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Title: CCN proteins as potential actionable targets in scleroderma

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Abstract: Systemic sclerosis (SSc) is a complex auto-immune connective tissue disease combining inflammatory, vasculopathic and fibrotic manifestations. Skin features, which give their name to the disease and are considered as diagnostic as well as prognostic markers, have not been thoroughly investigated in terms of therapeutic targets. CCN proteins (CYR61/CCN1, CTGF/CCN2, NOV/CCN3, and WISP1-2-3 as CCN4-5-6) are a family of secreted matricellular proteins implicated in major cellular processes such as cell growth, migration, differentiation. They have already been implicated in key pathophysiological processes of SSc, namely fibrosis, vasculopathy and inflammation. In this review, we discuss the possible implication of CCN proteins in SSc pathogenesis, with a special focus on skin features, and identify the potential actionable CCN targets.

Introduction

Systemic sclerosis (SSc), or scleroderma is defined as a systemic connective tissue disease characterized by auto-immune and vascular manifestations ultimately leading to organ fibrosis [1]. Like most auto-immune diseases, SSc develops on a genetically predisposed background (the polymorphisms that have been identified concern mostly the immune system), and is probably triggered by environmental factors. However, the interplay between the vascular, immune, and fibrotic components remains poorly understood and no real breakthrough has been made in terms of targeted treatments. The classical pathophysiological features as well as the underlying molecular processes are presented in Figure 1.

Skin manifestations, which give the name to the disease, are considered as diagnostic, subclassification, severity, and prognosis markers [2]. SSc skin effectively recapitulates the main pathogenic processes of the disease, namely fibrosis, vasculopathy and inflammation. Moreover, evidence emerges that key events initiating the disease could take place in the skin, whereas “inside-out” or even “outside-in” as in the field of atopic dermatitis [3]. Besides classically known features affecting the dermis, skin features also include various types of pigmentary disorders, which affect up to half of the patients, such as perifollicular depigmentation [4] or diffuse hyperpigmentation [5]. A recent study from our group found a significant association between diffuse hyperpigmentation and vascular involvement in

SSc, particularly digital ulcers [6]. Current knowledge of SSc epidermal features includes overexpression of PDGR-β, which was the starting point for targeting the PDGF-pathway [7]. Another aspect is abnormal terminal differentiation, with an altered expression of key proteins of the barrier, such as involucrin, loricrin, and filaggrin [8]. The skin barrier function could be altered through the increase of IL-31 [9], which has been shown in vivo to be responsible for transepidermal water loss (TEWL) [10]. However, TEWL seems not altered in SSc skin [11]. .

Paradoxically, skin manifestations have not been thoroughly investigated as possible indicators of actionable targets. Deciphering the molecular mechanisms behind skin features could be applicable to other organs and of importance for SSc, particularly from a therapeutic point of view.

In this context, taking aim at the CCN family of matricellular proteins could be promising, as they are implicated in key pathophysiological processes in SSc as well as in skin homeostasis. This family of six matricellular proteins plays critical regulatory roles in inflammation, angiogenesis and wound healing [12–16]. Initially identified as growth factors [17–19], they have been shown to be involved in several major cellular processes such as cell growth, adhesion, migration, and extracellular matrix homeostasis.

The first member of the CCN family (CYR61 for cysteine-rich angiogenic protein 61) was initially characterized in 1990 in the 3T3 murine cell line, as a an immediate early gene whose transcription was triggered within a few minutes upon serum stimulation [17]. Along this line, CTGF (connective tissue growth factor) was rapidly identified in human endothelial cells (HUVEC) using PDGF-IgG affinity chromatography [18]. NOV (nephroblastoma overexpressed) was discovered in chicken while sequencing the flanking sequences of a virus used to induce nephroblastoma [19]. The conserved primary sequence, as well as the similar tetramodular organization of CYR61, CTGF and NOV led to their designation as CCN (an acronym coined from the first letter of the three names) family of proteins [20]. Soon after, WISP1/ CCN4 (WNT1 inducible signaling pathway protein 1), WISP2/ CCN5, and WISP3/CCN6, were discovered as upregulated in a mouse mammary epithelial cell line after Wnt-1 induction [21]. Due to their similar multimodular structure (except CCN5, which lacks the C-terminal module), WISP proteins were integrated into the CCN family of proteins [22]. The common structure as well as binding sites for proteins of interest in SSc are presented in Figure 2.

