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# CCN1/CYR61: The Very Model of a Modern Matricellular Protein

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## Abstract

CCN1 (CYR61) is a dynamically expressed, multifunctional matricellular protein that plays essential roles in cardiovascular development during embryogenesis, and regulates inflammation, wound healing and fibrogenesis in the adult. Aberrant CCN1 expression is associated with myriad pathologies, including various cancers and diseases associated with chronic inflammation. CCN1 promotes diverse and sometimes opposing cellular responses, which can be ascribed, as least in part, to disparate activities mediated through its direct binding to distinct integrins in different cell types and contexts. Accordingly, CCN1 promotes cell proliferation, survival and angiogenesis by binding to integrins $\alpha_v\beta_3$, and induces apoptosis and senescence through integrin $\alpha_6\beta_1$ and heparan sulfate proteoglycans. The ability of CCN1 to trigger the accumulation of a robust and sustained level of reactive oxygen species underlies some of its unique activities as a matrix cell-adhesion molecule. Emerging studies suggest that CCN1 might be useful as a biomarker or therapeutic target in certain diseases.

### Keywords

Angiogenesis; apoptosis; fibrosis; integrins; reactive oxygen species; senescence; signal transduction; tumorigenesis; wound healing

## INTRODUCTION

The term “matricellular protein” was first introduced by Bornstein in 1995 to describe a group of extracellular matrix (ECM) proteins that play minimal roles in matrix structural integrity, but regulate a multitude of cellular responses with protean and sometimes opposing functions [1]. CCN1 (also named CYR61 or cysteine-rich 61) exemplifies many aspects of matricellular proteins by exhibiting diverse and at times seemingly conflicting functions in various cell types. Matricellular proteins are involved in wound healing without exception, and CCN1 is emerging as an important regulator of inflammation and wound repair [2–4]. First identified as a serum-inducible immediate-early gene product in mouse fibroblasts [5–8], CCN1 and other highly conserved homologs comprise the CCN protein family, which contains six members in mammals and nine members in zebrafish [9–11]. The CCN acronym is derived from the first three members of the family identified, namely CYR61, CTGF (connective tissue growth factor), and NOV (nephroblastoma overexpressed), and mammalian members of the family have been renamed CCN1–6 in order of their discovery [12]. CCN proteins share a modular structure, with an N-terminal secretory peptide followed by four conserved domains with sequence homologies to insulin-like growth factor-binding protein (IGFBP), von Willebrand factor type C repeat (vWC), thrombospondin type I repeat (TSR), and a carboxyl-terminal (CT) domain that contains a

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cysteine knot motif (Fig. 1). Each conserved structural domain is encoded by a separate exon, suggesting that CCN genes arose through exon shuffling [9,10]. A non-conserved central hinge region bisects the protein into two halves that bind distinct receptors and induce disparate cellular responses (Fig. 1). CCNs were initially thought to function primarily in regulating cell proliferation, as they were identified based on their induction by mitogenic growth factors, oncogenes, or transformation. However, early studies on CCN1 showed that it is tightly associated with the ECM upon secretion [13] and supports cell adhesion through direct binding to integrin receptors [14,15]. These findings, and the observation that CCN1 does not induce cell proliferation on its own but only enhances DNA synthesis induced by other mitogens [14], led to the appreciation that CCNs are matricellular proteins rather than classical growth factors [10].

CCN1 is remarkably versatile and has many functions, some seemingly at odds with each other. It promotes cell survival and yet triggers apoptosis, enhances cell proliferation but also induces cell-cycle arrest, and promotes tumor growth and yet suppresses tumorigenesis in various contexts. These disparate activities of CCN1 can now be attributed, in large parts, to its interaction with distinct integrins and heparan sulfate proteoglycans (HSPGs) in a cell-type and context-dependent manner (Fig. 1). Some of these activities are unique among cell-adhesion proteins of the ECM, and thus CCN1 serves as a model molecule for understanding novel aspects of matrix signaling. Current data have established CCN1 as being essential for cardiovascular development during embryogenesis, and critical for modulating inflammation, wound healing, and tissue repair in many tissues of the adult. Numerous gene-profiling studies have shown altered CCN1 expression in diverse pathological conditions, including various cancers, inflammatory diseases, and dysfunctional wound healing, suggesting that targeting CCN1 expression and signaling may hold promise in the development of diagnostic markers and therapeutics. Here, I seek to provide an update on the most recent findings on CCN1, focusing on its biological functions and mechanism of actions. Several earlier reviews afford a more comprehensive summary of research on the CCN family [9,10,16–18].

## TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL REGULATION

*CCN1* is an immediate-early gene expressed at a very low level in quiescent fibroblasts, but is transcriptionally activated within minutes of stimulation by serum growth factors without requiring *de novo* protein synthesis [5,6]. Exquisitely sensitive to a wide range of extracellular stimuli, *CCN1* is transcriptionally activated by platelet-derived growth factor and fibroblast growth factor 2 [8], transforming growth factor β1 (TGF-β1) [19], growth hormone [20], the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate, cAMP [8], vitamin $D_3$ [21], estrogen and tamoxifen [22], angiotensin II [23,24], hypoxia [25], UV light [26], and mechanical stretch [27,28]. Consistent with a role in inflammation, *CCN1* is also induced by bacterial and viral infections [29–32], and by inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α) [33]. *CCN1* is activated by agonists of G protein-coupled receptors (GPCRs) such as thrombin [34,35], prostaglandins $E_2$ and $F_{2\alpha}$ [33,36], and sphinogosine-1-phosphate [37]. Thrombin acts through the GPCR and RhoA signaling pathways to induce CCN1, which in turn interacts with integrins to promote cell proliferation [35]. Thus, CCN1 serves to connect the GPCR and integrin signaling pathways to enhance thrombin-induced cell proliferation [38].

