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RESEARCH ARTICLE

CCN2 induces cellular senescence in fibroblasts

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Abstract The expression of *Ccn2* (*CTGF*) has been linked to fibrosis in many tissues and pathologies, although its activities in fibroblastic cells and precise mechanism of action in fibrogenesis are still controversial. Here, we showed that CCN2 can induce cellular senescence in fibroblasts both in vitro and in vivo, whereupon senescent cells express an anti-fibrotic "senescence-associated secretory phenotype" (SASP) that includes upregulation of matrix metalloproteinases and downregulation of collagen. Mechanistically, CCN2 induces fibroblast senescence through integrin $\alpha_6\beta_1$-mediated accumulation of reactive oxygen species, leading to activation of p53 and induction of $p16^{INK4a}$. In cutaneous wound healing, *Ccn2* expression is highly elevated only during the initial inflammatory phase and quickly declines thereafter to a low level during the proliferation and maturation phases of healing when myofibroblasts play a major role. Consistent with this expression kinetics, knockdown of *Ccn2* has little effect on the rate of wound closure, formation of senescent cells, or collagen content of the wounds. However, application of purified CCN2 protein on cutaneous wounds leads to induction of senescent cells, expression of SASP, and reduction of collagen content. These results show that CCN2 can induce cellular senescence in fibroblasts and is capable of exerting an anti-fibrotic effect in a context-dependent manner.

Keywords CCN1/CYR61 · CCN2/CTGF · Integrins · Wound healing · Cellular senescence

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Introduction

Cellular senescence is a stress response to a variety of cellular insults that include DNA damage, mitochondrial dysfunction, and oncogenic activation (Campisi 2013; Wiley et al. 2016). Senescent cells lose the ability to proliferate even in the presence of growth factors and nutrients, although they remain viable and metabolically active. A hallmark of senescent cells is expression of the senescence-associated secretory phenotype (SASP) that includes upregulation of a number of cytokines, growth factors, and matrix metalloproteinases (MMPs), as well as downregulation of extracellular matrix (ECM) proteins and their regulators such as collagen and TGF-β (Coppe et al. 2008). Substantial evidence indicates that senescent cells play a role in physiological changes associated with degenerative phenotypes of aging, and in the suppression or promotion of cancer (Campisi 2013; Chang et al. 2016). Recent studies have also shown that senescent cells contribute to tissue injury repair (Kim et al. 2013; Krizhanovsky et al. 2008) and wound healing (Demaria et al. 2014; Jun and Lau 2010b), where senescent cells can play an anti-fibrotic role.

Wound healing is a complex process that includes three temporally overlapping but functionally distinct phases: it begins with an inflammatory response with infiltration of neutrophils and macrophages, followed by granulation tissue formation with deposition of ECM by myofibroblasts, and concludes with a maturation phase in which the provisional ECM is degraded and remodeled to rebuild the architecture of the original uninjured tissue (Gurtner et al. 2008). In cutaneous wound healing, cellular senescence occurs initially in the proliferation phase in fibroblasts and endothelial cells to promote the formation of granulation tissue by the secretion of mitogenic growth factors (Demaria et al. 2014), whereas in the maturation phase myofibroblasts undergo senescence to

initiate matrix remodeling and limit fibrosis through the secretion of MMPs and downregulation of collagen (Jun and Lau 2010b). The matricellular protein CCN1 (CYR61) plays a critical role in wound healing by inducing myofibroblast senescence in the maturation phase through interaction with integrin $\alpha_6\beta_1$, thus activating the anti-fibrotic SASP to limit fibrosis (Jun and Lau 2010a; Jun and Lau 2010b).

CCN1 and CCN2 (CTGF) are two highly homologous members of the CCN family of matricellular proteins that share common structural features including conservation of four structural domains, alignment of all 38 cysteine residues, and binding sites for a number of integrin receptors (Chen and Lau 2009; Jun and Lau 2011). Not surprisingly, CCN1 and CCN2 exhibit many similar biochemical activities and biological functions in various cell types. For example, both CCN1 and CCN2 induce integrin $\alpha_6\beta_1$-mediated adhesive signaling in fibroblasts (Chen et al. 2001) and $\alpha_v\beta_3$-dependent angiogenic functions in endothelial cells (Babic et al. 1999; Leu et al. 2002). However, *Ccn1* and *Ccn2* knockout mice show different phenotypes (Ivkovic et al. 2003; Mo et al. 2002), and various studies have suggested that *Ccn1* and *Ccn2* may serve distinct functions in diverse physiological and pathological contexts (Hall-Glenn and Lyons 2011; Kubota and Takigawa 2015; Lau 2011). Thus, it is plausible to hypothesize that some of the distinct biological functions of CCN1 and CCN2 may be due to differences in their expression patterns, including the cell type and/or time course of their expression in a given context. However, there has not been specific data to support this hypothesis to date.

Here we show that CCN2, similar to CCN1, is capable of inducing cellular senescence in human skin fibroblasts through integrin $\alpha_6\beta_1$-mediated accumulation of reaction oxygen species (ROS) and activation of p53 and $p16^{INK4a}$ (Jun and Lau 2010b). However, *Ccn2* does not play a significant role in senescence induction in cutaneous wound healing because its expression is induced only in the inflammatory phase and not in the proliferation and maturation phases when myofibroblasts form the granulation tissue and subsequently stimulate matrix remodeling. By contrast, *Ccn1* is highly expressed in the maturation phase when matrix remodeling occurs, during which CCN1 induces myofibroblast senescence (Jun et al. 2015; Jun and Lau 2010b). Remarkably, when purified CCN2 protein is applied on cutaneous wounds during the maturation phase, it is able to induce cellular senescence and elicit the senescence-associated anti-fibrotic response. These findings illustrate that CCN2 can play a context-dependent role in restricting fibrosis, and provide new insights into the function and biology of CCN2.

Materials and Methods

CCN proteins and reagents

Recombinant CCN1 and CCN2 proteins were produced in Sf9 insect cells using a baculovirus expression system and purified by ion-exchange chromatography (Chen et al. 2001; Kireeva et al. 1997). Immunoblots of purified proteins were probed with specific anti-CCN1 and anti-CCN2 antibodies (Fig. 1a). Rabbit polyclonal antibodies against CCN1 and CCN2 were raised against the CCN1 vWC domain (Leu et al. 2003) and a glutathione-*S*-transferase (GST)-CCN2 fusion protein (Kireeva et al. 1997), respectively. Function blocking monoclonal antibody against α_6 integrin (GoH3) were from Chemicon, and rabbit and mouse IgGs were from Sigma. HRP-conjugated anti-rabbit secondary antibodies were from Amersham Biosciences. Adenoviral vectors Ad-LacZ and Ad-CCN2 (Yoon et al. 2010) were kind gifts from Dr. Woo Jin Park (Gwangju Institute of Science and Technology, South Korea) and amplified in 293 T cells.

