[16] R.J. Lories, I. Derese, J.L. Ceuppens, F.P. Luyten, Bone morphogenetic proteins 2 and 6, expressed in arthritic synovium, are regulated by proinflammatory cytokines and differentially modulate fibroblast-like synoviocyte apoptosis, Arthritis Rheum. 48 (2003) 2807–2818.
[17] R.J.U. Lories, F.P. Luyten, Bone morphogenetic protein signaling in joint homeostasis and disease, Cytokine Growth Factor Rev. 16 (2005) 287–298.
[18] K. Bobacz, I.G. Sunk, S. Hayer, L. Amoyo, M. Tohidast-Akrad, G. Kollias, J.S. Smolen, G. Schett, Differentially regulated expression of growth differentiation factor 5 and bone morphogenetic protein 7 in articular cartilage and synovium in murine chronic arthritis: potential importance for cartilage breakdown and synovial hypertrophy, Arthritis Rheum. 58 (2008) 109–118.
[19] H. Iwata, S. Ono, K. Sato, T. Sato, M. Kawamura, Bone morphogenetic protein-induced muscle- and synovium-derived cartilage differentiation in vitro, Clin. Orthop. Relat. Res. 296 (1993) 295–300.
[20] N. Shintani, E.B. Hunziker, Chondrogenic differentiation of bovine synovium: bone morphogenetic proteins 2 and 7 and transforming growth factor beta1 induce the formation of different types of cartilaginous tissue, Arthritis Rheum. 56 (2007) 1869–1879.
[21] S. Yamane, A.H. Reddi, Induction of chondrogenesis and superficial zone protein accumulation in synovial side population cells by BMP-7 and TGF-beta1, J. Orthop. Res. 26 (2008) 485–492.
[22] M.B. Goldring, K. Tsuchimochi, K. Ijiri, The control of chondrogenesis, J. Cell. Biochem. 97 (2006) 33–44.
[23] V. Rosen, BMP and BMP inhibitors in bone, Ann. N. Y. Acad. Sci. 1068 (2006) 19–25.
[24] L.J. Brunet, J.A. McMahon, A.P. McMahon, R.M. Harland, Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton, Science 280 (1998) 1455–1457.

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CCN3 and bone marrow cells

Ken-ichi Katsube · Saki Ichikawa · Yuko Katsuki · Tasuku Kihara · Masanori Terai · Lester F. Lau · Yoshihiro Tamamura · Shin'ichi Takeda · Akihiro Umezawa · Kei Sakamoto · Akira Yamaguchi

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Abstract CCN3 expression was observed in a broad variety of tissues from the early stage of development. However, a kind of loss of function in mice (*CCN3* del VWC domain -/-) demonstrated mild abnormality, which indicates that CCN3 may not be critical for the normal embryogenesis as a single gene. The importance of CCN3 in bone marrow environment becomes to be recognized by the studies of hematopoietic stem cells and Chronic Myeloid Leukemia cells. CCN3 expression in bone marrow has been denied by several investigations, but we found CCN3 positive stromal and hematopoietic cells at bone extremities with a new antibody although they are a very few populations. We investigated the expression pattern of CCN3 in the cultured bone marrow derived mesenchymal stem cells and found its preference for osteogenic differentiation. From the analyses of in vitro experiment using an osteogenic mesenchymal stem cell line, Kusa-A1, we found that CCN3 downregulates osteogenesis by two different pathways; suppression of BMP and stimulation of Notch. Secreted CCN3 from Kusa cells inhibited the differentiation of osteoblasts in separate culture, which indicates the paracrine manner of CCN3 activity. CCN3 may also affect the extracellular environment of the niche for hematopoietic stem cells.

Keywords Nov · Notch · Stem cells · Niche · Hematopoiesis · Osteogenesis

K.-i. Katsube (✉) · S. Ichikawa · Y. Tamamura · K. Sakamoto · A. Yamaguchi
Oral Pathology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University,
1-5-45 Yushima, Bunkyo-ku,
Tokyo 113-8549, Japan
e-mail: ken-tmd@umin.ac.jp

Y. Katsuki · T. Kihara
Maxillofacial Surgery, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University,
1-5-45 Yushima, Bunkyo-ku,
Tokyo 113-8549, Japan

M. Terai · A. Umezawa
Department of Reproductive Biology,
National Institute for Child Health and Development,
2-10-1 Okura, Setagaya-ku,
Tokyo 157-8535, Japan

L. F. Lau
Department of Molecular Genetics,
University of Illinois at Chicago,
Chicago, IL 60607-7170, USA

S. Takeda
Department of Molecular Therapy,
National Institute of Neuroscience,
Ogawa-Higashi-cho, Kodaira,
Tokyo, Japan

Present Address:
S. Ichikawa
Terumo, .
2-44-1Hatagaya, Shibuya-ku,
Tokyo, Japan

Introduction

CCN3 (*Nov, Nephroblastoma overexpressed*) was originally identified as a gene that is responsible for nephroblastoma in chicken induced by the infection of a retrovirus (Soret et al. 1989) and now classified as a member of CCN family of genes (Brigstock et al. 2003). CCN is a Cysteine-rich small secretory protein that interacts with various extracellular and transmembrane proteins. In chicken embryogenesis, CCN3 expression starts from the early stage (Hamburger and Hamilton (HH) Stage 5) (Katsube et al. 2001). Initial

CCN3 expression was observed in the axial structure complex (notochord and floor plate), which guides the polarized differentiation of midline structure from the ventral side, such as neural tube, vertebra, and visceral endoderm. Since this body patterning stage, *CCN3* expression is observed in a wide variety of mesodermal and ectodermal cells such as osteoblasts, myoblasts, chondroblasts, epithelial cells and neuroblasts. Even in adult, several mesodermal tissues maintain the expression of CCN3. Like *CCN3*, *CCN1* and *CCN2* expression was observed in mesodermal tissues from the early stage development (Katsube et al. 2009), but their pattern is not identical. *CCN1* expression during development is observed in extraembryonic tissues such as the chorion and the allantois during the placenta formation (Mo et al. 2002). *CCN2* expression was observed in the developing notochord (Chiou et al. 2006; Erwin 2008), but its pattern is not precisely the same as *CCN3*.

The broad distribution of CCN3 in embryonic tissues let us expect drastic deformities in its knockout mice, but a recent investigation about a practical *CCN3* knockout mice (*CCN3* del VWC domain -/-) revealed that they are viable even after the birth (Heath et al. 2008). Although several mild changes in skeletogenesis and joint formation, myocardiopathy and lens degeneration of eye were found, whole morphogenesis is not much affected. CCN3 is not likely to be really critical as a single gene in the normal step of embryogenesis although there is an argument if this KO mice really represent a true loss of function of CCN3 (Perbal 2007). This fact is rather surprising because dysmorphic changes in both *CCN1* and *CCN2* knockout mice were apparent in the vasculogenesis and osteo/chondrogenesis, which lead to the lethal hemorrhage or skeletal abnormalities before or just after the birth(Mo et al. 2002) (Ivkovic et al. 2003).

The role of CCN3 in adult tissues remains elucidated, but several investigations suggest the role of CCN3 in hematopoiesis. Chronic myeloid leukemia (CML) occurs by a chromosomal translocation of t(9;22)(q34;q11), which creates a fused protein between BCR and ABL, a constitutively active form of tyrosine kinase. Before the stage of acute blastic crisis, the growth of CML cells requires the niche environment of hematopoiesis although they partially obtain the cell autonomous proliferation ability. The mechanism of acute blastic crisis is not yet fully understood, but initial potency of CML is believed to be due to the constitutive kinase activation of BCR-ABL gene product. Down-regulation of CCN3 was induced as a direct consequence of BCR-ABL kinase activity in FDCP-Mix primitive hematopoietic stem cells (McCallum et al. 2006). Administration of a tyrosine kinase inhibitor (Imatinib) upregulates CCN3 expression and transfection of *CCN3* inhibits proliferation and decreases clonogenic potential of BCR-ABL (+) CML cells. From these results, it may be possible to hypothesize that CCN3 directly regulates the proliferation and differentiation of myeloid precursor cells in the niche of normal hematopoiesis. In fact, transient upregulation of *CCN3* is essential for the development of hematopoietic stem cells derived from the umbilical cord vein or of FDCP-Mix cells (Gupta et al. 2007). Particularly, primitive stage of hematopoietic cells (CD34 (+)) is sensitive for CCN3 expression. Down-regulation by siRNA of *CCN3* abrogates their differentiation ability and upregulation of CCN3 either by CCN3 gene transfection or protein administration promotes their differentiation and proliferation. These investigations were done in culture in vitro of hematopoietic cells without the effect of stroma, in which self-regulation of hematopoietic cells is discussed for CCN3 expression. But it may be more possible that the neighboring cells to hematopoietic stem cells commit the regulation of CCN3 constructing the niche environment. In this issue, we focus on the role of CCN3 in the bone marrow environment.