Analysis of the mouse *Ccn1* promoter uncovered a serum response element (SRE) (–1912/–1933) to which the 67 kDa serum response factor (SRF) binds and mediates transcriptional activation by serum or platelet-derived growth factor in fibroblasts [39]. A 2 kb *Ccn1* promoter fragment that includes the SRE is sufficient to confer accurate developmental and tissue-specific expression in transgenic mice when linked to the β-galactosidase gene as a

reporter, thus defining the functional *Ccn1* promoter for transcriptional regulation *in vivo* [40]. Mechanical stretch induction of *CCN1* in human smooth muscle cells involves activation of the RhoA GTPase, which promotes actin remodeling and consequently recruitment of the transcription factor myocardin-related transcriptional activator (MRTF-A), a G-actin-binding SRF co-activator, to the SRE of the *CCN1* promoter [41]. Activation of p38 mitogen-activated protein kinase (MAPK), which enhances the histone acetyltransferase activity of CREB-binding protein (CBP) and its recruitment to the SRF–MRTF-A complex, enhances *CCN1* expression. The transcription factor Egr-1, although not sensitive to cytoskeletal remodeling, is also involved in stretch-induced activation of *CCN1* in vascular smooth muscle cells [42]. Consistent with its angiogenic functions, *CCN1* is highly induced under conditions of hypoxia, mediated through both hypoxia-inducible factor-1α (HIF-1α)-dependent and -independent mechanisms [25,43,44]. Recent genomic studies have also shown that *CCN1* is a target of the TAZ and YAP transcriptional co-activators, which act in concert with various transcription factors such as TEAD to regulate expression of genes related to proliferation and apoptosis [45,46].

Specific alternative splicing in *CCN1* was first reported in fibroblasts, causing an in-frame deletion within exon 4 (TSR domain) [47]. Another alternative splicing event retains intron 3, resulting in two stop codons that would terminate the CCN1 polypeptide within the central hinge region. However, this alternative splicing is blocked under hypoxic conditions in breast cancer cells, leading to the synthesis of full-length CCN1 [48]. Further post-transcriptional regulation involves microRNA-155, which appears to downregulate *CCN1* expression in pre-eclampsia [49].

The *CCN1* mRNA contains internal ribosome entry sites (IRES) that allow its preferential translation under conditions of stress, even when the concentration of the cap-binding complex eIF4F is low, for example upon infection by certain viruses [50]. Both the 5′-non coding region of the *Ccn1* mRNA and a highly GC-rich region encoding part of the IGFBP domain appear to contain IRES [50,51]. To date, information on post-translational regulation of CCN1 is scant. Although CCN1 is glycosylated, neither the chemical nature of the glycosylation nor its biological function is well understood. There is no direct evidence indicating whether CCN1 might function as a dimer or oligomer. Anecdotal observations of proteolytic processing have been made, although the biological significance of these modifications *in vivo* is currently unknown [14,52].

## MULTIPLE RECEPTORS AND DIVERSE FUNCTIONS

### Cell-surface signaling receptors: integrins and HSPGs

The functions of CCN proteins are mediated primarily through their binding to distinct integrins [53], which are bidirectional cell-surface signaling receptors capable of regulating diverse cellular functions [54]. That CCN proteins are ligand of integrins was first demonstrated by the direct binding of CCN1 to integrin $\alpha_v\beta_3$ to mediate endothelial cell adhesion [15]. Subsequent studies have shown that CCN1 supports cell adhesion by binding integrins $\alpha_6\beta_1$ and HSPGs in fibroblasts and smooth muscle cells [55,56], $\alpha_{IIb}\beta_3$ in activated platelets [57], $\alpha_M\beta_2$ in monocytes and macrophages [58,59], and $\alpha_D\beta_2$ in macrophage foam cells [60]. The functions of CCN1 mediated through integrins are listed in Table 1. CCN1 binds HSPGs with high affinity, which serve as co-receptors with integrins in some contexts [13,55]. Where examined, syndecan-4 has been identified as the HSPG critical for CCN1 functions [61,62]. The interactions between CCN1 and integrins–HSPGs have been reviewed in greater detail previously [10,18,53].

CCN1 does not contain the canonical RGD sequence that can bind a number of integrins [54]. Instead, specific non-canonical binding sites of CCN1 for several integrins have been

identified (Fig. 1) [63–66]. Mutational analysis showed that distinct integrin-binding sites of CCN1 can function independently of one another. CCN1 mutants that disrupt its $\alpha_6\beta_1$-binding sites abolish $\alpha_6\beta_1$-dependent activities without affecting $\alpha_v\beta_3$-mediated functions [66]. Likewise, disruption of the CCN1 $\alpha_v\beta_3$-binding site abolishes $\alpha_v\beta_3$-but not $\alpha_6\beta_1$-mediated activities [65]. However, the synergism of CCN1 with TNFα (see below) requires its interaction with both $\alpha_v\beta_5$ and $\alpha_6\beta_1$. Interestingly, whereas CCN1 mutants defective for either binding $\alpha_v\beta_5$ or $\alpha_6\beta_1$ are each incapably of functional synergism with TNFα, a combination of these two mutants can fully reconstitute wild-type activity [62]. This observation suggests that different CCN1 molecules can separately bind distinct integrins on the same cell and generate signaling events that converge inside the cell to produce specific biological outcome. Aside from integrins and HSPGs, CCN1 may potentially interact with other cell-surface receptors as well. The homologous CCN2 has been shown to bind LRP-1 [67] and TrkA [68], whereas CCN3 can bind Notch [69]. Assessing whether CCN1 can interact with these and other receptors will require further investigation.

## Cell adhesion, migration, DNA synthesis, and cell survival

Upon secretion, CCN1 is tightly but non-covalently associated with the ECM and the cell surface, an interaction that can be displaced by soluble heparin [13]. This observation led to the finding that CCN1 can support cell adhesion and induce adhesive signaling in many adherent cell types [18,53]. In human skin fibroblasts, CCN1 supports cell adhesion through $\alpha_6\beta_1$–HSPGs and results in the formation of focal adhesion complexes, activation of focal adhesion kinase, paxillin and Rac, actin cytoskeleton reorganization, and formation of structures critical for cell motility such as filopodia and lamellipodia [70]. A short CCN1 peptide containing the $\alpha_6\beta_1$–HSPG binding sites is sufficient to support cell adhesion and recapitulate the sustained activation of extracellular signal-regulated kinase (ERK) by immobilized CCN1 [66]. Furthermore, CCN1 stimulates cell migration in fibroblasts, smooth muscle cells and endothelial cells [56,71,72], and certain cancer cells [73–75]. Although CCN1 does not induce DNA synthesis on its own, it can synergize with other mitogenic growth factors to augment growth factor-induced DNA synthesis in both fibroblasts and endothelial cells through integrin $\alpha_v\beta_3$ [14,71]. This activity may contribute to its ability to promote growth factor-induced poliferation in PC3 prostate cancer cells [76,77] and ovarian carcinoma cells [78], and IL-17-stimulated proliferation in synoviocytes [79]. Consistent with cell-survival functions associated with cell adhesion, CCN1 promotes the survival of endothelial cells through integrin $\alpha_v\beta_3$ [72], and inhibits apoptosis by activation of Akt in pulmonary epithelial cells [80] or upregulation of XIAP in MCF7 breast cancer cells [81].

