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CCN4/WISP1 controls cutaneous wound healing by modulating proliferation, migration and ECM expression in dermal fibroblasts via α5β1 and TNFα

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Abstract

Understanding the mechanisms that control cutaneous wound healing is crucial to successfully manage repair of damaged skin. The goal of the current study was to uncover novel extracellular matrix (ECM) components that control the wound healing process. Full thickness skin defects were created in mice and used to show CCN4 up-regulation during wound-healing as early as 1 day after surgery, suggesting a role in inflammation and subsequent dermal migration and proliferation. To determine how CCN4 could regulate wound healing we used *Ccn4*-KO mice and showed they had delayed wound closure accompanied by reduced expression of *Col1a1* and *Fn* mRNA. Boyden chamber assays using *Ccn4*-deficient dermal fibroblasts showed they have reduced migration and proliferation compared to WT counterparts. To confirm CCN4 has a role in proliferation and migration of dermal cells, siRNA knockdown and transduction of CCN4 adenoviral transduction were used and resulted in reduced or enhanced migration of human adult dermal fibroblast (hADF) cells respectively. The induced migration of the dermal fibroblasts by CCN4 appears to work via α5β1 integrin receptors that further stimulates down-stream ERK/JNK signaling. The regulation of CCN4 by TNF-α prompted us look further at their potential relationship. Treatment of hADFs with CCN4 and TNF-α alone or together showed CCN4

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The authors declare no conflict of interest.

counteracted the inhibition of TNF-α on *COL1A1* and *FN* mRNA expression and the stimulation of TNF-α on *MMP-1* and *MMP3* mRNA expression. CCN4 appeared to counterbalance the effects of TNF-α by inhibiting downstream NF-κB/p-65 signaling. Taken together we show CCN4 stimulates dermal fibroblast cell migration, proliferation and inhibits TNF-α stimulation, all of which could regulate wound healing.

Introduction

Cutaneous wound healing is a highly orchestrated process involving several overlapping steps all of which can be influenced by the extra-cellular matrix (ECM) [1,2]. The first events begin with clot formation and subsequent hemostasis to stabilize the injured area. An inflammation phase follows that is characterized by infiltration of neutrophils and macrophages which produce cytokines and other immune regulators that will help prevent infection of the exposed area. The wounded area is closed over by the concerted differentiation, proliferation and migration of skin cell progenitors that control both re-epithelialization by keratinocytes or ECM formation by fibroblasts. The composition of repairing skin contains a multitude of ECM components including collagens, collagen crosslinking enzymes, proteoglycans, fibronectin, matricellular proteins [3] as well as molecules involved in ECM turnover including MMP's, ADAMTS and their inhibitors the TIMPs [4]. In certain pathological conditions, cutaneous wounds have delayed healing or fail to progress through the normal series of reparative events causing a disease burden [5]. Understanding more about the contribution of individual ECM components to cutaneous injury repair is crucial to establish a solid foundation for future developments designed to aid in wound healing.

One category of ECM molecules implicated to have roles in skin biology is the CCN family. It is named by its founding members CCN1/**C**yr61, CCN2/**C**TGF, CCN3/**N**ov. It was since expanded to include three more members: CCN4/WISP-1, CCN5/WISP-2, and CCN6/WISP-3 [6]. Each CCN protein is comprised of four distinct functional domains [7]. These domains have significant homology and functional similarity to other proteins and growth factors. Domain I has an insulin growth factor binding (IGFBP) like sequence; domain II has significant similarity to the core domain of the von WIllebrand factor type cII (VwfcII) and contains the consensus-binding motif for the BMP antagonist, chordin [8] as well as numerous integrin binding motifs [9]. Domain III has homology to the thrombospondin II domain while the fourth and final, C terminal (CT) domain, contains more integrin-binding motifs and a cysteine-knot region similar to those found in BMP/TGF-βs, and BMP/TGF-β inhibitors such as noggin [10]. Considering their complex modular structure, it is not surprising that these proteins play a role in a variety of biological processes such as embryonic development, tissue repair, and tumor growth and spread [3]. Previous studies showed that CCN1 and CCN2 regulate dermal fibroblast proliferation *in vitro* and are important in wound healing *in vivo* [9, 11–15]. While we and others showed that CCN4 is important in the musculoskeletal system where it regulates both osteogenesis and chondrogenesis [16–18], the role of CCN4 in cutaneous wound healing remains unclear.

In this study, we examined the expression pattern of all CCN family members during wound healing using a mouse model of full-thickness skin defect *in vivo*. Our findings showed a high level of CCN4 expression in early stages post-injury which prompted us to further investigate whether CCN4 could, possibly, regulate the wound healing process. In this report, we present evidence that CCN4 aids wound closure by regulating migration and proliferation of dermal fibroblasts through the actions of integrin α5β1 and subsequent ERK signaling. A modulatory role for CCN4 in TNF-α induced ECM mRNA expression was also uncovered pointing the way to new avenues for developing improved therapies for delayed wound closure relying on CCN4.

RESULTS

Ccn4 mRNA is up-regulated during skin wound healing

To determine the relative expression of the six Ccn family members we extracted mRNA from normal mouse skin and performed real-time RT-PCR. This showed that all *Ccn* family members were present, and that the expression level of *Ccn2* gene was the highest compared with the other members (Fig. S1). Next, we examined whether the expression levels of *Ccn* family members were altered during the wound healing process. Towards this goal we prepared a full-thickness skin defect in mice, extracted mRNA and carried out real-time RT-PCR as above. These experiments showed that the relative expression levels of *Ccn1*, *Ccn2* and *Ccn6* were not significantly changed (Fig. 1A,B,F) while *Ccn3*, *Ccn4* and *Ccn5* mRNA levels were significantly increased during wound healing but with different temporal patterns; *Ccn4* and *Ccn5* expression peaked by day 4, while *Ccn3* expression showed its highest level at day 14 (Fig. 1C–E). In comparison, the mRNA expression levels of inflammatory cytokines, *Tnf-α* and interleukin-1β (*Il1-β*), reached their peak on day 1 and decreased to normal levels at day 4, a time that corresponded to the rapid and early inflammation phase that ensues in this surgical model (Fig. 1G,H). Our data showed that *Ccn4* mRNA expression was dramatically up-regulated just after the inflammation phase, with a peak at day 4 (Fig. 1D). Next, we analyzed the expression pattern of type-I collagen (*Col1A1*) and fibronectin (*Fn*) genes, which are the major components of ECM in skin and are considered markers of dermal tissue healing [19]. As expected, the expression of these two genes was significantly increased in a time-dependent manner and achieved their peak at later stages, day 7 and day 4, respectively (Fig. 1I, J). At day 14, we found that the skin defect was completely regenerated, and that the mRNA levels of *Col1a1* and *Fn* returned to levels found in normal tissue (day 0).

