CCN2 modulates hair follicle cycling in mice

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ABSTRACT It is critical to understand how stem cell activity is regulated during regeneration. Hair follicles constitute an important model for organ regeneration because, throughout adult life, they undergo cyclical regeneration. Hair follicle stem cells—epithelial cells located in the follicle bulge—are activated by periodic β-catenin activity, which is regulated not only by epithelial-derived Wnt, but also, through as-yet-undefined mechanisms, the surrounding dermal microenvironment. The matricellular protein connective tissue growth factor (CCN2) is secreted into the microenvironment and acts as a multifunctional signaling modifier. In adult skin, CCN2 is largely absent but is unexpectedly restricted to the dermal papillae and outer root sheath. Deletion of CCN2 in dermal papillae and the outer root sheath results in a shortened telogen-phase length and elevated number of hair follicles. Recombinant CCN2 causes decreased β-catenin stability in keratinocytes. In vivo, loss of CCN2 results in elevated numbers of K15-positive epidermal stem cells that possess elevated β-catenin levels and β-catenin–dependent reporter gene expression. These results indicate that CCN2 expression by dermal papillae cells is a physiologically relevant suppressor of hair follicle formation by destabilization of β-catenin and suggest that CCN2 normally acts to maintain stem cell quiescence.

INTRODUCTION
Successful organ regeneration relies on organized and timely cross-talk among distinct cell niches. Insights into the molecular basis of such cross-talk can be obtained by examining the mammalian hair follicle, which serves as an example of such collaboration, notably between keratin 15 (K15) epithelial stem cells (EpSCs) located within the bulge and secondary hair germ and mesenchymal cells located within the dermal papilla (DP) and dermal sheath (Cotsarelis et al., 1990; Millar, 2002; Liu et al., 2003; Rendl et al., 2008). The DP is embedded within the hair bulb during the anagen phase and forms a compact ball during the telogen phase; conversely, dermal sheath line the outside of the epithelial follicle from the bulge to the base. In development, hair follicle morphogenesis takes place during the late embryonic and early neonatal period (Millar, 2002). Postnatally, hair follicle neogenesis can be induced in adult mouse skin in response to transgenic or wound-induced epidermal activation of Wnt/β-catenin (Lo Celso et al., 2004; Silva-Vargas et al., 2005; Ito et al., 2007; Lei et al., 2013). K15 EpSCs produce canonical Wnt ligands capable of activating hair follicle formation (Lei et al., 2013). Associated with new hair follicles are DP (Silva-Vargas et al., 2005; Ito et al., 2007; Lei et al., 2013), implying that cross-talk exists between Wnt-activated epidermis and signals emanating from the DP. However, the mechanism underlying this cross-talk is largely unknown.

Cells sense the microenvironment via signal transduction pathways, culminating in altered gene expression (Jewer et al., 2012). Candidate molecules secreted by the DP into the microenvironment that are in principle capable of mediating dermal/epidermal cross-talk are the so-called matricellular proteins. Matricellular proteins are secreted extracellular matrix–associated proteins that, through their ability to modify the activity of signaling molecules (Bornstein and Sage, 2002), are potential candidate molecules for mediating cellular cross-talk within niches. For example, the CCN family of matricellular proteins is increasingly being recognized as important in mediating pathological processes such as angiogenesis, oncogenesis, and...
Connective tissue growth factor (CTGF, CCN2), a member of the CCN family of secreted matricellular proteins, was discovered in 1991 during screening of a cDNA expression library from human vein endothelial cells (Bradham et al., 1991). CCN2 consists of four conserved domains: an insulin-like growth factor–binding protein domain, a von Willebrand factor type C domain, a thrombospondin type I repeat, and a C-terminal cysteine knot domain (Rachfal and Brigstock, 2005; Leask and Abraham, 2006; Jun and Lau, 2011; Perbal, 2013). CCN2 is expressed by fibroblasts in response to fibrogenic agents such as transforming growth factor-β (TGFβ; Holmes et al., 2001). CCN2 directly promotes cell adhesion and indirectly mediates angiogenesis and fibrogenesis by modulating the activity of other molecules, notably platelet-derived growth factor and TGFβ (Mori et al., 1999; Rachfal and Brigstock, 2005; Shi-wen et al., 2006). We showed, using a mesenchymal cell–specific knockout model, that CCN2 is required for fibrogenesis in skin (Liu et al., 2011); however, the overall in vivo role of CCN2 is largely unclear. For example, whether CCN2 expression by mesenchymal cells regulates hair follicle cycling is unknown.