Cell culture

Human BJ foreskin fibroblasts (ATCC# CRL-2522) were maintained at 37 °C in 5 % CO_2 in Eagle's minimum essential medium (EMEM) containing 10 % fetal bovine serum (FBS; Hyclone), 1 % penicillin/streptomycin, non-essential amino acids and sodium pyruvate. BJ cells stably infected with control lentivirus (LV) or lentivirus expressing shp53 or shp16 cells were as described (Jun and Lau 2010b). For adenoviral expression of CCN2, BJ cells were infected with recombinant adenoviral vectors for 2 h at a multiplicity of infection of 10 and cultured for 6 days. The cumulative population doubling (CPD) was calculated using the formula $n = (\log_{10}F - \log_{10}I)/\log_{10}2$, where F is the number of cells at the end of one passage, I is the number of cells that were seeded at the beginning of one passage, and n is the number of population doublings occurring in that passage. BJ cells were used between population doublings 30–45.

Analysis of cell proliferation and senescence

Senescence associated-β galactosidase (SA-βgal) assay was performed as described (Debacq-Chainiaux et al. 2009). Frozen wound tissues (6 μm) were fixed in 1 % formalin before staining, and then counterstained with Eosin. For growth curves, cells (1×10^4 cells per well) were grown in 12-well plates and treated as indicated. Cells were stained with 1 % Trypan blue (Invitrogen) and the total cell numbers counted. For Ki-67 immunostaining, cells in 8-well chamber slides (2×10^3 cells per well) were treated with CCN2 for 3 days and fixed using Carnoy's fixative (ethanol:acetic acid =3:1), washed and cells were stained with anti-Ki67 antibody

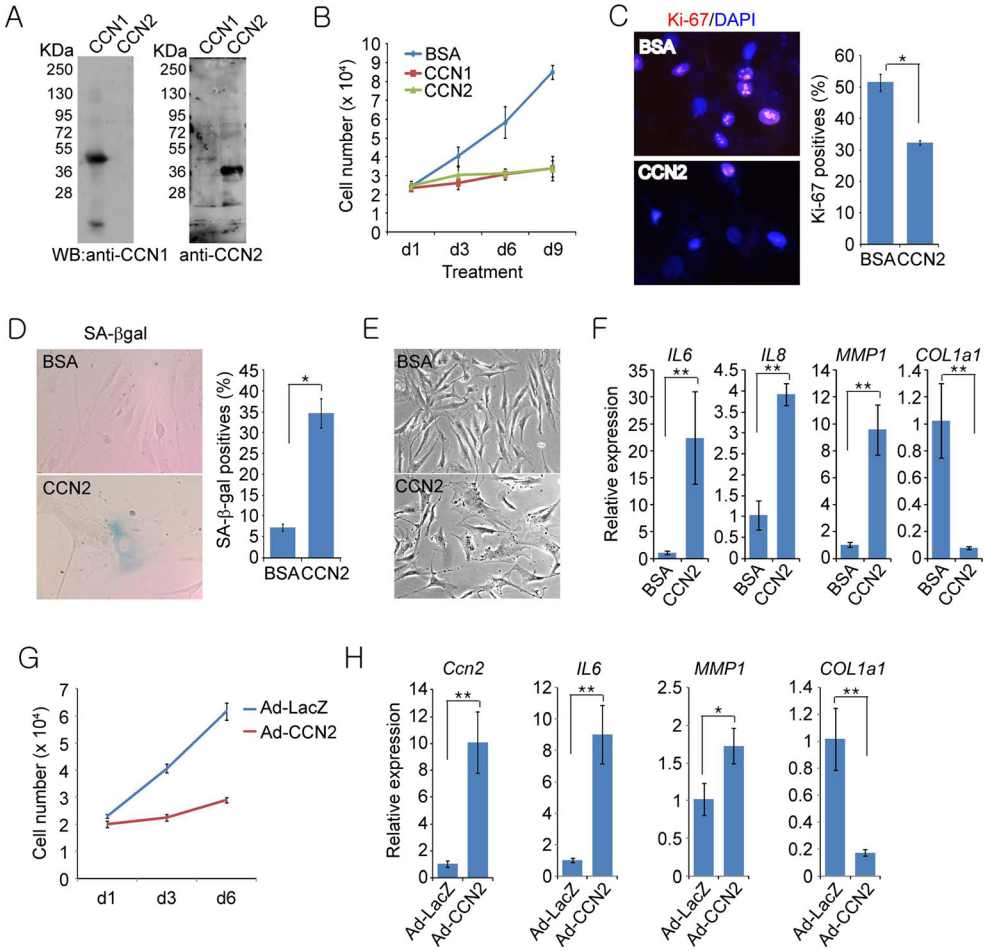

Fig. 1 CCN2 induces cellular senescence in normal human BJ fibroblasts. **a** Immunoblots of purified CCN1 and CCN2 protein probed with either anti-CCN1 or anti-CCN2 antibodies. **b** Human BJ fibroblasts treated with BSA, purified recombinant CCN1, or CCN2 (2.5 μg/ml each) were grown for indicated days and cell numbers counted using a haemocytometer. **c** After indicated treatment for 3 days, cells were subjected to immunostaining for Ki-67 and counterstained with DAPI. Cells positive for Ki-67 were counted in ten random fields and expressed as percentages of total cell number. **d** Cells were stained for SA-β-gal activity; positive cells were counted in ten random fields and expressed as percentages of total cell number. **e** Phase-contrast microscopy showing enlarged and flattened senescence morphology in cells treated with CCN2. **f** Total RNA was isolated from BJ cells treated with either BSA or CCN2 for 4 days and mRNAs for *IL6*, *IL8*, *MMP1*, and *COL1a1* were quantified by qPCR ($n = 4$ per time point). **g** BJ cells were infected with recombinant adenoviral vectors (Ad-LacZ and Ad-CCN2) at a multiplicity of infection of 10 for 2 h. Subsequently, cell numbers were counted at indicated days. **h** Gene expression at day 4 after adenoviral infection was quantified using qPCR ($n = 3$ per time point). Experiments were done in triplicates and data presented as means ± s.d. $^*p < 0.05$, $^{**}p < 0.01$

(Abcam), followed by Alexafluor594-conjugated anti-rabbit IgGs (Invitrogen). Fluorescence microscopy images were taken from five random fields in each well using a Leica DM4000B microscope with QImaging QIClick camera (Image pro Insight software) and Ki-67-positive cells were scored and normalized against 4,6-diamidino-2-phenylindole (DAPI; 1 mg/ml)-positive cells. All assays were done in triplicate, and approximately 300 cells were counted in each sample from randomly selected fields.