Materials and methods

Whole mount in situ hybridization

See the references for the detailed information about the used probes (chicken *CCN3* (Katsube et al. 2001), chicken *Sonic Hedgehog* (Watanabe and Nakamura 2000), chicken *Serrate1* (Sakamoto et al. 1998)). The digoxigenin-labeled cRNA antisense and sense probes were prepared using linearized plasmid DNA according to the manufacturer's protocol (Roche Diagnostics). Embryonic stage was determined by Hamburger and Hamilton staging (Hamburger 1992). Expression of each gene was visualized with antisense probes verifying the background coloration with sense probes (negative control). Coloration was stopped before the background staining started. The chick embryos were dissected and fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS). Whole mount in situ hybridization was performed in accordance with the protocol described in the reference (Papalopulu et al. 1991). Tissue section was made with 7 μm thickness after dehydration and embedding in paraffin.

Expression profiling

Mouse bone marrow derived mesenchymal stem cells were cultured with α-modified minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS). In semiconfluent stage, total RNA was extracted with Trizol (Invirogen) and approximately 1 μg of total RNA was reversetranscribed to cDNA by Superscript II

(Invitrogen). Detailed information about the analysis of genechip using cDNA is supplied by manufacturer's home page (Affymetrix, http://www.affymetrix.com/index.affx).

Anti CCN3 antibody

Rabbit polyclonal antibody against murine CCN3 was raised by three different synthetic epitope peptides (amino acid residue position; 83–95; CDRSADPNNQTGI-coNH2, 246–260; CEQEPEEVTDKKGKK-coNH2, 334–348; CPQNNEAFLQDLELK-coNH2). These amino acid sequences are well conserved among species. Raised antisera are purified with the peptide of 334–348; CPQNNEAFLQDLELK-coNH2. In this study, pre-absorption experiment of the antibody was performed with this peptide.

Immunohistochemistry

EDTA decalcified mouse adult (8 weeks after birth) femoral bone is embedded in paraffin. Tissue section was made by 5 μm thickness. The sections are deparaffinated by xylene and hydrated in PBS. Tissue section was incubated with anti-CCN3 antibody (diluted in 1:1000) overnight at 4°C. Antibody detection was performed by Envision rabbit antibody system. Coloration was performed with 3, 3'-diaminobenzidine and nuclear counterstain was done with Hematoxylin.

Western blot analysis

The cells were lysed with TNTE buffer (100 mM Tris-Cl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100). The samples were boiled in Laemmli loading buffer for 2 min, applied to 10% SDS-PAGE, and electroblotted onto Hybond-ECL nitrocellulose membrane (Amersham Biosciences). The proteins were identified using the raised anti CCN3 antibody and Envision rabbit antibody system (Dako-Cytomation). The chemiluminescent imaging was performed with ECL advanced western blot detection kit (Amersham Biosciences). Anti CCN1 antibody was raised in rabbit (Mo et al. 2002). Anti CCN2 antibody was purchased (rabbit anti-CTGF, Torrey Pines Biomedical). Anti CCN3 antibody is the same as used in immunohistochemistry.

Alkaline phosphatase induction in C2C12 cells in separate coculture

Kusa-A1 cells were cultured in α-MEM containing 10% FBS. C2C12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20% FBS. CCN3 and CCN3 ΔCT were transfected to Kusa-A1 by Flp-In system (Invitrogen) using FuGENE 6 Transfection Reagent (Roche Diagnostics). Kusa cells stably expressing CCN3 or CCN3 ΔCT were selected with 200 μg/ml Hygromycin (WAKO) and isolated several clones with single colony isolation. C2C12 cells are inoculated into the dish at semiconfluency. Recombinant human BMP2 (Osteogenetics) was administered at 500 ng/ml for one day to initialize the osteoblastic differentiation of C2C12. Then, the BMP2 containing culture media were removed and rinsed once with DMEM. Separate coculture started with Kusa-A1 cells using a membrane culture insert (Becton Dickinson). Separate coculture was maintained in DMEM containing 10% FBS for three days. Alkaline phosphatase (ALP) positive cells were visualized using NitroBlueTetrazolium and 5-Bromo-4-Chloro-3-Indolylphosphate-5-Toluidine salt after the fixation.

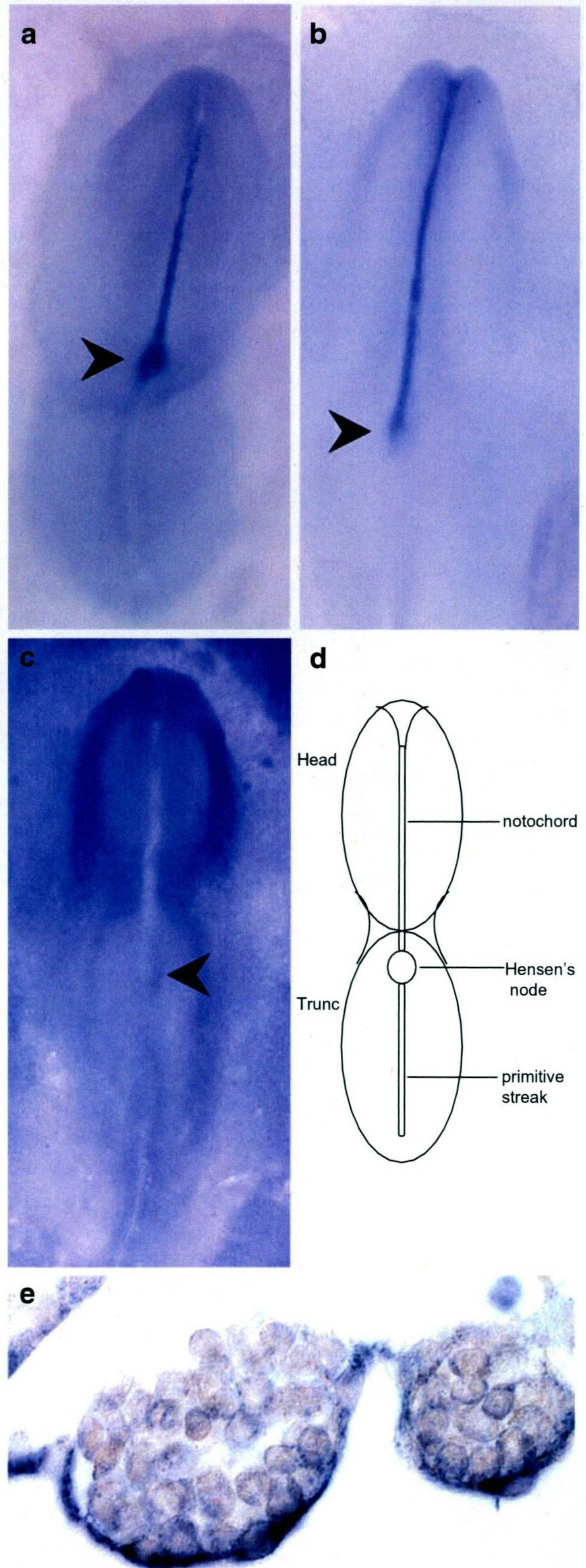
Results

Chicken CCN3 expression in embryonic and extraembryonic tissues

Expression of CCN3 in axial structure becomes visible in the presegmentation stage and reaches to the highest level at the beginning of somitogenesis (HH stage 7, Fig. 1a). Strong similarity between *CCN3* and *Sonic Hedgehog* was observed for the expression pattern in axial structure (notochord and floor plate), in which the left–right asymmetrical pattern in the node area is also conserved (Fig. 1b). However, *Sonic Hedgehog* expression is slightly broader than that of CCN3. Since CCN3 stimulates Notch signaling (Sakamoto et al. 2002), we investigated the expression of Notch signal genes. Among them, *Serrate1*, a ligand of Notch is unique because its expression is apparently a right deviated pattern in the node area (Fig. 1c). Another Notch ligand, *Delta1* shows a right deviated pattern, but is more caudal area of the node extending to the primitive streak (data not shown). The expression of *CCN3* in axial structure was followed by the presomitic mesoderm although it is diminished (Sakamoto et al. 2002).

