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REVIEW

The role of the CCN family of proteins in female reproduction

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Abstract The CCN family of proteins consists of six high homologous matricellular proteins which act predominantly by binding to heparin sulphate proteoglycan and a variety of integrins. Interestingly, CCN proteins are regulated by ovarian steroid hormones and are able to adapt to changes in oxygen concentration, which is a necessary condition for successful implantation. CCN1 is involved in processes of angiogenesis within reproductive systems, thereby potentially contributing to diseases such as endometriosis and disturbed angiogenesis in the placenta and fetus. In the ovary, CCN2 is the key factor for follicular development, ovulation and corpora luteal luteolysis, and its deletion leads to fertility defects. CCN1, CCN2 and CCN3 seem to be regulators for human trophoblast proliferation and migration, but with CCN2 acting as a counterweight. Alterations in the expression of these three proteins could contribute to the shallow invasion properties observed in preeclampsia. Little is known about the role of CCN4–6 in the reproductive organs. The ability of CCN1, CCN2 and CCN3 to interact with numerous receptors enables them to adapt their biological function rapidly to the continuous remodelling of the reproductive organs and in the development of the placenta. The CCN proteins mediate their specific cell physiological function through the receptor type of their binding partner followed by a defined signalling cascade. Because of their partly overlapping expression patterns, they could act in a concert synergistically or in an opposite way within the reproductive organs. Imbalances in their expression levels are correlated to different human reproductive diseases, such as endometriosis and preeclampsia.

Keywords CCN1 (CYR61) · CCN2 (CTGF) · CCN3 (NOV) · Ovary · Uterus · Placenta

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Introduction

Reproduction and the maintenance of pregnancy are crucial for the survival of every species. However, each step in the process of mammalian reproduction is susceptible to functional failures and is therefore relatively inefficient. Each of the various female reproductive organs, such as the ovaries, the fallopian tubes and uterus, must fulfill its own specific functions, such as ovulation, fertilisation, support of preimplantation of embryos, establishment of uterine receptivity and placental development and initiation of labour, via a distinct individual programme. Logically, a positive foetal outcome requires the coordination of all of these functions in a highly organised temporal and spatial manner and their tight interaction within one organ or across all reproductive organs.

Studies carried out in the recent years of the genomic era have revealed that numerous genes contribute to the proper functioning of the various reproductive organs and reproductive events. These genes specifically or ubiquitously express unique shared and redundant functions in the organs of the female reproductive tract. However, how these molecules interact and the scaffold arrangements, subcellular localisation and concentrations that are necessary for the development of a communication network that sets up a fully functional process are still completely unknown and pose a great challenge to future researchers.

During the last few years, studies on the phenotypes of various CCN protein knockout mice characterised by reproductive failures has focussed the attention of researchers on the roles played by this protein family. This had led to demonstration of the importance of members of the CCN family of matricellular proteins in various biological functions within a wide range of tissues. Matricellular proteins are a group of structurally diverse, extracellular matrix (ECM)-associated glycoproteins that play regulatory roles in cell–cell and cell–matrix interactions [1]. Because all of these CCN proteins use surface molecules to bridge the extracellular components, they form integrated constructions and scaffolds that serve to place the various actor molecules in the correct location for an appropriate cell physiological response [2–4].

The CCN genes are a small family with only six members. The name CCN is an acronym of the first three members to be discovered: cysteine-rich 61 (Cyr61, CCN1), connective tissue growth factor (CTGF, CCN2) and nephroblastoma overexpressed (NOV, CCN3) [5–7]. The subsequent identification of three additional members, namely, the Wnt-induced secreted proteins (WISP-1, -2 and -3, or CCN4, CCN5 and CCN6, respectively), completed the family as known today [8]. All CCN proteins are formed as multi-modular mosaic proteins with four conserved discrete modules, resulting in a similar (40–60 %) amino acid sequence (see Fig. 1) [9]. The four domains (modules) share homology with other extracellular domains of unrelated proteins. Module one is highly homologous to the insulin-like growth factor binding protein (IGFBP) domain module 2 resembles the von Willebrand factor type C repeat module (vWC), module 3 is the thrombospondin type-1 repeat (TSP1) and module four is the cysteine knot-containing module (CT), which is absent from the CCN5 gene [10]. All CCN proteins are characterised by an N-terminal secretory signal for extracellular release (for review see [11]). Module 1 contains high homology to theIGFBP, but IGF affinity is 100-fold lower than that for the full-length IGFBPs [11, 12]. As very little information is currently available on the exact role of the IGFBP domain in CCN function, this subject is open to considerable debate [12]. Zhang et al. [13] found evidence that the loss of the IGFBP module of CCN6, and to a lesser extent that of CCN4, promotes the response of mammary epithelial cells to the growth effects of IGF-1, thus indicating that the IGFBP is involved in tumourigenesis. The exact mode of the interaction between IGF and the CCN binding domain to limit tumour growth is unknown.

