



CCN2 (Cellular Communication Network factor 2) in the bone marrow microenvironment, normal and malignant hematopoiesis

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Abstract

CCN2, formerly termed Connective Tissue Growth Factor, is a protein belonging to the Cellular Communication Network (CCN)-family of secreted extracellular matrix-associated proteins. As a matricellular protein it is mainly considered to be active as a modifier of signaling activity of several different signaling pathways and as an orchestrator of their cross-talk. Furthermore, CCN2 and its fragments have been implicated in the regulation of a multitude of biological processes, including cell proliferation, differentiation, adhesion, migration, cell survival, apoptosis and the production of extracellular matrix products, as well as in more complex processes such as embryonic development, angiogenesis, chondrogenesis, osteogenesis, fibrosis, mechanotransduction and inflammation. Its function is complex and context dependent, depending on cell type, state of differentiation and microenvironmental context. CCN2 plays a role in many diseases, especially those associated with fibrosis, but has also been implicated in many different forms of cancer. In the bone marrow (BM), CCN2 is highly expressed in mesenchymal stem/stromal cells (MSCs). CCN2 is important for MSC function, supporting its proliferation, migration and differentiation. In addition, stromal CCN2 supports the maintenance and longtime survival of hematopoietic stem cells, and in the presence of interleukin 7, stimulates the differentiation of pro-B lymphocytes into pre-B lymphocytes. Overexpression of CCN2 is seen in the majority of B-acute lymphoblastic leukemias, especially in certain cytogenetic subgroups associated with poor outcome. In acute myeloid leukemia, CCN2 expression is increased in MSCs, which has been associated with leukemic engraftment *in vivo*. In this review, the complex function of CCN2 in the BM microenvironment and in normal as well as malignant hematopoiesis is discussed. In addition, an overview is given of data on the remaining CCN family members regarding normal and malignant hematopoiesis, having many similarities and some differences in their function.

Keywords CCN2 · CTGF · Connective tissue growth factor · Bone marrow · Hematopoiesis · Leukemogenesis

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Introduction

The bone marrow microenvironment

The bone marrow (BM) is the most important source of blood cells in the adult. It is the primary site where hematopoietic stem cells (HSCs) reside and give rise to more restricted progenitor cells that mature to become the different specialized blood cells. Besides hematopoietic cells, the BM contains mesenchymal stem cells, various more mature mesenchymal cells such as fibroblasts, adipocytes, osteoblasts, endothelial cells and perivascular stromal cells, as well neuronal cells and extracellular matrix (ECM), which together constitute the BM microenvironment. Integrins and selectins on the cell surface of stromal and hematopoietic cells mediate cell–cell and cell–matrix interactions (Anthony and Link 2014; Lindner et al. 2010).

One of the most important functions of the BM microenvironment is the formation of so called ‘niches’, local tissue microenvironments for the maintenance, regulation and differentiation of the HSCs (the stem cell niche) and other hematopoietic cells (Morrison and Spradling 2008). Different niches are recognized in the BM; the osteoblastic (endosteal) niche localized at the inner surface of the bone cavity where calcium levels are high, the (peri)vascular niches subdivided into a (peri)sinusoidal niche and a (peri)arteriolar niche containing high levels of stem cell factor (SCF) and CXC chemokine ligand-12 (CXCL12), the erythroid niche with a central macrophage, and a niche for lymphopoiesis located in the perisinusoidal region where both CXCL12 and interleukin 7 (IL-7) levels are high (Acar et al. 2015; Asada et al. 2017; Aurrand-Lions and Mancini 2018; Chasis and Mohandas 2008; Fujita et al. 2015; Ho and Méndez-Ferrer 2020; Lazzari and Butler 2018; Morrison and Scadden 2014; Pinho and Frenette 2019; Wei and Frenette 2018). Actively cycling, short-term HSCs as well as quiescent long-term HSCs are mainly localized in close proximity to vascular niches, but the different roles of the two vascular niches, arteriolar and sinusoidal, in HSC function are still not fully understood (Acar et al. 2015; Asada et al. 2017; Aurrand-Lions and Mancini 2018; Ho and Méndez-Ferrer 2020; Lazzari and Butler 2018; Wei and Frenette 2018). Mesenchymal cells, which include mesenchymal stem/progenitor cells and more differentiated mesenchymal cells such as perivascular stromal cells and osteoblasts, are important players in these niches as they secrete cytokines and other factors to support and regulate hematopoiesis (Morrison and Scadden 2014). When changed, the BM microenvironment can also support malignant hematopoiesis. Malignant niches differ from normal niches by the interaction with malignant cells. A role for malignant niche characteristics have been implicated in both myelodysplastic and myeloproliferative neoplasms as well as in lymphoid and myeloid leukemias (Arranz et al. 2014; Balderman et al. 2016; Battula et al. 2013; Blau et al. 2007; Boyerinas et al. 2013; Guerrouahen et al. 2011; Korn and Mendez-Ferrer 2017; Lazzari and Butler 2018; Li and Calvi 2017; Sangaletti et al. 2017; Schepers et al. 2013).

Homeostasis of the BM microenvironment is tightly regulated by a complex and not fully elucidated interplay between different cell types, structural ECM components and soluble factors such as cytokines, hormones, growth factors and matricellular proteins. Matricellular proteins are proteins secreted into the extracellular environment, modulating cell function and cell–matrix interactions by binding to cell-surface receptors, structural matrix proteins and other soluble matrix proteins (Bornstein 2009; Bornstein and Sage 2002). The Cellular Communication Network (CCN) family forms an important group of matricellular proteins (Chen and Lau 2009). Bork was the first to conceive the different

CCN proteins as members of a (single) family (Bork 1993), that now consists of six structural related proteins.

The CCN proteins act as central mediators of mechanotransduction and play important roles in, amongst others, angiogenesis, inflammation, connective tissue deposition, and a broad range of pathological processes including fibrosis and cancer (Chaour 2020; Leask 2020). The proteins of the CCN family might play as a team and ideally should be assessed together rather than individually (Peidl et al. 2019; Perbal 2018; Riser et al. 2009, 2010), but for most of them, little is known about their possible involvement in hematopoiesis and the BM microenvironment. Since CCN2 is by far the most studied CCN protein in this field, it will therefore be the focus of this review, and the more limited available knowledge on the other CCN proteins is included at the end.

CCN2 protein

Although CCN2 was originally thought to be a classical growth factor and named connective tissue growth factor (CTGF), no high-affinity classical growth factor receptor for CCN2 has been discovered (Lau 2016). Instead, CCN2 has the capacity to interact with a range of cell surface receptors, ECM macromolecules, growth factors and proteases, thereby directly or indirectly regulating cellular function, which are features common to all matricellular proteins (Bornstein 1995; Murphy-Ullrich and Sage 2014). Furthermore, all four structural domains of the CCN proteins are homologous to other ECM-associated proteins (Lau and Lam 1999). Therefore, the protein originally called CTGF is nowadays considered a matricellular protein and has been renamed CCN2 by the HUGO Gene Nomenclature Committee (Brigstock et al. 2003; Perbal et al. 2018).

CCN2 (Fig. 1) is a cysteine-rich 36–38 kDa (depending on the level of N-linked glycosylation) protein (Bradham et al. 1991; Yang et al. 1998), consisting of a secretory signal peptide and four functionally distinct and highly conserved domains/modules (Bork 1993; Holbourn et al. 2008). The N-terminal fragment of CCN2 is made up by an insulin-like growth factor binding protein (IGFBP) module and a von Willebrand factor C (VWC) module (Brigstock 1999; Lau and Lam 1999), which is linked by a hinge region to the C-terminal fragment consisting of a thrombospondin type 1 repeat (TSP1) module and a C-terminal cysteine knot (CT) module (Brigstock 1999; Lau and Lam 1999). Each module interacts with other extracellular proteins and/or proteoglycans (Perbal 2018). CCN2 is mainly secreted as an extracellular protein and has been identified in various human biological fluids (Bradham et al. 1991; Yang et al. 1998). Loss of its 2 kDa N-terminal secretory signal peptide leads to intracellular retention of the protein (Welch et al. 2015).

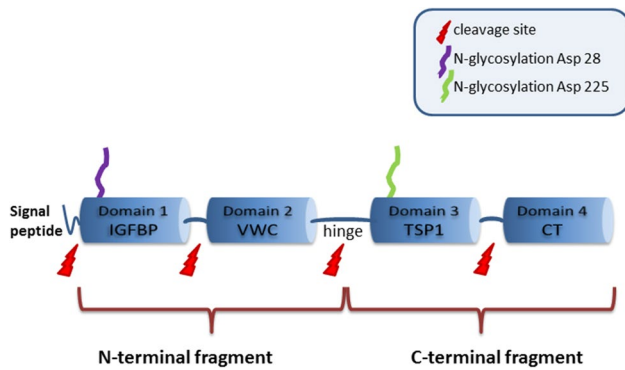


Fig. 1 CCN2 protein structure. Schematic representation of the full length CCN2 protein, which is made up by a signal peptide and 4 protein domains, depicted by the blue cylinders. Domain 1 consists of the insulin-like growth factor binding protein (IGFBP) module and domain 2 contains the von Willebrand factor C (VWC) module, together forming the N-terminal fragment of the protein. Domain 3 consists of the thrombospondin type 1 repeat (TSP1) module and domain 4 contains the C-terminal cysteine knot (CT) module, together forming the C-terminal fragment of the protein. The N- and C-terminal fragments are joint by a hinge region. Between the different domains, multiple cleavage sites are present, were CCN2 is cleaved by proteases, plasmin, chymotrypsin and matrix metalloproteinases. Loss of the signal peptide leads to intracellular retention of the protein. The protein contains 2 glycosylation sites. The functional relevance of glycosylation is, however, still unknown. Other (not depicted) posttranscriptional and posttranslational modifications to which the protein is subject to, are splicing, regulation by miRNAs and multimerisation

The cysteine-free hinge region between modules 2 and 3 contains multiple cleavage sites susceptible to proteolysis by a number of different proteases (de Winter et al. 2008; Grotendorst and Duncan 2005; Hashimoto et al. 2002). In addition, CCN2 can be cleaved between module 1 and 2 and between module 3 and 4 into fragments that represent the individual motifs (de Winter et al. 2008; Grotendorst and Duncan 2005; Hashimoto et al. 2002). The different modules and fragments have been associated with specific biological roles (Abd El Kader et al. 2014; Ball et al. 2003; de Winter et al. 2008; Grotendorst and Duncan 2005; Johnson et al. 2017; Kubota et al. 2006; Mokalled et al. 2016; Tong and Brigstock 2006). As truncated proteins missing one or more modules have even been reported to possess *enhanced* biological activities, CCN2 has been suggested to be a prepro-protein that may undergo proteolytic cleavage in order to release biologically more active fragments (Abd El Kader et al. 2014; Kaasboll et al. 2018). On the other hand, some of the biological activities of CCN2 require the intact, full length protein with the individual modules acting together (Kubota et al. 2006). Studying CCN2, however, remains challenging as it is difficult to purify larger amounts of native, biologically active CCN proteins in a stable form as pointed out by Perbal (2018).

CCN2 function and expression

CCN2 is expressed in a variety of tissues during embryonic development, with highest levels in vascular tissues and maturing chondrocytes (Hall-Glenn et al. 2012; Ivkovic et al. 2003). In the adult, CCN2 expression can be induced in various cell types, including endothelial cells (Bradham et al. 1991; Lee et al. 2015; Yan et al. 2014), vascular smooth muscle cells (Gao et al. 2007; Ko et al. 2012; Liu et al. 2008; Rodriguez-Vita et al. 2005), chondrocytes (Nakanishi et al. 1997), fibroblasts (Grotendorst 1997; Guo et al. 2011; Holmes et al. 2003; Igarashi et al. 1993) and mesangial cells (Goppelt-Struebe et al. 2001). CCN2 acts in an autocrine or paracrine fashion and its regulation and modes of action are complex and context dependent, depending on cell type, state of differentiation, and microenvironmental context (Cicha and Goppelt-Struebe 2009; Guo et al. 2011).