There is accumulating evidence of CCN3 commitment to hematopoiesis and therefore we investigated the CCN3 expression in extraembryonic tissues. Blood islands are formed in the yolk sac mesodermal area just between the visceral endodermal and ectodermal layers, which supply the red blood cells at the primary stage. The cells in blood islands are called hemangioblasts since they also differentiate into the endothelial cells of extraembryonic blood vessel structure. In chicken embryo, differentiating endothelial cells in the ventral part of blood islands express

Fig. 1 Expression of mRNA in early stage chicken embryo; CCN3 ▶ (**a**), Sonic Hedgehog (**b**) and Serrate1 (**c**) at the beginning stage of somitogenesis (Hamburger and Hamilton (HH) Stage 7). *Arrowheads* indicate Hensen's node. Demonstrates the schematic view of tissue organization at this stage (**d**). Expression of CCN3 mRNA in blood islands of chicken extraembryonic tissue (HH stage 10) (**e**). Ventral part of the endothelial precursor cells in blood islands is positive for CCN3 expression. Coloration was stopped before the background staining started in sense probes (negative control)



CCN3 from the initial stage (Fig. 1e). Erythroblasts in the center of blood islands (spherical cells in Fig. 1e) weakly express *CCN3* and their expression is mildly enhanced in later stage (data not shown).

CCN3 and mouse bone marrow cells

The importance of bone marrow stromal cells as the niche constituent for blood stem cells has been recognized from the investigation of in vitro culture of hematopoietic cells (Harigaya 1987). The stromal cells not only give the microenvironment for the installation of hematopoietic cells, but also produce several growth factors and membrane associated proteins to maintain hematopoiesis (Haylock and Nilsson 2005). In mammals and birds, bone marrow is the main place for tertiary stage of hematopoiesis, but the precise histological locality of hematopoietic stem cells has not been identified. In hematopoietic red bone marrow, many osteoblasts and stromal cells colocalize with hematopoietic cells, and histomorphologically, little difference is observed in whole area. In 2003, two independent research groups reported the precise locality of hematopoietic stem cells(Calvi et al. 2003; Zhang et al. 2003), in which a few "osteoblastic" populations (not termed as osteoblasts) in the bone extremities (endosteal tissue near the epiphysis of long bone) are charged for the niche formation. Also, the importance of several intercellular regulatory molecules is demonstrated from these reports such as Notch1/Jag1 and N-Cadherin. CCN3 expression in bone marrow has been expected from the analysis of in vitro hematopoiesis, but genechip analyses demonstrate that bone marrow tissue is empty for CCN3 expression (Refer to the web site of NCBI/Unigene; http://www.ncbi.nlm.nih.gov/ with Nov (=CCN3)). It has been difficult to detect the subtle expression of CCN3 by commercially available antibodies and we raised a new antibody in rabbit against mouse CCN3. We verified the specificity of this antibody by western blot analysis (Fig. 2a) using the protein extract of Kusa-A1. Raised CCN3 antibody detected two bands (major 45kd and minor 16kd) while the preincubated antibody with the epitope peptide had no specific bands. This CCN3 antibody was applied to the immunohistochemistry of mouse adult bone

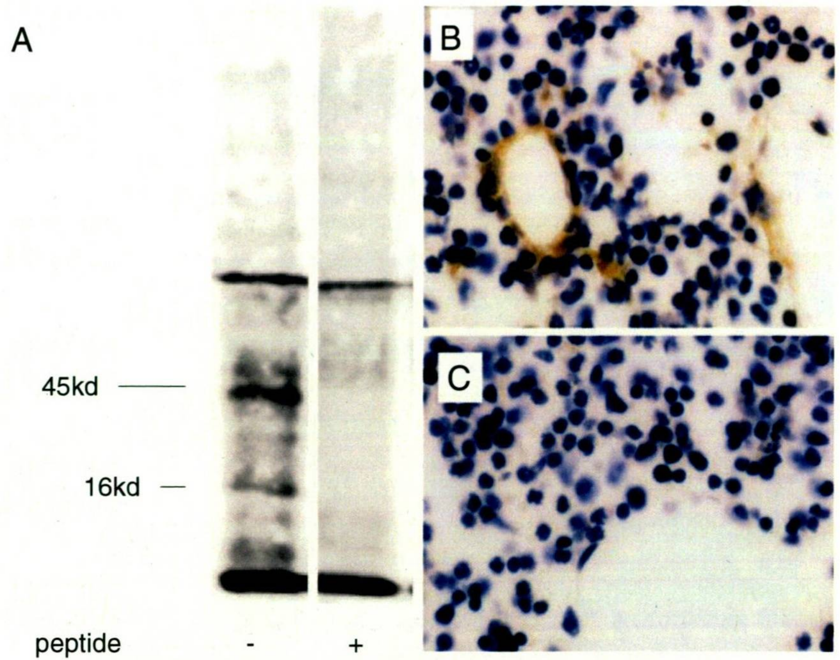


Fig. 2 Confirmation of the raised CCN3 antibody specificity (**a**) western blot analysis using CCN3 antibody. Protein extracts from Kusa-A1 were lorded and incubated with non-treated antibody or the antibody pre-incubated with the epitope peptide (**b**). CCN3 positive cells in mouse adult femoral bone marrow were detected with non-treated antibody (**c**). No positive cell is detected with the antibody preincubated with the epitope peptide

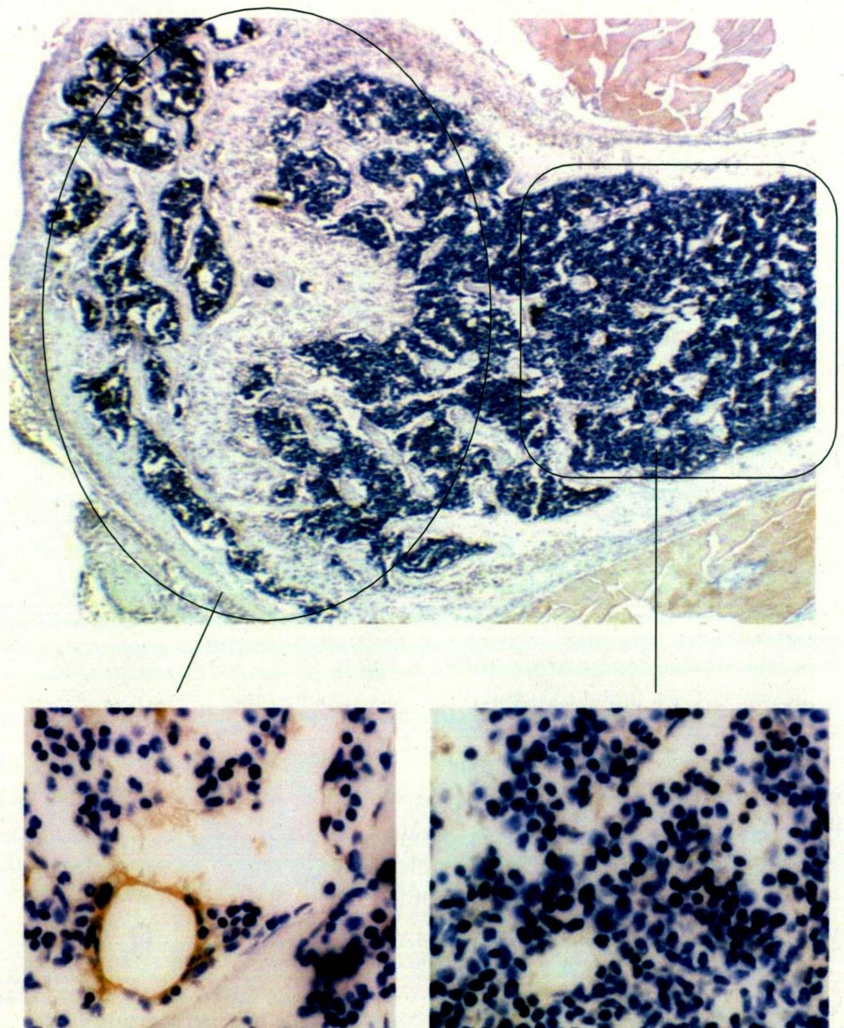


Fig. 3 CCN3 positive cells of the bone marrow are observed in the mouse femoral bone extremities. Endothelial cells of microvascular structure are positive for CCN3. Several surrounding cells (hematopoietic and spindle-shaped stromal cells) are also positive for CCN3. On the while, diaphysis bone marrow cells are negative

marrow tissue (Fig. 2b). Endothelial cells and several cells around the microvascular structure showed the immunoreactivity. On the while, the CCN3 antibody preincubated with the epitope peptide showed no positive cells (Fig. 2c). These CCN3 expressing cells exist only in the bone extremities (Fig. 3); endosteal region near the epiphysis including secondary ossifying center. Endothelial cells of small blood vessels are positively stained with CCN3 antibody in the bone extremities. Around these vessels, some spherical hematopoietic cells (including megakaryocytes) and polygonal or spindle-shaped stromal cells are also positive for CCN3. The spindle-shaped cells localized just between the trabecular bone tissue and microvascular structure. Some CCN3 positive spindle-shaped cells seem to be loosely contacted each other.