## Angiogenesis

The strong expression of *Ccn1* in endothelial cells during embryonic development in mice suggested a role in vessel growth [82]. Indeed, CCN1 has potent angiogenic activity, first demonstrated in a corneal micropocket implant assay [83] and subsequently confirmed in a rabbit ischemic hindlimb model [84]. The angiogenic activity of CCN1 can be attributed to its binding to integrin $\alpha_v\beta_3$, a major integrin expressed in endothelial cells responsible for mediating CCN1 function in promoting endothelial cell adhesion, migration, proliferation, survival, and tubule formation [72]. A mutant CCN1 protein specifically mutated in the $\alpha_v\beta_3$-binding site is devoid of angiogenic activity [65]. Consistent with an important role for CCN1 in angiogenesis *in vivo*, *Ccn1*-null mice are defective in placental vessel bifurcation and suffer severe cardiovascular defects [85,86]. In addition to its direct effects on endothelial cells, CCN1 can also regulate the expression of angiogenic factors such as VEGF-A and VEGF-C [85,87]. Furthermore, CCN1 stimulates the integrin-dependent recruitment of $CD34^+$ progenitor cells, thereby enhancing endothelial proliferation and neovascularization [88].

### Chondrogenesis and osteogenesis

*Ccn1* is expressed in pre-chondrocytic mesenchymal condensations of both mesodermal and neuroectodermal origins [82]. Consistent with a role in chondrogenesis, purified CCN1 protein accelerated the differentiation of mouse limb bud mesenchymal cells isolated from E10 embryos, enhanced the extent of differentiation, and lowered the threshold cell density for differentiation [89]. At later stages of skeletal development, CCN1 is highly expressed in the hypertrophic cartilage at the growth plate [40]. Thus, CCN1 may be involved in neovascularization of the hypertrophic cartilage, where vessel invasion provides conduits for osteoblasts. Moreover, CCN1 stimulates osteoblast differentiation through an $\alpha_v\beta_3$/ILK-dependent pathway but inhibits osteoclastogenesis, suggesting its role as a bifunctional regulator of bone remodeling that promotes bone formation but inhibits bone resorption [90–92].

### Potential nuclear functions

In contrast to its prominent localization to the ECM, CCN1 has also been detected in the nucleus of cells, despite the absence of a classical nuclear localization signal [93]. The specific roles of nuclear CCN1 are not well understood. Interestingly, CCN1 was identified in an expression screening of a λgt11 library for proteins that bind the human immunodeficiency virus type 1 (HIV-1) long terminal repeat [47]. Furthermore, recombinant CCN1 was shown to bind DNA with sequence specificity *in vitro*, and co-transfection of CCN1 expression plasmids inhibited transcription from HIV-1 long terminal repeat reporter constructs [47]. Although the functional implications of these observations have not been investigated further, they suggest the intriguing possibility that CCN1 may bind DNA and regulate transcription under certain conditions. A recent study showed that CCN5, a member of the family that uniquely lacks the CT domain, is able to act as a transcriptional repressor upon a GAL4-dependent reporter when linked to the GAL4 DNA-binding domain, presumably through association with the histone deacetylase HDAC1 [94]. The possibility that CCN1 might regulate nuclear gene transcription certainly warrants further investigation.

## CCN1 FUNCTIONS IN EMBRYONIC DEVELOPMENT AND PREGNANCY

*Ccn1* expression during mouse embryogenesis is tightly associated with development of the skeletal, cardiovascular, and neuronal systems [82], and targeted disruption of *Ccn1* in mice results in embryonic lethality. Approximately 30% of *Ccn1*-null embryos fail to form chorioallantoic fusion at E8.5 and die by E9.5, whereas the remaining embryos perish at mid-gestation from placental vascular insufficiency, loss of embryonic vascular integrity, hemorrhage, and severe cardiac atrioventricular septal defect (AVSD) [85,86]. At E8.5, *Ccn1* is highly expressed in the ectoplacental cone and in trophoblastic giant cells, suggesting a role in implantation and interaction with the decidua [82]. Despite normal vasculogenesis in *Ccn1*-null mice, specific angiogenic defects are observed. Vessel bifurcation is impaired in the chorionic plate of *Ccn1*-null embryos, resulting in a dearth of sprouting vessels that penetrate into the labyrinth, and thus an undervascularized placenta [85]. However, trophoblast differentiation and syncytiotrophoblast formation at the chorionic plate occur normally in the *Ccn1*-null placenta, forming the trilaminar trophoblast barrier between fetal vessels and maternal blood sinuses where fetal vessels are found. Therefore, the labyrinthine vascular deficiency in *Ccn1*-null mice is not due to trophoblast defects. The endothelium in large vessels of *Ccn1*-null embryos lack an intact basement membrane and the vascular cells are disorganized and apoptotic, leading to vessel rupture and hemorrhage [85].

In the human placenta, *CCN1* is most highly expressed in the second and third trimesters of pregnancy, especially in non-proliferating interstitial extravillous trophoblastic giant cells, vascular endothelial cells, and mesenchymal and stromal cells of the placental villi [95]. *CCN1* expression is greatly decreased in placentae associated with pre-eclampsia, a condition thought to result from a shallow invasion of the extravillous trophoblast into the decidua, followed by incomplete remodeling of the maternal vascular structures [95]. This finding is consistent with a role for CCN1 in placental angiogenesis and decidual tissue remodeling, and evidence suggests that serum levels of CCN1 may serve as a marker for early diagnosis of pre-eclampsia [96]. Additionally, CCN1 is upregulated in eutopic and ectopic endometria of women with endometriosis [33], a condition in which endometrial tissue proliferates outside of the uterine cavity, thought to occur most commonly on the ovaries as a result of retrograde menstruation. It is believed that the angiogenic activities of CCN1 may contribute to its role in the pathogenesis of endometriosis [33].

*Ccn1*-null embryos also exhibit severe defects in atrioventricular valvuloseptal morphogenesis, resulting in a common atrioventricular valve orifice that is the hallmark of complete AVSD [86]. The mesenchymal cells in the endocardial cushion tissue suffer premature apoptosis, preventing them from fusing properly with the atrial and ventricular septa to undergo apposite valvuloseptal morphogenesis. Although $Ccn1^{+/-}$ mice are largely viable, they exhibit persistent ostium primum atrial septal defects with 20% penetrance [86]. Human AVSD is a common group of congenital disorders often associated with Down's syndrome, whereas non-syndromic AVSDs are typically inherited with autosomal dominance [97]. The atrial septal defects due to *Ccn1* haploinsufficiency resemble those observed in some human patients with mutations in *AVSD1*, a susceptibility locus for non-syndromic AVSD identified by linkage analysis. The human *CCN1* gene and *AVSD1* both map within the same chromosomal location at 1p21–31 [97,98], suggesting that *CCN1* may be a candidate gene for human AVSD.