The vWC repeat module plays a major role in regulating the members of the small growth factor family of proteins, such as the bone morphogenic proteins (BMPs; e.g. BMP2, BMP4), transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF) and placenta growth factor (PLGF) (Fig. 1) [11, 12]. Because of these interactions, the CCN proteins mediate a variety of key functions that include TGF-β-mediated adhesion and tissue remodelling, induction of angiogenesis [2] and chondrogenic and skeletal development [14–16]. In addition to the multifunctional properties bestowed through this module, the different CCNs show diverse biological functions based on differences in substrate binding or binding capacity to the substrates of the vWC domain. Due to the capability of the vWC repeat model to interact with the different growth factors, this domain is believed to modulate the ECM components via binding to TGF-β1 [17] and—similar to the IGBP module—to be involved in tumourigenesis by regulating available amounts of growth factors [18].

In most CCN proteins the TSP1 domain plays a major role in cell attachment. The amino acid residue of this module is involved in the binding of CCNs to integrins (Fig. 1). Furthermore, the TSP domain is an important component of the angiogenic potential of the CCNs. In CCN proteins, both the TSP domain and the CT domain interact with VEGF, which is one of the key angiogenic growth factors (Fig. 1: CCN1, CCN2) [19]. Module 4 is a C-terminal domain that contains a cysteine knot (CT) [20]. Interactions between the CT domain and heparin, fibulin 1C [21] and several integrins, such as αvβ5, αvβ3, αMβ2 and α5β1, have been observed (Fig. 1); these are probably properties which are all needed for cell adhesion as it has been evidenced by constructs containing only the CT domain [22]. A further role of the CT domain, as shown for CCN3, is to regulate cell differentiation of mesenchymal cells and to suppress myogenesis by binding with Notch-1 at the CT domain [23]. Thus, each functional domain plays its distinct biological role by interacting with a variety of binding partners, and this interaction enables each domain to act not only individually but also in concert with one another (Fig. 1).

As described above, the binding partners for vWC, TSP1 and CT are predominantly integrins, but the interaction properties of the various integrin subtypes differ across the domains, and CT also binds heparin sulphate proteoglycan (HSPG) [9]. Through their interaction with the integrins, the CCNs can modulate the ECM and thereby mediate multifunctional cell physiological effects, which have been described to affect cell adhesion, migration and survival in various cell types, such as fibroblasts, endothelial cells, myoblasts, chondroblasts, osteoblasts and stem cells, and in a variety of tumour cells [24].

Since distinct integrins are differentially expressed in various cell types, CCN proteins can achieve functional variety in a cell type- and context-specific manner (reviewed in [25]). CCN1, CCN2 and CCN3 bind to specific integrins through distinct domains that have been mapped in peptide inhibition studies [26–31].

