-tethered luciferase activity in transfected NIH/3T3 fibroblasts and foetal bovine smooth muscle cells but not the two epithelial cell lines tested, namely HepG2 hepatocytes and human breast epithelial cells (14). The notion that CCN2 expression is confined to mesenchymal cells was challenged when a number of studies documented CCN2 expression in epithelial cells, although these initial results were too inconsistent to immediately reverse the traditional perception. Conflicting data regarding Grotendorst's HepG2 findings were reported, with additional studies showing no CCN2 mRNA expression (49), no appreciable CCN2 transcript staining (50) or marked CCN2 expression in hepatocytes (51). More recently, the ability of hepatocytes to synthesise CCN2 was confirmed by detailed cell culture studies that showcased hepatic CCN2 expression in a TGF-β1-free environment. These results were strengthened when the expression of CCN2 was found to be up-regulated by TGF-β1 as expected (25,52). Similarly, another key finding that has been heavily explored is that CCN2 is not expressed by human breast epithelial cells (14). In a follow-up study, Frazier and Grotendorst assayed human invasive mammary ductal carcinomas with in situ hybridisation for CCN2. Again, transcripts were found in the fibroblasts of the stroma but not in tumour epithelial cells or leukocytes (13). Studies by others have produced conflicting data, suggesting that CCN2 mRNA and protein expression occurs in both normal and malignant breast epithelial cells (53). Collectively, these more recent studies appear to suggest that CCN2 is expressed and plays a role in the cellular activities of epithelial cells, and the present study contributes to this observation in the context of the keratinocyte.

CCN2 holds great therapeutic potential not just for aberrant wound healing but also for the development of novel diagnostics and treatments for cancer. Studies have associated CCN2 with breast cancer (54), prostate cancer (55), glioma (56), lung cancer (57) and, most notably, pancreatic cancer (58) and metastatic melanoma (20). Clinical specimens of select pancreatic carcinomas display up to 50-fold over-expression of CCN2 secondary to aberrant TGF-β signalling (59,60). This over-expression manifests as a desmoplastic reaction that facilitates unregulated proliferation of the stromal microenvironment. The end result is a walled-off tumour with a reservoir of matricellular growth factors to sustain growth and development (61). Targeting the stromal microenvironment by modulating expression of CCN2 may thus offer a personalised approach for treatment and diagnosis. Similarly, clinical specimens of metastatic melanoma have also been associated with over-expression of CCN2 secondary to aberrant TGF-β signalling. One recent study found that melanoma cell lines with upregulated CCN2 displayed more invasive and migratory behaviour compared to melanoma cell lines expressing normal levels of CCN2 (19). Non-malignant melanocytes displayed similar changes in behaviour when transfected with recombinant CCN2, indicating that the presence or absence of CCN2 may orchestrate cellular activity in otherwise normal cells. Another study similarly found that CCN2 was up-regulated in the majority of tested melanoma cell lines, which were amenable to treatment with anti-CCN2 antibody FG-3019 in vivo (20).

The present study not only highlights the basic mechanism of TGF-β-induced CCN2 expression in keratinocytes but also underscores the potential implications for CCN2-centred therapies in wound healing and cancer. While CCN2 has great therapeutic potential, its diversity of functions and variable expression between cell types indicates that multiple molecular pathways are likely involved in regulating CCN2 activity. Additional studies focusing on the role of alternative molecular

pathways in keratinocyte as well as other cell types will be critical in developing therapeutic agents targeting CCN2.

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