Although *Ccn1* is prominently expressed in the skeletal system during embryogenesis [82], *Ccn1*-null mice succumb too early in development for its role in skeletal development to be assessed fully [85]. *Ccn1* is also highly expressed in the developing neuronal system; however, its role in this context has not been thoroughly investigated.

In *Xenopus laevis*, gain- and loss-of-function experiments have shown that precise control of CCN1 expression is required for normal gastrulation movements and modulation of Wnt signaling [99]. Either overexpression of CCN1 by injection of *Ccn1* RNA or CCN1 knockdown by antisense morpholino oligonucleotides caused defects in gastrulation, resulting in severe delay of blastopore closure. Ventral injection of *Ccn1* mRNA induces secondary axes formation in *Xenopus* and activates Wnt/β-catenin signaling, but can also inhibit *Wnt8*-induced secondary axis formation, indicating that CCN1 can stimulate or inhibit Wnt signaling in a context-dependent manner [99].

## UNUSUAL CELL ADHESIVE FUNCTIONS: ROLE OF REACTIVE OXYGEN SPECIES

### Apoptotic synergism with members of the TNF family

Cell adhesion to the ECM is thought to provide a cell-survival function, whereas detachment from the ECM induces rapid cell death by anoikis in many cell types. Although CCN1 promotes cell survival in endothelial cells and some cancer cells [72,81], it represents the first example of an ECM molecule that can induce apoptosis through the process cell adhesion [61]. In fibroblasts, CCN1 triggers apoptosis by binding $\alpha_6\beta_1$ and syndecan-4, leading to the p53-dependent activation of Bax and cytochrome *c* release, resulting in apoptosis [61]. This effect is relatively modest in normal fibroblasts, but is greatly enhanced

in cells that are defective for p21 and are thus more susceptible to apoptotic signals. More recent studies have found that other ECM proteins can also induce apoptosis, e.g. EMILIN 2 can trigger cell death through a death receptor-mediated mechanism [100].

Physiologically, the apoptotic activities of CCN1 may be most relevant when acting in combination with other apoptotic factors, notably members of the TNF family including TNF-α, FasL, and TRAIL [101]. TNF-α is a pro-inflammatory cytokine and a potent activator of the transcription factor NF-κB, which induces the expression of a multitude of pro-mitogenic, pro-inflammatory, and anti-apoptotic proteins, thus promoting cell proliferation and survival [102]. However, TNF-α is also a powerful apoptotic inducer *in vitro* when NF-κB signaling is blocked, typically by the addition of cycloheximide. Whereas TNF-α contributes to apoptotic tissue damage in inflammatory diseases, how it induces apoptosis *in vivo* is not clearly understood [102,103]. Remarkably, cell adhesion to CCN1 enables TNF-α to induce apoptosis without inhibiting NF-κB signaling or *de novo* protein synthesis, thus converting TNF-α from a survival-enhancing factor into a potent apoptotic agent [62]. Although CCN1 is a cell-adhesion molecule, these activities are observed when CCN1 is presented to cells either as an adhesion substrate or as a soluble factor. Mechanistically, CCN1 acts by binding integrins $\alpha_6\beta_1$, $\alpha_v\beta_5$ and syndecan-4, leading to the generation of reactive oxygen species (ROS) via 5-lipoxygenase and mitochondria (Fig. 2) [62]. The high level of ROS induced by CCN1/TNF-α counters the inhibition of JNK activation through the anti-oxidant effect of NF-κB and results in the biphasic activation of JNK necessary for apoptosis, most likely by oxidative inactivation of JNK phosphatases [104]. JNK targets c-FLIP, an inhibitor for the activation of caspase-8/caspase-10, for proteasomal degradation, thus allowing apoptosis to proceed [105]. In addition, CCN1 also strongly enhances the apoptotic activity of other apoptotic members of the TNF family, including FasL and TRAIL [77,106].

The importance of CCN1 in TNF-α- and FasL-induced apoptosis *in vivo* has been demonstrated using knockin mice (*Ccn1*$^{dm/dm}$) in which the genomic *Ccn1* locus has been replaced by an apoptosis-defective *Ccn1* allele with mutations at the $\alpha_6\beta_1$–HSPG-binding sites [62,101,106]. In contrast to the embryonic lethality of *Ccn1*-null mice, these knockin mice are viable, fertile, and do not exhibit any obvious developmental defects. *Ccn1*$^{dm/dm}$ mice are substantially resistant to concanavalin A-induced, TNF-α-dependent hepatocyte apoptosis [107,108]. Dermal apoptosis induced upon direct subcutaneous injection of TNF-α is also substantially reduced in *Ccn1*$^{dm/dm}$ mice compared with wild-type mice. Fas-mediated apoptosis is also reduced, as judged by using the monoclonal antibody Jo2 that recognizes and activates the Fas receptor and induces hepatic apoptosis [109], or by using ethanol gavage in mice, a system that mimics binge drinking in humans and triggers FasL-induced hepatocyte apoptosis [110]. In each of these experimental models for TNF-α- or Fas-mediated apoptosis, *Ccn1*$^{dm/dm}$ mice consistently show a >60% reduction in apoptosis compared with wild-type mice [62,106]. These results show that CCN1 is a physiological regulator of TNF-α- and Fas-mediated apoptosis *in vivo* [111].

## CCN1 induces cellular senescence

Another unusual activity of CCN1 as a matrix cell-adhesion molecule is the ability to induce cellular senescence [2]. Through binding to integrin $\alpha_6\beta_1$ and cell-surface HSPGs, CCN1 induces fibroblast senescence by activating RAC1 and the RAC1-dependent NADPH oxidase 1 (NOX1) to trigger a robust level of ROS that is sustained for many hours [2]. Consequently, CCN1 induces a DNA damage response, resulting in the activation of ATM, CHK1, CHK2, and p53 [2]. CCN1 also triggers the ROS-dependent hyperactivation of p38 MAPK and ERK, which in turn activate the p16$^{INK4a}$/pRb pathway to induce senescence (Fig. 2). Both p53 and p16$^{INK4a}$/pRb pathways, which are activated by CCN1, contribute to the induction of cellular senescence [112,113]. Cell adhesion to CCN1 induces a much

higher and more sustained level of ROS than cell adhesion to other ECM proteins such as collagen, fibronectin, and laminin, which do not induce senescence. Although CCN1 induces ROS through several pathways, NOX1, but not 5-lipoxygenase, is specifically required for the induction of senescence [2]. It is likely that CCN1 interacts with another receptor(s) in addition to $\alpha_6\beta_1$–HSPGs to elicit these responses, as other matrix proteins such as laminin can also bind $\alpha_6\beta_1$–HSPGs but do not induce sustained ROS accumulation or senescence.