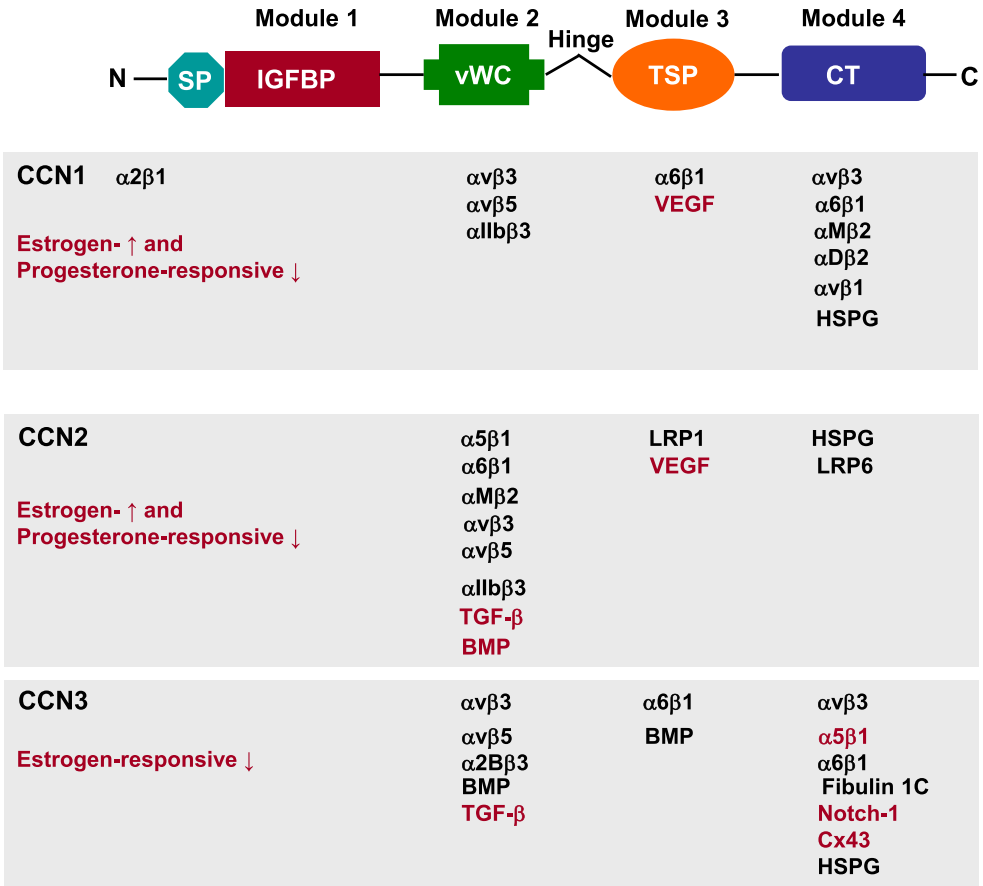

Fig. 1 Molecular interactions of CCN proteins (CCN1–3) via their molecular domains. The structure of CCN4 and CCN6 is the same as that of CCN1–3. CCN5 lacks the cysteine knot-containing (*CT*) domain. CCN5 is also known to be estrogen sensitive. The direction of estrogen- and progesterone-responsiveness of CCN proteins is marked by *arrows*. CCN4 and CCN5 bind biglycan and CCN4, in addition to transforming growth factor beta (*TGF-β*). Nothing is known about the binding properties of CCN6. The interaction partners of CCN1–3 and their known functions in reproductive organs are marked in *red*. *IGFBP* Insulin-like growth factor binding protein domain, *vWC* von Willebrand factor type C domain, *TSP1* thrombospondin type-1 repeat domain

CCN1 and CCN2 have been found to regulate angiogenesis through integrins αvβ3 and α6β1 [2]. CCN1 also interacts with HSPGs in combination with α6β1 to regulate adhesion and migration in smooth muscle cells [28]. CCN proteins can profoundly modify the activities of tumour necrosis factor alpha (TNF-α), which functions primarily to regulate inflammation and immunity, converting the latter from a proliferation-enhancing factor into a potent apoptotic agent [32]. Chen et al. [33] showed that the presence of CCN1, CCN2 or CCN3 can unmask the cytotoxicity of TNF-α without perturbing NF-κB signalling or inhibiting de novo protein synthesis; this unmasking leads to rapid apoptosis in otherwise resistant primary human fibroblasts. Juric et al. [34] revealed that the binding of CCN1 to integrins αvβ5, α6β1 and to cell surface HSPG4, is the key process to unmask the cytotoxicity of TNF-α and induce high levels of reactive oxygen species, which in turn activates JNK kinase as a precondition for apoptosis. Furthermore, CCN3 promotes adhesion through integrins αvβ3, α5β1, α6β1 and HSPGs, whereas cell migration is stimulated through integrins αvβ3 and α5β1 in the endothelial cells of human umbilical veins [29]. In general, integrins are essential for the adhesive, mitogenic and angiogenic function of CCN proteins and should be properly considered to be the functional receptors for this family [35–37].

There is a close relationship between CCNs and the Wnt signalling pathway. CCN1, CCN2, CCN4, CCN5 and CCN6 are upregulated by the Wnt/β-catenin pathway. This interaction regulates several differentiation pathways in embryogenesis and differentiation [38–40]. Direct interaction between CCN2 and the co-receptor of Wnt, LRP-6, is mediated by the CT domain and thus changes Wnt signalling [41].

This introduction to the CCN proteins provides only a rough overview of this multitasking matricellular protein family. A discussion of the full biochemical and physiological impact of the CCN proteins is beyond the scope

of this review, which will focus on the known functions of these proteins in the female reproductive organs. It has to be considered that in reproductive tissues, expression of CCN proteins as well as of their interaction partners differ among organs and different cell types within a tissue. Moreover, this pattern often changes in in vitro systems or in the tumour cell lines derived from reproductive tissues which are often taken as models. Altogether, this makes it difficult to point out the general functions of the CCN proteins.

Table 1 provides an overview of the expression and function of CCN proteins in reproductive organs and reproductive diseases. For the appropriate reorganisation and transformation of tissues, actions which are necessary during reproductive events, these modular proteins must successfully interact with various ligands. Such interactions are an important prerequisite for the fulfillment of multiple functions in a highly organised temporal and spatial manner.

CCN and the ovary

Ovarian follicular development is closely related to extensive remodelling of the extracellular matrix and to angiogenesis. In this context, CCN2 (CTGF) has been intensively investigated for its role in the growth and differentiation of granulosa cells and in the formation of the corpus luteum.

Like the other members of the CCN family, CCN2 is believed to play a role in such diverse processes as cellular proliferation, differentiation, migration, apoptosis and extracellular matrix remodelling, processes that are indispensable for follicular development and luteinisation [17]. Fundamental investigations have been performed by the group of Hillier, who found that CCN2 is continuously upregulated during follicular growth and that the highest amounts are found in the cumulus oophorus cells. This upregulation of CCN2 appears to be under the control of estrogen, but this stimulatory effect is negatively influenced by follicle stimulating hormone (FSH); thus, the FSH level is reduced in preovulatory follicles to support follicle maturation [42–44]. Similar results have been found in preantral follicles and in pigs, in which CCN2 expression in granulosa cells is downregulated by FSH during follicular development [45].

CCN2 is expressed in the granulosa lutein cells of rodents, pigs and humans [46, 47]. In these cells, human chorionic gonadotropin (hCG) has been shown to downregulate CCN2 [47], a finding that was confirmed by the observations of Duncan et al. [48]. These studies showed that CCN2 is less involved in luteinisation than in luteolysis.