As previously reviewed, CCN2 and its fragments have been implicated in the regulation of a multitude of biological phenomena, including cell proliferation, differentiation, adhesion, migration, cell survival, apoptosis and the production of ECM products (de Winter et al. 2008; Jun and Lau 2011; Takigawa 2018), as well as in embryonic development, angiogenesis, chondrogenesis, osteogenesis, fibrosis, mechanotransduction and inflammation (Chaour 2020; Jun and Lau 2011; Kubota and Takigawa 2013; Takigawa 2013, 2018). It should be noted, however, that at least several of these propositions have not been based on robust assays using sufficiently characterized and purified CCN2 and its fragments. As discussed by Leask, adhesion assays are probably the only robust, universally agreed-upon *in vitro* assays for assessing CCN activity, at least of full-length CCN proteins (Leask 2020).

CCN2 has the ability to interact with a wide variety of proteins and receptors by its different modules, and is considered to be active as a modifier of signaling activity of several different signaling pathways and as an orchestrator of their cross-talk (Leask 2020; Perbal 2018; Ramazani et al. 2018). CCN2 can regulate biological processes in various ways (Fig. 2): (1) It can bind to several cell surface receptors, thereby initiating signal transduction, (2) It can bind to growth factors, modulating their presentation and binding to cell-surface receptors, and subsequent initiation of downstream signaling pathways, (3) It has a modifying role in mediating matrix turnover by binding to (structural) ECM proteins, (4) It is involved in the regulation of the activity of cytokines and growth factors through modulation of crosstalk between signaling pathways, and (5) It has been reported to act intracellularly, after uptake into the cytosol via endocytic pathways and into the nucleus, where it may affect gene transcription (Kawata et al. 2006, 2012; Lau 2016; Ramazani et al. 2018; Takigawa 2018; Wahab et al. 2001).

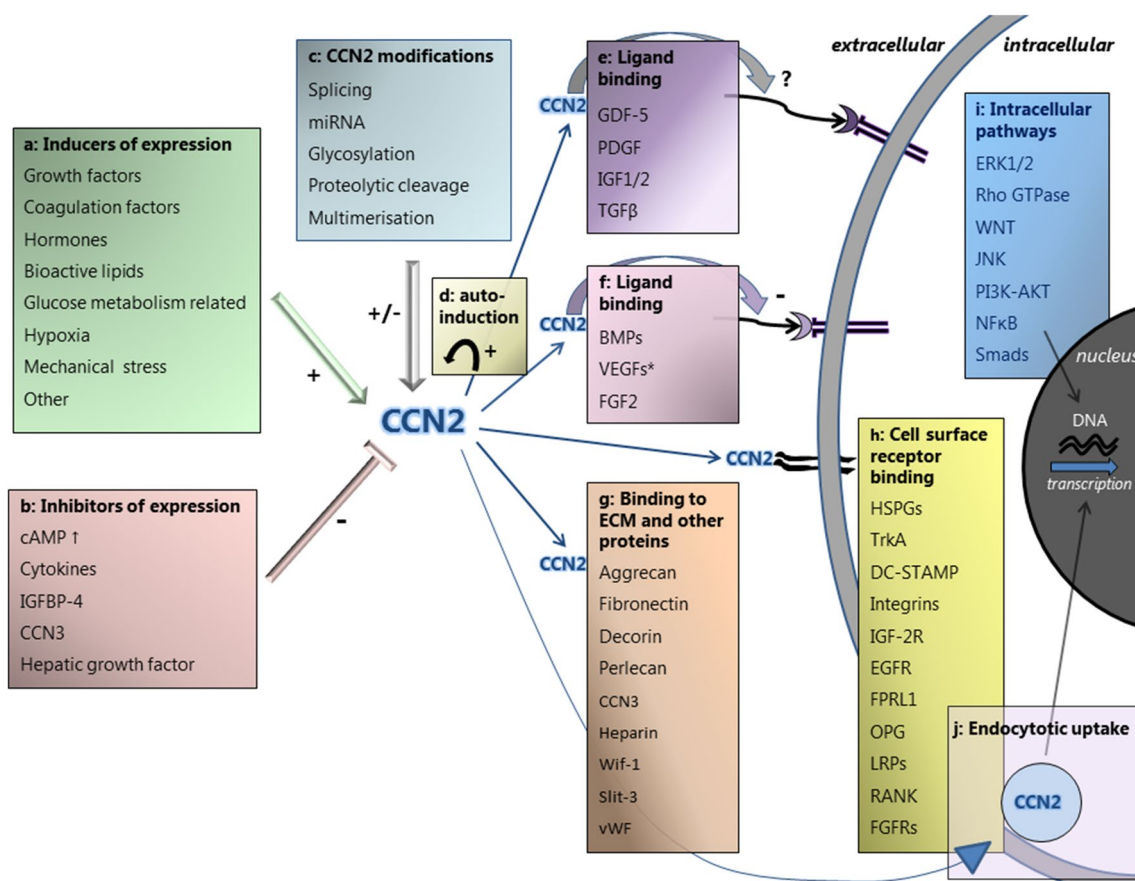


Fig. 2 Overview of the complex regulation of CCN2 expression, and the diverse actions of the CCN2 protein, as reported in literature. *Regulation of CCN2 expression.* **a** Factors inducing CCN2 expression include growth factors, coagulation factors, hormones, bioactive lipids, glucose metabolism related factors, hypoxia and mechanical stress. **b** Factors inhibiting CCN2 expression include increased levels of cyclic adenosine monophosphate (cAMP), cytokines, insulin-like growth factor binding protein-4 (IGFBP-4), CCN3 and hepatic growth factor. **c** CCN2 expression is modified by posttranscriptional and posttranslational factors, which includes splicing, regulation by miRNAs, glycosylation, proteolytic cleavage and multimerisation of the protein. **d** CCN2 can induce its own expression by auto-induction, resulting in a positive feedback loop. *Actions of the CCN2 protein:* CCN2 exerts its function by binding to growth factors and cell surface receptors, thereby affecting intracellular signaling, as well as by binding to extracellular matrix (ECM) and other proteins. **e** CCN2 binds to growth-differentiation factor 5 (GDF-5), platelet derived growth factor (PDGF), insulin-like growth factor-1 and 2 (IGF1/2) and transforming growth factor-β (TGF-β). The effect of CCN2 on the binding of these ligands to their putative receptors is not fully elucidated. **f** CCN2 binds to bone morphogenetic proteins (BMPs), vascular endothelial growth factors (VEGFs) and fibroblast growth factor 2 (FGF2), thereby modulating their presentation and binding to cell-surface receptors, resulting in inhibited signal trans-

duction. *Only full-length CCN2 inhibits VEGFA and VEGFC, not its proteolytic fragments. **g** CCN2 binds directly to structural ECM proteins, matricellular proteins and other proteins, including aggrecan, fibronectin, decorin, perlecan, CCN3, heparin, Wnt inhibitory factor 1 (Wif-1), Slit guidance ligand 3 (Slit-3), and von Willebrand factor (vWF). **h** CCN2 can bind directly to cell surface receptors, which include heparan sulphate proteoglycans (HSPGs), tropomyosin receptor kinase A (TrkA), dendritic cell-specific transmembrane protein (DC-STAMP), integrins, insulin-like growth factor 2 receptor (IGF-2R), epidermal growth factor receptor (EGFR), formyl peptide receptor-like 1 (FPRL1), osteoprotegerin (OPG), lipoprotein receptor-related proteins (LRPs), receptor activator of NF-κB (RANK), and fibroblast growth factor receptors (FGFRs). By CCN2 binding, intracellular signaling pathways and their cross-talk can be altered and gene transcription affected. **i** Intracellular signaling pathways that are affected by CCN2 include the extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway, the Rho GTPase pathway, the WNT pathway, the c-Jun N-terminal kinase (JNK) pathway, the phosphatidylinositol 3-kinase (PI3K)/AKT pathway and the nuclear factor κB (NFκB) pathway the signaling mother against dexamptaplegic peptides (Smad) pathway. **j** CCN2 has been reported to act intracellularly after being taken up into the cytosol via endocytic pathways and, after phosphorylation, into the nucleus where it may affect gene transcription

The reported binding partners of CCN2 are summarized in Table 1 and depicted in Fig. 2. It should be noted here that at least some of the interactions of CCN2 with other molecules have been studied only in ex vivo conditions

under circumstances that might not be fully representative of in vivo conditions. By binding to cell surface receptors, CCN2 can alter various intracellular signaling pathways, including the ERK pathway (Aoyama et al. 2012; Lee et al.

Table 1 Reported binding partners of CCN2

Factor	Abbreviation	References
<i>Cell surface receptors</i>		
Integrins		
Lipoprotein receptor-related protein-1 -4, and -6	LRP-1, LRP-4, LRP-6	Babic et al. (1999), Ball et al. (2003), Chen et al. (2001), Gao and Brigstock (2004), Gao and Brigstock (2005), Jedsadayanmata et al. (1999), Kiwanuka et al. (2013), Lau (2016), Liu et al. (2012), Rayego-Mateos et al. (2013), Schober et al. (2002)
Neurotrophin receptors: Tropomyosin receptor kinase A and P75 ^{NTR}	TrkA, P75 ^{NTR}	Edwards et al. (2011), Rayego-Mateos et al. (2013), Wahab et al. (2005), Wang et al. (2010)
Insulin-like growth factor 2 receptor/Cation-independent mannose-6-phosphate receptor	IGF-2-R/M6P	Blalock et al. (2012)
Fibroblast growth factor receptor 1, 2 and 3	FGFR-1, FGFR-2, FGFR-3	Aoyama et al. (2012), Nishida et al. (2011b)
Epidermal growth factor receptor	EGFR	Rayego-Mateos et al. (2013)
Receptor activator of NF-κB	RANK	Aoyama et al. (2015)
Dendritic cell-specific transmembrane protein	DC-STAMP	Nishida et al. (2011a)
Osteoprotegerin	OPG	Aoyama et al. (2015)
Formyl peptide receptor-like 1	FPRL1	Lee et al. (2015)
<i>Growth factors</i>		
Transforming growth factor beta	TGF-β	Abreu et al. (2002) and Khattab et al. (2015)
Bone morphogenetic protein 2, 4 and 7	BMP-2, BMP-4, MBP-7	Abreu et al. (2002), Maeda et al. (2009), Nguyen et al. (2008)
Platelet derived growth factor-B and -BB	PDGF-BB, PDGF-B	Khattab et al. (2015), Pi et al. (2012)
Vascular endothelial growth factors-A and -C	VEGF-A, VEGF-C	Heroult et al. (2004), Inoki et al. (2002), Khattab et al. (2015), Kinashi et al. (2017)
Fibroblast growth factor 2	FGF2	Nishida et al. (2011b)
Insulin-like growth factors 1 and 2	IGF1/2	Kim et al. (1997), Lam et al. (2003)
Growth-differentiation factor 5	GDF-5	Khattab et al. (2015)
<i>Structural matrix proteins</i>		
Perlecan		Nishida et al. (2003)
Aggrecan		Aoyama et al. (2009)
Fibronectin		Hoshijima et al. (2006), Pi et al. (2008)
Decorin		Vial et al. (2011)
<i>Other</i>		
Heparin		Ball et al. (2003), Brigstock et al. (1997), Frazier et al. (1996), Gao (2003), Kireeva et al. (1997)
Heparan sulphate proteoglycans (cell surface and matrix associated)	HSPGs	Ball et al. (2003), Chen et al. (2001), Gao (2003), Gao and Brigstock (2004), Kireeva et al. (1997), Nishida et al. (2003)
CCN2		Hoshijima et al. (2012)
CCN3		Hoshijima et al. (2012)
Wnt inhibitory factor 1 (Wif-1)	Wif-1	Surmann-Schmitt et al. (2012)
Slit guidance ligand 3	Slit-3	Pi et al. (2012)
von Willebrand factor	vWF	Pi et al. (2012)

2015; Rayego-Mateos et al. 2013; Yang et al. 2004), WNT pathway (Liu and Leask 2013; Mercurio et al. 2004; Rooney et al. 2011), JNK pathway (Aoyama et al. 2015; Yosimichi et al. 2006), NFκB pathway (Aoyama et al. 2015; Edwards et al. 2011) and others (Wahab et al. 2005; Yosimichi et al. 2006). Its binding to growth factors usually has an inhibitory

effect: Binding to bone morphogenetic proteins (BMPs) inhibits BMP signaling (Abreu et al. 2002; Maeda et al. 2009; Mundy et al. 2014; Nguyen et al. 2008), resulting in reduced phosphorylation of EKR and Smad 1/5/8 (Maeda et al. 2009; Mundy et al. 2014; Nguyen et al. 2008), and binding to vascular endothelial growth factor 165 (VEGF₁₆₅)

inhibits its angiogenic activity by interrupting binding to its major receptor VEGFR-2 (Heroult et al. 2004; Inoki et al. 2002). Binding of the CT module, but not full length CCN2, to fibroblast growth factor 2 (FGF2) inhibits its binding to fibroblast growth factor receptor-1 (FGFR1) and thereby its activation (Nishida et al. 2011b). Furthermore, there are indications that CCN2 has an inhibitory effect on insulin-like growth factor (IGF) signaling and WNT signaling (Smerdel-Ramoya et al. 2008). Binding of CCN2 to transforming growth factor beta (TGF- β) has been reported to enhance TGF- β signaling (Abreu et al. 2002). This interesting notion has, however, not been reproduced in more recent literature.