An important feature of senescent cells is the expression of the senescence-associated secretory phenotype (SASP), characterized by the increased expression of ECM-degrading enzymes such as matrix metalloproteinases (MMPs), inflammatory cytokines/chemokines, and down-regulation of ECM components such as collagen [112,114–116]. Consistently, CCN1-induced senescent fibroblasts express elevated levels of MMPs and downregulate collagen and TGF-β1, thus imposing a matrix-degrading phenotype [2]. These characteristics of senescent cells help to control fibrosis associated with cutaneous wound healing and possibly other pathologies (see below).

## CCN1 IN PATHOLOGIES

### Wound healing and fibrosis

In the adult, CCN1 expression is associated with many tissues undergoing inflammation and injury repair. Wound healing in virtually all mammalian organ systems is coordinated in three overlapping but distinct phases, initiating with an inflammatory response, followed by ECM deposition and granulation tissue formation, and finally tissue remodeling and maturation to complete the healing process [117–119]. Each of these steps must be tightly regulated for optimal wound healing. In cutaneous wound healing, *Ccn1* is highly expressed in myofibroblasts of the granulation tissue [40,87]. The myofibroblasts, derived from differentiation of resident activated fibroblasts or recruited fibrocytes, proliferate and rapidly synthesize ECM to maintain tissue integrity and help provide a barrier against microbes while the damaged tissue is being repaired, and they also express α-smooth muscle actin and promote wound contraction to accelerate wound closure [120]. However, if ECM synthesis in myofibroblasts is not kept in check, then excessive matrix deposition can lead to fibrosis, scarring, and loss of tissue function [120–122]. Fibrosis is a common problem associated with wound healing, particularly when inflammation and injury become chronic, leading to serious and potentially life-threatening conditions when it occurs in vital organs such as the liver, lung, kidney, and heart [118].

Recent studies show that, as myofibroblasts proliferate in the granulation tissue, CCN1 eventually accumulates to a sufficiently high level to drive the myofibroblasts themselves into senescence [2]. This CCN1-induced senescence switch in later stages of wound healing exerts a self-limiting effect on the synthesis and deposition of ECM by myofibroblasts in several ways (Fig. 3). First, senescent myofibroblasts no longer proliferate, thereby curbing the number of ECM-producing cells. Second, senescent cells express the anti-fibrotic SASP as described above, upregulating MMPs and downregulating collagen expression, leading to matrix degradation [2,70]. Third, senescent cells can be cleared by natural killer cells, thus accelerating wound resolution [123]. Hence, CCN1 turns the ECM-synthesizing myofibroblasts themselves into ECM-degrading senescent cells, thereby imposing a self-limiting effect on fibrogenesis and promoting wound resolution (Fig. 3) [124]. In knockin mice that express a CCN1 mutant unable to bind $\alpha_6\beta_1$–HSPGs and are thus defective for the induction of senescence, senescent cells do not accumulate in the granulation tissue during wound healing, resulting in exacerbated fibrosis [2]. Topical application of purified CCN1 protein to cutaneous wounds reverses these defects, further establishing the critical role of CCN1 in controlling myofibroblast senescence to limit fibrosis. In a mouse model of carbon

tetrachloride-induced liver fibrosis, the accumulation of senescent cells also functions to limit fibrosis [125]. Thus, the induction of cellular senescence during wound healing may be a general mechanism that controls fibrogenesis during wound repair [124,126].

In addition to cutaneous wound healing, CCN1 expression is elevated in remodeling cardiomyocytes after myocardial infarctions [24], in vascular injury [56], and in the long bones during fracture repair, notably in proliferating chondrocytes and osteoblasts [127,128]. Blockade of CCN1 by antibodies inhibits bone fracture healing in mice [129]. In the kidney, CCN1 is expressed in podocytes in normal adult and embryonic glomeruli, but expression is decreased in IgA nephropathy, diabetic nephropathy, and membranous nephropathy, particularly in diseased kidneys with severe mesangial expansion, suggesting that impaired CCN1 function may contribute to the progression of glomerular disease with mesangial expansion [130].

## Inflammation

Although the specific roles of CCN proteins in inflammation *in vivo* are currently not well understood, evidence for their participation in the inflammatory response is beginning to mount [31]. CCN1 expression is induced by inflammatory cytokines such as IL-1 and TNF-α [33,131], as well as by bacterial and viral infections [29–32]. As described above, CCN1 profoundly alters the activities of inflammatory cytokines such as TNF-α and FasL *in vitro* and *in vivo*, and significantly enhances the cytotoxicity of TRAIL [77,111]. Furthermore, CCN1 supports the adhesion of activated monocytes through integrin $\alpha_M\beta_2$, and reprograms macrophages towards M1 polarization through $\alpha_M\beta_2$-mediated activation of NF-κB [58,59]. These findings suggest that CCN1 can play important roles in inflammation, in part by acting on inflammatory cells such as macrophages, inducing angiogenesis, and collaborating with cytokines such as TNF-α.

CCN1 is upregulated in patients with Crohn's disease and ulcerative colitis, and in colons of mice treated with dextran sodium sulfate to induce inflammatory colitis [132]. Genomic studies have found that *CCN1* is among the most highly differentially expressed genes in rheumatoid arthritis in disease-discordant monozygotic twins [133]. CCN1 is highly expressed in collagen-induced arthritis in rodents, and inhibition of CCN1 expression correlates with suppression of inflammatory arthritis [134]. These findings suggest that CCN1 may play an important role in inflammatory diseases including colitis and rheumatoid arthritis. It is interesting to note that TNF-α is known to be critical in both inflammatory bowel disease and rheumatoid arthritis, and anti-TNF-α therapy is in clinical use for these diseases. The functional interaction between CCN1 and TNF-α may be important in these pathologies.