The breakthrough in research on the role of CCN2 in follicular development came from studies of CCN2 ovarian and uterine conditional knockout mice; these studies used ovarian- and uterine-specific Cre recombinase under the control of the anti-Mullerian hormone or progesterone receptor [49]. Both types of CCN2 conditional knockout mice exhibited a severe reduction in fertility because of multiple reproductive defects, including disrupted follicle development, decreased ovulation rates, increased numbers of corpora lutea and smaller but functionally normal uterine horns. In the follicle, CCN2 seems to interfere with the cell cycle, based on observations that a decrease in the proliferation of CCN2-deficient granulosa cells is combined with the downregulation of the cell cycle regulators Myc and CCND1; this downregulation could explain the increase in apoptosis of granulosa cells, which disrupts the development of follicles [49]. The observed reduction in fertility was caused not only by impairments in the process of follicular development and ovulation but also by the persistence of corpora lutea, which led to higher progesterone levels. These latter findings confirm the former observation by Duncan et al. [48] and by Phan et al. [47] that CCN2 is important for luteolysis.

Another important function of CCN2 in the rat ovary is the regulation of the development of primordial follicles. In newborn rats, primordial follicles are formed from oocyte nests, and the size of this initial pool determines the reproductive lifespan. It is likely that CCN2 can, both alone and in combination with TGF-β1, induce the assembly of ovarian primordial follicles [50].

Both CCN1 (CYR61) in the cow [51] and CCN2 in the pig [45] have been shown to be expressed in the corpus luteum, and their upregulation is correlated with the angiogenic switch of the corpus luteum. These findings support the conclusion that, as with other organ systems, both of these CCN family members are responsible for driving angiogenesis in the reproductive system [52].

In summary, these findings suggest that in the ovary CCN2 is an important multifunctional regulator of oocyte nest formation in primordial oocytes, of appropriate follicular development and of angiogenesis of the corpus luteum, as well as of luteolysis.

To date, except for CCN2, little is known about the expression pattern, regulation and function of CCN family members in the ovary. Further investigation is needed to determine their possible interaction in a synergistic or opposite role in follicular development, ovulation, luteinisation (especially angiogenesis) and luteolysis.

CCN in the endometrium, in endometriosis and in implantation

The uterus is a tissue with a great transforming capacity related to its reproductive function, especially with regard to the endometrium of species with a menstrual cycle. The

Table 1 Expression and function of CCN proteins in reproductive organs and human reproductive diseases

Reproductive organ	Expression and function of CCN proteins	Human reproductive disease	References
Ovary	CCN1: -upregulated in corpus luteum Function: increase of angiogenesis in corpus luteum CCN2: -expressed in granulosa cells, theca internal -upregulated during follicular growth by estrogen -CCN2 ovarian and uterine conditional KO mice: severe reduction in fertility Function: key factor in follicular development, ovulation and corpora lutea luteolysis CCN5: Function: role in oogenesis?	Unknown	Cow [51] Human [46–48] Mouse [49, 92] Pig [45] Rat [42–44]
Uterus	Endometrium: CCN1: -upregulated during proliferative phase and premenstrual period, upregulated by estrogen and low oxygen, downregulated by progesterone -upregulated in the uterine epithelium of the implantation chamber CCN1-KO mice: no implantation failure Function: proangiogenic CCN2: -expressed throughout cycle in human and mouse epithelium and endothelium -upregulation by estrogen, downregulation by progesterone -upregulated in the stroma in pregnancy -uterine CCN2-KO mice: no implantation failure Function: stromal remodeling and neovascularisation in early pregnancy CCN5: -expressed throughout cycle in mouse endometrium -upregulation by estrogen, downregulation by progesterone Function: unknown	Endometriosis: CCN1 is upregulated in endometriosis and in endometriotic lesions PCOS: Overexpression of CCN1 in the endometrium	Baboon [61] Human [55, 62, 64, 65, 69, 70, 79] Mouse [41, 49, 65] Rat [69]
	Myometrium: CCN1: -expressed in myometrial cells, upregulated by estrogen CCN5: -expressed in myometrium Function: growth inhibition	Leiomyomatas: CCN1, CCN2, CCN3, CCN4, CCN5 are down-regulated, no estrogen-responsiveness	

Table 1 continued

Reproductive organ	Expression and function of CCN proteins	Human reproductive disease	References
Placenta	CCN1 and CCN3: -expressed in EVT cells, in endothelial cells of placental vessels and in stromal cells -expression is increased during pregnancy -CCN1-KO mice: embryonic death in utero due to an impaired embryonic and placental angiogenesis -CCN1 and CCN3 are upregulated upon hypoxia via HIF-1α in trophoblast cells and first trimester placental explants Function: key regulatory molecule of the EVT cells by controlling proliferation and enhancing migration CCN2: -expressed in cyto- and syncytiotrophoblast cells, in extravillous trophoblast cells and in endothelial cells of third trimester placenta -expression is enhanced upon hypoxia in human term trophoblast cells Function: counterweight for CCN1/3 in human trophoblast differentiation?	Preeclampsia: CCN1 and CCN3 are decreased in placentas and sera of early-onset preeclamptic patients CCN2 is increased in maternal and fetal serum of women with severe preeclampsia and fetal growth restriction	Human [68, 83, 84, 88–91, 93] Mouse [74]

PCOS Polycystic ovary syndrome, *KO* knockout, *EVT* extravillous trophoblast, *HIF-1α* hypoxia-inducible factor 1-alpha

endometrial tissue constantly undergoes distinct hormonally regulated morphological alterations, and this transforming capacity is necessary for achieving the endometrial receptivity that allows implantation of an embryo and placentation. Impaired uterine receptivity concomitant with a failure in the decidualisation properties of the endometrial stroma is one of the main reasons for failure of implantation [53, 54].