To determine the localization of Ccn4 protein during wound healing process, we performed both H&E staining and immunohistochemistry of skin samples collected 1 day after surgery where an open wound is observed (Fig. 1K–N arrow). IHC analysis using antisera against Ccn4 revealed it was abundantly expressed, in a location coincident with inflammatory cells with characteristics of neutrophils in the healing dermis (Fig. 1N, arrowheads). Taken together, these results indicate that Ccn4 is up-regulated during the inflammation period of wound repair. Based on these results, we postulated that Ccn4 could be an important factor mediating the interplay between inflammatory, proliferative and remodeling phases of wound healing.

CCN4 regulates cutaneous wound healing and ECM production

To test the hypothesis that Ccn4 plays a role in cutaneous wound healing *in vivo*, we created uniform 15 mm incisions in the skin and evaluated wound healing by measuring the extent of wound closure in WT compared to *Ccn4*-KO mice (Fig. 2A). Our experiment showed that in *Ccn4*-KO mice wound closure was significantly reduced one day after wound healing and that this trend continued until 7 days post-surgery (Fig. 2B,C). The depletion of Ccn4 was confirmed with IHC showing that Ccn4 was not present in the skin in *Ccn4*-KO mice (Fig. 2D, arrows show normal expression in WT while the *Ccn*-KO had no comparable signal). To evaluate the extent of ECM production in WT and *Ccn4*-KO mice, real-time RT-PCR analysis was performed. This experiment showed that *Col1a1* and *Fn* mRNA expression levels were lower in *Ccn4*-KO mice compared to *WT* mice not only in normal skin (day 0), but also during wound healing (Fig. 2E,F). The most significant differences for *Col1a1* were found day 1 and day 7 after surgery and for *Fn* 4 days after surgery.

CCN4 promotes dermal fibroblasts migration and proliferation

Because dermal fibroblast migration and proliferation is known to play an important role in homeostasis and synthesis of ECM during wound healing in skin (19), we hypothesized that CCN4 might regulate their migration or proliferation. To test this, we collected adult dermal fibroblasts (mADFs) from the skin of both WT and *Ccn*4-KO mice and used an outgrow method to examine their cell behavior. As can be seen in Fig. 3A WT cells expanded a greater distance than the *Ccn*4-KO cells that was quantified in Fig. 3B. To test cell migration more directly, we next moved to Boyden chambers which confirmed that the relative migration of mADFs derived from *Ccn4*-KO mice was reduced compared to WT derived cells (Fig. 3C). In addition to defects in migration, the *Ccn4*-KO mADFs also had reduced proliferation compared to WT cells (Fig. 3D) which could have also contributed to the slower cell expansion shown in Fig. 3A. To further confirm our findings on the role of Ccn4 in ADF cell migration we silenced the *CCN4* gene in hADFs using siCCN4 or over-expressed CCN4 using adenovirus encoding CCN4 (adCCN4) loss and gain of function approaches respectively. Both approaches effectively lowered or raised CCN4 expression respectively judged by mRNA or protein production (Fig. S2). Altered cells were then subject to migration tests using the Boyden chamber or proliferation analysis using MTS assays. In agreement with our experiments with murine *Ccn4*-KO cells the migration and proliferation abilities of silenced hADFs were diminished compared to WT controls (Fig. 4A,C,E). Interestingly overexpression of CCN4 enhanced the hADF cell migration (Fig. 4B,D) but did not have an effect on proliferation (Fig. 4F). These data indicated that CCN4 regulated skin wound healing by controlling the migration and proliferation of dermal fibroblasts.

CCN4 promotes hADFs migration through α5β1-ERK directed signal transduction

To understand the mechanisms by which CCN4 could regulate hADFs migration we again employed a Boyden chamber assay in conjunction with recombinant human CCN4 protein (rhCCN4). We first determined the optimal concentration of rhCCN4 that would enhance of hADFs migration. These experiments confirmed that cell migration was upregulated by rhCCN4 treatment (Fig. 5A). In all subsequent experiments, 100 ng/mL of rhCCN4 was then

used. Previous work from our lab indicates that integrin α5β1 is one of the many functional receptors for CCN4 in human bone marrow stromal cell function [18]. To determine whether integrin α5β1 plays a role in CCN4-promoted hADFs migration, we selectively inhibited α5β1 using an integrin α5β1 antibody. Our data showed that the effect of CCN4 on cell migration was neutralized by treatment with integrin α5β1 antibody but not when anti-integrin α5β3 antibody was used (Fig. 5B). Because other members of the CCN family were proposed to regulate MAP kinase signaling pathways [20], we used western blotting to test whether CCN4 could activate the MAP kinase signaling pathway in hADFs. Stimulation with CCN4 enhanced p-ERK and p-JNK phosphorylation with maximum induction occurring within 15 minutes of treatment (Fig. 5C). Interestingly, p-ERK signaling stimulated by CCN4 was blocked by treatment with α5β1 antibody but not in IgG controls (Fig. 5D). CCN4 stimulated hADFs treated with the MEK/ERK inhibitor PD98059 showed reduced cell migration (Fig. 5E) providing further evidence for the importance of p-ERK signaling the CCN4 control of human dermal fibroblast migration. Taken together these data indicated that CCN4 promoted hADFs migration through an ERK-integrin α5β1 regulated signaling pathway.