## Vascular diseases

CCN1 is overexpressed in vascular smooth muscle cells of atherosclerotic lesions and in the neointima of restenosis after balloon angioplasty, both in rodent models and in humans [23,56,58,135]. Suppression of *CCN1* expression by either small interfering RNA or FOXO3a-mediated transcriptional repression results in reduced neointimal hyperplasia after balloon angioplasty, an effect that is reversed by delivery of CCN1 via gene transfer [136,137]. Overexpression of CCN1 from an adenoviral vector accelerates re-endothelialization after balloon injury, possibly because CCN1 can recruit $CD34^+$ endothelial progenitor cells to the endothelial monolayer and promote their differentiation [88,138]. In a mouse model of oxygen-induced retinopathy, overexpressing CCN1 by lentiviral vector delivery, or injecting CCN1-overexpressing hematopoietic stem cells into the vitreous humor, produced significant beneficial effects in repairing damaged vasculature [139]. These findings underscore a critical role for CCN1 in vascular injury repair.

## CCN1 in cancer: a double-edged sword?

CCN1 possesses two sets of divergent activities that can contribute to opposing effects on tumorigenesis (Fig. 1). First, CCN1 is a powerful angiogenic inducer *in vivo* [72,83], and angiogenesis is essential for the supply of oxygen and nutrients to nourish the growing tumor [140]. With the discovery of its angiogenic activity, CCN1 was shown to promote tumor growth and increase tumor vascularization when overexpressed in human gastric adenocarcinoma cells in SCID mice [83]. These findings demonstrated the ability of CCN1 to promote the growth of tumors generated by transformed cells, typically observed in xenografts transplanted into immunodeficient mice. In addition to angiogenesis, CCN1 can also promote cancer cell proliferation, invasion, survival, and metastasis [74,75,141]. Accordingly, subsequent studies have shown that forced expression of CCN1 enhanced tumor growth in xenografts of breast cancer cells [142], prostate cancer cells [74], ovarian carcinoma cells [78], and squamous carcinoma cells [143]. Consistently, silencing of CCN1 expression decreased tumor growth in xenografts of prostate cancer cells [74] and pancreatic cancer cells [144].

The clinical relevance of these observations is supported by the finding of a significant correlation between CCN1 expression and the stage, tumor size, lymph node positivity, and poor prognosis in several cancers, including breast cancer [142,145–148], prostate cancer [149], glioma [150], gastric adenocarcinoma [151], and squamous cell carcinoma [143]. In MCF7 breast cancer cells, which are dependent on estrogen for growth, overexpression of CCN1 renders these cells estrogen-independent and significantly resistant to apoptosis, in part through upregulation of the anti-apoptotic protein XIAP [81,152,153]. The anti-apoptotic functions of CCN1 in breast cancer cells are mediated through its receptor integrin $\alpha_v\beta_3$ [81,153], whose expression is also induced by CCN1 [154].

However, CCN1 can also induce apoptosis and cellular senescence [2,61,101], two well-established mechanisms of tumor suppression [155]. By inducing apoptosis or senescence, CCN1 may be able to suppress the initial phases of tumorigenesis by preventing the proliferation of damaged cells at risk of transformation. For example, whereas CCN1 can promote the proliferation of prostate cancer cells, CCN1 can also exacerbate apoptosis of these cells in the presence of the immune surveillance molecule TRAIL [74,76,77]. Interestingly, high levels of CCN1 expression are found in prostate cancer cell lines with a mutant or null p53 gene, but lower levels are found in cells expressing wild-type p53 [156]. This observation is consistent with the hypothesis that CCN1 and p53 together can promote tumor cell apoptosis or senescence, and thus high CCN1 levels are tolerated only in cells with mutant p53.

Several lines of evidence support the notion that CCN1 can suppress tumor growth. CCN1 expression is decreased in clinical specimens of non-small-cell lung cancer (NSCLC) compared with normal matched lung samples. NSCLC cells overexpressing CCN1 developed smaller tumors than parental cells in nude mice [157]. CCN1 inhibits NSCLC cell proliferation by upregulating p53, p21, and p130/pRb [158], suggesting that CCN1 may induce senescence through a p53 and pRb-dependent pathway [2]. Likewise, overexpression of CCN1 inhibits the proliferation of hepatocellular carcinoma cells, in part through p53 [159]. Furthermore, overexpression of CCN1 in endometrial adenocarcinoma cells decreased cell growth and increased apoptosis [160], and CCN1 expression in melanoma cells reduced tumor growth and metastasis and concomitantly increased apoptosis in tumors [161]. Thus, the role of CCN1 in tumorigenesis may be cell type- and context-dependent, and may hinge on whether the cell types are susceptible to CCN1-induced apoptosis or senescence, or whether angiogenic factors are limiting.

## CONCLUDING REMARKS AND FUTURE PROSPECTS

CCN1 is a multifunctional matricellular protein that is essential for life due its embryonic developmental functions, including placental angiogenesis, vascular integrity, and cardiac valvuloseptal morphogenesis. In addition to these developmental functions, in the adult it plays critical roles in inflammation, wound healing, and tissue repair, and is aberrantly expressed in myriad diseases associated with deregulation of these processes. How might CCN1 play such diverse roles in seemingly unrelated biological contexts? Based on current information discussed above, we speculate that the remarkable functional diversity of CCN1 may depend on several factors. First, through interaction with distinct integrins preferentially expressed in the target cell types, CCN1 can elicit diverse and at times opposing functions (Fig. 1). For example, interaction through $\alpha_v\beta_3$ and $\alpha_6\beta_1$ can promote either cell survival or cell death, respectively. Second, CCN1 interacts with multiple receptors, some of which may be yet unidentified, and the combination of these receptors may be required to generate the signaling events necessary for the biological responses. The modular domains of CCN1 contain binding sites for distinct receptors, and the amalgamation of various receptor-binding sites in a single polypeptide allows the activation of signaling pathways by multiple receptors that converge in the cell to trigger unique functions. Third, the biological responses to CCN1 may be significantly influenced by the presence of other growth factors, cytokines, and morphogens in the cellular microenvironment, as evidenced by the ability of CCN1 to alter the activities of TNF-α and modulate Wnt signaling. The possibility that CCN1 may interact with and thus modify the activities of other growth factors such as BMP, TGF-β, and VEGF, as has been demonstrated for the homologous protein CCN2, remains to be explored [16,18]. Thus, the biological outcome of CCN1 signaling may be highly context-dependent. Given that CCN1 interacts with inflammatory cells and modulates the activities of cytokines, the absence of an inflammatory response during early embryogenesis may underlie some of the differences in CCN1 activities in the embryo versus the adult.