In this context, researchers have investigated the role of the CCN family in this remodelling process that leads to the achievement of endometrial receptivity for early implantation and to the differentiation of the endometrial stroma into the decidua during peri-implantation and postimplantation.

CCN2 in the endometrium

One of the first CCN members detected in the uterus was the connective tissue growth factor CCN2. Uzumcu et al. [55] reported that CCN2 is strongly expressed in the human uterine epithelium and in the endothelium throughout the cycle, but not in the stromal compartment during the proliferative phase. In the late secretory phase, however, the induction of CCN2 shifts to decidualised areas and is found in the decidua of pregnant women. At least in human endometrial cell lines, prokineticin-1 (PROK1) seems to elevate CCN2 levels by activating phospholipase C, c-Src, epidermal growth factor receptor (EGFR) and finally the MAPK/ERK kinase pathway [56].

As in humans, in mice, CCN2 expression is predominantly found in the uterine epithelium during the very early stages of pseudopregnancy, but it shifts to the stromal compartment at approximately day 4 of pseudopregnancy [57]. Using various types of hormonal stimulation in ovarectomised mice, researchers found that the upregulation of CCN2 by estrogen is antagonised by progesterone. The same group found identical results in pigs: in the pig uterus, CCN2 is expressed predominantly in the uterine luminal epithelium during cycling but is enhanced in the stromal compartment during the initial phase of pregnancy [58]. The bovine endometrium is also characterised by an increase of CCN2 in the epithelial compartments in later stages of the cycle and in the stroma during pregnancy; however, in this species CCN2 expression seems to be under the control of progesterone [59].

All of these findings ascribe to CCN2 the function of stromal remodelling and neovascularisation during early pregnancy. In CCN2-knockout mice, however, the uterine tissues undergo no obvious changes, and their function appears to be normal because the endometrium responds to artificial decidualisation [49]. It needs to be clarified if this is due to the functional redundancy of CCN1 and CCN3 found also expressed in mouse endometrium.

CCN1 in endometriosis

The uterine disease endometriosis is a frequently encountered benign disease in women of reproductive age. It occurs when the menstrual endometrium implants on the peritoneum at various pelvic locations and forms small ectopic endometrial lesions. These lesions lead to chronic pelvic pain and dysmenorrhea and are related to subfertility [60]. They also cause inflammatory reactions that in turn can interfere with the programming of the endometrial differentiation process [61].

Our group has studied the role of CCN1 in normal human endometrial differentiation by performing gene array analysis of the endometrium of healthy women and of women with endometriosis. We found that CCN1, which is expressed in the uterine epithelium as well as in the endometrial endothelial cells, is aberrantly upregulated in the endometrium of women with endometriosis during the secretory phase; this upregulation is probably related to an increase in estrogen responsiveness [62]. The estrogen-responsiveness of CCN1 was demonstrated by elevated expression during the proliferation phase in normal endometrium and by its regulation of anti-estrogens in ectopic endometrial lesions. Using normal endometrium, we demonstrated that, like VEGF [63], CCN1 is upregulated not only during the proliferative phase but also during the premenstrual period, a finding suggesting the proangiogenic function of this molecule. Experiments using the benign endometrial epithelial cell line HES showed that CCN1 is upregulated by hypoxia-inducible factor (HIF)1-α and is enhanced synergistically with HIF1-α by various cytokines and prostaglandins, predominantly prostaglandin E2 [64]. All of these findings suggest that CCN1 plays a role in endometrial angiogenesis. MacLaughlan et al. [65] made similar observations about the expression level of CCN1 during the menstrual cycle. In addition, they found that CCN1 is overexpressed in the endometrium of women with polycystic ovary syndrome (PCOS), which is characterised by a heightened responsiveness to estrogen. Additional evidence has been obtained from investigations of endometriosis in a baboon model, in which the increase in expression of endometrial CCN1 indicated the presence of induced endometriotic lesions in a healthy animal [61]. Taken together, this evidence verifies the hypothesis of a loop model: healthy endometrium becomes diseased upon the presence of lesions with elevated CCN1 expression, which is a reliable marker for this programme change [65]. Moreover, in the ectopic lesions we found a clear relationship between elevated CCN1 expression and higher blood perfusion and increased expression of VEGF in these lesions [61, 64]. Thus, we surmise that CCN1 is predominantly involved in endometrial angiogenic processes that are stimulated by estrogen and factors involved in the inflammatory cascade [66]. It is not known whether CCN2 has any impact on endometriosis; Meola et al. [67] found no significant differences in gene expression profiles of CCN2 between ectopic and eutopic endometrium.