CCN4 regulates ECM mRNA expression with TNF-α treatment

Previous reports showed that TNF-α could induce CCN4 expression and, at the same time, attenuate TNF-α-induced collagen production and fibroblast proliferation in cardiac fibroblasts (21). In our current investigation, we found that *Ccn4* mRNA expression was up-regulated 4 days post injury (Fig. 1D), just after TNF-α reached peak expression and the inflammation phase (Fig. 1G). Based on these observations, we hypothesized that CCN4 could mediate TNF-α function in dermal fibroblasts. To test this hypothesis, we first examined the mRNA expression of CCN4 in hADFs before and after TNF-α stimulation. This experiment showed that TNF-α stimulated *CCN4* expression in a time-dependent manner, with the most significant induction seen 12 h after stimulation. Next, we analyzed the expression levels of ECM mRNA encoding *COL1A1* and *FN* as well as *MMP-1* and *MMP-3* in hADFs treated with TNF-α. TNF-α significantly suppressed the mRNA expression of *COL1A1* and *FN* after 24 h (Fig. 6B,C). While CCN4 on its own had little effect on the ECM when supplemented to hADFs (Fig. S3) we found that it could rescue the decreased mRNA expression of *COL1A1* and *FN* caused by TNF-α stimulation (Fig. 6B,C). Treatment of CCN4 also suppressed TNF-α-induced mRNA expression of *MMP-1* and *MMP-3* (Fig. 6D,E). To identify the mechanisms by which CCN4 may regulate TNF-α in hADFs, we analyzed the activation of the downstream pathways involved in TNF-α signaling: NF-κB/p-65. Our findings showed that NF-κB/p-65 phosphorylation provoked by TNF-α was inhibited by CCN4 in a dose-dependent fashion (Fig. 6F). Taken together, these data suggest that CCN4 regulates ECM remodeling by ameliorating TNF-α-induced *COL1A1* and *FN* repression and MMP-1 and MMP-3 induction.

Discussion

While much is known about the composition of ECM during cutaneous wound healing, many questions remain about the precise ways in which these components regulate each other and subsequently this biological process. For example, Type I collagen, the most

abundant ECM macromolecule in the skin [2] has clear structural roles that are modulated by associating partners such as decorin [21]. Decorin, in turn, can bind to CCN2 and by doing so inhibit its biological activity [22]. These observations illustrate the dynamic nature of the ECM, where there is interplay between different family members that could, in turn, control tissue repair and function. The goal of our investigation was to identify novel members of the ECM that could control cutaneous wound healing and determine their mechanism of action in this ECM regulatory interplay of function.

Previous studies suggested certain members of the CCN family could affect cutaneous wound healing [9, 11–15, 23,24]. To identify novel regulatory components in this family we examined the expression of all 6 CCN family members after skin incision and found that Ccn4 was significantly induced as soon as one day post-surgery. The upregulation of *Ccn4* mRNA expression in skin wounds was recently confirmed with using unbiased array approach in which myofibroblast enriched cell populations had 50-fold increase in Ccn4 mRNA expression 7 days post-injury [25]. To further assess CCN4 function in skin repair, we used *Ccn4* depleted mice to show they had delayed cutaneous healing that was accompanied by reduced expression of *Col1a1* and *Fn* mRNA lending support to the regulatory interplay which causes an “ECM cascade”. A major challenge is to understand what other factors regulate this cascade and to determine how this affects dermal cell and tissue function.

An important cellular event in the skin healing process is the proliferation and migration of dermal fibroblasts. Previous studies showed that CCN4 regulates the proliferation and migration of human vascular smooth muscle cells (VSMC) [26, 27] but its role in dermal fibroblasts had not been previously determined. In our current study, we used gain and loss of CCN4 function strategies in conjunction with migration assays to show CCN4 regulates the migration process in mouse and human dermal fibroblasts. Considering the early effects of CCN on skin healing it is possible that CCN4’s function is actually more important for migration than for regulating ECM expression however additional experiments are needed to verify this concept including a thorough analysis of ECM protein production in the presence and absence of CCN4.

It is generally accepted that integrins are key cell surface receptors for the CCN family [9]. To understand which integrin might be involved in dermal fibroblast function, integrin blocking experiments were performed and showed that α5β1 was needed and, further, required an active ERK/JNK pathway for its function. We do not know at this time how CCN4 functions at the cell surface and if, for example, it could compete or actually augment fibronectin binding to α5β1; this will be important to determine in the future. Interestingly, α5β1 is also needed for CCN4 stimulation of VSMC migration [26]. CCN4 was also found to control the migration of the prostate cancer cell line PC-3 [28], suggesting there could be a common role for CCN4 in enhancing cell mobility.

The ECM has critical roles in modulating growth factor and cytokine activity. One pro-inflammatory cytokine thought to be important early in the wound healing process is TNF-α. The exact role of TNF- α in the skin repair is not precisely known but is speculated to be involved in the epithelial mesenchymal transition in human skin [29]. TNF-α is an early

response gene in skin damaged by ultraviolet light [30] and appears to be regulated by a cis-responsive element containing a putative AP-1 site the in the TNF- α promoter [31]. TNF-α is also up-regulated in the autoimmune diseases cutaneous lupus erythematosus (CLE) discoid lupus erythematosus (DLE) and dermatomyositis (DM) [32]. How TNF-α works to regulate wound healing is not entirely clear but in this regard it is interesting to note that TNF-α regulates both CCN2 [33] and CCN4 (this study). Previous reports show that CCN4 mediates TNF-α induced proliferation of cardiac fibroblasts while, at the same time, inhibiting cardiomyocyte death [21]. In this study, we show that CCN4 can counterbalance the effects of TNF-α on *Col1a1*, *FN*, *MMP-1* and *MMP-3* mRNA expression by interfering with TNF-α induced p65 signaling. The localization of CCN4 to sites of inflammatory cell infiltration soon after injury supports the concept that it could have a role in the early inflammatory response to cutaneous damage. It will be important to next understand the exact nature of the inflammatory cells that localize with CCN4 during cutaneous wound healing and, further, determine how they might influence fibroblast behavior using co-culture systems containing polymorphonuclear cells (PMNs) and dermal fibroblasts.