Characterization of CCN proteins in human skin rapidly followed, both at the cellular and tissue level, as well as the transcriptional and protein level [23–25]. Total skin transcriptomic analysis showed that CCN5 is the most abundantly expressed CCN member in healthy skin in vivo, followed by CCN2, then CCN3 and CCN1 [24].

Roles of CCN proteins are difficult to characterize due to several factors: ubiquitous expression, multimodular organization, different isoforms; from a functional point of view, they can act on a wide

range of cellular processes, and sometimes present overlapping functions. To date, most studies in the scleroderma field focus on the first three family members (CCN1-3) and on specific cellular processes or tissues. A summary of the expression of CCN proteins in healthy and SSc skin can be found in Table 1.

In this review, we discuss the role of CCN proteins in the key pathophysiological processes of SSc, with a special focus on skin features, and their implication as potential new targets for therapies.

CCN proteins in fibrosis

One of the most studied actors in SSc is CCN2, a major mediator of various fibrotic conditions including skin, heart, lung, liver and kidney [26]. CCN2 is increased in SSc skin, both at the mRNA and protein level. CCN2 is also upregulated in SSc fibroblasts (mRNA) [27]. It is elevated in the serum [28] and correlated with the severity of skin and lung fibrosis. Moreover, polymorphisms of the CCN2 gene have been found to be associated with SSc [29,30]. CCN2 itself is not sufficient to induce fibrosis in a mouse model [31]. However, it is needed for the maintenance of fibrosis when induced by TGF-β [32,33], by promoting fibroblast adhesion to ECM components [34]. Indeed, CCN2 is part of an autocrine pro-fibrotic loop, independent of TGF-β, initiated by endothelin-1 and inducing fibroblasts to synthetize and contract ECM [14,27]. In this context, CCN2 acts by enhancing adhesive responses to TGF-β and endothelin-1 in fibroblasts, downstream of the anti-fibrotic protein PTEN (phosphatase and tensin homologue) [35]. PTEN, which is reduced in SSc skin fibroblasts [36], acts by suppressing PI3K-Akt signaling (phosphatidylinositol 3-kinase (PI3K) and Akt/Protein Kinase B) [37]. Its fibroblast-specific loss in an in vivo model induces collagen deposition in the lung in a CCN2-dependent manner [38]. Overall, inactivation of CCN2 is a promising lead – more specific than a targeted TGF-β therapy - in anti-fibrotic therapies: notably, fibroblast-specific ablation of CCN2 reduces skin fibrosis in the bleomycin mouse model [33].

CCN3 counteracts CCN2 profibrotic pathway, although this has never been demonstrated directly in the skin. In the kidney, CCN3 is upregulated in response to TGF-β signaling as shown by several studies of diabetic renal fibrosis, and is regulated in an opposite manner to CCN2 [39,40]. Treatment with recombinant CCN3 succeeded in reducing glomerular fibrosis in a mouse model of diabetic nephropathy, showing a capacity to reverse the disease [41]. The anti-fibrotic effect of CCN3 could work through the regulation of multiple pathways, possibly via repression of CCN2 but also CCN4 [42]. Moreover, CCN3 overexpression in a mouse fibroblast cell line blocked TGFβ- and Wnt-regulated profibrotic gene expression [12]. Of note, CCN3 mRNA has been found to be increased in SSc skin [12].

The role of CCN1 has not been extensively studied in fibrosis, although its role in promoting tissue repair in wound healing is becoming clearer [13]. One study has found that CCN1 is induced in dermal fibroblasts by TGF-β along with CCN2 and α-SMA [43].

CCN5 is the most abundant CCN in the dermis in terms of mRNA expression [25]. Contrary to CCN2, CCN5 has been found to be downregulated after TGF-β exposure in human skin fibroblasts [44]. Moreover, CCN2 and CCN5 present an opposite expression pattern in vascular smooth muscle cells (although they have not been studied together) [45]. However, no study has been conducted to characterize the expression of CCN5 in SSc.