The overlapping expression pattern of CCN1 and CCN2 in uterine tissues in several species raises the question of whether the two proteins could have compensating functions. This hypothesis will require testing in tissue-specific double-knockout mice.

CCN3 and CCN5 in the endometrium

To date, no published studies have reported CCN3 expression in the uterus. Our studies show that CCN3 transcript expression in human and mouse endometrial tissue is comparable to the high expression levels of CCN3 in the human placenta at term (unpublished results; Gellhaus et al. [68]).

Using ovariectomised rats, Mason et al. [69] investigated the distribution and expression of CCN5 during cycling and pregnancy and its hormonal regulation properties. Unlike that of the other CCN members, CCN5 expression was found throughout the endometrium and in the myometrium. Like that of CCN2, CCN5 expression was higher during the estrogen-dominated phase, strongly enhanced in ovariectomised mice after stimulation with estrogen and lower upon substitution of progesterone. Therefore, all three members of the CCN family seem to exert positive regulatory properties on high estrogen levels.

The responsiveness to ovarian steroid hormones could be mediated by their receptor binding properties in the CCN promoters. The CCN1 promoter has an estrogen-responsive elements at positions −1195 and −1219 and a progesterone receptor binding element at position −1592 to −1617 [70]. Studies using the MCF-7 cell line as a breast cancer model found that CCN3 was a direct target of estrogen. Indeed, CCN3 expression was repressed in MCF-7 cells after treatment with estrogen [71]. However, nothing is known about progesterone-responsive elements in the CCN3 promoter.

CCN in implantation

Successful implantation requires not only an endometrium appropriately transformed by ovarian steroid hormones but also a competent preimplantation embryo for precise cross-talk with the endometrium. This cross-talk includes several signalling molecules and brings about the local programme change in the endometrium that is required for the subsequent cell biological steps [72]: the blastocyst adheres and subsequently attached to the apical membrane of the uterine epithelium and then invades into the maternal compartment.

Among those genes induced in the endometrium upon blastocyst adhesion, CCN1 was found to be upregulated in the uterine epithelium of the implantation chamber; this upregulation could serve as a mediator for embryo implantation [73]. However, although ablation of the CCN1 gene in mice leads to problems in placental development (see below) it does not disturb implantation [74]. Targeted disruption of the CCN3 gene by deletion of exon 3 also seems not to impair implantation properties [75]. To the best of our knowledge, no other CCN family member has been evaluated for its role in implantation.

CCN in the myometrium and in the myometrial disease leiomyomata

Although several studies have investigated the role of CCN members in smooth muscle cell physiology [76–78], little is known about the role of CCN members in myometrial function, especially at term and at parturition; however, there is evidence that they play a role in the development and sustaining of leiomyomata. CCN1 has been shown to be highly expressed in myometrial cells of the human uterus throughout the cycle and is upregulated by estrogen. Interestingly, in leiomyomata, CCN1 expression is lower than in matched control cells and is not responsive to estrogen or basic fibroblast growth factor (bFGF) [70]. This phenomenon has been confirmed in members of the CCN family, such as CCN2, CCN3 and CCN4. All of these members are expressed in the myometrium, but their expression is lower in the corresponding leiomyomata [79]. Similar observations have been made with CCN5 [80]. CCN5 is completely downregulated in leiomyoma tissues, unlike normal human myometrium. Moreover, there is a strong reduction in proliferation among human myometrial and leiomyoma smooth muscle cells that overexpress CCN5. Thus, CCN5 may exert an important function in maintaining normally low-proliferating myometrial cells, and the loss of the anti-proliferative protein CCN5 may account for the pathogenesis of leiomyomata.

Taken together, the reduction of expression of all investigated CCN members in leiomyomata could contribute to the sustaining growth of these smooth muscle cells. Therefore, in this reproductive system CCN proteins seem to be involved in growth control but not in myometrial tissue remodelling.

CCN in placental development and disease

CCN in the mouse placenta

Implantation is followed by the development of the first embryonic organ, the placenta, to establish a route by which the embryo can receive nutrition from the mother. Among other cell biological events, the formation of a functional placenta includes the invasion of trophoblast cells into the maternal blood vessels and the development of an embryonic vascular system. As described above, the CCN proteins seem to be required for all of these cell biological processes. Thus, it is not surprising that Mo et al. [74] observed impairment of placental development and vessel integrity upon disruption of the CCN1 gene. In their study, approximately 30 % of CCN1-deficient mice exhibited a defect in the chorioallantoic fusion process and died around embryonic day (ED) 9.5. However, those embryos that did progress in terms of placental development exhibited impaired embryonic vascular development with a loss in vascular branching properties. As a consequence, the labyrinthine compartment of the placenta was not sufficiently vascularised, and the embryos died in utero from ED 10.5 onwards. Moreover, the vascular integrity of the entire embryo was disturbed, as demonstrated predominantly by leaky arteries that resulted in embryonic haemorrhages and oedema. Interestingly, these authors found that the loss of CCN1 was associated with a reduction in the expression of VEGF-C in the allantoic mesoderm. This phenomenon could account for the impairment of vessel morphogenesis [74]. No alterations in trophoblast migration into the decidua or into maternal spiral arteries were reported.