CCN3 and cultured bone marrow derived mesenchymal stem cells

To do the stable culture of hematopoietic cells, establishment of stromal cell line is indispensable. Many attempts have been done to get immortalized clones of stromal cells (Umezawa et al. 1991). During the analyses of these established cell lines, their own differentiation potential as stem cells has come to be recognized (Umezawa et al. 1992). It is not surprising that they have the potential to differentiate into osteoblasts or adipocytes since the bone marrow cavity are filled with the trabecular bone and adipose tissue in white bone marrow. But now it is known that some of the bone marrow derived mesenchymal stem cells differentiate into neurons or muscle cells, or even some endodermal cells. From the tracing analysis using genetic markers, some stromal cells in the bone marrow seem to originate from the neural crest, which may explain their pluripotency (Morikawa et al. 2009). This differentiation potential is actually highlighted from the point of view of their application to the human regeneration therapy, but in vivo, these cells are quiescent in the usual state. A few populations circulate in blood stream, which are seem to be recruited to the injured area to restitute the tissue organization. However, their proper regulatory mechanism in reorganization is still not well understood although their contribution is apparent.

Until now, a lot of clones of stem cells have been identified from the bone-marrow stromal cells. Each cell line of bone marrow stromal cells has different orientation for differentiation (Kohyama et al. 2001). We chose four independent clones of mouse stem cells and investigated their expression property of CCN family of genes by genechip analyses (Fig. 4). Examined cell lines are 9-15C, H123, Kusa-A1 and Kusa-O. 9-15C is multipotential, which includes osteogenic, chondrogenic, adipogenic, neurogenic, and cardio-myogenic differentiation (Sharov et al. 2003). H-123 is also multipotential, but strong preference for adipogenesis (Makino et al. 1999). Kusa-A1 and Kusa-O are sister cell lines and both osteogenic (Umezawa et al. 1992). However, Kusa-A1 has much stronger potential for osteogenesis and Kusa-O has relative tendency for adipogenic differentiation (Kawashima et al. 2005; Allan et al. 2003). Kusa-A1 also possesses the ability of neuronal differentiation (Kohyama et al. 2001). Among CCN family of genes, *CCN3* is highly expressed in Kusa-A1 and Kusa-O (Fig. 4c). On the while, CCN1 shows the tendency of high level expression for adipogenic orientation (Kusa-O and H-123) (Fig. 4a). Contrary to *CCN1*, *CCN2* expression is suppressed in adipogenic orientation (H-123 and Kusa A) (Fig. 4b). It may be possible to hypothesize that *CCN3* expression has relatively high preference for osteogenic differentiation as compared with other CCN family of genes. Western blot analysis revealed that the protein level almost corresponds to the mRNA level in each cell line (Fig. 4d).

CCN3 and osteogenic ability of bone marrow derived mesenchymal stem cells

Endogenous CCN3 expression in Kusa-A1 is high in the default state and moderately downregulated during the induction of mineralization (Kawashima et al. 2005). It is noteworthy that a relatively low osteogenic cell line of Kusa, Kusa-O maintains the continuous high expression of CCN3 (Kawashima et al. 2005). Therefore we investigated the role of CCN3 in Kusa-A1 (Katsuki et al. 2008). For this purpose, we created several deletion mutants of CCN3 to investigate its role from the aspect of Notch signal. CT domain binds to Notch extracellular domain and is indispensable for the Notch signal activation.

Constitutive expression of CCN3 significantly downregulated osteogenesis of Kusa-A1, but did not affect its neurogenesis. Anti-osteogenic activity of CCN3 was reduced by the deletion of the CT domain. This deletion abrogates the upregulation of authentic Notch signal downstream targets, Hes1 and particularly, Hey1. We also demonstrated that CCN3 could downregulate cell proliferation of Kusa-A1. We found a specific upregulation of p21 among several cell cycle regulators and confirmed that deletion of CT domain from CCN3 could cancel p21 upregulation, which suggests that CCN3 upregulates p21 through Notch signaling. In fact, inhibition of Notch signal by gamma secretase inhibitor also downregulated the CCN3 effect on p21 upregulation, strongly suggesting CCN3 mainly employs Notch signal for p21 upregulation. This finding is interesting from the point of view of the Notch signal because p21 activation by Notch was reported mainly in epithelial cells (Rangarajan et al. 2001). CCN3 seem to put default Kusa-A1 in a quiescent state suppressing both osteogenesis and proliferation (Figs. 4 and 5).