Little is known concerning the role of the other CCN proteins in skin. CCN4 has been identified in skin fibroblasts and seems to play role in a paracrine manner, by binding to decorin and biglycan [46]. Moreover, CCN4 has been shown to regulate wound healing by modulating proliferation, migration and ECM expression in dermal fibroblasts via α5β1 integrin and TNFα [47].

CCN proteins in angiogenesis and vasculopathy

CCN1, 2 and 3 are all known as pro-angiogenic. In particular, CCN1 and CCN2 are important actors in endothelial cells homeostasis, working at least partly through binding to cell surface integrins; their expression in endothelial cells is enhanced by VEGF [48]. However, although the role of CCN2 role in SSc is well-documented, CCN1 and CCN3 have been less studied in scleroderma.

CCN1 is known to enhance tubule formation in vitro via integrin α6β1 and integrin αvβ3 [49].CCN1 has been implicated in SSc impaired angiogenesis in a recent study [50], showing that CCN1 expression was markedly decreased in dermal microvessels of patients with SSc as well as in the serum of SSc patients with digital ulcers. Another recent study showed that simultaneous knock-down of histone deacetylase 5 and CCN1 inhibited in vitro angiogenesis, while overexpressing CCN1 in SSc endothelial cells led to increase in tube formation [51], suggesting that a decrease in CCN1 plays an important functional role in SSc impaired angiogenesis.

In vitro, CCN2 effectively recapitulates angiogenic events by promoting endothelial cell adhesion, migration, proliferation, and tubule formation [52]. In vivo, CCN2 enhances neovascularization in a mouse model of retinopathy [53]. However, recent in vitro studies using cells and tissues from the CCN2-null mouse eye did not indicate any effect of CCN2 deletion on neovascularization and angiogenesis. This finding suggests that CCN2 may be tissue-specific concerning angio/vasculogenesis, possibly via interaction with VEGF [54]. Moreover, the results from the knock-out (KO) studies could indicate that the upregulation of CCN2 observed in SSc is mainly responsible for enhanced fibrosis and is not sufficient to correct vascular dysfunction. Accordingly, we have observed a decrease of CCN2 expression in cultured SSc endothelial cells, whereas an increase was observed in SSc fibroblasts (unpublished data).

CCN3 has been shown to be pro-angiogenic in several studies: addition of recombinant CCN3 to rat corneas induces neovascularization [55]; CCN3 is highly expressed at a basal state in endothelial cells and is implicated in vascular repair as well as induced in HUVECs by laminar shear stress [15,56]. The

mechanism underlying its pro-angiogenic action is probably at least partly mediated by binding to several integrins such as $\alpha_v\beta5$ [57]. Interestingly, CCN3 and CCN1 are decreased in placental endothelial cells of women suffering from pre-eclampsia, a pathological pregnancy condition characterized by hypoxic vascular lesions [58]. The pathophysiology underlying pre-eclampsia is strikingly similar to SSc renal crisis. Unpublished data from an ongoing study of our group focusing on human microvascular dermal endothelial cells points towards an important role for CCN3 in angiogenesis in vitro.

Last, CCN4 promotes vascular smooth muscle cells migration in vitro via an integrin-dependent pathway [59], but its action has not been studied in the vascular SSc system.

CCN proteins in dermal inflammation and auto-immunity

A growing body of evidence supports the concept that CCN1 as a pro-inflammatory factor in skin. In the dermis, CCN1 is elevated during the inflammatory phase of wound healing and downregulated during the extra-cellular matrix remodeling phase [24]. CCN1 has also been shown to regulate macrophage function during inflammation in mice, by supporting macrophage adhesion as well as upregulating pro-inflammatory cytokines such as TNF-α [60]. Moreover, CCN1 is able to activate the cytotoxic potential of TNF-α and thus to induce fibroblast apoptosis [61]. Interestingly, CCN1 is highly produced by thymic epithelial cells and boosts T-cell production in mice [62]. Several studies have found an overexpression of CCN1 in the epidermis of inflammatory skin conditions such as psoriasis, where it promotes the production of pro-inflammatory mediators such as IL-8 [63], IL-1β[64] or CCL-20 [65].