ROS measurement

H_2DCF-DA (Invitrogen) was used for ROS measurement as described (Chen et al. 2007). Briefly, cells plated on 8-well chamber slides were treated with CCN2 for indicated times. Cells were then incubated with either H_2DCF-DA (10 μM) for 10 min at 37 °C, washed with 0.5 % BSA-containing HANKS' balanced salt solution (HBSS). The fluorescence images were acquired using Leica DM IRB microscope and fluorescence intensity was calculated using ImageJ software (NIH).

Animals, cutaneous wound healing, and wound analysis

All experimental procedures with animals were approved by the Institutional Animal Care Committee of the University of Illinois at Chicago, where mice were housed in an AAALAC-accredited barrier facility. *Ccn1*$^{dm/dm}$ mice were generated as

described (Jun and Lau 2010b). C57BL/6 mice and *Ccn1*$^{dm/dm}$ mice (10 to 12-week-old male mice with 25–28 g body weight) were used for cutaneous wound healing. Full thickness excisional wounds (6 mm) through the panniculus carnosus were created as described (Jun and Lau 2010b). Wound diameters were measured from digital images of wounds using Photoshop CS2 (Adobe). For histological analyses, wound tissues were snap-frozen in OCT compound (Tissue-Tek) and sectioned serially (6 μm) using a Leica CM1950 UV cryostat. Sections were stained for SA-β-gal activity for senescence or with Sirius-red (Sigma) for collagen deposition, and counterstained with Hematoxylin/Eosin (H&E; Sigma). The number of SA-β-gal positives in the wound tissues were counted in 5 randomly-selected high powered fields.

Measurement of collagen deposition in wounds

For hydroxyproline assay, skin wounds were collected at indicated days and dried in 110 °C oven overnight, placed in a screw-capped glass vessel and hydrolyzed in 6 *N* hydrochloric acid at 110 °C for 18 h. The lysates were processed and hydroxyproline content determined as described (Edwards and O'Brien 1980), using purified hydroxyproline (Sigma) to generate a standard curve. Alternatively, frozen section of wound tissues were processed for Sirius red staining (Sigma) and viewed under polarized light. The Sirius red positive areas were calculated using Image J software (NIH).

Antisense oligonucleotide treatment

Antisense oligonucleotide against *Ccn2* was prepared in PBS and injected intravenously (I.V.) (200 μg per injection) through the retro-orbital sinus starting from one day before wound creation to day 2 post-wounding. Sense oligonucleotides were used as control. The sequences of oligonucleotides are: antisense, 5′-CGACGGAGGCGAGCAT-3′ and sense, 5′-ATGCTCGCCTCCGTCGC-3′.

Total RNA isolation and qRT-PCR

BJ cells or skin wound tissues were homogenized using TRIzol reagent (Invitrogen) and total RNA from each sample was isolated using RNeasy® Mini Kit (Quiagen). After reverse transcription using MMLV-Reverse Transcriptase (Promega), qRT-PCR was performed with the iCycler Thermal Cycler (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). The specificity of qRT-PCR was confirmed by agarose gel electrophoresis and melting-curve analysis. A housekeeping gene (*CyclophilinE*) was used as an internal standard. Primers for qRT-PCR are as follows: *IL6*: forward 5′-AAATTCGGTACATCCTCGACGGCA-3′, reverse 5′-AGTGCCTCTTTGCTGCTTTCACAC-3′; *IL8*: forward 5′-AGCCTTCCTGATTTCTGCAGCTCT-3′, reverse 5′-AATTTCTGTGTTGGCGCAGTGTGG-3′; *MMP1*: forward 5′-AGTGACTGGGAAACCAGATGCTGA-3′, reverse 5′-TCAGTGAGGACAAACTGAGCCACA-3′; *COL1A1*: forward 5′-ACGAAGACATCCCACCAATCACCT-3′, reverse 5′- AGATCACGTCATCGCACAACACCT-3′; *Cyclophillin E (human)*: forward 5′-TTCACAAACCACAATGGCACAGGG-3′, reverse 5′-CACCCTGACACATAAACCCTG-3′; *Ccn2*: forward 5′-TGAGGCTGAGTCCAGCTGTTCTTT-3′, reverse 5′-ACTTGCCACAAGCTGTCCAGTCTA-3′; *Ccn1*: forward 5′-GGAGGTGGAGTTAACGAGAAAC-3′, reverse 5′-GTGGTCTGAACGATGCATTTC-3′; *Mmp2*: forward 5′-TCTGGTGCTCCACCACATACAACT-3′, reverse 5′-CTGCATTGCCACCCATGGTAAACA-3′; for *Mmp3*: forward 5′-TGGAACAGTCTTGGCTCATGCCTA-3′, reverse 5′- TGGGTACATCAGAGCTTCAGCCTT-3′; *Mmp9*: forward 5′- TGAGCTGGACAGCCAGACACTAAA-3′, reverse 5′- TCGCGGCAAGTCTTCAGAGTAGTT-3′, for *Tgfb1*; forward 5′-GTGCGGCAGCTGTACATTGACTTT-3′, reverse 5′- TGTACTGTGTGTCCAGGCTCCAAA-3′; *Cyclophilin E (mouse)*: forward 5′- TTCACAAACCACAATGGCACAGGG-3′, reverse 5′- TGCCGTCCAGCCAATCTGTCTTAT-3′.

Topical CCN2 treatment during wound healing

Recombinant CCN2 protein was diluted in PBS to 0.1 μg/μl, and 50 μl of protein or saline control was directly applied daily to each full-thickness excisional wound in *Ccn1*$^{dm/dm}$ mice. Wound tissues were harvested 9 days post-wounding and processed for analysis.