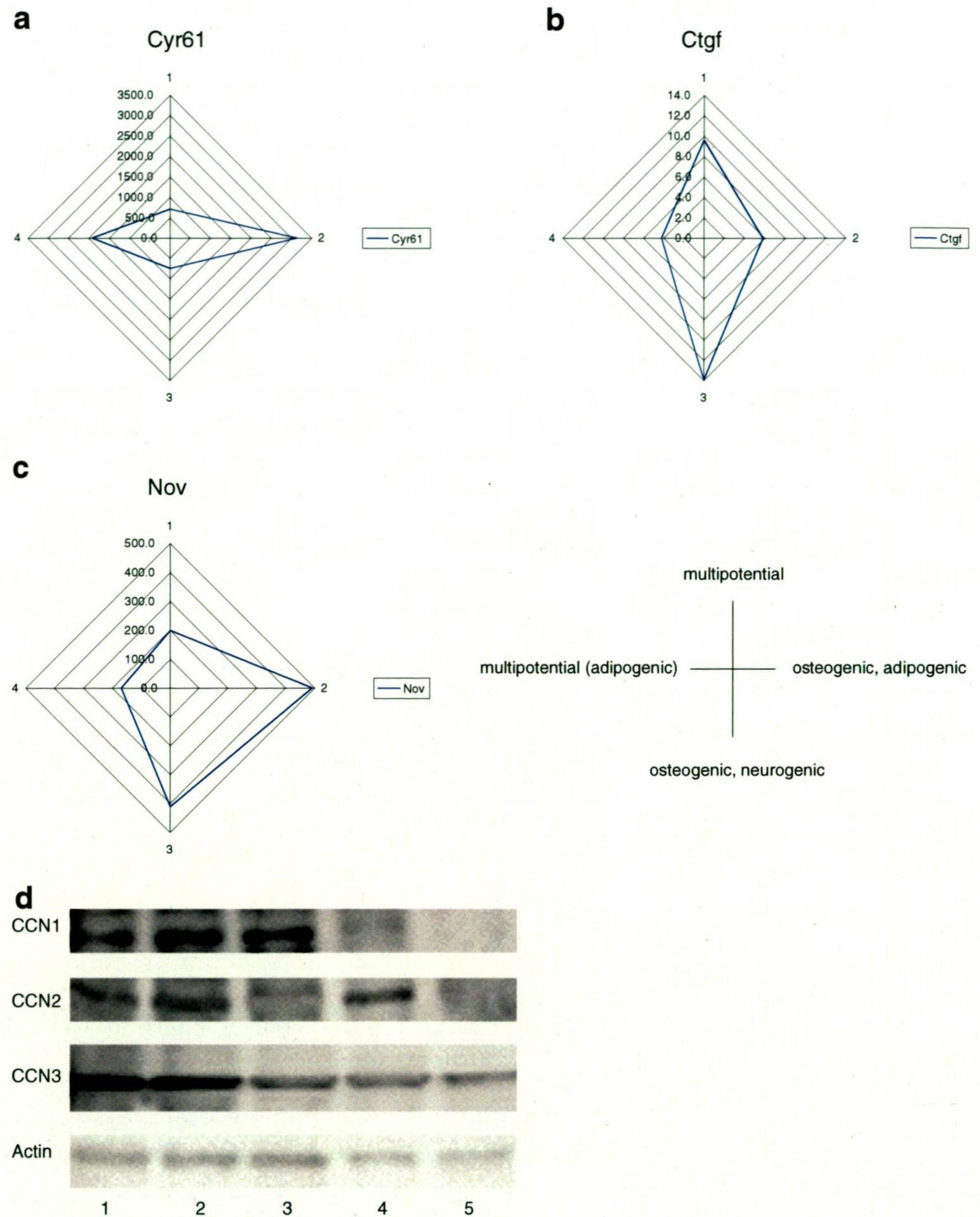


Fig. 4 Expression profile of *CCN1* (**a**), *CCN2* (**b**), and *CCN3* (**c**) mRNAs in four different mouse bone marrow derived mesenchymal stem cell lines. Top (1): 9-15c, right (2): Kusa-O bottom (3): Kusa-A1, left (4): H-123. Normalization of relative expression level was done by the comparison with a set of house keeping genes in the genechip of Affymetrix. All quantitative analyses were done by Affymetrix Gene Spring with normalization with several house-keeping genes. (**d**) Western blot analysis with anti-CCN1, CCN2 and CCN3. 1: Kusa-A1, 2: Kusa-O, 3: 9-15C, 4: H-123, 5; MC3T3-E1

The role of CCN3 in the microenvironment of bone marrow is still not clear. The anti-osteogenetic ability of CCN3 in osteoblast cell lines are demonstrated in vitro culture (Minamizato et al. 2007; Parisi et al. 2006; Rydziel et al. 2007), but CCN3 expression is under detectable in most of the osteoblasts in vivo (Fig. 3). And also there has been an argument that interaction of CCN3 with the relating molecules is an intracellular event or an extracellular event (cell-autonomous or non autonomous). It may be possible that CCN3 is not expressed by osteoblasts themselves but by other surrounding cells such as stromal cells. To clarify these matters, we tried the separate coculture experiment using Kusa-A1 and C2C12 that was initiated into osteoblastic differentiation (Fig. 6a). The induction of alkaline phosphatase (ALP) in C2C12 was inhibited by secreted CCN3 from Kusa-A1 (Fig. 6b). Kusa-LacZ transfected with full length CCN3 by Flp-In vector clearly reduced the number of ALP positive cells. On the while, Kusa-LacZ transfected with CT deleted CCN3 moderately reduced the number of ALP positive cells. These results indicate that anti-osteogenetic activity of CCN3 is intercellularly mediated probably by Notch stimulation and BMP suppression.

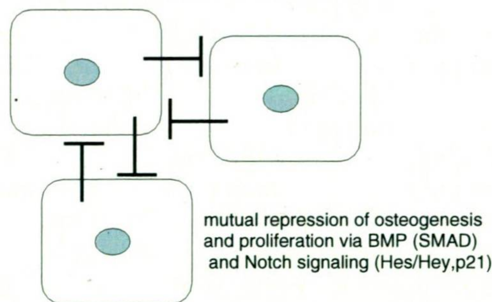


Fig. 5 Osteogenic mesenchymal stem cells negatively regulate osteogenesis and proliferation by CCN3

Discussion

CCN3 expression is detected from the early stage of embryogenesis including the hematopoiesis in the extraembryonic tissue. Its expression in the ventral part of blood islands is interesting because visceral endodermal cells of yolk sac are considered to be essential for hemangiogenesis (angiogenetic cell clustering), secreting Indian Hedgehog (Byrd et al. 2002) and VEGF (Caprioli et al. 2001). Indian Hedgehog expression is also observed in mesodermal cells between visceral endoderm and embryonic ectoderm (Grabel et al. 1998). High similarity in spatial distribution pattern between CCN3 and the Hedgehog family gene is also observed in Hensen's node. Preliminary experiment using the bead carrying recombinant CCN3 protein demonstrated that CCN3 upregulates *Sonic Hedgehog* in the nodal area (data not shown) during gastrulation. This indicates that there exists the direct interaction for the coexpression of CCN3 and Hedgehog gene family. Serrate1 expression in Hensen's node is also interesting because expression of Jag1, the murine equivalent of Serrate1 is similar to that of CCN3 in bone marrow. We do not know the significance of the mirror-imaged symmetrical expression pattern of *CCN3* and *Serrate1* in the node, but Notch signal is reported to participate in left–right asymmetry under the influence of calcium signal (Raya et al. 2003).

In adult mouse, a very few populations exhibit CCN3 expression in the extremity of bone marrow, whose area is responsible for the stem cell niche of hematopoietic cells. Previous investigations by gene chip analysis all failed to detect CCN3 expression in bone marrow, but it may be due to the limited locality of CCN3 positive cells (Bone extremities are usually cut off to elute the bone marrow cells.). As mentioned, distribution of CCN3 positive cells in bone marrow is rather similar to that of Jag1 (=murine Serrate1) positive cells although CCN3 positive cells are much limited. CCN2 (CTGF) expression is previously

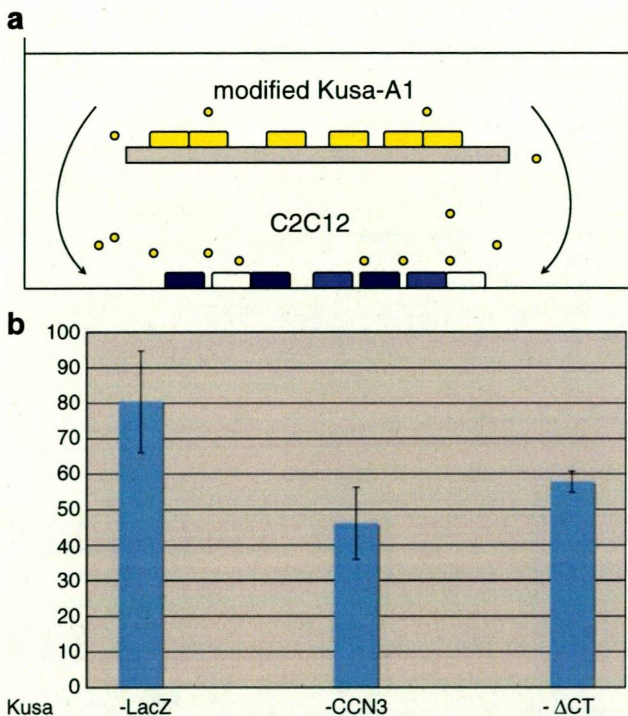


Fig. 6 CCN3 functions as a secreting inhibitor in alkaline phosphatase (ALP) induction ofC2C12 cells. **a** Schematic view of the separate coculture experiment. Kusa-A1 and C2C12 cells were cultured in the same dish, but separated by a thin membrane insert (Culture separate, Becton Dickinson). The medium freely circulates, but no direct contact between two separate cultures. **b** Effect of Kusa cells stably transfected with CCN3 and its deleted form is measured by counting ALP positive cells in C2C12 (cells/0.01 mm^2)

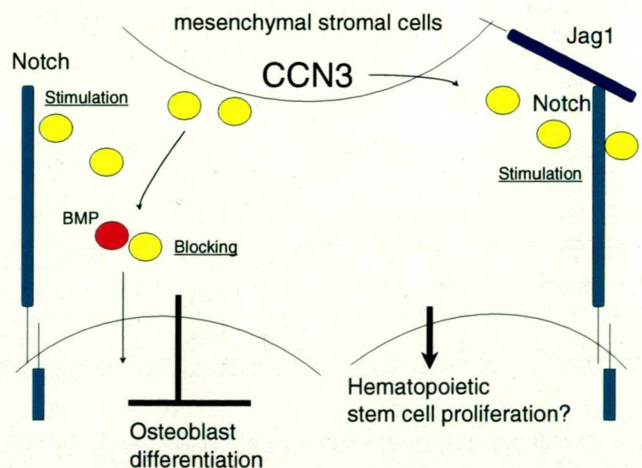


Fig. 7 Scheme of a hypothetical role of CCN3 in the bone marrow niche. CCN3 (*yellow*) suppresses the BMP (*red*) activity by direct binding. On the while, CCN3 binds to Notch receptor, which results in the signal stimulation. Notch signal stimulation is principal for the maintenance of stem cell property, blocking the terminal differentiation. CCN3 secreted by stromal stem cells modulates the growth and differentiation of neighboring osteoblasts and hematopoietic stem cells

reported in the chondrocytes near the secondary ossifying center (Oka et al. 2007), but there is no report about the expression of CCN family of genes in the secondary ossifying center itself.

