CCN2 is required for recruitment of Sox2-expressing cells during cutaneous tissue repair

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Abstract Connective tissue growth factor (CTGF/CCN2), a member of the CCN family of matricellular proteins is upregulated in both fibrosis as well as tissue repair. Recently, we showed that, in mice, CCN2 expression by fibroblasts was required for dermal fibrogenesis, but not for cutaneous tissue repair. Lineage tracing analysis linked the ability of CCN2 to promote fibrosis to the requirement for CCN2 to recruit cells expressing the progenitor cell marker Sox2 to fibrotic connective tissue and for differentiating these cells into myofibroblasts. Herein, we show that although loss of CCN2 expression by Sox2-expressing cells does not impair cutaneous tissue repair, CCN2 was required for recruitment of cells derived from Sox2-expressing cells to the wound area. Collectively, these results are consistent with the notion that neither CCN2 nor Sox2-expressing progenitor cells are essential for cutaneous tissue repair and that CCN2 represents a specific anti-fibrotic target.

Keywords CTGF · Connective tissue growth factor · CCN2 · Wound healing · Sox2 · Dermis · Progenitor cell

Introduction

The CCN family of six matricellular proteins (CCN1–6) is dynamically regulated and believed to contribute to tissue remodeling and repair and well as the abnormal activation of

connective tissue in pathological diseases such as fibrosis and cancers (Lau and Lam 1999; Leask and Abraham 2006; Perbal 2013). As an example, in adult connective tissue, CCN2 is not normally expressed unless induced (Igarashi et al. 1993). One of the most potent inducers of CCN2 is transforming growth factor β (TGF β) (Igarashi et al. 1993; Holmes et al. 2001). Consequently, an initial hypothesis was proposed that CCN2 is essential for TGF β to promote both tissue repair and fibrosis (Grotendorst 1997). Indeed, we now know that CCN2 is not required in dermal fibroblasts for TGF β to induce either collagen or α -SMA expression; instead, CCN2 appears to act as a cofactor to enhance the fibrotic effects of TGF β either by promoting adhesive signaling or Smad1/ERK (Mori et al. 1999; Shi-wen et al. 2006; Wang et al. 2011; Nakerakanti et al. 2011).

There is no universally agreed-upon therapy for fibrosis, in part because the fundamental, underlying mechanisms are unclear. For example, although the α -smooth muscle actin (SMA)-expressing myofibroblast is known to be the effector cell in fibrosis (and wound repair), the origin of the myofibroblast is unclear; however, an emerging consensus is that these cells are derived from the recruitment and differentiation of progenitor cells (Hinz et al. 2012; Duffield 2014). Indeed, it has been long suggested that pericyte-like progenitor cells contribute to excessive scarring and scleroderma fibrosis (Sundberg et al. 1996; Rajkumar et al. 1999). Indeed, in bleomycin-induced skin fibrosis, the overwhelming number of myofibroblasts stain positive with the pericyte/progenitor cell (PC) marker NG2 (Liu et al. 2010). These cells are also largely positive for CCN2 and the PC marker Sox2 (Liu et al. 2014a). CCN2 expression by type I collagen- or Sox2-expressing cells appears to contribute to bleomycin-induced skin fibrosis by being essential for the recruitment of these pericyte-like PCs to

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fibrotic tissue (Liu et al. 2011, 2014a). Conversely, in normal tissue repair, myofibroblasts do not appear to originate from pericyte-like PCs, in that they are largely NG2 negative (Kapoor et al. 2008). Indeed, CCN2 expression by type I collagen-expressing cells was not required for normal, cutaneous wound healing, even through loss of CCN2 resulted in the failure of NG2-positive cells to appear within the wound (Liu et al. 2014b).

Collectively, these results suggest the intriguing possibility that, whereas CCN2-expressing PCs contribute to skin fibrosis, this mechanism is not required for normal tissue repair; that is, the mechanisms underlying how myofibroblasts originate differs between tissue repair and fibrosis. In this report, we investigate this issue by assessing the effect of loss of CCN2 from Sox2-expressing cells on the kinetics of cutaneous repair as well as PC recruitment. Our results give new and valuable insights into the contribution of CCN2 and PCs to skin biology.

Methods

Generation of transgenic mice

Mice hemizygous for a tamoxifen-dependant Cre recombinase under the control of a sox2 promoter [Sox2-Cre(ER)-T] (Institut Clinique de la Souris) were bred with mice containing a double-fluorescent reporter transgene (mT/mG) integrated into the Gt(ROSA) 26Sor locus (Jackson Laboratories) that results in the expression of membrane-targeted tdTomato prior to Cre-mediated excision and membrane-targeted GFP after excision. These mice were subsequently bred, or not, with mice harboring a floxed CCN2 allele to produce reporter mice with (K/K) and without (C/C) a Sox2-specific deletion of CCN2. To induce Cre recombinase, a stock solution of tamoxifen in ethanol (100 mg/ml) was diluted in corn oil to 10 mg/ml. Three week old littermate mice were given 0.1 ml intraperitoneal injections of the tamoxifen solution for five consecutive days. All experimental animals were genotyped by polymerase chain reaction (Institut Clinique de la Souris, Jackson Labs). All animal protocols were approved by the animal care committee at the University of Western Ontario

Wound surgery

Two weeks post-cessation of tamoxifen, C/C and K/K mice were subjected to a 4 mm dermal punch wounding assay as previously described (Liu et al. 2014a).