Statistics

Results are expressed as mean ± standard deviation (S.D.), and statistical analysis was performed by one-way ANOVA analysis of variance and Student's *t*-test. A $p < 0.05$ was considered significant. All experiments were performed in triplicate unless otherwise indicated.

Results

CCN2 induces cellular senescence in human skin fibroblasts

To determine whether CCN2 can induce cellular senescence in fibroblasts, we assessed the effect of purified CCN2 protein on the proliferation of human BJ skin fibroblasts cultured in 10 % serum with CCN1 as a positive control. CCN2 inhibited the proliferation of BJ fibroblasts with similar efficiency as CCN1 based on cell numbers counted over several days, whereas cells treated with BSA continued to proliferate as

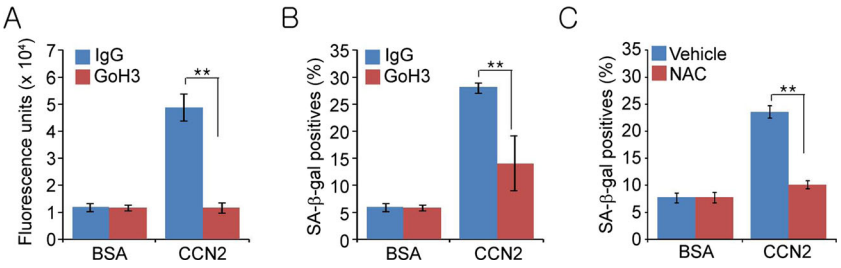

Fig. 2 CCN2 induces cellular senescence through α_6 integrin-mediated ROS accumulation. **a** BJ cells were pre-treated with either IgG or function-blocking monoclonal antibodies against integrin α6 (GoH3, 50 μg/ml) for 1 h, followed by the addition of BSA or CCN2 for an additional 1 h and then stained with H_2DCF-DA (10 μM). ROS was quantified by fluorescence measurements using ImageJ (NIH). **b** Three days after addition of BSA or CCN2, cells positive for SA-β-gal were counted in ten random fields and expressed as percentages of total cell number. **c** Cells were treated with either NAC (2.5 mM) or vehicle 1 h prior to BSA or CCN2 addition and SA-β-gal activity was assayed 3 days thereafter. Experiments were done in triplicates and data presented as means ± s.d. ** indicates $p < 0.01$

expected (Fig. 1b). Consistently, CCN2-treated fibroblasts exhibited >40 % reduction in Ki-67-positive cells (Fig. 1c), indicating that CCN2 inhibited BJ cell proliferation. Nearly 35 % of cells treated with CCN2 expressed the lysosomal enzyme senescence-associated β-galactosidase (SA-β-gal)(Fig. 1d), a marker for cellular senescence (Rodier and Campisi 2011), compared to ~6 % in BSA-treated control. BJ cells treated with CCN2 also exhibited enlarged and flattened cell morphologies, characteristics of senescent cells (Fig. 1e). Furthermore, CCN2 treatment of BJ fibroblasts induced the expression of SASP, with up-regulation of cytokines (*IL6* and *IL8*) and matrix metalloproteinase 1 (*MMP1*) and down-regulation of collagen (*COL1a1*; Fig. 1f). To confirm these results further, we also expressed *Ccn2* in BJ cells via an adenoviral vector, which increased *Ccn2* mRNA by ~10 fold in BJ cells (Fig. 1g,h). Adenoviral expression of *Ccn2* in BJ cells also inhibited cell proliferation and induced expression of SASP, showing upregulation of *IL6* and *MMP1* but down-regulation of *COL1a1*. Taken together, these results indicate that CCN2 can induce cellular senescence in human BJ skin fibroblasts.

CCN2 induces reactive oxygen species (ROS) through integrin $\alpha_6\beta_1$, leading to cellular senescence

CCN1 was previously shown to induce cellular senescence through its binding to integrin $\alpha_6\beta_1$, leading to the accumulation of ROS (Jun and Lau 2010b). Thus, we assessed the potential role of α_6 integrin and ROS in CCN2-induced senescence. We found that fibroblasts treated with CCN2 for 1 h showed high levels in ROS as measured using H_2-DCFDA, and ROS accumulation was efficiently blocked when cells were pre-treated with a function-blocking mAb against integrin α_6 (GoH3) but not by treatment with control IgG (Fig. 2a). Importantly, pretreatment with either GoH3 or N-acetyl cysteine (NAC), a ROS scavenger, abrogated CCN2-induced cellular senescence (Fig. 2b,c). These results are consistent with previous observation that CCN2 can induce ROS

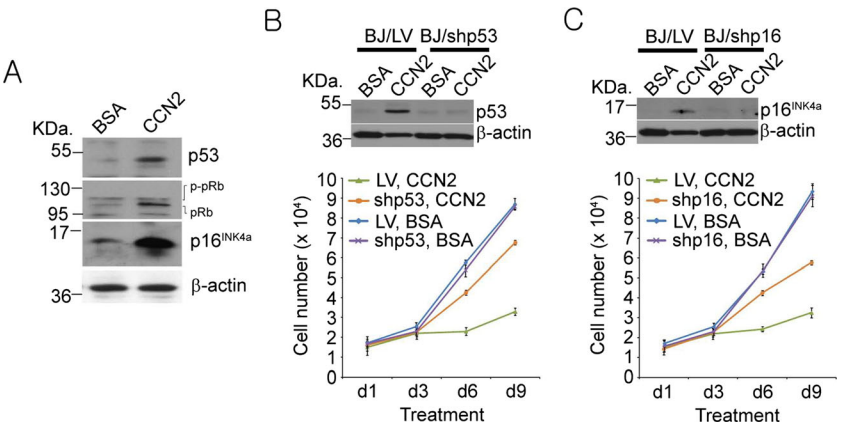

Fig. 3 CCN2-induced senescence requires p53 and $p16^{INK4a}$. **a** Cell lysates from BJ fibroblasts treated with either BSA or CCN2 were electrophoresed and immunoblotted to show increases in p53, $p16^{INK4a}$, and hypophosphorylated pRb. **b** Cells were infected with either empty lentivirus (LV) or lentivirus driving expression of shRNAs against p53; cells were then treated with BSA or CCN2 for indicated times before cell numbers counted. **c** Cells were infected with either LV or lentivirus driving expression of shRNAs against $p16^{INK4a}$; cells were then treated with BSA or CCN2 for indicated times and cell numbers counted. Knockdown effects of shp53 and shp16 were confirmed using Western blotting. Experiments were done in triplicates and data presented as means ± s.d

accumulation in human skin fibroblasts as measured by flow cytometry (Juric et al. 2009). ROS can initiate a DNA damage response that may include senescence (Catalano et al. 2005).