RESULTS

CCN2 is not readily detectable in adult mouse dermis unless it is induced, for example, in tissue repair and fibrosis (Kapoor et al., 2008; Liu et al., 2010). Previously, we used a tamoxifen-dependent cre recombinase under the control of a collagen type I promoter/enhancer to show that mice deleted for CCN2 in collagen type I–producing cells were resistant to bleomycin-induced skin fibrosis (Liu et al., 2011; Zheng et al., 2002). However, the specific cells in which the activity of the collagen promoter/enhancer is active in uninjured adult skin, as well as the expression pattern of CCN2 within the hair follicle, are unclear. To address this issue, we generated col1A2-cre(ER)T/0; Ccn2fl/fl mice after tamoxifen injection. (A) To identify cells in the skin that expressed the col1a2 promoter, Col1a2-cre(ER)T/0; ROSA-STOPfl/fl-lacZ/0 mice were generated. Three-week-old mice were treated with tamoxifen as described in Materials and Methods. As a result, β-galactosidase is expressed only in cells in which the Col1a2 promoter is active at the time of injection. Two weeks after cessation of tamoxifen injection, tissue sections were stained with anti-CCN2 and anti–β-galactosidase antibodies, as indicated. Six mice were analyzed, and representative images are shown. Note colocalization (yellow) of β-galactosidase and CCN2 staining. (B, C) As described in Materials and Methods, Col1a2-cre(ER)T/0; Ccn2fl/fl mice were generated and treated with tamoxifen or corn oil to generate mice deleted (K/K) or not (C/C) for CCN2 in Col1a2-expressing cells (DP niche). (B) Tissue sections of C/C mice undergoing hair follicle cycling were subjected to indirect immunofluorescence with anti-CCN2 and anti-FSP1 antibodies. N = 4; representative images. (C) Tissue sections of C/C and K/K mice undergoing hair follicle cycling were subjected to indirect immunofluorescence with anti-CCN2 and anti-NCAM antibodies. Note the early appearance of NCAM staining, indicating DP activation, in K/K mice. N = 6; representative images are shown.

FIGURE 1: CCN2 expression and col1a2 promoter activity colocalize to the dermal papillae (DP), and CCN2 in DP was deleted in Col1a2-cre(ER)T/0; Ccn2fl/fl mice after tamoxifen injection. (A) To identify cells in the skin that expressed the col1a2 promoter, Col1a2-cre(ER)T/0; ROSA-STOPfl/fl-lacZ/0 mice were generated. Three-week-old mice were treated with tamoxifen as described in Materials and Methods. As a result, β-galactosidase is expressed only in cells in which the Col1a2 promoter is active at the time of injection. Two weeks after cessation of tamoxifen injection, tissue sections were stained with anti-CCN2 and anti–β-galactosidase antibodies, as indicated. Six mice were analyzed, and representative images are shown. Note colocalization (yellow) of β-galactosidase and CCN2 staining. (B, C) As described in Materials and Methods, Col1a2-cre(ER)T/0; Ccn2fl/fl mice were generated and treated with tamoxifen or corn oil to generate mice deleted (K/K) or not (C/C) for CCN2 in Col1a2-expressing cells (DP niche). (B) Tissue sections of C/C mice undergoing hair follicle cycling were subjected to indirect immunofluorescence with anti-CCN2 and anti-FSP1 antibodies. N = 4; representative images. (C) Tissue sections of C/C and K/K mice undergoing hair follicle cycling were subjected to indirect immunofluorescence with anti-CCN2 and anti-NCAM antibodies. Note the early appearance of NCAM staining, indicating DP activation, in K/K mice. N = 6; representative images are shown.