The involvement of other members of the CCN family in mouse placental development has not yet been determined. Although CCN2, similar to the other CCN members, is involved in processes such as angiogenesis, CCN2-deficient mice die directly after birth because of a respiratory defect but they are born at the expected Mendelian rate, a finding that indicates a functional intact placenta [81]. During early pregnancy in pigs, the amount of CCN2 combined with TGFβ increases in the placental membranes during early pregnancy; however, no functional analysis has yet been performed [58].

CCN in the human placenta and in the placental disease preeclampsia

Most studies of the CCN members focus on their expression, regulation and function in the human placenta.

We became interested in the role of CCNs, predominantly CCN3, during our investigations on the importance of Connexin (Cx) 43 for the control of proliferation in a human malignant trophoblast cell line, Jeg3. We found that induction of the gap junction protein Cx43 led to an upregulation of CCN3 [82] and that this upregulation was responsible for the growth reduction observed in these Cx43-transfected cells. In studying the role of CCN3 in the human placenta and in placental diseases, such as

preeclampsia, we found that CCN3 has a defined expression pattern during placental development but that this pattern is deregulated in early-onset preeclampsia [68]. The pathogenesis of this placental disease is ascribed to shallow invasion by the extravillous trophoblast, which results in immature remodelling of the vessel walls of maternal spiral arteries. Both CCN1 and CCN3 are highly upregulated in placental tissues during pregnancy, and their localisation is restricted to embryonic endothelial cells and extravillous trophoblast cells (Fig. 2). The hormonal regulation of CCN protein expression by estrogen, progesterone and hCG during placental development is still elusive. Recent studies have revealed that there is no difference in CCN1 and CCN3 protein levels between the serum of pregnant women with high levels of progesterone and hCG and non-pregnant women [83]. This is surprising since it is known that the levels of the CCN1–3 proteins increase with higher estrogen levels in the endometrium, that CCN1 and CCN3 exhibit estrogen-responsive elements and that CCN1 is additionally a progesterone receptor binding element. This non-responsiveness needs further investigation.

The expression of both CCN1 and CCN3 is significantly decreased in early preeclamptic placentas and in the sera of patients with early-onset preeclampsia [68, 83], a phenomenon that could contribute to the failure of trophoblast invasion and the resultant impairment in uterine vascular remodelling.

To clarify which molecular mechanisms are involved in the reduction of CCN levels in preeclampsia, Wolf et al. [84] investigated the regulation properties of CCN1 and CCN3 at various oxygen levels. These authors observed that low physiological oxygen concentrations of 1–3 %, which represent the oxygen condition during early placentation, increased the levels of CCN1 and CCN3 proteins and that the upregulation of CCN1 and CCN3 was directly dependent on HIF1-α stabilisation and further positively modulated by TGF-β3. These findings again support the hypothesis that CCN and TGF-β3 interact as partners in determining regulatory properties in reproductive tissues.

Taken together, these results indicate that HIF1-α is a potent enhancer of CCN1 and CCN3 under conditions of low oxygen concentrations over a short time period but that these proangiogenic proteins are downregulated during a chronic hypoxic situation, such as preeclampsia. It is known that other proangiogenic factors, such as VEGF-A and PLGF, exhibit contradictory behaviour to a low oxygen environment depending on the exposure time. HIF1-α upregulates both VEGF-A and PLGF in normal placentas but the latter are downregulated in preeclamptic placentas [85–87]. Interestingly, CCN2 exhibits a similar distribution pattern in the human placenta and is enhanced by hypoxia in human term trophoblast cells and placental tissues [88]. A recent study by Chen et al. [89] supported this finding by demonstrating the overlapping expression of CCN1 and CCN2 in extravillous trophoblast and endothelial cells of