CCN2-induced senescence requires p53 and p16^{INK4a}

Cellular senescence is known to be established and maintained by pathways involving either p53 or p16^{INK4a}, or both (Campisi and d'Adda di Fagagna 2007; Collado et al. 2007). Immunoblot analysis showed that CCN2 treatment in BJ fibroblasts led to the activation of p53 and induction of p16^{INK4a} (Fig. 3a). p16^{INK4a} can in turn activate pRb by inhibiting Cdk4 and Cdk6, leading to accumulation of hypophosphorylated pRb (Fig. 3a). To further confirm the functional role of p53 and p16^{INK4a} in CCN2-induced senescence, we expressed short hairpin RNAs (shRNAs) against p53 and p16^{INK4a} using the lentiviral expression system (Fig. 3b,c). Whereas CCN2 efficiently blocked cell proliferation in cells infected with control lentivirus (LV), knockdown of p53 restored cell proliferation by more than 80 % (Fig. 3b). Likewise, p16^{INK4a} knockdown also mitigated CCN2-induced growth arrest (Fig. 3c). These results indicate that CCN2-induced senescence is dependent on activation of p53 and p16^{INK4a}.

CCN2 is dispensable for cellular senescence during cutaneous wound healing

Since myofibroblast senescence occurs as part of the healing process in skin wounds (Demaria et al. 2014; Jun and Lau

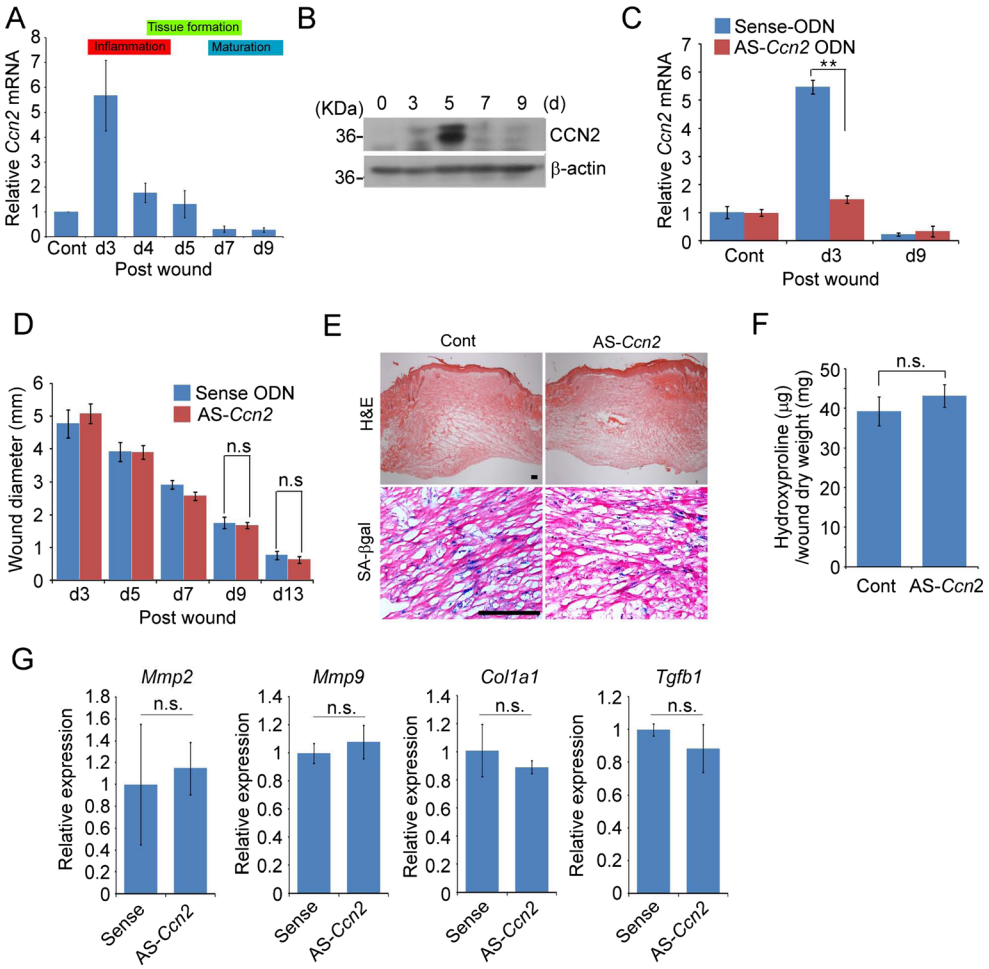

Fig. 4 CCN2 is dispensable for cellular senescence during skin wound healing. Cutaneous wounds were created in mice using a 6 mm biopsy punch and healing process observed. **a** *Ccn2* mRNA was quantified by qPCR in total RNA isolated from wounds of WT C57BL/6 mice at indicated days post wounding (n = 4 per time point). **b** Immunoblots of protein lysates from wounds of C57BL/6 mice probed with anti-CCN2 or anti-β actin antibodies. **c** *Ccn2* mRNA levels were quantified in wounds treated daily with sense or antisense ODN against *Ccn2* (AS-*Ccn2*). **d** Wound diameters were measured in C57BL/6 mice treated with sense or antisense *Ccn2* ODN; $n = 6$. **e** Wounds treated as above were harvested at day 9, sectioned, and stained for SA-β-gal activity and counterstained with H&E. **f** Hydroxyproline content of wounds treated with sense or antisense *Ccn2* oligonucleotides was measured 9 days post-wounding. $n = 4$. **g** Relative expression of *Mmp2*, *Mmp9, Col1a1*, and *Tgfb1* in wounds at day 9 was quantified using qPCR and normalized to *Cyclophilin E* (n = 4). $^{*}p < 0.05$, $^{**}p < 0.01$; n.s., not statistically significant. Scale bar = 100 μm