Despite significant advances in recent years, many questions concerning the functions and mechanisms of action of CCN1 remain. Biochemically, the large number of cysteine residues portends a specific pattern of disulfide bonding and tertiary structure, and suggests potential roles for some of the cysteines in metal chelation or sensing oxidative stress. Much remains to be learned about the structure–function relationships of CCN1, including the significance of any post-translational modification. On the organismal level, CCN1 is likely to participate in the development of the neuronal and skeletal systems and in various other organs, and careful analysis of tissue-specific knockout of *Ccn1* will be necessary to assess its roles in the development and functions of various organs and tissues. The specific roles of CCN1 in inflammation, wound healing, and tissue repair are just beginning to emerge, and understanding its functions in these contexts is key to unlocking how CCN1 contributes to the myriad pathologies in which CCN1 is aberrantly expressed. Future investigations may validate the potential utility of CCN1 as a biomarker or therapeutic target for diseases associated with its deregulation.

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## ABBREVIATIONS

**AVSD** atrioventricular septal defects

| | |
|---|---|
| **CTGF** | connective tissue growth factor |
| **CYR61** | cysteine-rich 61 |
| **ECM** | extracellular matrix |
| **ERK** | extracellular signal-regulated kinase |
| **GPCR** | G protein-coupled receptor |
| **HIF-1α** | hypoxia-inducible factor-1α |
| **HIV-1** | human immunodeficiency virus type 1 |
| **HSPG** | heparan sulfate proteoglycan |
| **IL** | interleukin |
| **IRES** | internal ribosome entry sites |
| **MMP** | matrix metalloproteinase |
| **MRTF-A** | myocardin-related transcriptional activator |
| **NSCLC** | non-small-cell lung cancer |
| **NOV** | nephroblastoma overexpressed |
| **ROS** | reactive oxygen species |
| **SASP** | senescence-associated secretory phenotype |
| **SRE** | serum response element |
| **SRF** | serum response factor |
| **TGF-β** | transforming growth factor β |
| **TNF-α** | tumor necrosis factor α |
| **TSR** | thrombospondin type I repeat |

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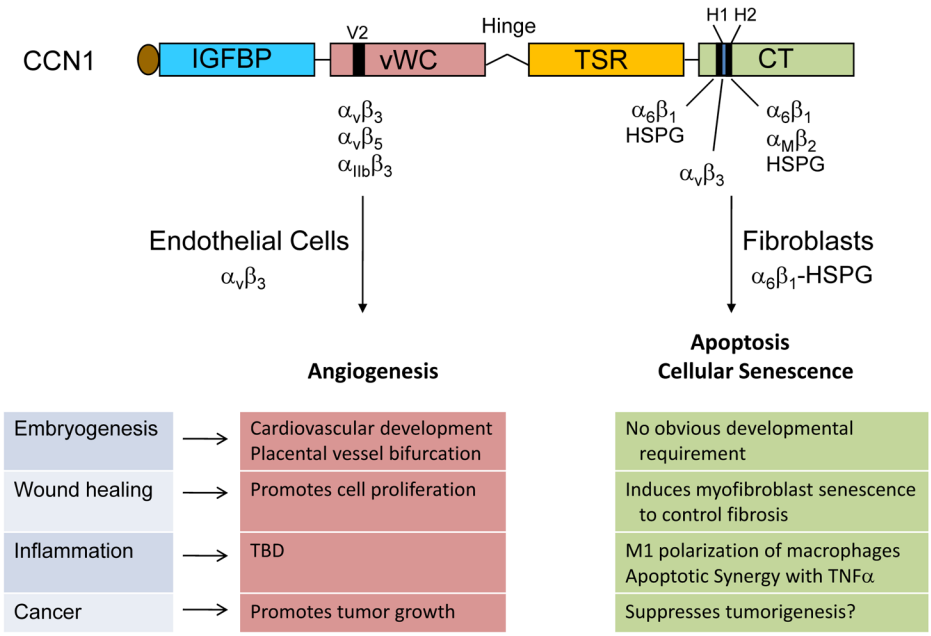


**Figure 1. Schematic diagram of CCN1, its receptor-binding sites, and biological activities**

The modular domain structure of CCN1 and the locations of several identified integrin-binding sites are illustrated. The interaction of CCN1 with $\alpha_v\beta_3$ in endothelial cells is critical for its angiogenic activities, which underlie biological functions in embryonic development, cell proliferation, and tumor growth. The interaction of CCN1 with $\alpha_6\beta_1$–heparan sulfate proteoglycans (HSPGs) in fibroblasts induces apoptosis or cellular senescence, and may function to regulate the inflammatory response, control fibrosis during wound healing, and suppress tumorigenesis. TBD, to be determined.