Despite the progress that has been made in connecting CCN4 to wound healing many biological questions remain. To begin, other than TNFα, (shown in the current study and previously by Liu et al., [26]) not much is known about what regulates the expression of CCN4 with wound healing. mRNA profiling of human skin cells treated with ascorbic acid (AA) shows that in addition to *COL1A1* mRNA there is a parallel and accompanying increase in CCN4 in response to AA. In pulmonary fibrosis, *CCN4* mRNA is upregulated by TGF-β and can be reduced by miR-92a [34]. *CCN4* is also upregulated by wnt activation which is the origin of its former name (WISP1/wnt induced secreted protein) [35, 36]. In this context, it is interesting to note that in addition to being a target of wnt signaling, CCN4 also controls wnt activation, leading to a biological “feed forward” biological loop [17]. While there are reports that CCN4 might control cell survival by regulating human marrow stromal cells apoptosis [37] or autophagy in traumatic brain injury [38] it is unknown whether CCN4 is involved in these cell processes during cutaneous wound healing.

In summary, we show that CCN4 is needed for cutaneous wound healing and that it regulates proliferation and migration of dermal fibroblasts via a mechanism that requires functional α5β1 receptors. Considering CCN4 it is upregulated in both skin and in oral mucosa wounds [39] it is possible that WISP1/CCN4 could be potentially used to treat wounds with delayed healing caused by underlying genetic factors or other pathological conditions such as diabetes, infection, obesity or poor nutrition [5].

Experimental Procedures

Animal experiments

Ccn4-knock out (*Ccn4*-KO) mice were generated by inserting a 1.7 kb PGK neo cassette in exon 2 as described previously [17]. The creation and expansion of the *Ccn4*-KO mice was carried out in the Gene Targeting Facility of the NIDCR, NIH, under an institutionally approved protocol (#12-655). All wound healing and cell isolation experiments were performed using protocols and guidelines approved by the Animal Care and Use Committee at the NIDCR of the NIH (#12-655). Eight-week-old female mice (C57BL/6 WT mice and

Ccn4-KO mice) were anesthetized and the dorsal skin was shaved aseptically. For analysis of gene expression patterns during skin wound healing, full-thickness skin defect of 6 mm diameter was created using a tissue punch in the center of the back. Using the same size punch samples were collected at 0, 1, 4, 7 and 14 days after surgery, skin tissues of the wound healing process were corrected using same size of tissue punch. For the comparisons of dermal wound healing in WT-mice vs, *Ccn4*-KO mice, a 15-mm straight incision was made in the skin located on the back of the mice (See Figure 2A). At 1, 4, 7, 10 and 12 days post-surgery, a photo of wound healing site was taken, and the level of skin wound healing was measured using ImageJ (ImageJ, JAVA-based free software) in a blinded fashion. The wounded skin including 5 mm margin around healing site was collected for mRNA isolation and real time RT-PCR analysis described below.

Histological analysis

Skin samples were fixed with 4% Paraformaldehyde (PFA) and embedded in paraffin and 5 μm sections were stained with hematoxylin and eosin. For immunohistological analysis of Ccn4, deparaffinized and hydrated sections were blocked with 3% hydrogen peroxide to quench endogenous peroxidase activity, then blocked with 5% normal goat serum (Life Technologies, Carlsbad, CA, USA) for 60 min at room temperature. Sections were incubated at 4°C overnight with primary antibody against Ccn4 (1:100 dilution; LF-187; Dr. Larry W. Fisher, NIH) and SuperPicture HRP Polymer Conjugate Broad Spectrum kit (AEC) (ZYMED Laboratories, Inc., Carlsbad, CA, USA) was used for detection. Samples were counterstained with hematoxylin.

Cells and cell culture

Human adult dermal fibroblasts (hADFs) were purchased from DS Pharma Biomedical (Osaka, Japan). hADFs were cultured in high glucose Dulbecco's Modified Eagle's Medium (D-MEM, Life technologies, Carlsbad, CA, USA) containing 15% FBS (Life technologies, Carlsbad, CA, USA), 2 mM L-glutamine (Life technologies, Carlsbad, CA, USA), 100 units/mL penicillin (SIGMA, St. Louis, MO, USA),100 mg/mL streptomycin (SIGMA) (culture medium) at 37°C in 5% CO_2. Cells at the 5–8th passages were used for the experiments.

For the knockdown of *CCN4* mRNA expression, 10 nM of siRNA targeting the *CCN4* gene (StelthTM SiCCN4; Life Technologies) was transfected into hADFs using Lipofectamine RNAiMAX (Life Technologies), following the manufacturer's instructions. StelthTM RNAi negative control high GC Duplex (Life Technologies) was used as the negative control. For the overexpression of the *CCN4* gene, hADFs were transfected with adenovirus expressing human CCN4 (adCCN4) as reported previously [16–18]. Adenovirus with a CMV promoter minus the CCN4 coding sequence (adCMV) was used as the negative control. After 48h, transfected hADFs were used for the analysis.

Mouse adult dermal fibroblasts (mADFs) were collected using an outgrow method. Briefly, the hypodermis layer and associated blood vessels were scraped off the collected skin using a scalpel and the dissected skin was cut into 2–3 mm pieces. 4–5 pieces were placed in a culture dish and incubated for 3 min to allow attachment. After the incubation, culture

medium was gently added to each dish. Photographs of the expanding cells were taken to estimate relative cell migration from the skin sample and 10 days after cultivation, mADFs were collected by Trypsin EDTA (Life technologies) for proliferation and migration analysis.