2010b), we assessed whether *Ccn2* contributes to senescence in wounds. Interestingly, *Ccn2* expression in cutaneous wound healing was greatly induced only in the early inflammatory phase (day 3) and is substantially decreased thereafter to a low level in the proliferation phase (day 4–7) and below the uninjured control in the maturation phase (day 6–9; Fig. 4a). Consistently, immunoblot analysis showed that CCN2 protein accumulation peaked at day 5 post-wounding and declined thereafter (Fig. 4b). The low level of *Ccn2* expression during the maturation phase suggests that *Ccn2* may not play an important role in cellular senescence induction during matrix remodeling. Indeed, although antisense oligonucleotides (AS-ODN) effectively knocked down *Ccn2* expression over the 9 day experimental period (Fig. 4c), they did not affect the kinetics of wound closure nor reduce cellular senescence as judged by SA-β-gal activity staining (Fig. 4d,e). Furthermore, *Ccn2* knockdown had no effect on collagen accumulation during the maturation phase, as shown by measurements of hydroxyproline content (Fig. 4f). Expression of SASP genes, including *Mmp2, Mmp9*, *Tgfb1*, and *Col1a1* was not affected by *Ccn2* knockdown as analyzed by qPCR (Fig. 4g). Thus, consistent with the low level of *Ccn2* expression in the proliferation and maturation phases of wound healing, knockdown of *Ccn2* had no appreciable effect on the kinetics of wound closure, accumulation of senescent cells, or collagen content of the wounds.

Administration of CCN2 induces cellular senescence and associated anti-fibrotic phenotype in vivo

To test whether CCN2 is capable of inducing cellular senescence in vivo, we applied purified recombinant CCN2 protein directly on cutaneous wounds. For this purpose, we have used *Ccn1*$^{dm/dm}$ knockin mice, which encode a mutant CCN1 unable to bind integrin $\alpha_6\beta_1$ and therefore defective for senescence induction, to eliminate CCN1-mediated senescence in skin wounds (Jun and Lau 2010b). The expression of *Ccn2* during wound healing in *Ccn1*$^{dm/dm}$ mice, as measured by qPCR analysis, was similar to that in WT mice (Fig. 5a). As expected, *Ccn1*$^{dm/dm}$ mice showed a dearth of cellular senescence during the maturation phase at day 9, concomitant with a substantial accumulation of collagen as shown by Sirius red staining (Fig. 5b). Strikingly, application of CCN2 on the wounds resulted in a large increase (~8-fold) in the number of senescent cells as well as decreased collagen accumulation (Fig. 5b). Direct measurement of hydroxyproline content confirmed and quantified the decreased of collagen accumulation as a results of CCN2 treatment (Fig. 5c). CCN2-treated wounds also exhibited greatly increased expression of *Mmp2, Mmp3,* and *Mmp9,* and decreased expression of *Col1a1* and *Tgfb1,* characteristics of SASP (Fig. 5d,e). Together, these results indicate that CCN2 is capable of inducing cellular senescence in cutaneous wounds and thereby exerting an anti-fibrotic effect.

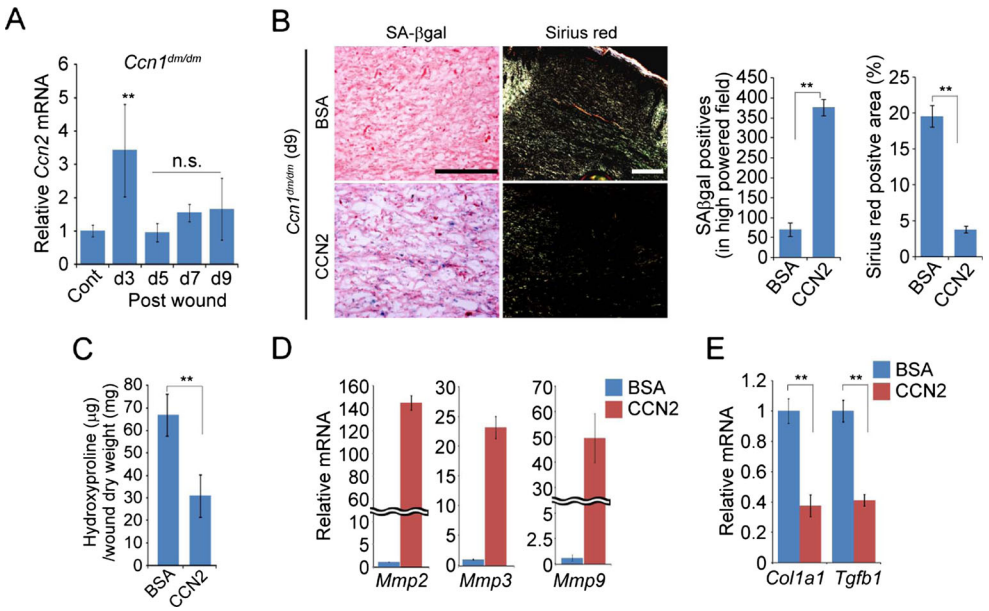

Fig. 5 Administration of CCN2 in skin wounds induces senescence and reduces fibrosis. **a** Expression of *Ccn2* in *Ccn1*$^{dm/dm}$ mice during wound healing was quantified using qPCR. **b** *Ccn1*$^{dm/dm}$ knockin mice were subjected to cutaneous excisional wounds, and purified recombinant CCN2 protein (0.1 mg/ml; 50 μl per dose) in PBS was applied topically daily. Wounds were sectioned and stained for SA-β-gal activity, or stained with Sirius red and visualized under polarized light. Scale bar = 100 μm. **c** Wounds treated with CCN2 as above were harvested at day 9 and hydroxyproline content was quantified and normalized over the total dry weight (n = 4). **d** Relative expression of *Mmp2*, *Mmp3* and *Mmp9* in day 9 wounds, as judged by qPCR analysis, was greatly increased in mice treated with CCN2 protein vs. BSA control. **e** Expression of *Col1a1* and *Tgfb1* from wounds at day 9 from mice treated with CCN2 or BSA was quantified using qPCR and normalized to *Cyclophilin E* (n = 4). Data are expressed as mean ± s.d in triplicate determinations.*$p < 0.05$, **$p < 0.01$. n.s., not statistically significant

Discussion

Many matricellular proteins genes are highly expressed at sites of tissue injury and wound repair (Kubota and Takigawa 2015; Roberts and Lau 2011). Although the expression of both *Ccn1* and *Ccn2* are associated with wound repair and fibrosis, these homologous genes appear to have distinct biological effects. Here we show that CCN2, like CCN1, is capable of inducing fibroblast senescence and the anti-fibrotic SASP. However, their divergent roles in cutaneous wound healing appear to be dictated in part by their distinct expression kinetics.