CCN3 is present at a high levels in the supernatant of T regulatory lymphocytes in the central nervous system, where it promotes oligodendrocyte differentiation and myelination [66]. Preliminary data from our team indicat high production of CCN3 by lymphocyte infiltrating the dermis in vitiligo. Moreover, CCN3 expression is regulated by TNF-α and IL-1 [67], and CCN3 physically interacts with IL33 [68], a T-helper-2 associated cytokine able to induce fibrosis in SSc [69].

Similar to CCN3, CCN6 gene expression is induced by TNF-α and IL-1; of note, CCN6 is overexpressed in the synovial tissue of patients suffering from rheumatoid arthritis [70]. However, such observations have not been made in the skin.

CCN proteins and accelerated aging

There is increasing evidence that SSc skin presents several hallmarks of cellular aging, such as enhanced production of reactive oxygen species (ROS), methylation abnormalities and impaired autophagy [71]. CCN1 has been associated with skin aging in several studies [72–75]. Notably, it has been shown to upregulate ROS production and induce skin fibroblasts senescence by binding to integrin α6β1 and heparin sulfate proteoglycan during wound repair [75]. Of note, knock-in (KI) mice for a mutant

CCN1 lacking the α6β1 integrin-binding (so-called senescence-defective CCN1) showed exacerbated fibrosis in wound healing. Another study implicates CCN1 in aberrant collagen homeostasis associated with dermal fibroblasts senescence, suggesting an important role for CCN1 in collagen loss [72]. Indeed, CCN1 is a known regulator of type I collagen production and degradation [73].

CCN proteins in pigmentation

CCN3 mRNA is the most highly expressed CCN gene in the epidermis [25]. Its role in epidermal homeostasis and particularly pigmentation regulation remains unclear. Its epidermal expression seems to be increased in pigmented phototypes [76] (interestingly, CCN5 seems to vary inversely). CCN3 plays a major role in melanocyte homeostasis and is implicated in melanocyte adhesion [67]. Conversely, downregulation of CCN3 in melanocytes results in cell detachment from the epidermis in vitro and could be implicated in pigmentary disorders such as vitiligo [77]. To date, however, the implication of CCN3 in the development of pigmentary changes often observed in SSc has not been proven.

UV radiation upregulates CCN1 and CCN2 expression in whole skin in vivo, whereas UV radiation downregulates CCN3, 4, 5 and CCN6 expression, at the mRNA level [24]. Interestingly, CCN1 has recently been found to stimulate melanogenesis through integrin α6β1 binding as well as p38 MAPK and ERK1/2 signaling [78]. The authors suggested that CCN1 is a fibroblast-derived melanogenic paracrine mediator, secreted under UVB irradiation. This finding, along with the role of CCN1 in accelerated aging, could be consistent with the "photo-aging" pattern observed in some SSc patients.

CCN proteins in epidermal differentiation

CCN2 is increased in the epidermis of SSc patients, particularly in the basal membrane [8]. This increase was predominantly seen in recent SSc (< 2 years). Accordingly, CCN2 expression has been shown to be induced by TGF-β in human keratinocytes [79]. CCN2 is also thought to regulate keratinocyte migration via the RAS-MEK-ERK pathway [80].

Differential expression of CCN3 and CCN5 within epidermal layers also suggest that these factors are associated with epidermal differentiation, although the precise mechanism is unknown to date [25]. CCN3 expression is reported as nuclear or peri-nuclear in basal keratinocytes, as opposed to cytoplasmic in the upper layers, whereas CCN5 expression is reported weak and perinuclear in the basal layer and strong and cytoplasmic in the upper layers.

CCN proteins in animal models: what can we learn about key pathophysiological processes of SSc?

Knock-out (KO) and knock-in (KI) animal models provide relevant information concerning the role of CCN proteins in key pathophysiological processes of SSc. The KO models interestingly point out the major role of CCN1 and CCN2 (and to a lesser extent CCN3 and CCN5) in vasculogenesis. Moreover, CCN1 and CCN2 seem to play a prominent role in fibrosis regulation, CCN1 being responsible for wound healing resolution and CCN2 for a persistent fibrotic phenotype as shown by the KI models. CCN5 also appears as an anti-fibrotic molecule. Lastly, CCN1 seems to be an important actor in inflammatory processes. Surprisingly, CCN4 may come off both as a pro-angiogenic and anti-fibrotic protein, which makes it an interesting actor in scleroderma. A summary of the phenotypes of transgenic mice models can be found in Table 2.