Real-time RT-PCR analysis

The collected skin was homogenized using Lysing Matrix D (MP Biomedicals, Illkrich, France) and processed using a FastPrep™ FP120 instrument (Funakoshi, Tokyo, Japan). After homogenization, total RNA was extracted using TRIzol® Reagent (Life technologies) using conventional procedures and purified using a PureLink® RNA Mini Kit (Life technologies). The total RNA from cultured cells was extracted and purified using a PureLink® RNA Mini Kit.

RNA samples were reverse-transcribed using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Real time RT-PCR was performed to quantify the expression of the target genes using KAPA Sybr Fast qPCR Master Mix (KAPA Biosystems, Wilmington, MA, USA) and a CFX96 thermo cycling system (Bio Rad, Hercules, CA, USA). Expression levels were normalized to that of the reference gene S29. Primer sequences for both human and mouse target genes are shown in Table S1.

MTS assay

To estimate the proliferation capacity of the dermal fibroblast, an MTS assay that measures the number of viable cells was used following manufacturer's guidelines (CellTiter 96 AQueous One Solution, Promega, Madison, WI, USA). Specifically, cells were plated and cultured for 3 days and the MTS reagent applied for 1 hour after which the proliferation efficiency was determined by measuring the optical absorbance at 490 nm with a microplate reader (Bio Rad, Hercules, CA, USA).

Migration assays

To examine cell migration Boyden chamber assays were carried out using multi-well chemotaxis chambers (pore size 8 μm: BD, Franklin Lakes, NJ, USA). The mADFs or hADFs in 0.1% FBS DMEM (5×10^4 cells/well) were added to the upper chamber. 0.1% FBS DMEM with or without recombinant human CCN4 (rhCCN4; Pepro Tech, Rocky Hill, NJ) was added to the lower chamber. Six hours after plating, the upper surface of the filter of each well was rubbed with a cotton swab to remove the cells that had attached but had not migrated onto the bottom surface. The filter was fixed in 4% PFA for 10 min, stained with diamidino-2-phenylindole (DAPI: Life technologies, Waltham, USA). The number of migrated cells was counted in four randomly selected fields using fluorescence microscope (Biozero BZ-8000, Keyence Corp., Osaka, Japan).

For the inhibition assays, hADFs were pre-incubated for 30 min with either 10 ug/mL of anti-integrin α5β1 antibody (JBS5; Millipore, Temecula, CA, USA), 10 ug/mL anti-integrin αVβ3 antibody (LM609; Millipore) or with 10 μM of MEK/ERK inhibitor (PD 98059; Calbiochem, San Diego, CA, USA). Cell migration assay and western blotting assay were carried out as described.

Western blotting analysis

Total cellular protein was prepared by lysing cells in M-PER (Mammalian Protein Extraction Reagent; Thermo, Waltham, MA, USA) supplemented with a Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA). 20 microgram protein samples were separated by SDS–PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 1% BSA/TBS, each membrane was incubated over night at 4 °C with one of the following antibodies: anti-ERK (1:2000; Cell Signaling Technology, Boston, MA, USA), p38 MAPK (1:2000; Cell Signaling Technology), JNK (1:2000; Cell Signaling Technology), NF-κB p65 (1:2000; Cell Signaling Technology), Phospho-ERK (1:2000; Cell Signaling Technology), Phospho-p38 MAPK (1:2000; Cell Signaling Technology), Phospho-JNK (1:2000; Cell Signaling Technology), Phospho-NF-κB p65 (1:2000; Cell Signaling Technology) and β-actin (Sigma, St. Louis, MO, USA) which was used as the loading control. Membranes were then washed, and incubated with goat anti-rabbit lgG-HRP (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat anti-mouse lgG-HRP (1:2000; Santa Cruz Biotechnology) for 1 h at room temperature. The blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce-Thermo Scientific, Rockford, IL, USA), and visualized with Image Quant LAS 4000 mini (Fujifilm, Tokyo, Japan).

Statistical analysis

The results obtained from quantitative experiments were reported as the mean values ± SD. Statistical analyses were performed with one-way factorial ANOVA followed by Tukey's multiple comparison tests, two-way factorial ANOVA followed by Tukey's multiple comparison tests or Student's unpaired t-tests. $P < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *CCN4* is up-regulated during cutaneous wound healing
- *Ccn4* deficient mice have delayed wound healing accompanied by reduced expression of Co1A1 and Fibronectin
- *CCN4* regulates migration and proliferation of dermal fibroblasts in culture
- CCN4 mediated dermal cell migration requires functional α5β1receptors and pERK signaling
- TNF-α stimulates CCN4 which in turn modulates TNF-α regulation of *Col1A1*, fibronectin, *MMP-1* and *MMP-3*