Wounds were imaged at 0, 3, 7 and 10 days post-wounding using a Sony D-9 digital camera (Toronto, ON, Canada). Wound area was measured using ImageJ 1.46 (National Institutes of Health, Bethesda, MD, USA) software, and wound closure was expressed as a percentage of initial wound size ($n=11$). Subsets of mice were sacrificed at day 7 for histological analysis.

Immunohistochemistry and assessment

Sections (0.5 μ m) were cut using a microtome (Leica, Concord, ON), collected on Superfrost Plus slides (Fisher Scientific, Ottawa, ON), de-waxed in xylene and rehydrated by successive immersion in descending concentrations of alcohol. To assess the effect of gene deletion on tissue repair, sections were stained with Hemotoxylin and Eosin (H&E; Fisher Scientific); Granulation area was measured using Northern Eclipse software (Empix). Alternatively, to assess the effects of gene deletion on collagen synthesis, semi-quantitative analysis using Masson's trichrome stain was employed. Immunolabeling of α -SMA, CCN2, CCN1, NG2 and PCNA was also conducted by indirect immunofluorescence analysis. Tissue sections were blocked by incubated with 5 % donkey serum for 1 h and washed with phosphate-buffered saline (PBS). Sections were then incubated with primary antibodies overnight at 4 °C under humidified conditions. Primary antibodies used were: goat anti-CCN2 (anti-CTGF; 1:200 dilution, Santa Cruz, Santa Cruz, CA), rabbit anti-CCN1 (anti-Cyr61; 1:200 dilution, Santa Cruz), rabbit anti-PCNA (anti-PCNA; 1:500 dilution, Abcam, Toronto, ON), mouse anti-NG2 (1:100 dilution, Millipore), and mouse anti-alpha-smooth muscle actin (anti- α -SMA, 1:500 dilution, Sigma, St Louis, MO). After primary antibody incubation, sections were washed with PBS and incubated for 1 h at room temperature with appropriate fluorescent secondary antibodies (Jackson Immunoresearch, West Grove, PA). Sections were washed with PBS, mounted using 4',6-diamidino-2-phenylindole (DAPI) (Vector Labs, Burlington, ON), and photographed using a Zeiss fluorescence microscope and Northern Eclipse software (Empix, Mississauga, ON, Canada). Average fluorescence intensity was calculated, using image analysis software (Northern Eclipse, Empix), by determining the fluorescence/cell in an area of fixed dimensions within a field.

Cell culture and western blot analysis

Primary dermal fibroblasts were isolated from explants (4–6 week old animals). Cells were lysed in RIPA buffer, proteins

Fig. 1 Loss of CCN2 expression by Sox2-expressing cells does not affect cutaneous tissue repair in vivo (**A**) Kinetics of wound closure. Wild type mice (C/C) and mice made deficient for CCN2 in Sox2-expressing cells (K/K) were generated and subjected to the dermal punch model of cutaneous tissue repair, as described in methods. Mice from at least two different litters were assessed. *Six mice per group (4 wounds per mouse) were analyzed.* Data represent averages and standard deviation from all these mice. (**B**) Deletion of CCN2 from Sox2-expressing cells. Unwounded areas of mice with day 7 wounds were examined. Sections were stained with DAPI (to detect nuclei), anti-GFP (to detect cells derived from Sox2-expressing cells; left hand two panels) and anti-CCN2 antibody (red; right hand two panels). Representative sections are shown

quantified (Pierce) and subjected to Western blot analysis as described using anti-CCN1, anti-CCN2, anti- α -SMA and anti- β -actin (Sigma) antibodies. Blots were then probed with

appropriate HRP-conjugated secondary antibodies (1:5000, Jackson ImmunoResearch) and developed using a chemiluminescent reagent according to the manufacturer (Pierce).