In addition to its direct effects on cell surface receptors and growth factors, CCN2 can increase the level of matrix metalloproteinases (MMPs), a large family of enzymes playing a central role in the ECM by the degradation of specific ECM components and cleavage of growth factors and their binding proteins, by upregulating their gene expression in fibroblasts and endothelial cells (Chen et al. 2001; Fan and Karnovsky 2002; Kondo et al. 2002). On the other hand, CCN2 itself is a substrate of several MMPs, by which it can be cleaved in the hinge region (Dean et al. 2007; Hashimoto et al. 2002; Tam et al. 2004). For an overview and more detail of CCN2 binding partners and intracellular signaling pathways we like to refer to several recent reviews (Chaour 2020; Lau 2016; Ramazani et al. 2018; Takigawa 2018).

Regulation of CCN2 expression

TGF- β is a powerful and well-known inducer of CCN2 transcription (Brunner et al. 1991; Grotendorst et al. 1996; Holmes et al. 2001; Igarashi et al. 1993; Yang et al. 1998), but many other factors, summarized in Table 2 and depicted in Fig. 2, can also directly or indirectly induce CCN2 mRNA expression through the initiation of signaling pathways and the activation of transcription factors, as previously reviewed (Chaour 2020; Jun and Lau 2011; Ramazani et al. 2018; Takigawa 2018). For example, on mechanical stress, the transcriptional regulators YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif) induce CCN2 gene transcription (Dupont et al. 2011; Nagasawa-Masuda and Terai 2017; Preisser et al. 2016; Raghunathan et al. 2014). FAK (focal adhesion factor) is an important factor in mechanotransduction, which controls the nuclear translocation and activation of YAP and subsequent CCN2 gene expression in response to mechanical activation (Lachowski et al. 2018). In addition, CCN2 can induce its own expression by auto-induction, resulting in a positive feedback loop (Riser et al. 2000; Shimo et al.

2001b). Other factors, summarized in Table 3 and depicted in Fig. 2, can directly or indirectly inhibit CCN2 expression. For involvement of specific regulatory elements in the CCN2 gene we refer to Leask et al. (2009). Furthermore, CCN2 expression can be regulated at the posttranscriptional and posttranslational level by various factors, including VEGF (Kondo et al. 2006), hypoxia (Kondo et al. 2002), tumor necrosis factor α (TNF- α), interferon gamma (IFN- γ) (Cooker et al. 2007; Laug et al. 2012), and a host of different microRNAs (miRNAs) (Cai et al. 2018; Che et al. 2019; Chen et al. 2016, 2019; Ernst et al. 2010; Fox et al. 2013; He et al. 2017; Mu et al. 2016; Qiao et al. 2017; Sun et al. 2016). For example, CCN2 and miRNA-21 are components of a positive feedback loop in pancreatic stellate cells, that may serve as an amplification mechanism for enhanced collagen production (Charrier et al. 2014a). On the other hand, CCN2 can increase the expression of miRNA-302, which targets the TGF β type II receptor and thereby decreases its expression, constituting a negative feedback loop (Faherty et al. 2012).

CCN2 and disease

CCN2 has been implicated in the pathophysiology of many diseases; increased CCN2 expression has been demonstrated in the tissue of a range of diseases that are accompanied by fibrosis such as fibrotic lung diseases, scleroderma, chronic pancreatitis, renal fibrosis, liver cirrhosis, myocardial infarction, and Crohn's disease (Abou-Shady et al. 2000; di Mola et al. 1999, 2004; Ito et al. 1998; Ohnishi et al. 1998; Pan et al. 2001; Shi-wen et al. 2000), as well as in diabetic retinopathy and in the osteoarthritic cartilage of patients with osteoarthritis (Omoto et al. 2004; Tikellis et al. 2004). In addition, increased levels of CCN2 cleavage products have been demonstrated in human extracellular fluids, including plasma, urine, dermal interstitial fluid and vitreous fluid, of patients with fibrotic diseases, correlating with the severity of fibrosis (Leask et al. 2009). Furthermore, increased CCN2 plasma levels have been associated with cardiac dysfunction and increased risk of cardiovascular events in patients with atherosclerotic disease (Behnes et al. 2014; Gerritsen et al. 2016; Koitabashi et al. 2008), and altered CCN2 expression has been demonstrated in more than 25 different forms of cancer, with deregulation of CCN2 expression usually correlating with worse clinical outcome (Wells et al. 2015). Besides altered CCN2 expression in tumor cells, elevated CCN2 expression in stromal fibroblasts is implicated in the desmoplastic response in various cancer types, and CTGF expression in stromal cells can advance tumor growth or promote invasion as reviewed by Wells et al. (2015).

Table 2 Reported (direct or indirect) inducers of CCN2 mRNA expression

Factor	References
<i>Growth factors</i>	
TGF- β	Gao and Brigstock (2005), Goppelt-Struebe et al. (2001), Grotendorst et al. (1996), Holmes et al. (2003), Igarashi et al. (1993), Nakanishi et al. (1997), Riser et al. (2000), Wunderlich et al. (2000)
PDGF	Gao and Brigstock (2005)
Epidermal growth factor (EGF)	Wunderlich et al. (2000)
Basic fibroblast growth factor (bFGF)	Wunderlich et al. (2000)
BMP-2	Nakanishi et al. (1997)
VEGF	Lee et al. (2015), Suzuma et al. (2000)
<i>Hormones</i>	
<i>Steroids</i>	
	Dammeier et al. (1998), Kubota et al. (2003), Moritani et al. (2005), Okada et al. (2006), Pereira et al. (2000)
Angiotensin II	Gao et al. (2007), Gu et al. (2012)
Endothelin-1	Kemp et al. (2004), Rodriguez-Vita et al. (2005), Shi-Wen et al. (2007)
Aldosteron	Lee et al. (2004)
<i>Coagulation factors</i>	
Thrombin	Bai et al. (2013), Chambers et al. (2000)
Factor Xa	Chambers et al. (2000)
<i>Glucose metabolism related</i>	
Glucose	Lam et al. (2003), Murphy et al. (1999), Paradis et al. (2001), Riser et al. (2000)
Glycolysis—via adenosine triphosphate (ATP)	Akashi et al. (2018)
Advanced glycosylation end products	Twigg et al. (2001)
Insulin	Paradis et al. (2001)
<i>Cytokines</i>	
Tumor necrosis factor alpha (TNF- α) ^a	Cooker et al. (2007)
<i>Other</i>	
Bioactive lipids	Chowdhury and Chaqour (2004), Goppelt-Struebe et al. (2001), Muehlich et al. (2004)
Ethanol and acetaldehyde	Charrier et al. (2014b), Gao and Brigstock (2005)
UV-light	Kafi et al. (2004), Quan et al. (2009)
Mechanical stress (shear and cell stretch)	Guo et al. (2011), Honjo et al. (2012), Kessler et al. (2001), Riser et al. (2000), Schild and Trueb (2002), Schild and Trueb (2004), Wong et al. (2003), Yamashiro et al. (2001)
Hypoxia—via hypoxia-inducible-factor-1 (HIF-1 α)	Higgins et al. (2004), Kondo et al. (2002), Shimo et al. (2001a), Valle-Tenney et al. (2020)
Nitric oxide (NO)	Tsai et al. (2014)
CCN2	Bradham et al. (1991), Kawaki et al. (2008), Parada et al. (2013), Riser et al. (2000), Shimo et al. (2001b)
Insulin-like growth factor-binding protein 5 (IGFBP-5)	Nguyen et al. (2018)

^aTNF- α has also been reported to inhibit CCN2 mRNA expression (see Table 3)

CCN2 and the normal BM microenvironment (Fig. 3)

CCN2 expression and function in mesenchymal stem and stromal cells

Mesenchymal stem cells are essential for the maintenance of the BM microenvironment; they can self-renew and have the capacity to differentiate into other mesenchymal cell types, including chondrocytes, adipocytes, fibroblasts and

osteoblasts. They also stimulate the production of the ECM (Huang et al. 2016), and maintain hematopoiesis by the secretion of cytokines that stimulate proliferation of hematopoietic progenitor cells (Huang et al. 2016). Mesenchymal stem cells are rare, constituting only 1 in 3.4×10^4 nucleated BM cells (Wexler et al. 2003). As it is difficult to identify mesenchymal stem cells on a per cell basis, mesenchymal stem cell-enriched cell populations of both true mesenchymal stem cells and more differentiated mesenchymal stromal cells are used in most experimental settings (Lindner et al.

Table 3 Reported (direct or indirect) inhibitors of CCN2 mRNA expression

Factor	References
<i>Growth factors</i>	
Hepatocyte growth factor	Inoue et al. (2003), Kroening et al. (2009)
<i>Cytokines</i>	
Tumor necrosis factor alpha (TNF- α) ^a	Abraham et al. (2000), Dammeier et al. (1998), Laug et al. (2012), Lin et al. (1998), Moritani et al. (2005)
Interferon-gamma (IFN- γ)	Laug et al. (2012)
Interleukin 1 beta (IL-1 β)	Masuko et al. (2010)
<i>Elevators of cyclic adenosine monophosphate (cAMP) levels</i>	
Cholera toxin	Duncan et al. (1999), Kothapalli et al. (1998), Masuko et al. (2010)
Prostaglandin E2	Masuko et al. (2010), Ricupero et al. (1999)
<i>Other</i>	
CCN3	Kawaki et al. (2008)
Insulin-like growth factor binding protein-4 (IGFBP-4)	Su et al. (2019)

^aTNF- α has also been reported to induce CCN2 mRNA expression (see Table 2)