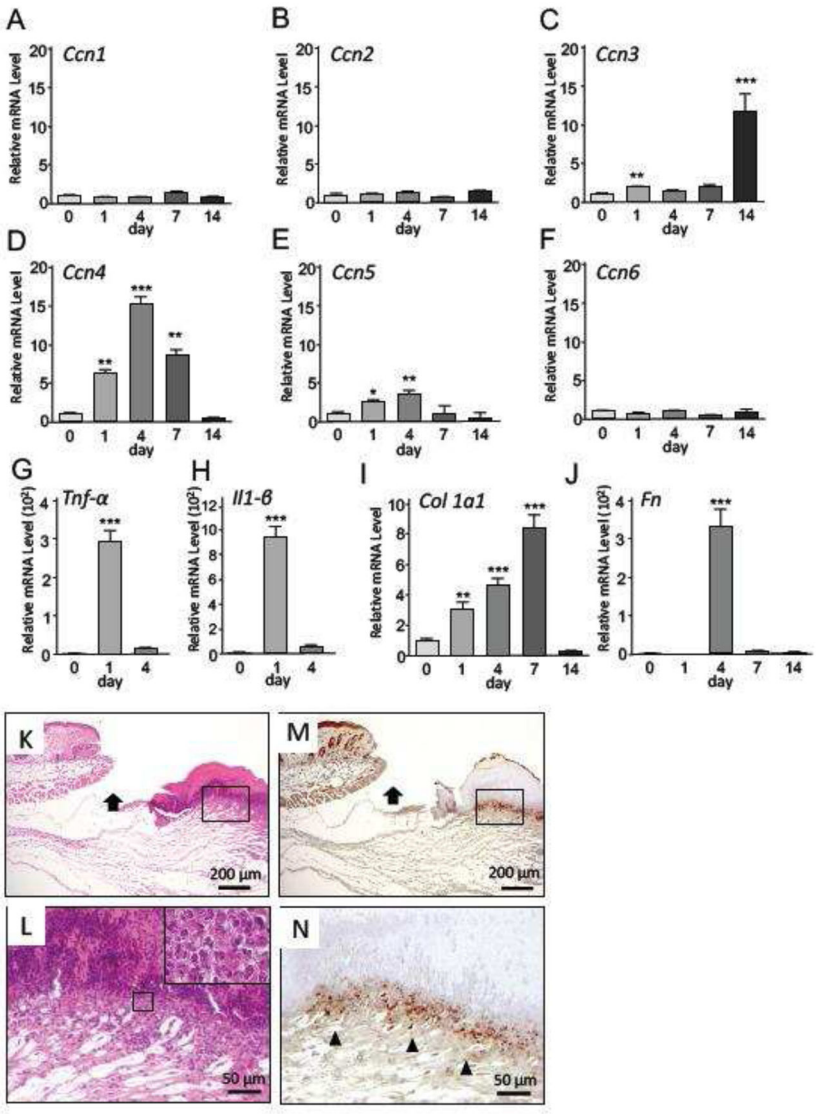

Figure 1. Enhanced expression of CCN4 during skin wound healing

A–J: Relative mRNA expression of *Ccn* family genes **(A–F)**, inflammation markers **(G–H)** and ECM components **(I–J)** following full-thickness skin wounds in mice. Skin samples were obtained from normal skin (day 0) and at 1, 4, 7 and 14 days post-surgery. Gene expression was normalized to the ribosomal protein *S29*. Bars represent the means +/− SD (n=3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (One way ANOVA/Tukey), compared to day 0 sample. **K–N:** Skin samples were obtained at 1 day after full-thickness skin wounds were created, and analyzed by H & E staining **(K–L)** and for localization of CCN4 by immunohistochemistry **(M–N)**. Black arrows show the edge of the wound healing site. Arrowheads show the localization of CCN4 at the healing site, specifically surrounding neutrophils in the dermis. Results are representative of at least three independent experiments.

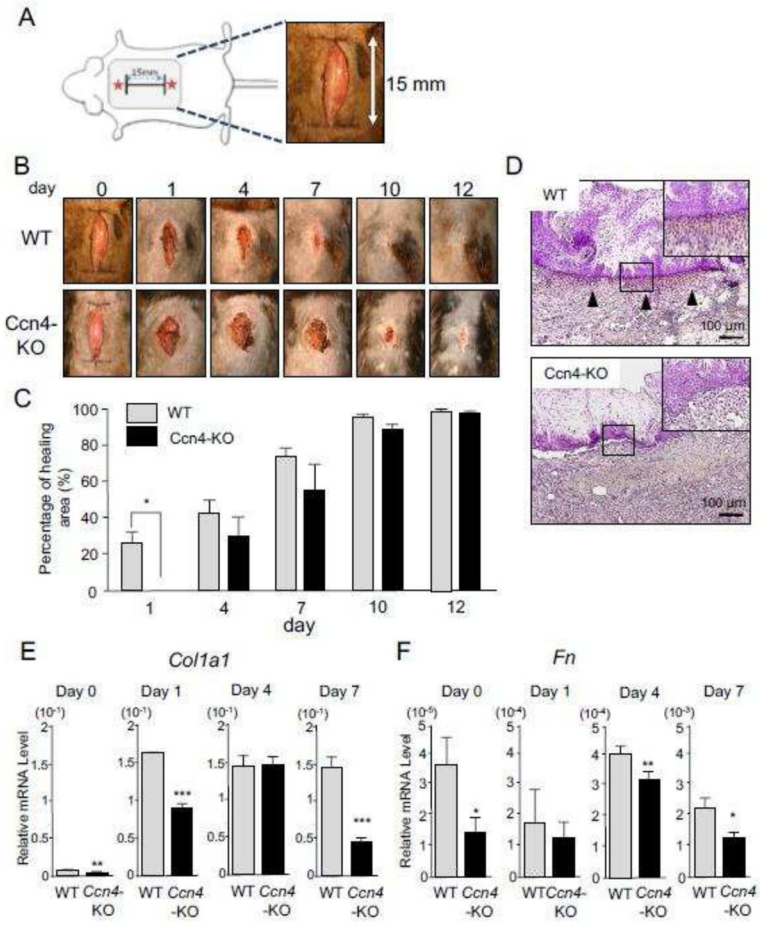

Figure 2. Delayed cutaneous wound healing in *Ccn4*-KO mice

(A) Schematic illustration of this wound healing model. **(B)** Representative photograph of the skin wound healing site after 1, 4, 7, 10 and 12 days. **(C)** The relative percentage of healing is shown for each time point **(D)** Skin samples obtained 7 days after full-thickness skin wounds analyzed for localization of CCN4 protein by immunohistochemistry. Arrowheads show the localization of CCN4 at the healing site only found in WT mice. **(E–F)** Expression pattern of ECM components following full-thickness skin wounds. Skin samples were obtained from normal skin (day 0) and from wounded skin at 1 and 7 days post-surgery. The gene expression levels were normalized to *S29*. Bars represent the means +/− SD (n=3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (student t-test).