fibrosis (Leask and Abraham, 2006; Jun and Lau, 2011; Perbal, 2013). However, the role of the CCNs in normal tissue homeostasis is unclear.
(FSP1) antibody, which detects fibroblasts, revealed that CCN2, but not FSP1, was readily detected in the DP niche, whereas both CCN2 and FSP1 colocalized to the ORS (Figure 1B), suggesting that fibroblasts were largely absent from the DP.

To assess whether DP expression of CCN2 could affect hair follicle cycling, we generated col1A2-cre(ER)-T/0; Ccn2<sup>fl/fl</sup> mice. As described in Materials and Methods, 3-wk-old mice were injected with tamoxifen or corn oil to generate mice deleted (K/K) or not (C/C) for CCN2 in fibroblasts. We synchronized hair follicles by depilation at 1 wk after cessation of tamoxifen injection (i.e., the mice were in telogen). Note the absence of CCN2 expression in the DP in KO mice (Figure 1C). Note also that neural cell adhesion molecule (NCAM), a marker of hair follicle induction (Müller-Röver et al., 1998), showed premature induction in anagen II (Figure 1C). Moreover, the length of the resting phase between cycles was reduced in CCN2 knockout mice (Figure 2A). To assess the effect of loss of CCN2 in fibroblasts on normal hair cycling, we generated an additional set of mice deleted (K/K) or not (C/C) for CCN2 in fibroblasts. These were not subjected to depilation. Instead, 6 mo after cessation of tamoxifen injection, skin was removed from mice and the number of hair follicles was assessed; K/K mice had more hair follicles than wild-type (C/C) mice (Figure 2B).

Wnts modulate hair follicle cycling (Lei et al., 2013). Hence we assessed whether CCN2 could, in principle, alter the Wnt signaling pathway in epithelial cells. Wnt signals by binding to its receptor LRP6 (Angers and Moon, 2009). To begin to assess the potential effect of CCN2 on Wnt signaling, we conducted an immunoprecipitation assay using rCCN2, rWnt3a, and rLRP6. We found that rWnt3a could bind LRP6, and this interaction was competed by increasing amounts of rCCN2 (Figure 3A). These data are consistent with prior observations that rCCN2 binds LRP6 in an active site of the latter molecule (Mercurio et al., 2004). Moreover, addition of rCCN2 to cultured keratinocytes resulted in reduced steady-state levels of endogenous β-catenin (Figure 3B). We then generated col1A2-cre(ER)-T/0; Ccn2<sup>fl/fl</sup> TOPGAL mice. We injected 3-wk-old mice with tamoxifen or corn oil to delete CCN2 or not in cells in which the col1A2 promoter was active. Two months after cessation of tamoxifen injection, loss of CCN2 resulted in an increased β-catenin accumulation and β-catenin reporter activity in epithelial cells (Figure 4, A and B). A subset of β-catenin accumulation colocalized with K15, suggesting that EpSCs showed increased β-catenin steady-state levels (Figure 4C).

As an independent confirmation that CCN2 expression by the DP and ORS niche influences the Wnt signaling pathway in target cells, we examined the effect of CCN2 on melanocytes, as Wnt signaling coordinately promotes epithelial and melanocyte stem cell activation in hair regeneration (Rabbani et al., 2011). To assess this question, we used col1A2-cre(ER)-T/0; Ccn2<sup>fl/fl</sup> TOPGAL mice. Loss of CCN2 resulted in increased association of tyrosinase-related protein (trp) 2, a melanocyte marker (Yavuzer and Goding, 1994; Rabbani et al., 2011), with the hair follicle that colocalized with β-catenin reporter activity, suggesting that loss of CCN2 resulted in increased melanocyte differentiation (Figure 5). Collectively these data suggest that CCN2 controls the number of hair follicles within the skin.