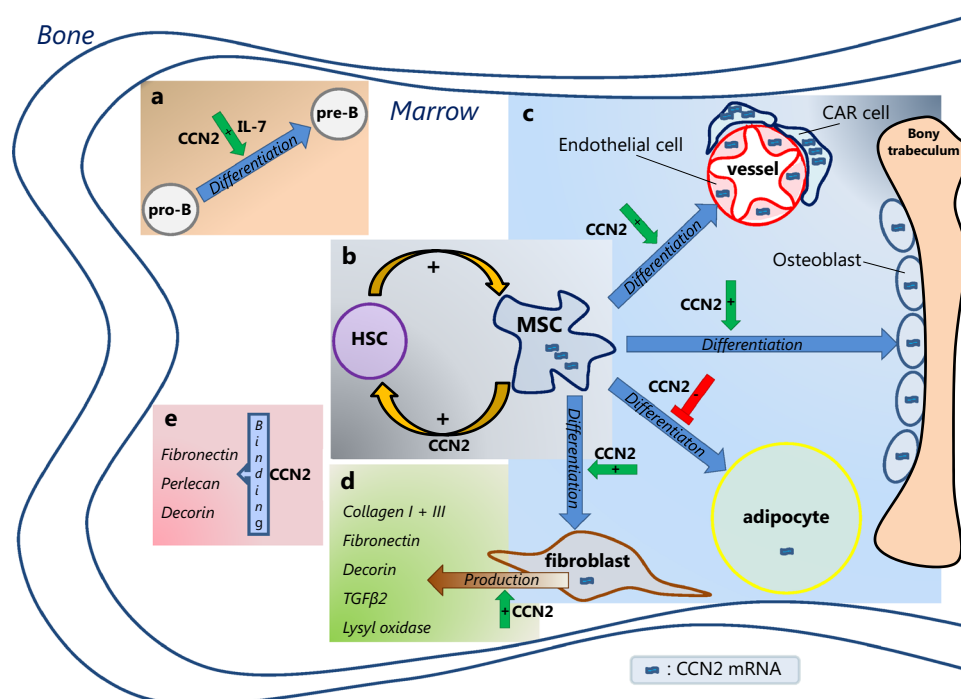


Fig. 3 CCN2 in the bone marrow microenvironment. CCN2 mRNA, depicted by ■, is present in different bone marrow (BM) mesenchymal cells, including endothelial cells, osteoblasts and fibroblasts, with highest levels (■■■) reported in mesenchymal stem/stromal cell (MSC) and CXCL12-abundant reticular (CAR) cells. CCN2 exerts different actions in the BM. **a** In the presence of interleukin 7 (IL-7), CCN2 promotes pro-B cell to pre-B cell differentiation. **b** CCN2 produced by MSCs affects the (long-term) qualities of hematopoietic stem cells (HSCs). HSCs, in turn, upregulate CCN2

expression by MSCs. **c** CCN2 enhances the differentiation of MSCs into endothelial cells, osteoblast and fibroblasts, but has an inhibitory effect on the differentiation of MSCs into adipocytes. **d** CCN2 might induce the production of the ECM proteins collagen type I and type III, fibronectin, decorin, TGF β -2 and lysyl oxidase by fibroblast. **e** CCN2 binds to fibronectin, perlecan and decorin, known constituents of the BM extracellular matrix. The effects hereof in the BM are yet unknown.

2010). The mesenchymal stem and stromal cells from the cited studies are therefore further referred to as mesenchymal stem/stromal cells (MSCs).

BM-derived MSCs show high expression of CCN2 mRNA (Battula et al. 2013, 2017; Cheung et al. 2014; Djoud et al. 2007; Igarashi et al. 2007; Ren et al. 2011;

Schutze et al. 2005; Shinde et al. 2014), which can be induced in vitro by Wnt3a and BMPs (Luo et al. 2004). CCN2 is important for MSC function as indicated by several in vitro and mouse studies. Although cell numbers and proportions of MSCs isolated from the BM of newborn CCN2-knockout mice were unchanged compared with those from wild-type (WT) mice (Cheung et al. 2014), MSCs from tissues of CCN2 homozygous knockout mice were not able to generate colony-forming unit fibroblast-like cells in vitro (Battula et al. 2013), implying a functional defect. Indeed, a role for CCN2 in MSC cell growth has been demonstrated, as CCN2 is shown to enhance proliferation and inhibit apoptosis of MSCs in vitro (Battula et al. 2013; Wang et al. 2009; Wells et al. 2016). In addition, CCN2 enhances MSC cell migration and recruitment, shown by either the addition of exogenous CCN2 or through plasmid induced expression of CCN2 (Luo et al. 2004; Wang et al. 2009). Furthermore, CCN2 affects MSC differentiation. Although knockdown of endogenous CCN2 expression in MSCs did not affect their capacity to differentiate into osteoblasts or chondrocytes (Battula et al. 2013), increase of CCN2 by either exposure to recombinant CCN2 (rCCN2) or transfection of CCN2-expressing plasmids did enhance the differentiation of human BM MSCs into osteoblasts and chondrocytes as well as fibroblasts in culture (Lee et al. 2010; Nishida et al. 2004; Wang et al. 2009). MSCs can also be differentiated into myofibroblasts by the addition of rCCN2, but only when stimulated subsequently with TGF- β (Lee et al. 2010; Wang et al. 2009). On the other hand, knockdown of CCN2 expression in MSCs enhanced adipocytic differentiation (Battula et al. 2013), suggesting an inhibitory effect of CCN2 on adipogenesis. When MSCs differentiate into these progenitor cells with lineage commitment, CCN2 expression was shown to decrease (Luo et al. 2004; Schutze et al. 2005).

CCN2 expression and function in endothelial and perivascular cells

Endothelial cells and perivascular stromal cells are part of the (peri)vascular niche and contribute to the microenvironment by the production of SCF and other growth factors, cytokines, chemokines and adhesion molecules such as E-selectin and CXCL12 (Ding and Morrison 2013; Sacchetti et al. 2007; Sipkins et al. 2005; Sugiyama et al. 2006; Winkler et al. 2012; Zhao et al. 2019).

CCN2 has been shown to induce neovascularisation and to promote the adhesion, migration, proliferation and survival of vascular endothelial cells in vitro (Babic et al. 1999; Shimo et al. 1998, 1999). CCN2 mRNA expression in endothelial cells increases in vitro by the addition of bioactive lipids such as sphingosine-1-phosphate and lysophosphatidic acid (Muehlich et al. 2004). Also, addition of freshly isolated platelets to endothelial cells upregulates

their CCN2 mRNA expression (Muehlich et al. 2004), possibly due to the release of constituents of lipoproteins, TGF- β and other CCN2-inducing compounds by platelets.

As expected, CCN2 mRNA is present in endothelial cells derived from the BM as well (Cheung et al. 2014). The perivascular region of the BM contains a heterogeneous population of stromal cells characterized by very high CXCL12 expression, including the CXCL12-abundant reticular (CAR) cells, which are mesenchymal progenitor cells important for the maintenance of both HSC and B-cells (Eltoukhy et al. 2016; Sugiyama et al. 2006). These CAR-cells were shown to have the highest expression of CCN2 mRNA of all investigated BM stromal cells (Cheung et al. 2014). The cell numbers and the proportions of endothelial cells and CAR cells isolated from *CCN2*^{+/-} and *CCN2*^{-/-} newborn mice are unchanged compared with those from WT mice (Cheung et al. 2014), but their function has not been studied in these knock-out models. The role of CCN2 in the perivascular niche thus remains to be established.

CCN2 expression and function in osteoblasts

Osteolineage cells contribute to the BM microenvironment by secreting factors such as granulocyte colony stimulating factor (G-CSF) (Taichman and Emerson 1994), thrombopoietin (TPO) (Yoshihara et al. 2007), and CXCL12 (Jung et al. 2006), although their effect on hematopoiesis is not fully determined (Ho and Méndez-Ferrer 2020). Osteolineage cells also express CCN2 (Luo et al. 2004; Safadi et al. 2003; Xu et al. 2000).

CCN2 is long known for its importance in endochondral ossification, and crucial for normal development, growth and regeneration of bone (Ivkovic et al. 2003; Kanyama et al. 2003; Lambi et al. 2012; Xu et al. 2000; Yamaai et al. 2005). Its mRNA and protein expression have been detected in normal long bones during the period of growth and (re) modeling, and have been located to osteoblasts lining metaphyseal trabeculae and those lining active, osteogenic surfaces in fracture callus (Safadi et al. 2003). CCN2 null mice show severe skeletal abnormalities involving both cartilage and bone, and die shortly after birth due to respiratory distress and cyanosis caused by severe rib cage malformations as well as by disruption of normal lung development due to reduced proliferation and increased apoptosis of pulmonary cells (Baguma-Nibasheka and Kablar 2008; Cheung et al. 2014; Falke et al. 2020; Ivkovic et al. 2003; Lambi et al. 2012; Yamaai et al. 2005).

As discussed above, increased CCN2 expression enhances the differentiation of human BM MSCs into osteoblasts (Wang et al. 2009). The osteoblast lineage-specific differentiation of MSCs is at least in part regulated by Wnt signaling and osteogenic BMPs, especially BMP-9 (Luo et al. 2004). Of note, in MSCs stimulated by Wnt3a and

osteogenic BMPs, CCN2 was among the most significantly up-regulated genes (Luo et al. 2004). Prolonged CCN2 expression, in turn, inhibited both Wnt3a and BMP induced osteogenic differentiation, suggesting a regulatory role for CCN2 in normal osteogenesis (Luo et al. 2004).

The differentiation of pre-osteoblasts into bone forming osteoblasts encompasses the following phases in development: 1. proliferation, 2. maturation and extra-cellular matrix synthesis, and 3. matrix mineralization (Neve et al. 2011). A bimodal pattern of CCN2 mRNA levels is observed in primary osteoblast cultures; high CCN2 levels during the proliferative phase of early osteoblast progenitor cells (pre-osteoblasts), diminished CCN2 expression as the progenitor cells show terminal differentiation towards committed osteoblasts (Luo et al. 2004; Xu et al. 2000), and again high CCN2 mRNA levels during the mineralization phase (Safadi et al. 2003). Several studies showed that addition of rCCN2 increases the proliferation as well as mineralization of osteoblasts (Nishida et al. 2000; Safadi et al. 2003; Wang et al. 2009). Furthermore, delivery of rCCN2 into the femoral marrow cavity induced osteogenesis *in vivo*, which was associated with increased angiogenesis (Safadi et al. 2003; Wang et al. 2009).

Thus, CCN2 expression is important in the proliferation and differentiation of BM osteoblasts and is thereby at least indirectly of importance for the formation and maintenance of the BM microenvironment. A direct effect of CCN2 expression by osteoblasts on hematopoiesis still needs to be determined.

CCN2 expression and function in adipocytes

Several studies indicate a regulatory role of adipocytes in hematopoiesis, although their effect might be context-dependent. Some studies indicate an inhibitory effect of BM adipocytes on hematopoiesis, as adipocyte-rich marrow spaces in mice contain less HSCs and hematopoietic progenitor cells, and adipocytes inhibit hematopoietic recovery (Ambrosi et al. 2017; Naveiras et al. 2009; Zhu et al. 2013). Another study, in contrast, shows that BM adipocytes express high levels of SCF and have a stimulatory effect on hematopoiesis, increasing hematopoietic recovery after irradiation (Zhou et al. 2017). The latter, however, seems location dependent as adipocytes in long bones promote hematopoietic recovery after irradiation, while those in the caudal vertebrae inhibit hematopoietic recovery, despite SCF production (Zhou et al. 2017). The factors responsible for these disparate observations remain to be identified.

The effect of CCN2 on adipogenesis seems inhibitory; when CCN2 is knocked down in MSCs, they differentiate

into adipocytes at a six fold higher rate (Battula et al. 2013). Furthermore, when CCN2 knockdown MSCs are used to form humanized extramedullary bone, this contains less cortical bone and more adipose-like marrow tissue when compared with that derived from normal MSCs (Battula et al. 2013). The possible impact hereof on hematopoiesis still remains to be elucidated.

CCN2 expression and function in the extracellular matrix

The ECM is a fibrillar basement network that plays a key role in cell proliferation, differentiation and migration, as well as in interactions between cells (Midwood et al. 2004). The BM ECM is composed of structural and non-structural (soluble) proteins. The structural matrix proteins include collagens, proteoglycans and other glycoproteins, the most abundant being collagens I-XI, fibronectin, laminin, tenascin, thrombospondin and elastin (Klamer and Voermans 2014). The soluble ECM proteins include growth factors, cytokines, hormones and matricellular proteins, including those of the CCN family.