As for cultured bone marrow-derived mesenchymal stem cells, CCN3 expression is highly maintained in the default state of an osteogenic cell line, Kusa-A1. In human bone marrow derived stem cells, the expression level of CCN family was examined with regard to several orientation of differentiation (adipogenesis, osteogenesis, chondrogenesis) (Schutze et al. 2005). From their investigation, CCN3 expression is not detectable, but high expression of CCN1 and CCN2 was observed in the default state. Their expression was downregulated in the course of differentiation, but there is no particular correlation with differentiation orientation. Direct evidence about the role of CCN in the human mesenchymal stem cells is not confirmed from their investigation, but it is hypothesized that CCN is a negative regulator of differentiation. Another report demonstrated that the induction of hepatocyte-like cells from human bone marrow derived stem cells downregulated CCN4 and CCN5 expression (Shimomura et al. 2007) while no significant change is observed in CCN1, CCN2 and CCN3. They tried downregulation of CCN4 or CCN5 by siRNA, which resulted in the promotion of the hepatocyte-like differentiation. We observed that Kusa-A1 downregulated endogenous CCN3 expression after the induction of mineralization while low osteogenic Kusa-O continuously upregulates CCN3 even after the treatment (Kawashima et al. 2005). Continuous CCN3 upregulation in Kusa-A1 downregulated the osteogenesis, but did not touch its neurogenic potency (Katsuki et al. 2008). These facts may indicate the CCN3 specifically affects the osteogenic differentiation in murine bone marrow mesenchymal stem cells.

It may be intriguing to hypothesize that Kusa-A1 is a core descendent of stem cell niche constituents of hematopoiesis because the stromal cells of hematopoietic niche are "osteoblastic" (Calvi et al. 2003). Interaction of bone marrow stromal cells with hematopoietic cells is critical as they regulate the microenvironment of niche and maintain the stemness and homing of hematopoietic stem cells. From this point of view, p21 upregulation by CCN3 is interesting. p21 is important not only for cell proliferation, but also terminal differentiation of mesenchymal stem cells (van den Bos et al. 1998). Also, stem cell maintenance of PML-RAR activated hematopoietic cells requires p21 to prevent DNA damage and excess proliferation (Viale et al. 2009). Recent investigation of bone marrow niche demonstrates that hematopoietic stem cells dynamically change their locality with differentiation (Lo Celso et al. 2009). CCN3 may regulate these complicated behaviors since it also affects cell attachment and migration (Fukunaga-Kalabis et al. 2008). CCN3 expression suppresses not only the self-osteogenic activity, but also the osteoblasts in separate culture. This paracrine manner of CCN3 expression may supply the bone cavity space enough to reside the niche environment of hematopoiesis. The verification of the effect of CCN3 in adult bone marrow would provide a new way of thinking about hematopoiesis (Fig. 7). Analyses using knockout mice of CCN3 are really necessary to give an answer about this question.

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References

Allan EH, Ho PW, Umezawa A, Hata J, Makishima F, Gillespie MT, Martin TJ (2003) Differentiation potential of a mouse bone marrow stromal cell line. J Cell Biochem 90(1):158–169

Brigstock DR, Goldschmeding R, Katsube KI, Lam SC, Lau LF, Lyons K, Naus C, Perbal B, Riser B, Takigawa M, Yeger H (2003) Proposal for a unified CCN nomenclature. Mol Pathol 56 (2):127–128

Byrd N, Becker S, Maye P, Narasimhaiah R, St-Jacques B, Zhang X, McMahon J, McMahon A, Grabel L (2002) Hedgehog is required for murine yolk sac angiogenesis. Development 129 (2):361–372

Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, Martin RP, Schipani E, Divieti P, Bringhurst FR, Milner LA, Kronenberg HM, Scadden DT (2003) Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 425(6960):841–846

Caprioli A, Minko K, Drevon C, Eichmann A, Dieterlen-Lievre F, Jaffredo T (2001) Hemangioblast commitment in the avian allantois: cellular and molecular aspects. Dev Biol 238(1):64–78

Chiou MJ, Chao TT, Wu JL, Kuo CM, Chen JY (2006) The physiological role of CTGF/CCN2 in zebrafish notochond development and biological analysis of the proximal promoter region. Biochem Biophys Res Commun 349(2):750–758

Erwin WM (2008) The Notochord, Notochordal cell and CTGF/CCN-2: ongoing activity from development through maturation. J Cell Commun Signal 2(3–4):59–65

Fukunaga-Kalabis M, Martinez G, Telson SM, Liu ZJ, Balint K, Juhasz I, Elder DE, Perbal B, Herlyn M (2008) Downregulation of CCN3 expression as a potential mechanism for melanoma progression. Oncogene 27(18):2552–2560

Grabel L, Becker S, Lock L, Maye P, Zanders T (1998) Using EC and ES cell culture to study early development: recent observations on Indian hedgehog and Bmps. Int J Dev Biol 42(7):917–925

Gupta R, Hong D, Iborra F, Sarno S, Enver T (2007) NOV (CCN3) functions as a regulator of human hematopoietic stem or progenitor cells. Science 316(5824):590–593

Hamburger V (1992) The stage series of the chick embryo. Dev Dyn 195(4):273–275

Harigaya K (1987) Fibrocytic bone marrow stromal cells and hematopoiesis. Nippon Ketsueki Gakkai Zasshi 50(8):1499–1509