Are CCN proteins potential druggable targets in SSc?

Since CCN proteins are downstream effectors, they could be therapeutically targeted without affecting major upstream signaling pathways. However, very few studies have reported the use of anti-CCN antibodies in pre-clinical studies, and the proof of concept remains to be obtained. Figure 3 summarizes the potential roles of CCN proteins in SSc pathways and suggested targets for therapy.

Recently, a monoclonal antibody against CCN2 (FG-3019) has been shown to inhibit skin fibrosis in the angiotensin-II induced SSc mouse model [81]. In this study, the intra-peritoneal injection of FG-3019 (concomitant to Ang-II injection) significantly reduced dermal thickness and collagen content in skin from Ang-II challenged mice, as well as the number of αSMA-positive cells, PDGFRβ and procollagen expression in the upper dermis. Of note, the same antibody has been tested in an open-label phase II clinical trial designed for patients suffering from idiopathic pulmonary fibrosis [82]. For one third of treated subjects, pulmonary function improved as well as lung fibrosis. A randomized placebo-controlled phase 2 clinical trial is currently underway.

Since CCN1 is pivotal for many pathophysiological pathways of SSc, a CCN1-based therapy, whether topical or systemic, could also be promising for SSc. Topical application of CCN1 has indeed shown to reverse the enhanced fibrosis of cutaneous wounds in the α6β1 integrin-binding defective CCN1-KI mice [75]. Conversely, a neutralizing anti-CCN1 polyclonal antibody inhibited angiogenesis in an oxygen-induced retinopathy mouse model [83] as well as a bone fracture mouse model [84]. A monoclonal neutralizing antibody against CCN4 has also been used in a murine model of pulmonary fibrosis and showed promising results in reducing the expression of genes implicated in fibrosis and epithelial-to-mesenchymal transition [85]. However, these two antibodies have never been used, to our knowledge, in the skin or for scleroderma. Finally, a recombinant CCN3 has been used successfully in a mouse model of renal fibrosis [41], and could be considered for skin issues.

In conclusion, knowledge gained from studies of the roles of CCN proteins in dermal and epidermal biological processes can be applicable for other organs. Moreover, due to multiple functions of CCN protein, effective therapeutic strategies may rely on downregulating or upregulating specific CCN proteins in SSc. Based on emerging evidence, there is a growing need to systematically determine the roles of all CCN family members in the pathophysiological pathways that are characteristic of scleroderma skin. SSc models provide an ideal opportunity to test the concept of therapeutic targeting of CCN proteins for the treatment of SSc.

Author contribution

PH wrote the original draft. MET, GF, AT, MC reviewed and edited the manuscript. All authors have read and approved the final manuscript.

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Conflicts of interest

The authors have declared no conflict of interest.

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CCN member	Synonym (human)	Healthy skin		SSc skin	
		Skin expression (mRNA level) [25]	Main producing skin cells in vivo [25,76]	Skin expression (mRNA level)	Expression in SSc skin cells