CCN2 is expressed by various BM stromal cells, which include mesenchymal stem cells as well as more differentiated mesenchymal cells such as osteoblasts and CAR cells (Battula et al. 2013, 2017; Cheung et al. 2014; Igarashi et al. 2007; Istvanffy et al. 2015; Luo et al. 2004; Ren et al. 2011; Safadi et al. 2003; Schutze et al. 2005; Xu et al. 2000). Furthermore, BM stromal cells show a high level of CCN2 binding through the low density lipoprotein receptor-related protein-1 (LRP1) (Segarini et al. 2001), a well-known binding partner of CCN2. CCN2 is expected to bind many other factors in the BM as it contains many known binding partners of CCN2, such as integrins, heparan sulphate proteoglycans and growth factors. CCN2 might also induce the production of BM ECM proteins, as one study reports that BM stromal cells incubated with recombinant human CCN2 (rhCCN2) show increased expression of genes associated with ECM synthesis, including collagen type I and type III, lysyl oxidase, fibronectin, decorin and TGF β -2 (Wells et al. 2016). It should be noted here that, typically, it has been difficult to show such activity of rCCN2 *per se* in other experiments.

Thus, as a matricellular protein, CCN2 can be expected to regulate intercellular signaling in the BM ECM. Furthermore, the relevance of ECM-characteristics for HSC biology and blood cell maturation underscores that CCN2 regulation might indirectly affect hematopoiesis through ECM-modification (Durand et al. 2018; Ho and Méndez-Ferrer 2020; Klamer and Voermans 2014).

CCN2 and normal hematopoiesis

CCN2 expression and function in hematopoietic stem cells, myelopoiesis and lymphopoiesis

CCN2 has *not* been detected in HSCs, and transplant studies showed that HSCs of *CCN2*^{-/-} or WT neonatal mice transplanted into WT recipients had similar HSC properties after transplantation, indicating that CCN2 indeed has minimal cell-autonomous effects in HSCs (Cheung et al. 2014). HSCs, however, *are* able to upregulate CCN2 expression in BM *stromal* cells (Istvanffy et al. 2015) and there is substantial evidence that *stromal* CCN2 does affect hematopoiesis.

The effects of stromal CCN2 on hematopoiesis have been investigated by *in vitro* and *in vivo* studies. Murine HSCs co-cultured on stroma with decreased CCN2 protein content (*shCCN2* stroma) showed reduced colony formation with increased number of hematopoietic stem and progenitor cells in G₀ phase and senescence, and delayed time to first cell division, indicating that stromal CCN2 supports the growth and proliferation of HSCs and hematopoietic progenitor cells (Istvanffy et al. 2015). Stromal CCN2 seems to regulate the G₀/G₁ transition in murine HSCs by concerted action on TGF-β and WNT signaling pathways (Istvanffy et al. 2015). Furthermore, HSCs cultured on *shCCN2* stroma and subsequently transplanted into recipient WT mice in a competitive setting, showed normal initial engraftment, but at later time points (10 and 16 weeks), there was a significant decline of myeloid and B-lymphoid but not T-lymphoid engraftment (Istvanffy et al. 2015). Adding rCCN2 to these cultures partly compensated for the diminished CCN2 production by stromal cells, significantly enhancing both the number of B- and T-cells, whereas the number of myeloid cells did not change (Istvanffy et al. 2015). In addition, when the donor HSCs isolated from primary recipients were again transplanted in equal numbers into secondary recipients, none of the secondary recipients of HSCs from *shCCN2* stromal co-cultures engrafted more than 1% in the peripheral blood, BM, and spleen in contrast to control co-cultures (Istvanffy et al. 2015). Thus, co-culturing HSCs with CCN2-deficient stroma affects HSC properties in mice, with stromal CCN2 supporting HSC maintenance and longtime survival as well as supporting both myelopoiesis and B-lymphopoiesis.

CCN2 involvement in myelopoiesis and B-lymphopoiesis is also demonstrated by another study, showing that myeloid cell numbers in newborn *CCN2*^{-/-} mice were *decreased* in the liver, although *unaltered* in BM and spleen, whereas B-cell numbers in the same mice were *increased* in the liver, while *decreased* in BM and spleen

(Cheung et al. 2014). T-cells were the same in all the compartments compared with WT mice (Cheung et al. 2014). The opposite effects of CCN2 on myelopoiesis and B-lymphopoiesis in BM and spleen versus liver might relate to differences in microenvironmental context in these compartments, although this has not been further explored. In the associated transplant study, the authors found similar results for B-cells; mice receiving liver-derived cells containing high numbers of HSCs, showed significantly *decreased* numbers of B-cells in both BM and spleen when these cells were derived from *CCN2*^{-/-} neonatal mice compared with that from WT mice, indicating that presence of CCN2 in the microenvironment of developing HSCs supports B-lymphopoiesis in BM and spleen (Cheung et al. 2014). As for the lower numbers of B-cells in the BM of mice receiving *CCN2*^{-/-} cells, pre-B and later differentiation stages were herein most affected, while pro-B populations remained unchanged and overall B-cell function was not affected (Cheung et al. 2014). This effect on B-lymphopoiesis is supported by *in vitro* studies, showing that CCN2, in the presence of IL-7, could potentiate B-cell proliferation and promote pro-B to pre-B cell differentiation, but not the further differentiation into sIgM + B cells (Cheung et al. 2014). In contrast to B-cells, the myeloid population in the BM of recipients transplanted with cells from *CCN2*^{-/-} mice was more abundant, which points to a possible inhibitory effect of CCN2 on myelopoiesis in this compartment (Cheung et al. 2014). The apparent discrepancy with the aforementioned study by Istvanffy et al., reporting that HSCs cultured on *shCCN2* stroma transplanted into recipient WT mice showed a decline of myeloid engraftment, suggesting a supportive effect of stromal CCN2 on myelopoiesis (Istvanffy et al. 2015), might relate to the differences in experimental set up.

In all, it can be concluded that stromal/environmental CCN2 is important for maintenance and longtime survival of HSCs and affects both myelopoiesis and B-lymphopoiesis. The effect on the latter two is likely to be highly dependent on the local microenvironment of the different tissue compartments and the precise effect of CCN2 on especially the myelopoiesis needs to be further determined.

CCN2 expression in peripheral blood, megakaryopoiesis and erythropoiesis

Normal mononuclear cells derived from peripheral blood show very low to undetectable CCN2 mRNA levels (Kim et al. 1997; Sala-Torra et al. 2007; Vorwerk et al. 2000). Plasma and serum mainly contain the N-terminal fragment of CCN2, while the full-length protein is abundant in platelets and released upon platelet stimulation (Cicha et al. 2004; Kubota et al. 2004; Miyazaki et al. 2010; Roestenberg et al.

2004). The CCN2 content of a single platelet is estimated to be more than 20-fold higher than that of any other growth factor reported in platelets such as TGF- β , insulin-like growth factor-1 (IGF1), and platelet derived growth factor (PDGF)-AB (Kubota et al. 2004).

Platelets do not produce CCN2 themselves as CCN2 transcripts are absent in platelets (Gnatenko et al. 2003). Also, normal megakaryocytes do not seem to contain or produce CCN2 as neither cells from the megakaryocytic CMK cell line nor megakaryocytes differentiated from human HSCs showed any detectable CCN2 protein production or gene expression in vitro (Sumiyoshi et al. 2010). In addition, no appreciable CCN2 was detected in normal megakaryocytes in vivo (Astrom et al. 2015; Cicha et al. 2004; Sumiyoshi et al. 2010), although immunohistochemical staining indicated strong expression of CCN2 in megakaryocytes of primary myelofibrosis and in a subpopulation of megakaryocytes in patients with X-linked thrombocytopenia with thalassemia (Astrom et al. 2015). Thus, platelets are likely to take up CCN2 from the environment via endocytosis, similar to other molecules (Escobar et al. 2008), which is supported by the observation that human platelets are able to absorb exogenous CCN2 in vitro (Sumiyoshi et al. 2010). Possible receptors for endocytosis of CCN2 by platelets are LRP1 and integrin α IIb β 3, which are both known to bind CCN2 and to mediate endocytosis of their ligands (Jedsadayanmata et al. 1999; Kawata et al. 2006, 2012). The source of this platelet CCN2 is hypothesized to come from mesenchymal cells in the microenvironment, including chondrocytes and stromal fibroblasts. The transcription factor Myeloid Zinc Finger 1 (MZF1) can directly regulate CCN2 gene expression of BM stromal cells by binding to its promoter (Piszczatowski et al. 2015), and in vitro treatment of stromal fibroblasts with either vitamin A or D activates the MZF1 pathway, which increases CCN2 production and results in enhanced loading of CCN2 into developing platelets (Rozado et al. 2014). Whether other cells and factors are involved in CCN2 loading of platelets is undetermined.

To our knowledge, no data are available on the role of CCN2 in erythropoiesis.

CCN2 and malignant hematopoiesis

CCN2 has been implicated in more than 25 different forms of cancer, mostly based on correlations (either positively or negatively) with clinical outcome (Wells et al. 2015). Altered CCN2 expression has been reported in tumor cells as well as in supporting stromal cells (Wells et al. 2015).

Several studies have investigated the role of CCN2 in leukemia. Lymphoblast from both pediatric and adult B-acute lymphoblastic leukemias (B-ALL) show moderate to highly increased CCN2 mRNA expression compared with control

cells (often CD34 positive cells, CD19⁺igM⁻ cells or mononuclear cells) in the majority (60–80%) of cases (Boag et al. 2007; Sala-Torra et al. 2007; Vorwerk et al. 2000, 2002). MSCs isolated from the BM of acute myeloid leukemia (AML)-bearing mice showed increased CCN2 expression compared with MSCs from control mice (Battula et al. 2017). CCN2 expression has occasionally been described in chronic myeloid leukemia (CML) cells (Vorwerk et al. 2000), although this has not been confirmed by later studies. CCN2 gene amplification or mutations have not been reported.

CCN2 expression, effect and regulation in acute lymphoblastic leukemias

CCN2 expression and prognostic effect in ALL

High CCN2 mRNA expression is frequently observed in the lymphoblasts of B-ALL, but rarely in T-ALL (Advani et al. 2010; Boag et al. 2007; Gandemer et al. 2007; Kang et al. 2010; Lu et al. 2014; Sala-Torra et al. 2007; Tesfai et al. 2012; Vorwerk et al. 2002, 2000; Welch et al. 2013, 2015). It could be hypothesized that this is related to the fact that CCN2 plays a role in normal B-cell development while no effects in T-cell development have been reported (Cheung et al. 2014).

Several studies investigated the prognostic effect of CCN2 expression in pediatric and adult B-ALL. In pediatric B-ALL, increased CCN2 expression has been associated with certain cytogenetic subgroups; B-ALL with BCR-ABL, ETV6-RUNX1 (TEL-AML1) or translocations of MLL showed high CCN2 expression (Boag et al. 2007; Gandemer et al. 2007; Tesfai et al. 2012), while those with hyperdiploidy showed low CCN2 expression, and B-ALL with an E2A-PBX1 translocation showed hardly any CCN2 expression (Boag et al. 2007). Thus, with the exception of ETV6-RUNX1, high CCN2 expression is associated with poor prognostic cytogenetics, and low/no CCN2 expression with favorable cytogenetics. In addition, high CCN2 gene expression was part of the high risk profile in a study on pediatric ALL patients with high risk features (Kang et al. 2010). This study used a 38-gene expression classifier predictive of relapse-free survival (RFS) to distinguish 2 groups, one with low relapse risk (81% 4-year RFS) and one with high relapse risk (50% 4-year RFS). Patients with very high-risk features (BCR-ABL1 or hypodiploidy) were excluded, as well as those with low-risk features (trisomies of chromosomes 4 or 10; t[12;21][ETV6-RUNX1]) unless they had central nervous system disease or testicular localization. In an earlier study on pediatric B-ALL, CCN2 expression of lymphoblasts at diagnosis was not found to be predictive of relapse, as the same number of patients with a relapse had CCN2 expressing lymphoblasts at diagnosis

as those in continued remission (Vorwerk et al. 2002). This study, however, differed from the later published study in that it did not select for high-risk patients. Furthermore, it merely looked at absence or presence of CCN2 expression without taking the level of CCN2 expression into account. As discussed above, low CCN2 expression has been associated with favorable cytogenetics. Therefore, the level of CCN2 expression, and not merely its presence or absence, seems to be important for its prognostic value.