Haylock DN, Nilsson SK (2005) Stem cell regulation by the hematopoietic stem cell niche. Cell Cycle 4(10):1353–1355
Heath E, Tahri D, Andermarcher E, Schofield P, Fleming S, Boulter CA (2008) Abnormal skeletal and cardiac development, cardiomyopathy, muscle atrophy and cataracts in mice with a targeted disruption of the Nov (Ccn3) gene. BMC Dev Biol 8(1):18
Ivkovic S, Yoon BS, Popoff SN, Safadi FF, Libuda DE, Stephenson RC, Daluiski A, Lyons KM (2003) Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. Development 130(12):2779–2791
Katsube K, Chuai ML, Liu YC, Kabasawa Y, Takagi M, Perbal B, Sakamoto K (2001) The expression of chicken NOV, a member of the CCN gene family, in early stage development. Brain Res Gene Expr Patterns 1(1):61–65
Katsube K, Sakamoto K, Tamamura Y, Yamaguchi A (2009) Role of CCN, a vertebrate specific gene family, in development. Dev Growth Differ 51(1):55–67
Katsuki Y, Sakamoto K, Minamizato T, Makino H, Umezawa A, Ikeda MA, Perbal B, Amagasa T, Yamaguchi A, Katsube K (2008) Inhibitory effect of CT domain of CCN3/NOV on proliferation and differentiation of osteogenic mesenchymal stem cells, Kusa-A1. Biochem Biophys Res Commun 368 (3):808–814
Kawashima N, Shindo K, Sakamoto K, Kondo H, Umezawa A, Kasugai S, Perbal B, Suda H, Takagi M, Katsube K (2005) Molecular and cell biological properties of mouse osteogenic mesenchymal progenitor cells, Kusa. J Bone Miner Metab 23 (2):123–133
Kohyama J, Abe H, Shimazaki T, Koizumi A, Nakashima K, Gojo S, Taga T, Okano H, Hata J, Umezawa A (2001) Brain from bone: efficient "meta-differentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent. Differentiation 68(4–5):235–244
Lo Celso C, Fleming HE, Wu JW, Zhao CX, Miake-Lye S, Fujisaki J, Cote D, Rowe DW, Lin CP, Scadden DT (2009) Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. Nature 457(7225):92–96
Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S (1999) Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 103(5):697–705
McCallum L, Price S, Planque N, Perbal B, Pierce A, Whetton AD, Irvine AE (2006) A novel mechanism for BCR-ABL action: stimulated secretion of CCN3 is involved in growth and differentiation regulation. Blood 108(5):1716–1723
Minamizato T, Sakamoto K, Liu T, Kokubo H, Katsube K, Perbal B, Nakamura S, Yamaguchi A (2007) CCN3/NOV inhibits BMP-2-induced osteoblast differentiation by interacting with BMP and Notch signaling pathways. Biochem Biophys Res Commun 354 (2):567–573
Mo FE, Muntean AG, Chen CC, Stolz DB, Watkins SC, Lau LF (2002) CYR61 (CCN1) is essential for placental development and vascular integrity. Mol Cell Biol 22(24):8709–8720
Morikawa S, Mabuchi Y, Niibe K, Suzuki S, Nagoshi N, Sunabori T, Shimmura S, Nagai Y, Nakagawa T, Okano H, Matsuzaki Y (2009) Development of mesenchymal stem cells partially originate from the neural crest. Biochem Biophys Res Commun 379(4):1114–1119
Oka M, Kubota S, Kondo S, Eguchi T, Kuroda C, Kawata K, Minagi S, Takigawa M (2007) Gene expression and distribution of connective tissue growth factor (CCN2/CTGF) during secondary ossification center formation. J Histochem Cytochem 55 (12):1245–1255
Papalopulu N, Clarke JD, Bradley L, Wilkinson D, Krumlauf R, Holder N (1991) Retinoic acid causes abnormal development and segmental patterning of the anterior hindbrain in Xenopus embryos. Development 113(4):1145–1158
Parisi MS, Gazzerro E, Rydziel S, Canalis E (2006) Expression and regulation of CCN genes in murine osteoblasts. Bone 38(5):671–677
Perbal B (2007) CCN3-mutant mice are distinct from CCN3-null mice. J Cell Commun Signal 1(3–4):229–230
Rangarajan A, Talora C, Okuyama R, Nicolas M, Mammucari C, Oh H, Aster JC, Krishna S, Metzger D, Chambon P, Miele L, Aguet M, Radtke F, Dotto GP (2001) Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. Embo J 20(13):3427–3436
Raya A, Kawakami Y, Rodriguez-Esteban C, Buscher D, Koth CM, Itoh T, Morita M, Raya RM, Dubova I, Bessa JG, de la Pompa JL, Belmonte JC (2003) Notch activity induces Nodal expression and mediates the establishment of left–right asymmetry in vertebrate embryos. Genes Dev 17(10):1213–1218
Rydziel S, Stadmeyer L, Zanotti S, Durant D, Smerdel-Ramoya A, Canalis E (2007) Nephroblastoma overexpressed (Nov) inhibits osteoblastogenesis and causes osteopenia. J Biol Chem 282 (27):19762–19772
Sakamoto K, Nakamura H, Takagi M, Takeda S, Katsube K (1998) Ectopic expression of lunatic Fringe leads to downregulation of Serrate-1 in the developing chick neural tube; analysis using in ovo electroporation transfection technique. FEBS Lett 426 (3):337–341
Sakamoto K, Yamaguchi S, Ando R, Miyawaki A, Kabasawa Y, Takagi M, Li CL, Perbal B, Katsube K (2002) The nephroblastoma overexpressed gene (NOV/ccn3) protein associates with Notch1 extracellular domain and inhibits myoblast differentiation via Notch signaling pathway. J Biol Chem 277 (33):29399–29405
Schutze N, Noth U, Schneidereit J, Hendrich C, Jakob F (2005) Differential expression of CCN-family members in primary human bone marrow-derived mesenchymal stem cells during osteogenic, chondrogenic and adipogenic differentiation. Cell Commun Signal 3(1):5
Sharov AA, Piao Y, Matoba R, Dudekula DB, Qian Y, VanBuren V, Falco G, Martin PR, Stagg CA, Bassey UC, Wang Y, Carter MG, Hamatani T, Aiba K, Akutsu H, Sharova L, Tanaka TS, Kimber WL, Yoshikawa T, Jaradat SA, Pantano S, Nagaraja R, Boheler KR, Taub D, Hodes RJ, Longo DL, Schlessinger D, Keller J, Klotz E, Kelsoe G, Umezawa A, Vescovi AL, Rossant J, Kunath T, Hogan BL, Curci A, D'Urso M, Kelso J, Hide W, Ko MS (2003) Transcriptome analysis of mouse stem cells and early embryos. PLoS Biol 1(3):E74
Shimomura T, Yoshida Y, Sakabe T, Ishii K, Gonda K, Murai R, Takubo K, Tsuchiya H, Hoshikawa Y, Kurimasa A, Hisatome I, Uyama T, Umezawa A, Shiota G (2007) Hepatic differentiation of human bone marrow-derived UE7T-13 cells: effects of cytokines and CCN family gene expression. Hepatol Res 37 (12):1068–1079
Soret J, Dambrine G, Perbal B (1989) Induction of nephroblastoma by myeloblastosis-associated virus type 1: state of proviral DNAs in tumor cells. J Virol 63(4):1803–1807
Umezawa A, Tachibana K, Harigaya K, Kusakari S, Kato S, Watanabe Y, Takano T (1991) Colony-stimulating factor 1 expression is down-regulated during the adipocyte differentiation of H-1/A marrow stromal cells and induced by cachectin/tumor necrosis factor. Mol Cell Biol 11(2):920–927
Umezawa A, Maruyama T, Segawa K, Shadduck RK, Waheed A, Hata J (1992) Multipotent marrow stromal cell line is able to induce hematopoiesis in vivo. J Cell Physiol 151(1):197–205
van den Bos C, Silverstetter S, Murphy M, Connolly T (1998) p21 (cip1) rescues human mesenchymal stem cells from apoptosis

induced by low-density culture. Cell Tissue Res 293(3): 463–470

Viale A, De Franco F, Orleth A, Cambiaghi V, Giuliani V, Bossi D, Ronchini C, Ronzoni S, Muradore I, Monestiroli S, Gobbi A, Alcalay M, Minucci S, Pelicci PG (2009) Cell-cycle restriction limits DNA damage and maintains self-renewal of leukaemia stem cells. Nature 457(7225):51–56

Watanabe Y, Nakamura H (2000) Control of chick tectum territory along dorsoventral axis by Sonic hedgehog. Development 127 (5):1131–1140

Zhang J, Niu C, Ye L, Huang H, He X, Tong WG, Ross J, Haug J, Johnson T, Feng JQ, Harris S, Wiedemann LM, Mishina Y, Li L (2003) Identification of the haematopoietic stem cell niche and control of the niche size. Nature 425(6960):836–841

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Review

Role of CCN, a vertebrate specific gene family, in development

Ken-ichi Katsube,* Kei Sakamoto, Yoshihiro Tamamura and Akira Yamaguchi

Oral Pathology, Graduate School of Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549 Japan

The CCN family of genes constitutes six members of small secreted cysteine rich proteins, which exists only in vertebrates. The major members of CCN are *CCN1* (*Cyr61*), *CCN2* (*CTGF*), and *CCN3* (*Nov*). *CCN4*, *CCN5*, and *CCN6* were formerly reported to be in the Wisp family, but they are now integrated into CCN due to the resemblance of their four principal modules: insulin like growth factor binding protein, von Willebrand factor type C, thrombospondin type 1, and carboxy-terminal domain. CCNs show a wide and highly variable expression pattern in adult and in embryonic tissues, but most studies have focused on their principal role in osteo/chondrogenesis and vasculo/angiogenesis from the aspect of migration, growth, and differentiation of mesenchymal cells. CCN proteins simultaneously integrate and modulate the signals of integrins, bone morphogenetic protein, vascular endothelial growth factor, Wnt, and Notch by direct binding. However, the priority in the use of the signals is different depending on the cell status. Even the equivalent counterparts show a difference in signal usage among species. It may be that the evolution of the CCN family continues to keep pace with vertebrate evolution itself.

Key words: Notch, skeletogenesis, stem cells, vasculogenesis, wound repair.