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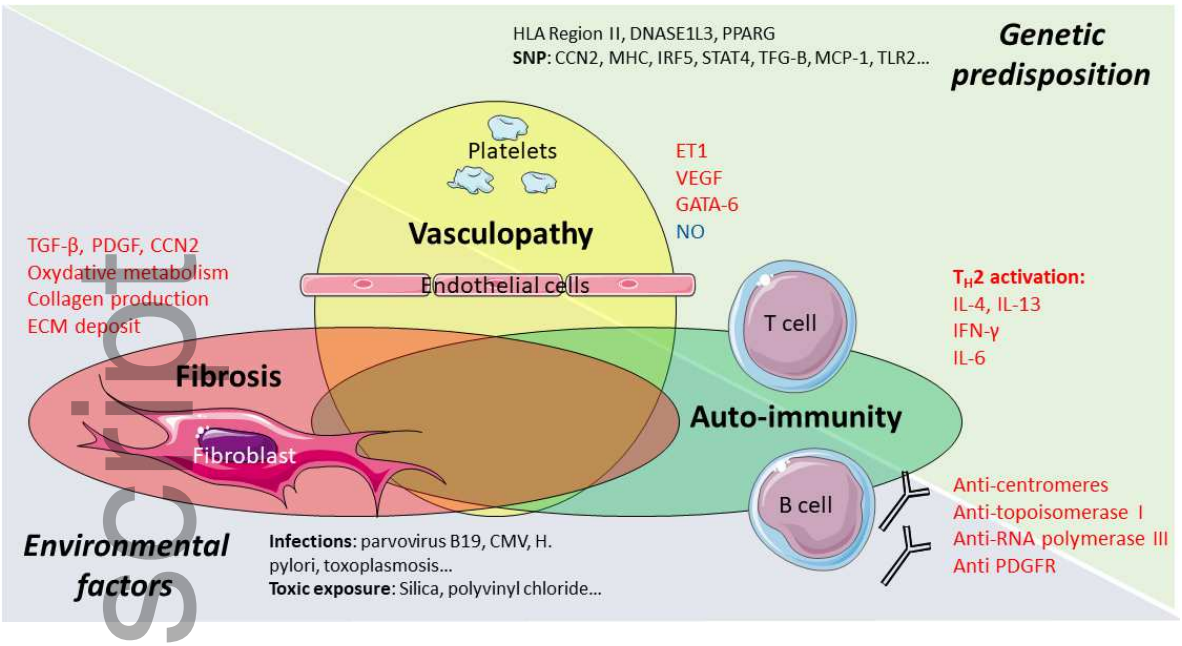
CCN1	CYR61	Weak in epidermis and dermis	Fibroblasts, endothelial cells	Decreased [50]	Decreased in endothelial cells in situ, but unchanged in fibroblasts in vitro [50]
CCN2	CTGF	Strong in dermis> Epidermis	Melanocytes > Fibroblasts, endothelial cells	Increased [86]	Increased in fibroblasts, endothelial cells and keratinocytes in situ and in vitro [8,87,88]
CCN3	NOV	Dermis> Epidermis	Keratinocytes > Fibroblasts, endothelial cells, eccrine sweat glands Melanocytes	Increased [12] or unchanged [86]	Decreased in endothelial cells in situ and in vitro, and in fibroblasts in vitro (unpublished data)
CCN4	WISP1	Weak in epidermis and dermis	Fibroblasts, endothelial cells (?) [47,89]	ND	ND
CCN5	WISP2	Strong in dermis> Epidermis	Differentiated keratinocytes > Endothelial cells, fibroblasts	Decreased? [86]	ND
CCN6	WISP3	Weak in epidermis and dermis	ND	ND	ND

Table 1. CCN proteins in human skin and expression in SSc.
mRNA=messenger RNA, ND = not described.
Expression in SSc skin cells: compared to healthy control cells.

CCN member	Knock-in (KI)				Knock-out (KO)				Ref
	Fibrosis	Vasculopathy	Inflammation	Other	Fibrosis	Vasculopathy	Inflammation	Other	
CCN1	Increased fibrosis	ND	Lung alveolitis, decreased TNF-α-mediated apoptosis	Weight loss and higher mortality; (KI of senescence-defective mutant CCN1)	ND	Placental vascular insufficiency and compromised vessel integrity	ND	Embryonic lethal, chorio-allantoic fusion (30% mice)	[61, 75,90,91]
CCN2	Increased cardiac fibrosis in response to pressure overload KI fibroblast-specific: skin, lung, kidney, and small arteries fibrosis	ND	ND	Embryonic lethal if ninefold overexpression	ND	Abnormal growth plate angiogenesis	ND	Perinatal lethal: severe skeletal and pancreatic abnormalities, pulmonary hypoplasia	[92 – 97]
CCN3	ND	ND	ND	Overexpression in fibroblasts: Osteopenia (skin and vessels not analyzed)	ND	Vascular congestion in kidney and liver; enhanced neointimal hyperplasia in response to endothelial injury	ND	Viable: modest skeletal and cardiac abnormalities, muscle atrophy and cataract	[98 – 100]