In adult B-ALL, higher CCN2 expression levels have also been associated with worsening of overall survival (Advani et al. 2010; Sala-Torra et al. 2007). In a study on 79 adult ALL specimens, a higher CCN2 expression level in blood or BM lymphoblasts was an independent negative predictor of survival in a multivariate proportional hazards model and correlated with the percentage of CD34 expressing blasts, although there was no correlation between CCN2 expression levels and rate of complete remission or resistant disease (Sala-Torra et al. 2007). There were also no significant differences in CCN2 expression when analyzed according to sex, age, French–American–British (FAB) classification, SWOG performance status, white blood cell counts, and number of blasts in peripheral blood or BM (Sala-Torra et al. 2007). Similar findings were reported in a smaller study on 33 adult ALL patients with relapsed or refractory disease; CCN2 expression was not predictive for complete remission rate nor for resistant disease, but there was a trend for patients with higher expression of CCN2 in circulating lymphoblasts to have an inferior overall survival (Advani et al. 2010).

Several *in vitro* and *in vivo* studies support the notion that CCN2 has a pro-leukemic effect in B-ALL. *In vitro*, knockdown of CCN2 in B-ALL cell lines reduces leukemia cell growth due to reduced proliferation as well as increased apoptosis (Lu et al. 2014; Wells et al. 2016). The reduced proliferation is likely due to inhibition of the G₁/S transition, associated with decreased levels of phospho-AKT and increased levels of p27, whereas the increased apoptosis is associated with increased levels of the pro-apoptotic BCL-2 family protein BIM (Lu et al. 2014). *In vivo*, mice injected with genetically engineered B-ALL cells with overexpressed CCN2 showed reduced survival (Wells et al. 2016). Mice injected with B-ALL with knocked down CCN2 showed less engraftment in the BM compared with mice transplanted with control cells (B-ALL cells with empty vector) (Wells et al. 2016). Thus, reduced survival associated with elevated CCN2 expression seems related to increased engraftment, proliferation, and apoptosis resistance (Wells et al. 2016).

B-ALL cells can also secrete CCN2 (Boag et al. 2007; Welch et al. 2015; Wells et al. 2016), and addition of rhCCN2 promotes adhesion of B-ALL cells to stromal cells *in vitro*, which induces them to overexpress genes associated

with cell cycle, intracellular transport and ECM synthesis (Wells et al. 2016). Therefore, CCN2 secreted by B-ALL cells might also enhance leukemia engraftment due to its modifying effects on the microenvironment and ECM interactions (Wells et al. 2016).

Seemingly in contrast to the above, one study showed *increased* leukemic engraftment when CCN2 was knocked down in MSCs, which suggests a protective effect of stromal CCN2, diminishing leukemic outgrowth (Battula et al. 2013). This study used CCN2 knockdown MSCs to form humanized extramedullary BM (EXM-BM) in WT mice. Increased leukemic engraftment of ALL cells was observed in this EXM-BM compared with that in the control EXM-BM derived from normal MSCs (Battula et al. 2013). This disparity suggests that not only the effects of CCN2 can be cell-type dependent, but also that the source of CCN2 might be critical (leukemic blast versus stromal cell). Another explanation might relate to the fact that actions of CCN proteins are context dependent. In the latter study, the CCN2 knockdown MSCs are used to induce newly formed bone and BM when injected together with human endothelial colony-forming cells (Battula et al. 2013). The EXM-BM derived from these CCN2 knockdown MSCs proved to be more adipocyte-rich, attributed to an inhibitory effect of CCN2 on adipogenesis, and expressed significantly higher levels of CXCL12 and of the adipocyte growth factor leptin than the EXM-BM derived from normal MSCs (Battula et al. 2013). Thus, there is not merely a down-regulation of CCN2 expression in the knockdown MSCs, but a profound change in the microenvironment they contribute to, which can be attributed to the effects of CCN2 on MSC differentiation. In particular, the enhanced leukemic engraftment in this setting might be due to the enhanced fat content of the BM with increased leptin and CXCL12 expression, possibly overruling a negative effect of CCN2 deficiency, as leptin enhances leukemic cell growth (Konopleva et al. 1999; Tabe et al. 2004) and CXCL12 is a known homing factor for leukemia cells (Möhle et al. 2000).

Epigenetic and post-transcriptional regulation of CCN2 in acute lymphoblastic leukemia

Both epigenetic regulation and post-transcriptional regulation of CCN2 might play a role in the pathophysiology of ALL.

DNA methylation is the only epigenetic modification of CCN2 studied in ALL. The CCN2 locus contains a dense CpG island at the 5' end of the coding region and demethylation of this region was shown to be a common feature of pediatric B-ALL; mononuclear cells extracted from BM of these patients showed this locus to be largely unmethylated, regardless of the level of CCN2 gene expression (Welch

et al. 2013). Remarkably, CD34+ cells from normal BM also showed extensive hypomethylation of the CCN2 locus, while BM lymphoblasts from T-ALL patients, not expressing detectable levels of CCN2 mRNA, showed hypermethylation focused at either end of the CCN2 CpG island (Welch et al. 2013). In B-ALL cell lines, an inverse correlation between the methylation state of the CCN2 locus and CCN2 gene expression was found: B-ALL cell lines with unmethylated CCN2 CpG islands showed high levels of CCN2 expression, while those with methylated CCN2 CpG islands showed no measurable CCN2 expression (Welch et al. 2013). In conclusion, while in B-ALL cell lines, demethylation of the CCN2 locus was associated with increased CCN2 expression, demethylation in ALL BM samples was commonly present but not related to CCN2 gene expression. Further studies are needed to elucidate the exact role of the CCN2 methylation status in ALL.

Alternative splicing of CCN2 in B-ALL is described by one study, which showed several novel short CCN2 mRNA isoforms (alternative splice forms) in B-ALL cell lines and B-ALL specimens (Welch et al. 2015). The splice forms all exhibited variable loss of sequences corresponding to exons 1–3, and in some cases loss of exon 4, but always full retention of exon 5 containing the CT domain (Welch et al. 2015). The short isoform encoding only the CT domain was the most frequently observed CCN2 alternative splice form, being present in 70% of the investigated B-ALL specimens expressing full length CCN2 (Welch et al. 2015). The shorter transcripts (but not the full length transcript) showed higher expression during the most active phase of cell growth, suggesting that they may be associated with the proliferation of B-lineage ALL cells (Welch et al. 2015). The truncated CCN2 protein with only the CT domain can still strongly bind to heparin, mediate cell adhesion and induce proliferation (Ball et al. 2003; Brigstock et al. 1997; Holbourn et al. 2009), but differences in biological activity of alternative splice products, compared to those of full length CCN2, largely remain to be elucidated.

MiRNAs play an important regulatory role in hematopoiesis and leukemogenesis (Fernandes 2017; Grobbelaar and Ford 2019; Schotte et al. 2009; Yeh et al. 2016; Yendamuri and Calin 2009; Zhang et al. 2018). The miRNA-17–92 cluster is essential for B-cell development, regulating pro-B to pre-B cell development and apoptosis of B-cells, and amplification of the miR-17–92 coding region has been associated with lymphoproliferative disease (Koralov et al. 2008; Ventura et al. 2008; Xiao et al. 2008). CCN2 is a predicted target of several miRNAs, including the miRNA-17–92 cluster (Chen et al. 2016; Ernst et al. 2010; Fox et al. 2013). It is, however, still unknown if and how miRNAs might relate CCN2 expression to ALL biology.

CCN2 expression and effect in acute myeloid leukemia

CCN2 expression has *not* been demonstrated in AML blast cells. But similar to normal HSCs, AML cells can induce CCN2 expression in MSCs, which relies on BMP-mediated signaling (Battula et al. 2017; Li et al. 2019). The CCN2 gene in MSCs from AML-bearing mice is upregulated 12- to 33-fold across various AML genotypes compared with that in MSCs from non-AML bearing control mice (Battula et al. 2017).

The effect of stromal CCN2 on AML engraftment is not fully established. One study indicates a pro-leukemic effect; transgenic mice overexpressing CCN2 in stromal cells, injected with AML cells, show: (1) a fourfold (time-dependent) enhancement of leukemia engraftment, (2) a higher percentage of leukemia cells in the peripheral blood, and (3) more leukemia engraftment in spleens compared with WT mice (Battula et al. 2017). Another study, however, suggests an opposite effect of stromal CCN2. Increased leukemic engraftment was observed in humanized extramedullary BM (EXM-BM) in mice when this BM was formed from CCN2 knockdown MSCs (Battula et al. 2013). As has been discussed above in more detail in the section on CCN2 and ALL, the EXM-BM derived from CCN2 knockdown MSCs proved to be more adipocyte-rich, which can be attributed to the inhibitory effect of CCN2 on the adipogenic differentiation of MSCs, and expressed higher levels of leptin and CXCL12 than the EXM-BM derived from normal MSCs (Battula et al. 2013). Thus, there is not merely a down-regulation of CCN2 expression in MSCs, but a complete change in microenvironment, with more adipose tissue and higher levels of leptin and CXCL-12, factors known to enhance leukemic growth and homing, possibly overruling a negative effect of CCN2.

CCN2 expression and effect in myeloid neoplasms with fibrosis

As described above, CCN2 enhances differentiation of cultured human BM MSCs into (myo)fibroblasts when stimulated subsequently with TGF- β (Lee et al. 2010). CCN2 is required for the differentiation of progenitor cells into contractile myofibroblasts through the regulation of extracellular matrix, cytoskeleton, cell adhesion, and cell migration genes, at least in dermal fibroblasts, as well as for the recruitment of progenitor cells to the fibrotic lesion in response to bleomycin, as has been shown by two different mouse models (Liu et al. 2014; Tsang et al. 2020; Liu et al. 2014). Another mouse fibrosis model has shown that either CCN2 mRNA or an application of exogenous CCN2 protein seems required for the development of persistent fibrosis (Mori et al. 1999). Although initially there has been some misconception that

CCN2 would be merely a down-stream mediator of TGF- β , it has now been shown that CCN2 rather acts as cofactor mediating and amplifying the profibrotic actions of TGF- β through domain-specific interactions with TGF- β and its receptor (Holmes et al. 2001; Khankan et al. 2011; Mori et al. 2008), and that TGF- β and CCN2 have overlapping and distinct fibrogenic effects (Gore-Hyer et al. 2002). In a wide variety of in vivo systems, CCN2 is required for experimental fibrosis. CCN2 is important as a key central mediator of the feed-forward system that both initiates and perpetuates fibrosis since adhesive signaling/mechanotransduction, mediated by FAK and YAP/TAZ, is required for fibrosis and CCN2 activates this pathway (Dupont et al. 2011; Lachowski et al. 2018).

Higher mRNA levels of TGF- β and CCN2 have been demonstrated in BM of myelodysplastic syndrome (MDS) with fibrosis than in MDS without fibrosis (Hussein et al. 2018), suggesting a role for both in its pathophysiology. The TGF- β pathway has already been implicated in the pathogenesis of many BM disorders, including myeloid neoplasms (Bataller et al. 2019), and TGF- β is known to play a central role in the induction of BM fibrosis in myeloproliferative neoplasms (Agarwal et al. 2016). The role of CCN2 in myeloproliferative neoplasms or other BM diseases with fibrosis still needs to be elucidated.

CCN2 expression and effect in plasma cell neoplasia

Only 1 study investigated the role of CCN2 in plasma cell neoplasia. This study reported significantly lower plasma levels of whole CCN2 in multiple myeloma (MM) patients compared with healthy controls, and in MM patients *with* bone disease compared with those *without* (Munemasa et al. 2007). Therefore, lowered CCN2 might be an indicator of bone disease in MM patients.