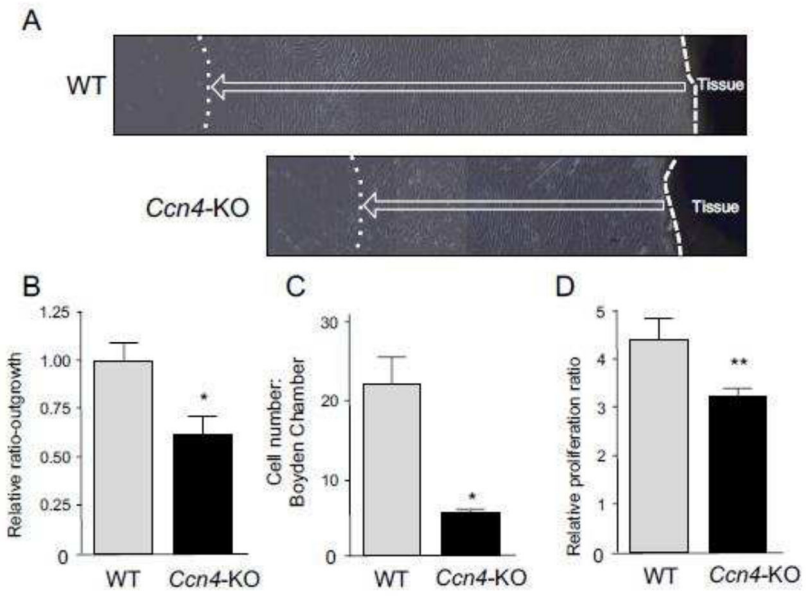

Figure 3. Decreased cell migration and proliferation of mADFs derived from *Ccn4*-KO mice

(A) Cell expansion assay showing relative migration of WT vs *Ccn4-KO* derived dermal fibroblasts from right (tissue source) to left (where cells migrated to). **(B)** Quantification of the relative migration of *Ccn-4-KO* compared to WT cells using an outgrowth assay. **(C)**. Migration of mADFs WT and *Ccn4*-KO mADFs using Boyden chamber assay. **(D)** Relative proliferation of mADFs WT and *Ccn4*-KO mADFs estimated by an MTS assay. Data are representative of 2 independent experiments performed. Bars represent the means +/− SD (n=3). * $p < 0.05$, ** $p < 0.01$ (student t-test).

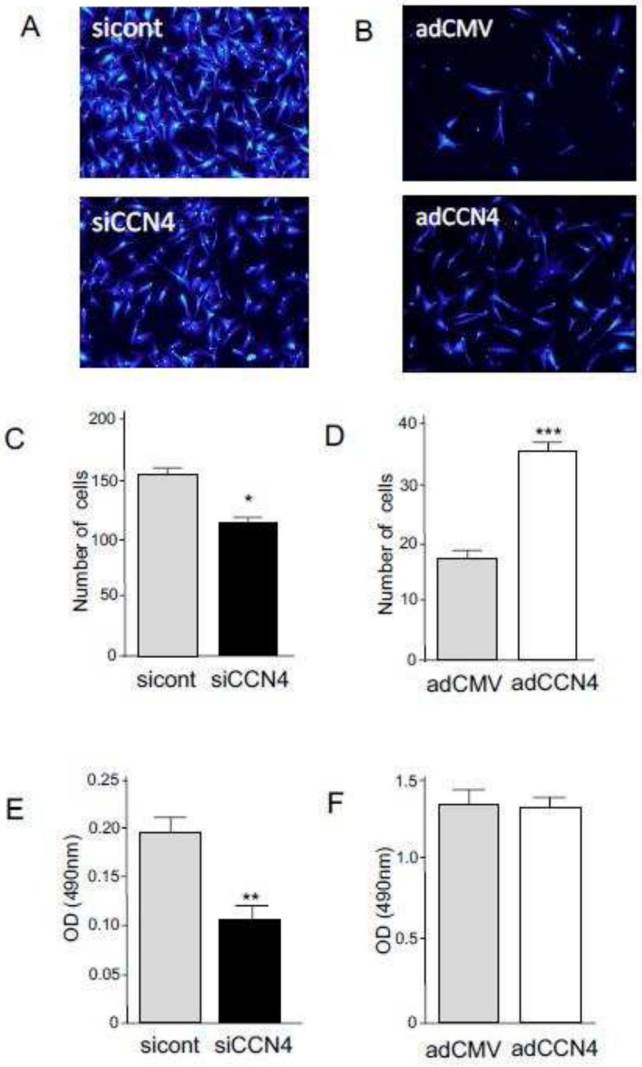

Figure 4. Effects of CCN4 knockdown and overexpression on dermal cell migration and proliferation

Human adult dermal fibroblasts (hADFs) were transduced with sicont (silence control) or siCCN4 **(A, C, E)** or adCMV (control) or adCCN4 **(B, D, F)** and cultured for two days. The degree of cell migration was evaluated using a Boyden Chamber **(A–B**, representative images; **C–D** quantification of migrated cells) and the degree of cell proliferation was evaluated by MTS assay **(E, F)**. Data are representative of 3 independent experiments. Bars represent the means +/− SD (n=3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (student t-test).