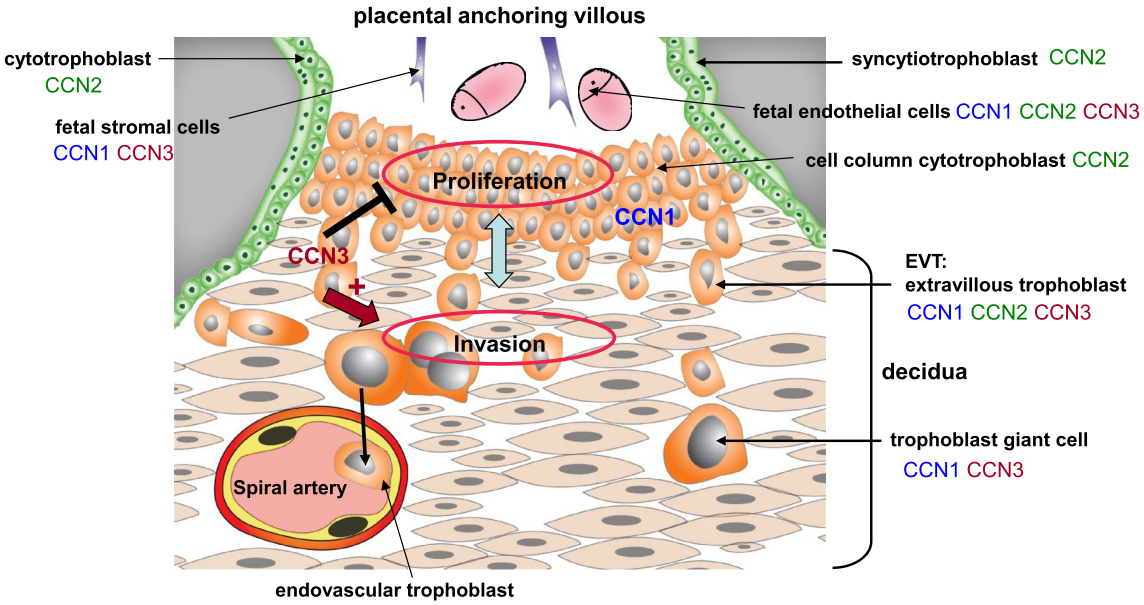

Fig. 2 Schematic overview of the expression and function of CCN1, CCN2 and CCN3 proteins in the human placenta built up by different cell types, such as trophoblast subtypes, endothelial and stromal cells. A placental anchoring villous attached to the maternal decidua is shown with the proliferating cytotrophoblast cells of the cell column. The extravillous trophoblast cells must stop proliferation and increase migration/invasion if they are to invade the spiral arteries and to transform into polyploidy giant cells. Here CCN3 is shown to be involved in this important step in trophoblast differentiation leading to reduced proliferation and at the same time to increased migration properties

human placentas. In addition, CCN2 is also expressed in the villous trophoblast cell populations composed of syncytiotrophoblast and underlying cytotrophoblast cells. In preeclamptic placentas, however, only CCN1 is downregulated, whereas CCN2 is upregulated, a finding suggesting that the two CCNs play opposite roles in placental development and preeclampsia [89].

Taken together, all of the findings from our group and from others indicate that CCN1, CCN2 and CCN3 are regulated by hypoxia and that deregulation contributes to the condition of placental disease preeclampsia. Although CCN proteins play various roles in placental function and dysfunction, all three CCN members are strongly regulated by hypoxia, even though their reactions to hypoxia differ.

One reason for the upregulation of the CCNs by hypoxia could be to provide support for trophoblast invasion into the decidua. The proximal extravillous trophoblast cells of the trophoblast columns must proliferate if they are to establish a cell pool for the invading cells. However, before trophoblast cells are released into the invasive pathway, they exit from the cell cycle to avoid acquiring tumour-like properties (Fig. 2). Several studies of CCN3 [84, 90, 91] have provided evidence that CCN3 can regulate the cessation of proliferation and can support the migration properties in the cell column that are dependent on the receptors. The extravillous trophoblast cells express CCN3, which activates the Notch pathway and results in the reduction of proliferation, whereas trophoblast migration properties are stimulated by the binding of CCN3 to integrin α5β1, followed by the activation of Akt kinase [91]. Taken together, these results show that CCN3 is a key regulatory protein of the extravillous trophoblast cells because it supports the exit from the cell cycle of trophoblast cells located at the proximal column and at the same time enhances the migration properties of the invasive trophoblasts, which begin to express integrin α5β1 while detaching from the column (Fig. 2). These findings support the hypothesis that CCN family members have multifunctional properties that depend on their binding to various receptors and thus on their subsequent various signalling cascades that in turn can lead to specific temporal and spatial changes in the programme of the same cell types.

Although many research studies have examined the roles of the first three CCN members in the human placenta, nearly nothing is known about the roles of CCN4, CCN5 and CCN6 in placental development or disease.

Conclusions

Most of the documented functions of CCN proteins in the female reproductive tract have come from studies of the first three members identified: CCN1 (CYR61), CCN2 (CTGF) and CCN3 (NOV). All of the investigated CCN family members are regulated by ovarian steroid hormones and by oxygen, and they can adapt their expression levels to the various hormonal or oxygen levels of the reproductive systems. Moreover, they mediate environmentally dependent signalling between various tissue compartments and cell types within the reproductive systems and thereby regulate distinct cell biological changes.

This protein family serves tissue remodelling and development, including angiogenesis, in a relatively short time frame. These quick adaptations and tissue reorganisation or organ development are especially needed in all of the reproductive organs as they react to hormonal stimulation, inflammatory cytokines and hypoxia. Their modular structure and their numerous and various binding properties may explain their highly adaptive function in the different reproductive organs and even within one organ or cell type. The contributions of the CCN family members to human reproductive diseases, such as endometriosis, leiomyoma and preeclampsia, support this notion because the regulation of CCNs is impaired during the pathogenesis of these diseases. An increase in our knowledge of the coordinated multifunctional properties of the CCNs within one organ and their defined signalling cascades would provide researchers with an advantage in that it may then become possible to correct multiple impaired pathways in reproductive diseases by interfering with only one molecule.

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