Other CCN protein family members

CCN2 is just one representative of the family of closely related CCN genes, which also includes CCN1 (Cysteine rich 61/Cyr61), CCN3 (Nephroblastoma overexpressed/NOV), CCN4 (Wnt-inducible-secreted protein (WISP)-1), CCN5 (WISP-2) and CCN6 (WISP-3) (Brigstock et al. 1997; Perbal 2018). The CCN family members can work in concert to orchestrate a multitude of biological processes in similar but also partly opposite ways (Peidl et al. 2019; Perbal 2018; Riser et al. 2009, 2010). Therefore, the CCN family genes should ideally be studied together rather than separate. Unfortunately, the limited availability of well characterized tools including purified individual CCN proteins and the high complexity of their individual biologies have largely prevented such studies thus far.

As discussed in the reviews on CCN proteins in cancers and their tumor microenvironment, the role of a particular CCN protein in either potentiating or inhibiting tumor progression is related to the tumor type, and altered protein expression can be observed in either tumor cells or in tumor associated stromal cells (Wells et al. 2016; Yeger and Perbal 2016). With respect to their role in the BM microenvironment and in normal or malignant hematopoiesis, we have found no integrative studies involving multiple CCN genes. The available data on the individual non-CCN2 protein family members are discussed below and their expression in hematologic malignancies of the BM is summarized in Table 4.

CCN1

High levels of CCN1 mRNA and protein have been demonstrated in human BM stromal cells (Djouad et al. 2007; Johnson et al. 2014; Li et al. 2012; Long et al. 2015; Schutze et al. 2005). The highest CCN1 expression is observed in MSCs, which decreases during osteogenic, adipogenic and chondrogenic differentiation (Djouad et al. 2007; Schutze et al. 2005).

In (T- and B-) ALL and CML, CCN1 expression has been detected in cell lines, and increased CCN1 levels have been demonstrated in BM-derived mononuclear cells, BM aspirate supernatant, and plasma samples of ALL and CML patients (Cao et al. 2019; Song et al. 2019; Zhu et al. 2016). In ALL, BM and plasma CCN1 levels correlate with the percentage

Table 4 Expression of CCN proteins in hematologic malignancies of the bone marrow

	CCN1	CCN2	CCN3	CCN4
<i>B-ALL</i>				
Tumor cell	↑	↑	n/r	↑
Stromal cell	n/r	n/r	n/r	n/r
<i>T-ALL</i>				
Tumor cell	↑	-	n/r	n/r
Stromal cell	n/r	n/r	n/r	n/r
<i>CML</i>				
Tumor cell	↑	n/r	↓	n/r
Stromal cell	n/r	n/r	n/r	n/r
<i>AML</i>				
Tumor cell	↑	n/r	n/r	n/r
Stromal cell	↑	↑	n/r	n/r
<i>MM</i>				
Tumor cell	-/↑ ?	n/r	n/r	n/r
Stromal cell	↑	n/r	n/r	↓

Increased (↑), decreased (↓), or unaltered (-) expression of CCN proteins in tumor cells and tumor associated stromal cells as far as reported in literature

N/R not reported, *ALL* acute lymphoblastic leukemia, *CML* chronic myeloid leukemia, *AML* acute myeloid leukemia, *MM* multiple myeloma

of blasts (Zhu et al. 2016). In both ALL and CML, CCN1 enhances leukemic cell survival in vitro by decreasing apoptosis through enhanced BCL2 expression via the NF- κ B pathway (Song et al. 2019; Zhu et al. 2016). In ALL, AKT but not ERK1/2 affects in vitro NF- κ B signaling by CCN1

(Zhu et al. 2016), whereas in CML, AKT nor ERK1/2 are involved in in vitro NF- κ B signaling by CCN1 (Song et al. 2019). Direct and indirect effects of CCN1 in CML and ALL are depicted in Fig. 4a, b, respectively. CCN1 inhibition increases Imatinib-induced apoptosis of CML cells in vitro

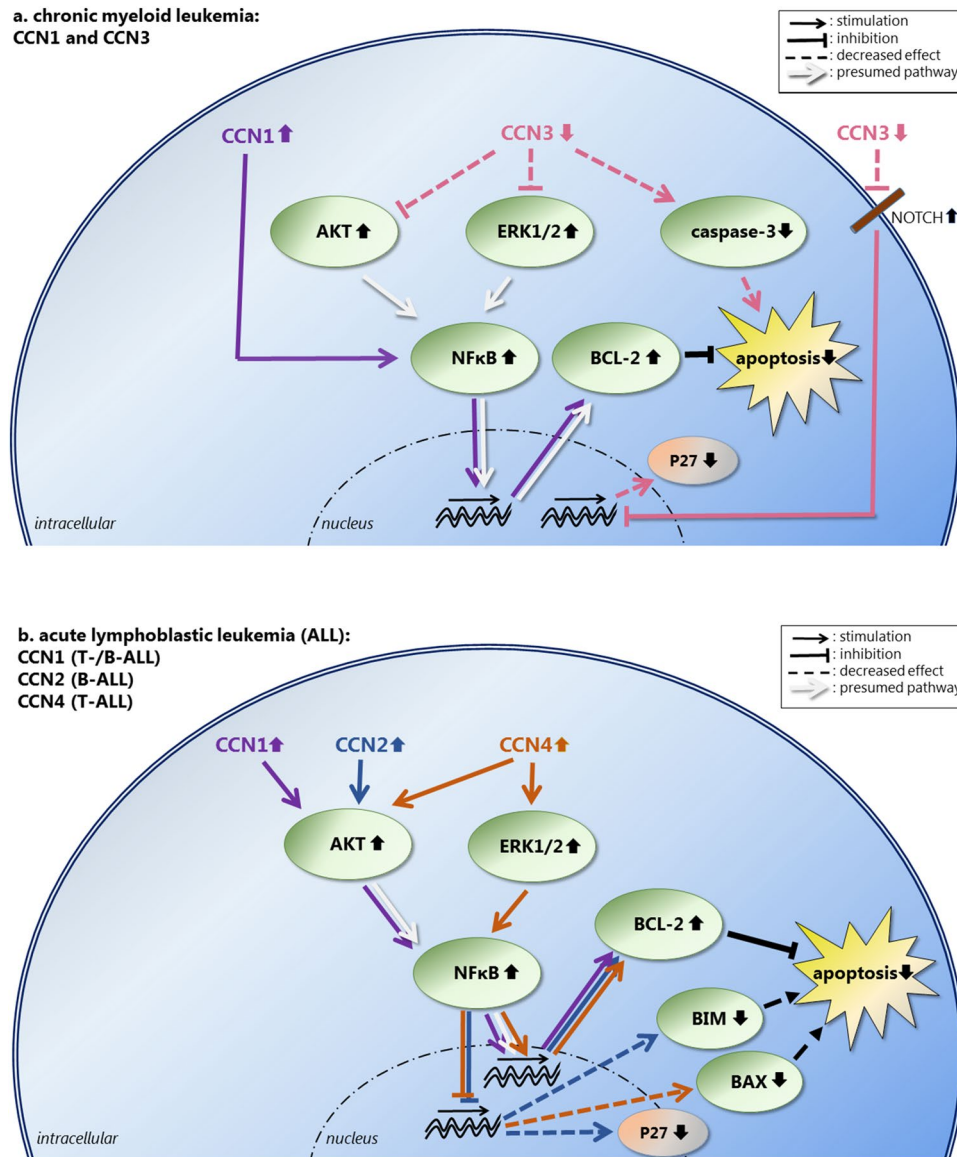


Figure 4 a Direct and indirect intracellular effects of CCN proteins in chronic myeloid leukemia (CML). CCN1 expression is increased in CML cells, inhibiting apoptosis through enhanced expression of the anti-apoptotic protein BCL2 via the NF- κ B pathway, without involvement of AKT or ERK1/2. CCN3 expression is decreased in CML cells, decreasing its inhibitory effect of CCN3 on ERK and AKT phosphorylation, resulting in elevated levels of phosphorylated ERK and AKT. This leads to less apoptosis, presumably via the NF- κ B pathway. In addition, decreased CCN3 levels result in less caspase 3 cleavage, thereby also reducing apoptosis. Furthermore, decreased CCN3 levels lead to less inhibition of NOTCH1 signaling, resulting in higher levels of NOTCH. This results in decreased expression of p27, disrupting cell cycle regulation. **b** Direct and indirect intracel-

ular effects of CCN proteins in acute lymphoblastic leukemia (ALL). The effects have been demonstrated for CCN1 in B- and T-ALL, for CCN2 in B-ALL and for CCN4 in T-ALL cells. In these cells, CCN1, CCN2 as well as CCN4 expression is increased, all inhibiting apoptosis through enhanced expression of the anti-apoptotic protein BCL2. Both CCN1 and CCN2 activate AKT, whereas CCN4 activates AKT as well as ERK1/2. Involvement of the NF- κ B pathway has been demonstrated for CCN1 and CCN4. In addition, increased CCN2 and CCN4 levels lead to decreased expression of the pro-apoptotic proteins BIM and BAX, respectively, both resulting in less apoptosis. Furthermore, increased CCN2 levels are associated with decreased p27 expression, thereby affecting cell cycle regulation

and restores the sensitivity of CML cells to Imatinib *in vivo* (Song et al. 2019). Similarly, CCN1 decreases Cytarabine chemosensitivity in ALL cells via NF- κ B pathway activation, and inhibition of CCN1 can restore ALL cell response to Cytarabine *in vitro* (Cao et al. 2019). Thus, CCN1 expression of leukemic cells seems to enhance tumor cell growth as well as drug resistance in both CML and ALL.

In AML, CCN1 expression has been demonstrated in leukemic cells as well as in stromal cells (Long et al. 2015; Niu et al. 2014). CCN1 expression in AML cells was upregulated by co-culturing them with BM stromal cells (Long et al. 2015). The amount of CCN1 expression in AML cells varies, with some BM samples and cell lines showing high expression, while others having low or no detectable expression (Niu et al. 2014). CCN1 promotes growth and survival of AML cells through the MEK/ERK pathway, with up-regulation of c-Myc and Bcl-xL, an anti-apoptotic protein of the Bcl2-family, and down-regulation of Bax, a pro-apoptotic member of the Bcl-2 family (Niu et al. 2014). The β -catenin/survivin pathway does not seem to be involved (Niu et al. 2014). Inhibition of *stromal* CCN1 partially reverses the stroma-induced resistance to mitoxantrone by increasing the mitoxantrone-induced apoptosis by AML cells (Long et al. 2015), suggesting a role for CCN1 in stroma-mediated chemoresistance. Spleen tyrosine kinase (SYK) is involved in this CCN1 signaling (Long et al. 2015). Thus, in AML, CCN1 seems to have a pro-leukemic effect in both tumor and stromal cells. CCN1 gene polymorphisms have been associated with either a lowered or increased risk of AML (Niu et al. 2014).

Increased CCN1 protein and gene expression have also been demonstrated in BM of patients with a plasma cell neoplasia (Johnson et al. 2014; Liu et al. 2017). Contradicting results, however, have been reported regarding cell type (plasma cells versus stromal cells) expressing CCN1 and its effect on tumor cell growth. Johnson et al. found high CCN1 gene expression in multiple myeloma (MM) associated BM *stromal* cells, but no CCN1 expression in cultured (normal or malignant) plasma cells (Johnson et al. 2014), whereas Dotterweich et al. *did* demonstrate CCN1 mRNA and protein in the cells of their MM cell line, which could be markedly increased by MSC contact or addition of recombinant CCN1 (Dotterweich et al. 2014). And whereas Johnson et al. showed recombinant CCN1 to inhibit growth of MM cells (Johnson et al. 2014), the viability of primary myeloma cells in the study of Dotterweich et al. increased significantly after CCN1 incubation, implying a pro-myeloma effect of CCN1 (Dotterweich et al. 2014). The contradicting results of CCN1 on MM cells might be explained by the use of different cell lines (H929 versus INA-6 MM cell line), difference between cell lines versus patient samples, and differences in experimental conditions as the effects of CCN proteins are known to be dependent on the micro-environment including the presence of certain cytokines.