Given the high degree of homology between CCN1 and CCN2, it is not surprising that they share similar biochemical activities, including $\alpha_6\beta_1$-mediated functions in fibroblasts (Chen et al. 2001; Chen et al. 2007). Early studies have shown that both CCN1 and CCN2 support fibroblast adhesion through direct binding to integrin $\alpha_6\beta_1$ and heparin sulfate proteoglycans, thus inducing the formation of filopodia and lamellipodia, $\alpha_6\beta_1$-containing focal adhesion complexes, activation of focal adhesion kinases (FAK), paxillin, Rac, and p42/p44 MAPK (Chen et al. 2001). It was also shown that fibroblast adhesion to CCN2 induces expression of MMP1 and MMP3 (Chen et al. 2001), which are now appreciated to be part of the SASP signature (Coppe et al. 2008). The mechanism by which CCN2 induces senescence involves $\alpha_6\beta_1$-dependent accumulation of ROS, leading to p53 activation and $p16^{INK4a}$ induction (Figs. 2 and 3), similar to the signaling pathway for CCN1-induced senescence (Jun and Lau 2010b).

Previous studies showed that deletion of *Ccn2* in fibroblasts had no effect on the kinetics of cutaneous wound healing, the collagen content, or accumulation of α-smooth muscle actin-positive myofibroblasts in the wounds (Liu et al. 2014b). Our results with *Ccn2* knockdown in cutaneous wounds are in agreement with these observations, showing that depletion of *Ccn2* in the wound tissue had no effect on the wound closure rate, collagen content, induction of cellular senescence, or expression of the SASP (Fig. 4). These findings may reflect high expression of *Ccn2* only in the initial inflammatory phase of wound healing (day 3); *Ccn2* expression rapidly declines thereafter in the proliferation and maturation phases when myofibroblasts are most active (Fig. 4a). Thus, it is not surprising that antisense knockdown (Fig. 4) or deletion of *Ccn2* (Liu et al. 2014b) had little effect on myofibroblast functions during granulation tissue formation or matrix remodeling.

Despite the strong association of *Ccn2* with fibrosis, the precise mechanism by which CCN2 promotes fibrogenesis is not well understood (Jun and Lau 2011; Lipson et al. 2012). Given the ability of CCN2 to directly induce cellular senescence in fibroblasts, the pro-fibrotic effects of CCN2 may be mediated through cell types different from, or in addition to, myofibroblasts. For example, *Ccn2* has been found to promote fibroblast differentiation from mesenchymal stem cells, although it does not by itself induce myofibroblast differentiation in these cells (Lee et al. 2010). *Ccn2* plays an important role in the recruitment of *Sox2*-expressing cells to sites of cutaneous wound healing or bleomycin-induced skin injury, and these cells serve as progenitors for myofibroblasts (Liu et al. 2014a; Tsang and Leask 2015). Thus, it is possible that recruitment of *Sox2*-expressing progenitor cells by *Ccn2* in cutaneous wounds occurs only when *Ccn2* is highly expressed; however, *Ccn2* expression declines to a low level when differentiated myofibroblasts proliferate in granulation tissue and progress to matrix remodeling in the maturation phase (Fig. 4a). In this model, *Ccn2* is pro-fibrotic by recruiting progenitors for myofibroblasts (Liu et al. 2014a; Tsang and Leask 2015), but retains the capability to trigger cellular senescence in myofibroblasts and induce the associated anti-fibrotic genetic program (Fig. 5). This dichotomy of pro-fibrotic and anti-fibrotic functions may be found in CCN1 as well, which has been shown to promote matrix remodeling and limit fibrosis by inducing myofibroblast senescence in cutaneous wound healing, liver injuries, and mouse models of cardiac diseases (Borkham-Kamphorst et al. 2014; Jun and Lau 2010a; Kim et al. 2013; Meyer et al. 2016). However, *Ccn1* has also been reported to exhibit pro-fibrotic properties in lung fibrosis (Kurundkar et al. 2016), suggesting a context-dependent effect on fibrogenesis.

It is of interest that direct application of purified CCN2 protein on skin wounds is able to induce cellular senescence and the associated SASP, including upregulation of MMPs and downregulation of collagen and TGF-β, resulting in reduced accumulation of collagen (Fig. 5). These findings suggest a therapeutic potential for CCN2 for the treatment of fibrotic conditions when delivered during specific stages in fibrotic progression.

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References

Babic AM, Chen C-C, Lau LF (1999) Fisp12/mouse connective tissue growth factor mediates endothelial cell adhesion and migration through integrin αvβ3, promotes endothelial cell survival, and induces angiogenesis in vivo. Mol Cell Biol 19:2958–2966

Borkham-Kamphorst E, Schaffrath C, Van de Leur E, Haas U, Tihaa L, Meurer SK, Nevzorova YA, Liedtke C, Weiskirchen R (2014) The anti-fibrotic effects of CCN1/CYR61 in primary portal myofibroblasts are mediated through induction of reactive oxygen species resulting in cellular senescence, apoptosis and attenuated TGF-beta signaling. Biochim Biophys Acta 1843:902–914

Campisi J (2013) Aging, cellular senescence, and cancer. Annu Rev Physiol 75:685–705

Campisi J, d'Adda di Fagagna F (2007) Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol 8:729–740

Catalano A, Rodilossi S, Caprari P, Coppola V, Procopio A (2005) 5-lipoxygenase regulates senescence-like growth arrest by promoting ROS-dependent p53 activation. EMBO J 24:170–179

Chang J, Wang Y, Shao L, Laberge RM, Demaria M, Campisi J, Janakiraman K, Sharpless NE, Ding S, Feng W, Luo Y, Wang X, Aykin-Burns N, Krager K, Ponnappan U, Hauer-Jensen M, Meng A, Zhou D (2016) Clearance of senescent cells by ABT263 rejuvenates aged hematopoietic stem cells in mice. Nat Med 22:78–83

Chen C-C, Lau LF (2009) Functions and mechanisms of action of CCN matricellular proteins. Int J Biochem Cell Biol 41:771–783

Chen C-C, Chen N, Lau LF (2001) The angiogenic factors Cyr61 and CTGF induce adhesive signaling in primary human skin fibroblasts. J Biol Chem 276:10443–10452

Chen CC, Young JL, Monzon RI, Chen N, Todorovic V, Lau LF (2007) Cytotoxicity of TNFalpha is regulated by integrin-mediated matrix signaling. EMBO J 26:1257–1267

Collado M, Blasco MA, Serrano M (2007) Cellular senescence in cancer and aging. Cell 130:223–233