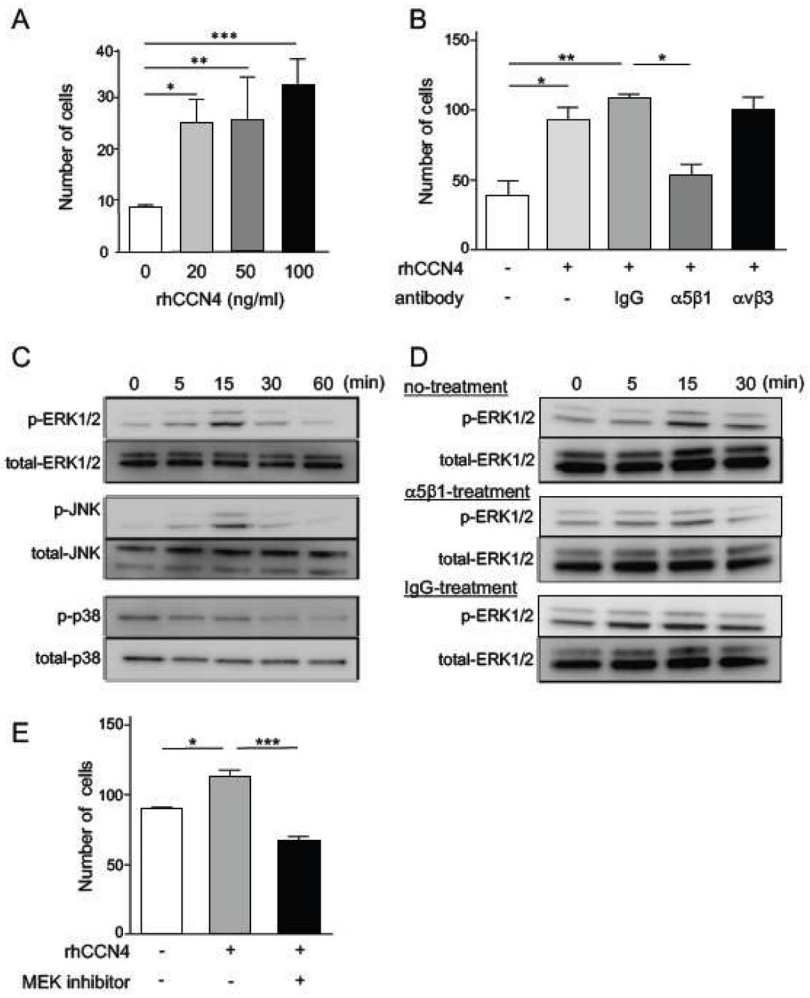

Figure 5. CCN4 promotes hADFs migration through integrin α5β1-ERK signaling

(A) Boyden chamber assays were performed on hADFs with several concentrations of rhCCN4. **(B)** Cells were pre-incubated with functional blocking antibodies against either the integrin α5β1 or integrin α5β3 for 30 min, and cell migration analyzed using Boyden chambers. Mouse IgG was used as control. **(C)** hADFs were cultured with serum free medium prior to the addition of 100 ng/mL of CCN4, and western blotting performed to assess phosphorylation of ERK1/2, JNK and p38. **(D)** hADFs were pre-treated for 30 min with 10 μg/mL of anti-integrin α5β1 antibodies followed by 100 ng/mL of rhCCN4 followed by and western blotting **(E)** Cells were pre-incubated for 30 min with 10 μM of MEK inhibitor followed by Boyden chamber analysis. Data are representative of 3 independent experiments. Bars represent the means +/− SD. * $p < 0.05$, *** $p < 0.001$ (One way ANOVA/Tukey).

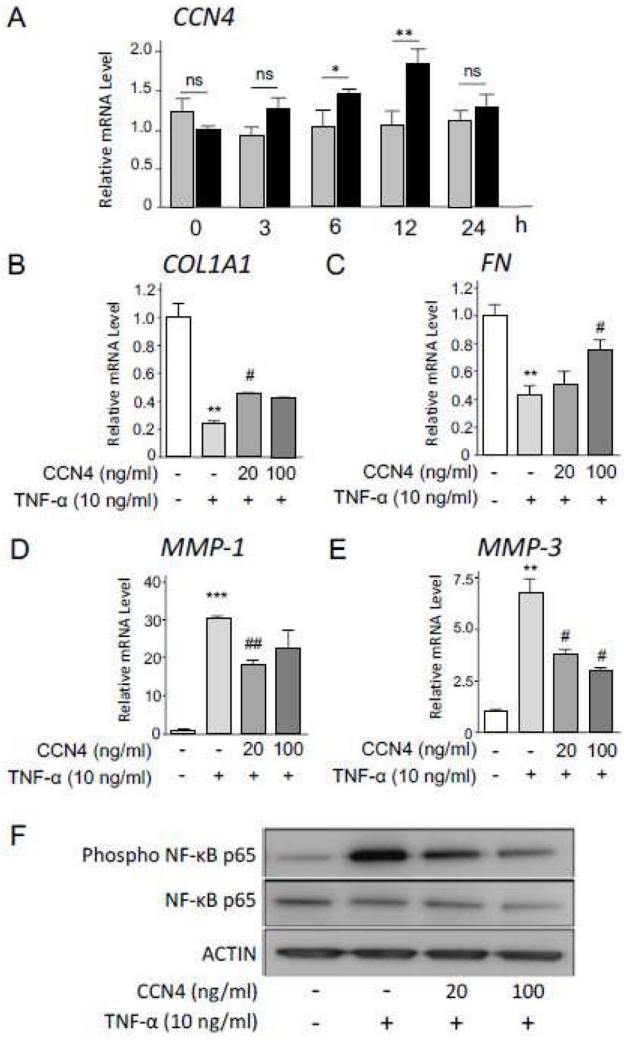

Figure 6. The effect of TNF-α on CCN4 expression in hADFs

(A) hADFs were treated with 10 ng/mL of TNF-α for 3, 6, 12 and 24 h. Expression level of *CCN4* was measured by real time RT-PCR. Bars represent the mean values and standard deviation +/− SD (n=3). ** $p < 0.01$, *** $p < 0.001$ (One way ANOVA/Tukey), compared to non-treatment sample. **(B–E)** hADFs were treated with or without TNF-α for 24 h. Expression levels of *COL1A1*, *FN*, *MMP-1* and *MMP-3* were measured 24 h post-treatment by real time RT-PCR. Bars represent the means +/− SD (n=3). ** $p < 0.01$, *** $p < 0.001$ (One way ANOVA/Tukey), compared to control sample. # $p < 0.05$, ## $p < 0.01$ (One way ANOVA/Tukey), compared to TNF-α-treated group. **(F)** hADFs were treated with CCN4 with TNF-α and or without CCN4 and cellular proteins collected at 30 min after stimulation. NF-κB p65 signals were detected using western blotting. Results are representative of at least two independent experiments.