Two studies imply that CCN1 has a favorable clinical effect in plasma cell neoplasms; Johnson et al. showed elevated serum CCN1 levels to be associated with a longer time to progression of monoclonal gammopathy of undetermined significance (MGUS) to overt MM and as such with a superior overall survival (Johnson et al. 2014), and Liu et al. showed increased CCN1 protein levels in the BM to be inversely associated with the severity of myeloma associated bone lesions (Liu et al. 2017). In addition, both studies confirmed in mouse models that overexpressed CCN1 in engrafted MM cells results in reduced bone disease (Johnson et al. 2014; Liu et al. 2017). This effect seems opposite to that of CCN2, showing decreased levels in MM patients with bone disease (Munemasa et al. 2007).

CCN3

CCN3 gene expression has been demonstrated in HSCs and hematopoietic progenitor cells (Bruno et al. 2004; Gupta et al. 2007; Ishihara et al. 2014; Kimura et al. 2010) as well as in MSCs (Djouad et al. 2007). In MSCs, CCN3 expression increases two- to threefold after chondrogenesis (Djouad et al. 2007). In HSCs, CCN3 is essential for self-renewal, which seems to be at least in part due to its effect on cell cycling and NOTCH signaling (Gupta et al. 2007).

Similar to the other CCN family members, expression of CCN3 is strongly influenced by cytokines from the micro-environment. Without stimulation, HSCs show low levels of CCN3, but its expression can increase over 100-fold upon stimulation (Kimura et al. 2010). Interleukin 3 (IL-3) is the key cytokine for CCN3 induction in HSCs, directly increasing CCN3 expression by inducing STAT5A/B binding to a γ -interferon-activated sequences site in the CCN3 gene promoter (Kimura et al. 2010). Furthermore, exogenous CCN3 can induce endogenous expression of CCN3 in HSCs by binding to integrin $\alpha\beta$ 3 on their cell surface, thereby affecting the long-term repopulating activity of HSCs (Ishihara et al. 2014). This process is context dependent; TPO mediates CCN3 binding to integrin $\alpha\beta$ 3, inducing CCN3 expression likely through STAT5 activation, thereby supporting the long-term repopulating activity of HSCs (Ishihara et al. 2014). IFN- γ , however, impairs this TPO-induced expression of CCN3, likely through the activation of STAT1, inhibiting the long-term repopulating activity of HSCs (Ishihara et al. 2014).

In the transplant setting, exposure of umbilical cord blood to CCN3 can enhance engraftment by increased recruitment of cells with the highest long-term HSCs function, as these are preferentially bound to CCN3 through integrin α 6 (a.k.a. CD49f) (Gupta et al. 2007).

In CML, the CCN3 gene is down-regulated and its protein expression decreased as a direct consequence of the BCR-ABL tyrosine kinase activity, mediated at least in

part by microRNAs 130a/b (McCallum et al. 2006; Suresh et al. 2011). The decreased cellular CCN3 protein content was shown to correlate with increased CCN3 secretion (McCallum et al. 2006). Treatment with Imatinib increases CCN3 expression (McCallum et al. 2006). On the other hand, overexpression of CCN3 by transfection or treatment with the recombinant protein, significantly reduces tumor cell growth by enhancing Imatinib induced apoptosis (McCallum et al. 2009, 2012). CCN3 induced reduced cell growth and enhanced apoptosis is associated with enhanced caspase 3 cleavage and reduced phosphorylation of ERK and AKT (McCallum et al. 2009, 2012). Furthermore, CCN3 upregulates the expression of β 4 integrin, which might affect the re-installation of growth control mechanisms (McCallum et al. 2012). In addition, CCN3 inhibits NOTCH1 signaling in CML, which is associated with increased expression of p27, thereby contributing to the restoration of cell cycle regulation (Suresh et al. 2013). Thus, the oncogenic BCR-ABL protein in CML enhances cell survival at least in part through its inhibitory effect on CCN3, resulting in reduced apoptosis and enhanced cell growth. These direct and indirect effects of CCN3 in CML are depicted in Fig. 4a.

A possible pathogenic role for the increased amount of secreted CCN3 needs to be determined. Furthermore, methylation of the CCN3 promoter was significantly increased in peripheral blood samples of CML patients compared with healthy controls, but this was unaffected by Imatinib treatment (Vatanmakanian et al. 2019). Whether hypermethylation of CCN3 is important in the pathophysiology in CML needs to be further investigated.

CCN4–6

CCN4, CCN5 and CCN6 expression has been demonstrated in MSCs (Djouad et al. 2007; Schutze et al. 2005). Like CCN3, CCN4 expression increases after chondrogenesis, whereas CCN6 expression decreases (Djouad et al. 2007; Schutze et al. 2005). CCN5 expression declines during adipogenic differentiation (Schutze et al. 2005).

Two studies investigated CCN4 in malignant hematopoiesis. The first describes a threefold decrease in mRNA expression of CCN4 in BM MSCs in MM compared with healthy age-matched controls, which meaning remains to be established (Corre et al. 2007). The other study shows variable and sometimes high mRNA and protein expression of CCN4 in T-ALL cell lines (Zhang et al. 2015). Knockdown of CCN4 in the cell line with the highest expression inhibited proliferation and induced apoptosis by down-regulating expression of, amongst others, p-AKT, p-ERK and Bcl-2, and upregulation of Bax, suggesting a possible pro-leukemic effect of CCN4 in T-ALL (Fig. 4b) (Zhang et al. 2015). We

found no studies on CCN5 or CCN6 with regard to normal or malignant hematopoiesis.

Summary of CCN protein family members

In summary, the collected data on CCN1-6 with respect to BM microenvironment and normal or malignant hematopoiesis show, as far as the different experimental approaches allow such a comparison, many similarities and some differences between the CCN family members. In normal BM, expression of all CCN proteins have been demonstrated in MSCs, often at high levels and declining during lineage commitment and further differentiation, whereas only CCN3 expression has been found in HSCs. CCN3 directly affects the maintenance of HSCs, while CCN2 indirectly supports it through its secretion by MSCs. In hematologic malignancies of the BM, the CCN family members are often altered in either tumor cells or in the stromal cells (Table 4), usually, but not always, displaying pro-tumor effects. Many aspects of the CCN proteins in the various hematologic malignancies, however, remain to be elucidated.

CCN2 therapeutic options

Targeting CCN2 may be a therapeutic option for diseases associated with increased CCN2 expression, malignant as well as non-malignant. CCN2 can be inhibited by:

1. The use of an anti-CCN2 antibody (Aikawa et al. 2006; Alapati et al. 2011; Barbe et al. 2020a, b; Bickelhaupt et al. 2017; Dornhofer et al. 2006; Finger et al. 2014; Makino et al. 2017; Moran-Jones et al. 2015; Neeße et al. 2013; Ohara et al. 2018; Raghu et al. 2016; Richeldi et al. 2020; Sakai et al. 2017),
2. Gene expression silencing by antisense oligonucleotides (ASOs) or small interfering RNAs (siRNAs) (Chen et al. 2014; Gale et al. 2018; Gibson et al. 2017; Jensen et al. 2018; Kang et al. 2020; Li et al. 2006; Okada et al. 2005; Sisco et al. 2008; Sung et al. 2013; Yokoi et al. 2004; Yoon et al. 2016),
3. Drugs that indirectly (and less specifically) inhibit CCN2 expression, for example by targeting Sirtuin 1 (Sirt1) (Ren et al. 2017), peroxisome proliferator-activated receptor gamma (PPAR γ) (Sun et al. 2006; Zhao et al. 2006), the CCN2 transcriptional regulators YAP and TAZ (Ji et al. 2018), or proteins involved in signaling pathways affecting CCN2 transcription such FAK (Peidl et al. 2019),
4. The use of CCN3, as it can antagonize the effects of CCN2 (Peidl et al. 2019; Riser et al. 2009, 2010, 2014).

Of the above, only the use of an anti-CCN2 antibody and the use of ASOs and siRNAs have made it to clinical trials for their direct effect on CCN2 (Gale et al. 2018; Jensen et al. 2018; <https://www.prnewswire.com/news-releases/rxi-pharmaceuticals-announces-positive-results-from-phase-12-trial-with-rxi-109-for-retinal-scarring-300690078.html>; <http://www.sciencedirect.com/science/article/pii/S0190962214008147>). The PPAR γ agonist Rosiglitazone is an FDA approved drug for the treatment of type 2 diabetes mellitus and has anti-fibrotic effects with in vitro and in vivo reduction of CCN2 expression in different organs (Gao et al. 2007; Guo et al. 2012; Ihm et al. 2010; Jeon et al. 2015). The FAK inhibitor GSK2256098 is and has been tested in phase 1 and 2 studies for pulmonary hypertension and solid tumors, respectively (<http://www.clinicaltrials.gov>), whereas the FAK inhibitor PF573228 blocks CCN2 expression in vitro (Peidl et al. 2019). Several Sirt1 activators have been tested in clinical trials as reviewed by Dai et al. (2018), although their suppressive effects on CCN2 expression have only been demonstrated in animal models (Ren et al. 2017). The Hippo pathway is important in oncogenesis and many currently used drugs restrict YAP/TAZ activities, whereas several novel YAP/TAZ inhibitors are under development, as is reviewed by Pobbati and Hong (2020). The use of CCN3 as a counter-regulator and a potential therapeutic agent has thus far been tested in experimental models (Riser et al. 2014).

The only therapeutic option for targeting CCN2 that has been studied in BM diseases, is the use of the humanized monoclonal anti-CCN2 antibody FG-3019 (Pamrevlumab), which, in combination with conventional chemotherapy, significantly prolonged the survival of mice injected with a primary xenograft of B-ALL cells (Lu et al. 2014). Pamrevlumab showed a very favorable clinical safety profile in a phase 2 study of idiopathic pulmonary fibrosis (Raghu et al. 2016; Richeldi et al. 2020). Two other phase 2 studies, on Pamrevlumab in Duchenne muscular dystrophy [<https://clinicaltrials.gov/ct2/show/NCT02606136>] and on Pamrevlumab in hospitalized patients with acute COVID-19 disease [<https://clinicaltrials.gov/show/NCT04432298>], are ongoing. Furthermore, three phase 3 studies, including one on locally advanced pancreatic cancer, have started [<https://clinicaltrials.gov/ct2/show/NCT03955146>; <https://clinicaltrials.gov/ct2/show/NCT04371666> and <https://clinicaltrials.gov/ct2/show/NCT03941093>], while a fourth one is announced [<https://clinicaltrials.gov/ct2/show/NCT04419558>]. Pamrevlumab has neither been tested in clinical trials on ALL, nor in other BM diseases.

Concluding remarks

The BM microenvironment is a complex and not fully unraveled milieu made up of many different cell populations, structural matrix proteins and soluble factors communicating

with each other and forming different niches essential for normal hematopoiesis. It is subject to modulation by many different factors. One of these is CCN2, a matricellular protein with a wide variety of functions, known to be important in ECM for both the production of ECM proteins and the coordination of signaling pathways. CCN2 is produced by MSCs and other mesenchymal cells of the BM. It is involved in many different aspects of MSC biology, including proliferation, migration and differentiation. In addition, it plays a role in normal B-cell development and has been implicated in the maintenance and longtime survival of HSCs. Furthermore, CCN2 is shown to be overexpressed in leukemic cells of B-ALL, the most studied BM disease in this regard, in which it is associated with reduced overall survival. In AML samples, increased CCN2 expression is demonstrated in MSCs, which also affects leukemic engraftment, but in a way that still needs to be determined.

All other CCN family members are expressed in MSCs as well, whereas only CCN3 expression has been demonstrated in HSCs. Except for CCN5 and CCN6, which have not been studied in this context, all CCN protein family members have been associated with hematologic malignancies and often, but not always, their expression is increased in either tumor cells or in stromal cells, mostly displaying pro-tumor effects.

With this review, the authors hope to increase awareness of the CCN proteins, especially CCN2, as important players in the BM and as attractive subject for further studies on the BM microenvironment and BM diseases. These studies should include the use of anti-CCN therapies for their effect in neoplastic diseases such as ALL, but also for their possible disease modifying activities, for example on BM fibrosis.

Compliance with ethical standards

Conflict of interest The authors report no conflict of interest.

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