**DISCUSSION**

Cross-talk between epithelial and mesenchymal niches governs hair follicle cycling; however, the underlying mechanism is unclear (Ohyama et al., 2010). It is likely that factors secreted from the mesenchymal niches govern the action of epithelial stem cells; because of their abilities to modulate cell signaling, matricellular proteins are likely to play a significant role in this process (Bornstein and Sage, 2002).

The CCN family of secreted matricellular proteins is emerging as a critical modulator of pathological processes such as cancer and fibrosis (Leask and Abraham, 2006; Jun and Lau, 2011), but the precise role of this family in normal tissue is unclear. Here we uncover a heretofore unappreciated role for CCN2 in modulating hair follicle cycling; CCN2 acts as a key regulator that controls niche signals and hence acts as an essential regulator of mesenchymal–epithelial cross-talk that orchestrates the number of hair follicles. In particular, we show that loss of CCN2 expression postnatally affects normal hair cycling; moreover, loss of CCN2 in collagen type I-producing cells results in overall increases in hair follicle number concomitant with activation of β-catenin reporter activity. Our results are consistent
with previous data identifying CCN2 mRNA as being expressed in hair stem cells, associated with elevated hedgehog signaling (Tumbar et al., 2004; Rittie et al., 2009). In addition to their role in modulating adhesion (Babic et al., 1999; Leask and Abraham, 2006), CCN proteins also bind extracellular ligands, modifying their activity (Perbal, 2013). In this article we show that CCN2 blocks Wnt3a binding to the Wnt receptor LRP6. These data suggest that CCN2, by blocking the ability of Wnt to bind its receptor, normally suppresses hair follicle cycling. In the future, CCN2 agonists/antagonists might be used to alter hair stem cell function and hence hair regeneration. For example, CCN3, which is known to antagonize CCN2 activity (Riser et al., 2010; Perbal, 2013), might be used to promote hair growth.

MATERIALS AND METHODS

Animal studies

Mice deleted or not for CCN2 in collagen type I–expressing cells were generated as previously described (Liu et al., 2011). Briefly, mice (C57BL/6) hemizygous for Col1a2-Cre (ER)-T and homozygous for Ccn2fl/fl mice were generated by crossing. Three-week-old mice were injected with corn oil or tamoxifen to generate mice deleted or not for CCN2 in fibroblasts. When indicated, mice were generated that were also hemizygous for TOPGAL reporter (C57BL/6) mice, which express β-galactosidase as a reporter in the presence of the lymphoid enhancer binding factor 1/transcription factor 3–mediated signaling pathway and activated β-catenin (Jackson Labs, Bar Harbor, ME). When indicated, depilation of mid dorsal hair follicles was achieved on anaesthetized mice 1 wk after cessation of tamoxifen injection to provide a proliferative stimulus and to synchronize

FIGURE 3: CCN2 alters Wnt-dependent signaling in vitro. (A) CCN2 competes for Wnt3a binding to LRP-6. rCCN2, rWnt3a, and rLRP-6 were combined in vitro and subjected to immunoprecipitation assays, as described in Materials and Methods. (B) CCN2 destabilizes β-catenin in epithelial cells. As described in Materials and Methods, A549 epithelial cells were exposed to different amounts of rCCN2. Top, 0–200 ng/ml of rCCN2 incubated with cells for 24 h. Bottom, 20 ng/ml of rCCN2 incubated with cells for 0–12 h.