Coppe JP, Patil CK, Rodier F, Sun Y, Munoz DP, Goldstein J, Nelson PS, Desprez PY, Campisi J (2008) Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS Biol 6:2853–2868

Debacq-Chainiaux F, Erusalimsky JD, Campisi J, Toussaint O (2009) Protocols to detect senescence-associated beta-galactosidase (SA-betagal) activity, a biomarker of senescent cells in culture and in vivo. Nat Protoc 4:1798–1806

Demaria M, Ohtani N, Youssef SA, Rodier F, Toussaint W, Mitchell JR, Laberge RM, Vijg J, Van Steeg H, Dolle ME, Hoeijmakers JH, de Bruin A, Hara E, Campisi J (2014) An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. Dev Cell 31:722–733

Edwards CA, O'Brien J (1980) Modified assay for determination of hydroxyproline in tissue hydrolyzate. Clin Chim Acta 104:161–167

Gurtner GC, Werner S, Barrandon Y, Longaker MT (2008) Wound repair and regeneration. Nature 453:314–321

Hall-Glenn F, Lyons KM (2011) Roles for CCN2 in normal physiological processes. Cell Mol Life Sci 68:3209–3217

Ivkovic S, Yoon BS, Popoff SN, Safadi FF, Libuda DE, Stephenson RC, Daluiski A, Lyons KM (2003) Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. Development 130:2779–2791

Jun JI, Lau LF (2010a) Cellular senescence controls fibrosis in wound healing. Aging (Albany NY) 2:627–631

Jun JI, Lau LF (2010b) The matricellular protein CCN1 induces fibroblast senescence and restricts fibrosis in cutaneous wound healing. Nat Cell Biol 12:676–685

Jun JI, Lau LF (2011) Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets. Nat Rev Drug Discov 10:945–963

Jun JI, Kim KH, Lau LF (2015) The matricellular protein ccn1 mediates neutrophil efferocytosis in cutaneous wound healing. Nat Commun 6:7386. doi:10.1038/ncomms8386

Juric V, Chen CC, Lau LF (2009) Fas-mediated apoptosis is regulated by the extracellular matrix protein CCN1 (CYR61) in vitro and in vivo. Mol Cell Biol 29:3266–3279

Kim KH, Chen CC, Monzon RI, Lau LF (2013) The matricellular protein CCN1 promotes regression of liver fibrosis through induction of cellular senescence in hepatic myofibroblasts. Mol Cell Biol 33: 2078–2090

Kireeva ML, Latinkic BV, Kolesnikova TV, Chen C-C, Yang GP, Abler AS, Lau LF (1997) Cyr61 and Fisp12 are both signaling cell adhesion molecules: comparison of activities, metablism, and localization during development. Exp Cell Res 233:63–77

Krizhanovsky V, Yon M, Dickins RA, Hearn S, Simon J, Miething C, Yee H, Zender L, Lowe SW (2008) Senescence of activated stellate cells limits liver fibrosis. Cell 134:657–667

Kubota S, Takigawa M (2015) Cellular and molecular actions of CCN2/CTGF and its role under physiological and pathological conditions. Clin Sci (Lond) 128:181–196

Kurundkar AR, Kurundkar D, Rangarajan S, Locy ML, Zhou Y, Liu RM, Zmijewski J, Thannickal VJ (2016) The matricellular protein CCN1 enhances TGF-beta1/SMAD3-dependent profibrotic signaling in fibroblasts and contributes to fibrogenic responses to lung injury. FASEB J 30:2135–2150

Lau LF (2011) CCN1/CYR61: the very model of a modern matricellular protein. Cell Mol Life Sci 68:3149–3163

Lee CH, Shah B, Moioli EK, Mao JJ (2010) CTGF directs fibroblast differentiation from human mesenchymal stem/stromal cells and defines connective tissue healing in a rodent injury model. J Clin Invest 120:3340–3349

Leu S-J, Lam SC-T, Lau LF (2002) Proangiogenic activities of CYR61 (CCN1) mediated through integrins $\alpha v\beta 3$ and $\alpha 6\beta 1$ in human umbilical vein endothelial cells. J Biol Chem 277:46248–46255

Leu S-J, Liu Y, Chen N, Chen CC, Lam SC, Lau LF (2003) Identification of a novel integrin $\alpha 6\beta 1$ binding site in the angiogenic inducer CCN1 (CYR61). J Biol Chem 278:33801–33808

Lipson KE, Wong C, Teng Y, Spong S (2012) CTGF is a central mediator of tissue remodeling and fibrosis and its inhibition can reverse the process of fibrosis. Fibrogenesis Tissue Repair 5:S24

Liu S, Herault Y, Pavlovic G, Leask A (2014a) Skin progenitor cells contribute to bleomycin-induced skin fibrosis. Arthritis Rheum 66: 707–713

Liu S, Thompson K, Leask A (2014b) CCN2 expression by fibroblasts is not required for cutaneous tissue repair. Wound Repair Regen 22: 119–124

Meyer K, Hodwin B, Ramanujam D, Engelhardt S, Sarikas A (2016) Essential role for premature senescence of myofibroblasts in myocardial fibrosis. J Am Coll Cardiol 67:2018–2028

Mo FE, Muntean AG, Chen CC, Stolz DB, Watkins SC, Lau LF (2002) CYR61 (CCN1) is essential for placental development and vascular integrity. Mol Cell Biol 22:8709–8720

Roberts DD, Lau LF (2011) Matricellualr Proteins. In: Mecham RP (ed) The extracellular matrix: an overview. Springer-Verlag, Berlin, pp. 369–413

Rodier F, Campisi J (2011) Four faces of cellular senescence. J Cell Biol 192:547–556

Tsang M, Leask A (2015) CCN2 is required for recruitment of Sox2-expressing cells during cutaneous tissue repair. J Cell Commun Signal 9:341–346

Wiley CD, Velarde MC, Lecot P, Liu S, Sarnoski EA, Freund A, Shirakawa K, Lim HW, Davis SS, Ramanathan A, Gerencser AA, Verdin E, Campisi J (2016) Mitochondrial dysfunction induces senescence with a distinct secretory phenotype. Cell Metab 23:303–314

Yoon PO, Lee MA, Cha H, Jeong MH, Kim J, Jang SP, Choi BY, Jeong D, Yang DK, Hajjar RJ, Park WJ (2010) The opposing effects of CCN2 and CCN5 on the development of cardiac hypertrophy and fibrosis. J Mol Cell Cardiol 49:294–303