Fig. 2 Conditional deletion of CCN2 in Sox2-expressing cells: immunochemical analysis of day 7 wounds. Mice as described in Fig. 1 were also positive for ROSA^{mT/mG} to permit lineage tracing analysis. Frozen sections were examined to permit detection of cells not expressing cre (red) and cells derived from cells expressing cre at the time of tamoxifen

injection (green). Note appearance of GFP positive cells in control mice (C/C), but not mice deleted for CCN2 in Sox2-expressing cells (K/K). Seven control wild-type mice, 8 wounded wild-type mice, 7 KO controls, 9 KO wounds, 6 KKO controls and 7 Three representative images are shown

Results

Deletion of CCN2 from Sox2-expressing cells does not affect the cutaneous tissue repair kinetics

To assess whether loss of CCN2 from Sox2-expressing cells resulted in altered cutaneous tissue repair in mice, we used Cre; CCN2^{fl/fl} (K/K) or Cre; CCN2^{+/+} (C/C) mice; i.e., mice either harboring a CCN2 allele bordered by loxP sites or a wild-type CCN2 allele and tamoxifen-dependent cre recombinase gene located downstream of a Sox2 promoter/enhancer (Liu et al. 2014a). Mice were also hemizygous for a ROSA^{mT/mG} allele to allow lineage tracing and identification of cells expressing cre at the time of tamoxifen injection (i.e., cells that were GFP positive). Mice deleted (K/K) or not for CCN2 (C/C) were then subjected to the dermal punch model of cutaneous tissue repair. Wound closure kinetics was unaffected by loss of CCN2 (Fig. 1A). Immunofluorescence staining of hair follicles in unwounded areas confirmed loss of CCN2 expression in Sox2 expressing cells in Cre; CCN2^{fl/fl} (K/K) but not Cre; CCN2^{+/+} (C/C) mice (Fig. 1B).

Deletion of CCN2 affects recruitment of Sox2-expressing cells to the wound

In models of fibrosis, CCN2 plays an essential role in myofibroblast differentiation at least in part by being required for the recruitment of cells derived from a Sox2-expressing population (Liu et al. 2014a). Examination of frozen sections of day 7 wound dermis of control mice (C/C) revealed the presence of the occasional GFP-positive cell (i.e., those derived from cells expressing Sox2 at the time of tamoxifen injection); conversely, CCN2-deficient (K/K) mice essentially lacked GFP-positive cells (Fig. 2). Thus, in response to wounding, CCN2 appeared to be required for the recruitment of cells derived from Sox2-expressing cells to the repairing connective tissue.

A subset of myofibroblasts are derived from Sox2-expressing cells

Indirect immunofluorescence analysis of day 7 wounds revealed abundant myofibroblasts in C/C and K/K mice

Fig. 3 Conditional deletion of CCN2 in Sox2-expressing cells fibroblasts results in impaired recruitment of myofibroblasts derived from Sox2-expressing cells. Mice 7 days post-wounding were measured as described in Methods using PCNA and α -SMA antibody to detect myofibroblasts in wild-type mice (K/K) or mice deleted for CCN2 in Sox2-expressing cells (C/C). For all assays, 6 mice per group were

analyzed. Sections were stained with anti-GFP antibody to detect cells derived from Sox2-expressing cells and α -SMA antibody to detect myofibroblasts in wild-type mice (C/C), and mice deleted for CCN2 in Sox2-expressing cells (K/K). For all assays, 6 mice per group were analyzed. $*=p<0.05$, One way ANOVA

(Fig. 3). A subset of α -SMA expressing myofibroblasts also stained positive with an anti-GFP antibody, indicating that a small number of myofibroblasts were derived from Sox2-expressing cells; loss of CCN2 impaired the recruitment of these cells to the wound area (Fig. 3). Collectively, these results are consistent with the hypothesis that CCN2 is required for recruitment of PCs to wounded or fibrotic tissue, but that this activity appears not to significantly contribute to normal tissue repair.

Discussion

For many years, CCN2 has been known to be induced in wound healing and in fibrogenesis (Igarashi et al. 1993; Leask and Abraham 2006). However, until recently the precise in vivo role of CCN2 has remained elusive. To address this issue, we have used conditional knockout strategies. Confirming a role for CCN2 in skin fibrosis, mice lacking CCN2 in cells expressing a fibroblast-specific type I collagen promoter/enhancer showed resistance to

bleomycin-induced fibrosis and the progressive skin fibrosis caused by loss of phosphatase and tensin homolog (PTEN) expression in fibroblasts (Liu et al. 2011, 2013).

CCN2 appears to contribute to fibrosis by being required for dermal progenitor cells to differentiate into myofibroblasts (Liu et al. 2014a). Such a mechanism seems not to contribute substantially to cutaneous tissue repair (Liu et al. 2014b), a process dependent on TGF β signaling likely due to its ability to induce myofibroblast differentiation of resident fibroblasts. Specifically, we found in our current study that although loss of CCN2 resulted in the failure to recruit cells derived from Sox2-expressing progenitors to the wound area, the overall impact of this defect was not detected. It is possible that CCN3, a member of the CCN family that antagonizes CCN2 action (Riser et al., 2009), might block the recruitment of progenitor-derived cells to the wound.

Thus, our studies have revealed that CCN2 expression by progenitor cells is responsible for the development of skin fibrosis in the bleomycin model of skin fibrosis but not cutaneous tissue repair. Thus CCN2 may be a selective anti-fibrotic target.

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