FIGURE 4: CCN2 alters Wnt-dependent signaling in vivo. (A) Fibroblast-specific CCN2 knockout mice show elevated β-catenin expression. As described in Materials and Methods, mice hemizygous for Col1a2-cre(ER)T and homozygous for Ccn2fl/fl were generated. Three-week-old mice were treated with tamoxifen or corn oil to generate mice deleted (K/K) or not (C/C) for CCN2 in Col1a2-expressing cells (i.e., DP niche). Two months after cessation of tamoxifen injection, tissue sections were stained with anti–β-catenin and anti-CCN2 antibodies. (B) Fibroblast-specific CCN2 knockout mice show elevated TOPGAL (TCF/LEF) reporter activity. As described in Materials and Methods, mice hemizygous for Col1a2-cre(ER)T and for the TOPGAL reporter that were also homozygous for Ccn2fl/fl were generated. Three-week-old mice were treated with tamoxifen or corn oil to generate mice deleted (K/K) or not (C/C) for CCN2 in Col1a2-expressing cells (i.e., DP niche). Two months after cessation of tamoxifen injection, whole-mount β-galactosidase activity was stained as described in Materials and Methods. Tissue was then sectioned and stained with anti-CCN2 antibodies. (C) Elevated TOPGAL (TCF/LEF) reporter activity in fibroblast-specific CCN2 knockout mice exists in K15+ EpPCs. As described in Materials and Methods, mice hemizygous for Col1a2-cre(ER)T and for the TOPGAL reporter that were also homozygous for Ccn2fl/fl were generated. Three-week-old mice were treated with tamoxifen or corn oil to generate mice deleted (K/K) or not (C/C) for CCN2 in Col1a2-expressing cells (i.e., DP niche). Two months after cessation of tamoxifen injection, tissue sections were stained with anti-β-gal and anti-K15 antibodies.
β-proteinase inhibitors (Roche). Cell lysates were subjected to SDS–PAGE and immunoblotted with anti–β-catenin antibody (1:5000; Sigma). The ECL+ detection technique was used for detection.

**Immunohistochemistry**

Paraffin-embedded tissue sections (0.5 μm) were cut using a microtome (Leica, Richmond Hill, Canada), collected on Super-frost Plus slides (Thermo Fisher, Ottawa, Canada), dewaxed in xylene, and rehydrated by immersion in descending concentrations of alcohol. Tissue sections were blocked by incubation with 5% donkey serum for 1 h and then incubated with primary antibodies overnight at 4°C under humidified conditions. Primary antibodies used were as follows: goat anti-CCN2 (1:200 dilution; Santa Cruz Biotechnology), mouse anti–β-galactosidase (1:100 dilution; Abcam), anti-K15 (1:200 dilution; NeoMarker, Thermo Fisher, Ottawa, ON, Canada), anti-trp1 (1:200 dilution; Santa Cruz Biotechnology), and anti-FSP1 (1:100 dilution; Abcam, Cambridge, UK). After primary antibody incubation, sections were washed with PBS and incubated with appropriate fluorescent secondary antibodies (Jackson ImmunoResearch, West Grove, PA) at room temperature. Sections were mounted using 4',6-diamidino-2-phenylindole (DAPI), and imaged using fluorescence microscopy and Northern Eclipse software (Empix, Mississauga, Canada).

For whole-mount β-galactosidase staining, skin samples were fixed in 0.2% glutaraldehyde/1% formaldehyde/0.02% Nonidet P-40 in PBS overnight at 4°C. After washing with PBS for 3 x 10 min at room temperature, tissues were incubated in X-gal staining solution (5 mM K2Fe(CN)6, 5 mM K4Fe(CN)6, 2 mM MgCl2, 1 mg/ml X-gal in PBS) overnight at room temperature on a rocking platform. Tissues were washed in PBS for 3 d to allow for the stain to develop. The staining was then stopped by 4% paraformaldehyde, embedded in paraffin, and subsequently sectioned for histology. The sections were dewaxed in xylene, rehydrated by immersion in descending concentrations of alcohol, and stained with eosin. Images were taken using fluorescence microscopy and Northern Eclipse software.

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