CCN4	ND	Increased intimal thickening due to smooth muscle cell migration	ND	ND	Delayed wound healing, reduced collagen expression	ND	ND	Impaired motor coordination	[47, 59, 101]
CCN5	Decreased cardiac fibrosis in response to pressure overload	ND	ND	ND	ND	Hyperproliferation of vascular smooth muscle cells in response to injury	ND	Both reported: early embryonic lethality and normal phenotype	[45, 97, 102, 103]
CCN6	Normal	Normal	Normal	Normal phenotype	Normal	Normal	Normal	Normal phenotype	[104, 105]

Table 2. Phenotypes of genetically-modified mouse models.
ND = not described



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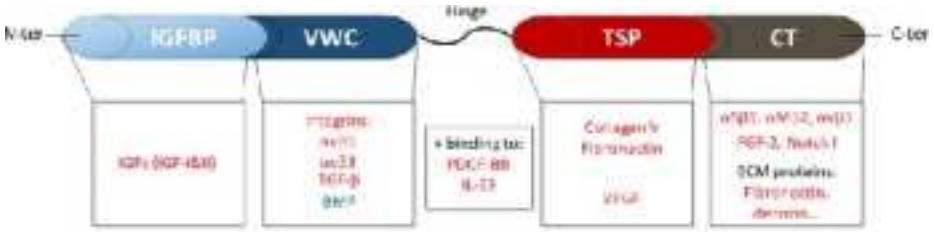

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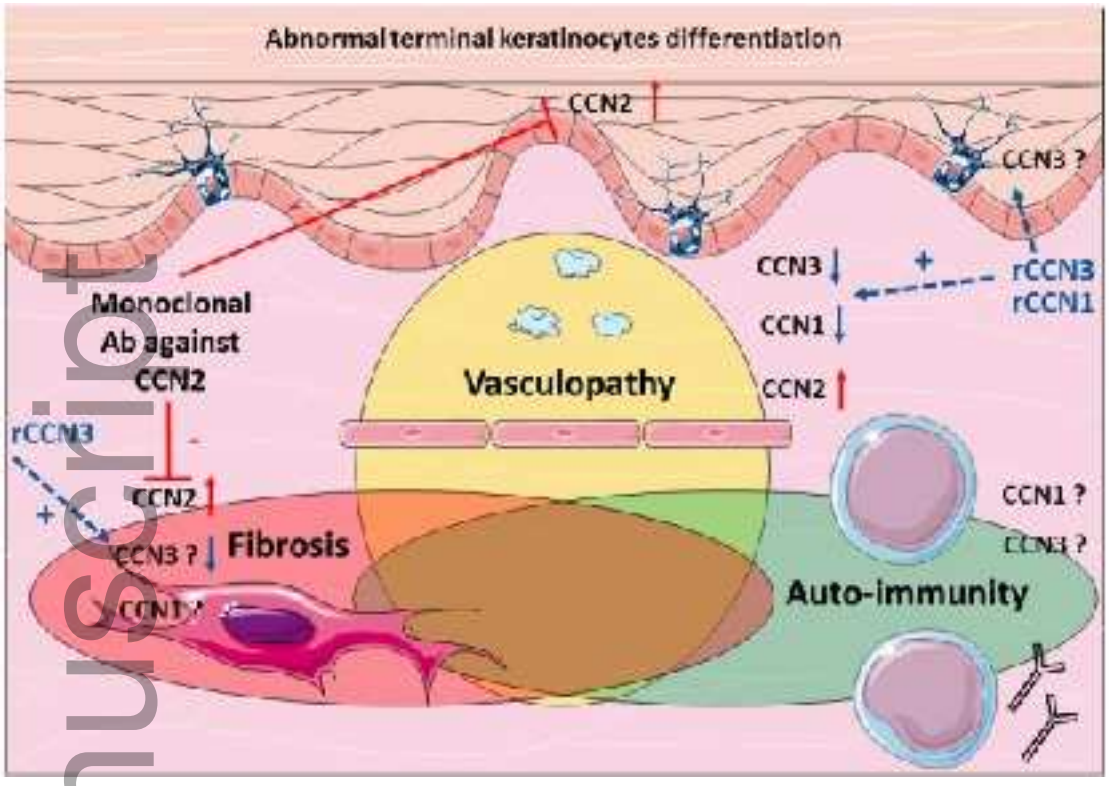

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