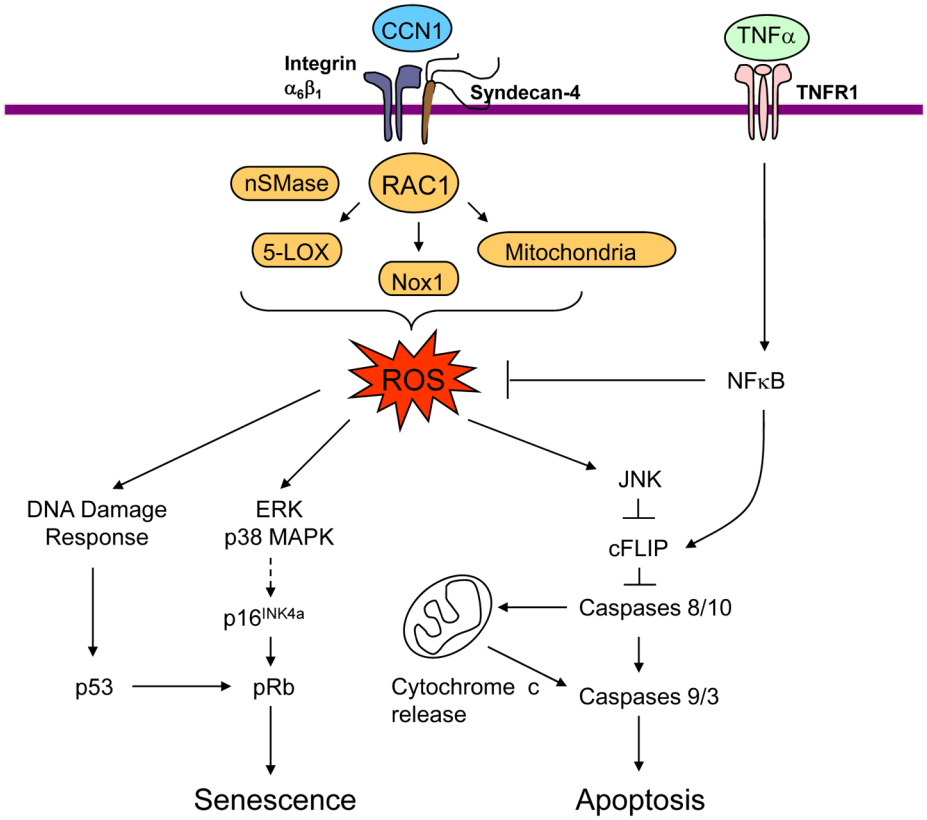


**Figure 2. Signaling pathways mediated through CCN1-induced ROS accumulation**

The interaction of CCN1 with $\alpha_6\beta_1$ and HSPGs (syndecan-4) is required to trigger the RAC-1-dependent accumulation of ROS through several pathways, including 5-lipoxygenase (5-LOX), NADPH oxidase, and the mitochondria. Neutral sphingomyelinase (nSMase) also contributes to CCN1-induced ROS. CCN1-induced ROS lead to the biphasic activation of JNK, allowing TNF-α to induce apoptosis by targeting c-FLIP for degradation. The high and sustained level of ROS induced by CCN1 also triggers a DNA damage response, leading to p53 activation. ROS also lead to the hyperactivation of ERK and p38 MAPK, which in turn induce $p16^{INK4a}$ and activate pRb. Both p53 and $p16^{INK4a}$/pRb contribute to senescence.

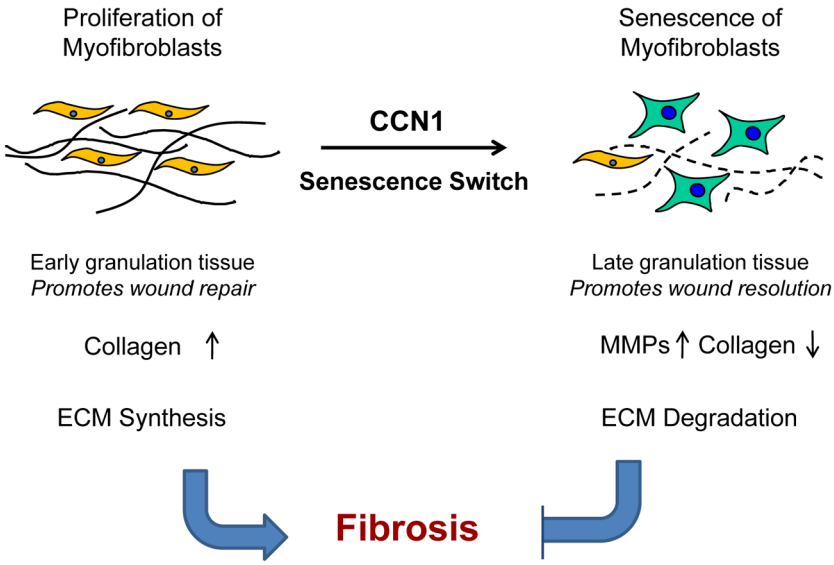


**Figure 3. CCN1 controls fibrosis in wound healing by inducing cellular senescence**

In cutaneous wound healing, myofibroblasts are recruited to form the granulation tissue, where they proliferate and rapidly synthesize ECM to provide tissue integrity during repair. At later stages of wound healing, these myofibroblasts are driven into senescence by CCN1, whereupon they express an ECM-degrading phenotype and limit fibrosis. Thus, CCN1 functions as an anti-fibrotic molecular switch that converts ECM-producing myofibroblasts into ECM-degrading senescent cells, thereby imposing a self-limiting control on fibrogenesis during wound healing.

## TABLE I

Cellular activities of CCN1 and their mediating receptors

| Cellular responses stimulated by CCN1 | Cells types and cell-surface receptors involved |
|---|---|
| Cell adhesion | Fibroblasts – $\alpha_6\beta_1$ and HSPGs [55]<br>Activated endothelial cells – $\alpha_v\beta_3$; unactivated endothelial cells – $\alpha_6\beta_1$ [15,72]<br>Smooth muscle cells – $\alpha_6\beta_1$ and HSPGs [56]<br>Platelets – $\alpha_{IIb}\beta_3$ [57]<br>Monocytes – $\alpha_M\beta_2$ [58]<br>Macrophages – $\alpha_M\beta_2$ [59]<br>Macrophage foam cells – $\alpha_D\beta_2$ [60] |
| Migration | Fibroblasts – $\alpha_v\beta_5$ [71]<br>Microvascular endothelial cell chemotaxis – $\alpha_v\beta_3$ [83]<br>Smooth muscle cell chemotaxis – $\alpha_6\beta_1$ and HSPGs[56] |
| DNA synthesis/proliferation | Fibroblasts – $\alpha_v\beta_3$ [71]<br>Astrocytoma cells – $\alpha_5$, $\alpha_6$, and $\beta_1$ [35] |
| Survival | Endothelial cells – $\alpha_v\beta_3$ [72]<br>Breast cancer cells – $\alpha_v\beta_3$ [81,153]<br>Cardiac myocytes – $\beta_1$ [162] |
| Apoptosis | Fibroblasts – $\alpha_6\beta_1$ and syndecan 4 [61]<br>Synergism with TNF-α in fibroblasts – $\alpha_6\beta_1$, $\alpha_v\beta_5$, and syndecan-4 [62]<br>Synergism with FasL in fibroblasts – $\alpha_6\beta_1$ and HSPGs [106]<br>Synergism with TRAIL in PC3 cells – $\alpha_v\beta_3$, $\alpha_6\beta_4$ and syndecan-4 [77] |
| Differentiation | Osteoblastic differentiation – $\alpha_v\beta_3$ [92]<br>Endothelial tubule formation – $\alpha_v\beta_3$ [72] |
| Gene expression | Chondosarcoma cells, MMP13 expression – $\alpha_v\beta_3$ [163]<br>Macrophages, M1 genetic program – $\alpha_M\beta_2$ [59]<br>MCF7 breast cancer cells – $\alpha_v\beta_3$ [81] |
| Invasiveness | Gastric adenocarcinoma cells – $\alpha_v\beta_3$ [151]<br>Oral squamous cell carcinoma line – $\alpha_v\beta_5$ [141] |
| Senescence | Fibroblasts – $\alpha_6\beta_1$ and HSPGs [2] |