Discovery of the CCN family of genes

CCN is the name of a small gene family whose protein products are approximately 40-kDa cysteine-rich secreted proteins, and expressed in a broad spectrum of vertebrate tissues. The name *CCN* is an acronym of three members of the family: *Cysteine rich 61* (*Cyr61*, *CCN1*), *Connective tissue growth factor* (*CTGF*, *CCN2*), and *Nephroblastoma overexpressed* (*Nov*, *CCN3*) (Brigstock *et al.* 2003). These three genes were identified independently and have several synonyms (Table 1). *CCN1* was cloned as one of the immediate-early genes that are induced in fibroblasts in response to serum or growth factors such as PDGF or EGF (Lau & Nathans 1987). This gene was the 61st identified and thus named *cysteine rich protein-61* (*Cyr-61*). CCN2 was identified by chance as a PDGF associating factor expressed in human umbilical vein endothelial cells (HUVEC). This novel gene was termed connective tissue growth factor because of its *in vivo* mitogenic activity of connective tissue (Bradham *et al.* 1991). *CCN3* was cloned from one of the proviral DNA insertion sites

Some of the communications in this review are provided in the 5th International meeting of CCN family of genes (19–22 October 2008, Toronto). Those who are interested in the CCN family are welcome to the next meeting at Belfast, 2010. Further information is available from the website (http://ccnsociety.com/index.html).

Abbreviations: AVSD, atrial and ventricular septum defects; BMP, bone morphogenetic protein; Cyr61, cysteine rich 61; CT, carboxy-terminal domain; CTGF; connective tissue growth factor; Dan; differential screening-selected gene aberrant in neuroblastoma; EGF, epidermal growth factor; EST, expressed sequence tag; FGF; fibroblast growth factor; GDF; growth/differentiation factor; HSP; heparan sulfate proteoglycan; IGF; insulin like growth factor; IGFBP; IGF binding protein; LRP, low density lipoprotein receptor-related protein; MMP, matrix metalloproteinase; NICD, notch intracellular domain; Nov, nephroblastoma overexpressed; PDGF, platelet-derived growth factor; PPD, progressive pseudorheumatoid dysplasia; SOST, Sclerostin; TGF, transforming growth factor; TSG, Twisted gastrulation; TSP1, thrombospondin type 1; VEGF, vascular endothelial growth factor; VWC, von Willebrand factor type C; Wisp, Wnt1-inducible signaling pathway protein; Wise, Wnt inhibitor secreted protein.

*Author to whom all correspondence should be addressed.
Email: katsube.mpa@tmd.ac.jp

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Table 1. Synonyms of the CCN family of genes and relative genes

CCN1	Cyr61, CEF10
CCN2	CTGF, Fisp12, Hcs24
CCN3	Nov
CCN4	ELM-1, Wisp-1
CCN5	rCOP-1, Wisp-2, CTGF-L
CCN6	Wisp-3
Sost	Sclerostin
Wise	Ectodin, USAG-1, Sostdc1

of a retrovirus (myeloblastosis-associated virus type 1, MAV1) (Perbal *et al.* 1985), which specifically induces nephroblastoma in chicken.

Other members of the CCN family include the Wnt1-inducible signaling pathway proteins (Wisp), which had been previously called by other names (*ELM-1* and *rCOP-1*). Pennica *et al.* proposed to call them *Wisp-1* and *Wisp-2* because of upregulation by Wnt1 (transforming), but not by Wnt4 (non-transforming); hence Wisp (Pennica *et al.* 1998). By screening EST databases, *Wisp-3* was identified as a homologous gene to Wisp1. Since the Wisp family has a close relationship to CCN, they are now allocated to *CCN4* (*Wisp1*), *CCN5* (*Wisp2*), and *CCN6* (*Wisp3*) (Brigstock *et al.* 2003).

Since the discovery of these genes, most CCN researchers have focused on their role in cell proliferation *in vitro* and in tumorigenesis. Other aspects of this gene family were elucidated after the establishment of knockout mice of CCN members. Several distinct roles of CCNs in development have also been unveiled. In this review, we also focus on unresolved questions for future research.

CCNs interact with various types of extracellular and transmembrane proteins

CCNs have four principal domains led by a signal peptide sequence: IGFBP motif, VWC domain, thrombospondin type 1 (TSP1) domain, and the CT domain that contains several cystine-knot motifs. Each domain of CCN has binding motifs, but CCNs do not seem to have their own binding counterparts. For example, CCN2 has been called connective tissue 'growth factor', but as of now no specific receptor has been identified. In the case of CCN1, 2, and 3, co-immunoprecipitation or pull-down experiments revealed that VWC, TSP1, and CT domains bind to integrin, which is a heterodimeric (α/β) transmembrane protein (Perbal & Takigawa 2005). However, the binding integrin subtype is not identical; for example, VWC to $\alpha v\beta 3$, TSP1 to $\alpha 6\beta 1$, CT to $\alpha 6\beta 1$/HSPG or to $\alpha M\beta 2$/HSPG. Integrin interacts with various extracellular and intracellular proteins such as collagen, laminin, fibronectin, tetraspanin, and focal adhesion kinase. CCNs do not constitute specific counterparts to each integrin and should instead be considered as constituents of these integrin interacting protein complexes. The combination of the CCN/integrin complex will lead to a variety of cellular effects. So far, it has been reported that CCN/integrin association influences cell adhesion, migration, proliferation, survival, and protein production in mesenchymal cells such as fibroblasts, myoblasts, osteoblasts, chondroblasts, and endothelial cells (Babic *et al.* 1999; Gao & Brigstock 2006; Pi *et al.* 2008). These CCN/integrin interactions strongly affect angiogenesis and osteo/chondrogenesis at the tissue organization level. CCN1/integrin interactions enhance proangiogenesis by regulating the adhesiveness and motility of endothelial cells and smooth muscle cells (Leu *et al.* 2003; Chen *et al.* 2004). In primary cultures of chondrocytes, CCN1 promotes sulfate incorporation into HSPG and enhances cell–cell aggregations in the initial step in chondrogenesis, resulting in larger cartilage nodules. It is suggested that CCN2 stimulates extracellular matrix production through integrin signals in chondrocytes (Nishida *et al.* 2007). CCN2 directly interacts with fibronectin and enhances cell adhesion of chondrocytes through integrin $\alpha 5\beta 1$ (Hoshijima *et al.* 2006). Likewise, CCN3 has integrin-dependent enhancing activities for angiogenesis (Lin *et al.* 2003). Through these effects on chondrogenesis and angiogenesis, CCNs promote skeletogenesis by both endochondral and membranous ossification, which is discussed in the following section.

CCN interaction with LRP is important in development. LRP5/6 is now known to be a coactivator (coreceptor) in the Wnt signaling pathway to enhance receptivity of Frizzled by direct association. CCN1 binds directly to LRP6 and upregulates the Wnt signal (Mercurio *et al.* 2004). Likewise, CCN2 binds to LRP1, but this complex is rapidly internalized (Segarini *et al.* 2001). This is a similar effect of DKK against LRP5/6 (Hendrickx & Leyns 2008). It has been reported that LRP1 interaction with Frizzled-1 downregulates the Wnt signaling pathway (Zilberberg *et al.* 2004), and the effect of CCN2 would be interpreted to be removal of the corepressor (LRP1) of the Wnt signal. Thus, CCN may enhance the Wnt signal by both positive and negative interactions.

CCNs can bind to growth/cytokine factors such as the BMP family (BMP2, BMP4, TGFβ, etc.) and VEGF (Perbal & Takigawa 2005). For the BMP family, CCNs have an inhibitory effect like chordin or noggin (Abreu *et al.* 2002; Minamizato *et al.* 2007; Rydziel *et al.* 2007). CCNs probably regulate the cell surface localization of BMP, and may modulate their manner of interaction with its receptor although the locality of the CCN/BMP complex (either extracellular or intracellular) has not yet been fully investigated. CCN2 also binds to VEGF and

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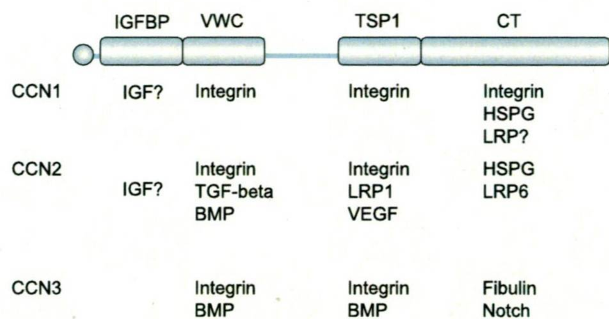

Fig. 1. Interacting proteins (extracellular and transmembrane type) and correspondence to modules of CCN1, CCN2 and CCN3. CCN has signal peptide sequence and four principal modules; insulin-like growth factor binding protein (IGFBP) motif, von Willebrand factor type C (VWC) domain, thrombospondin type 1 (TSP1) domain, and carboxy-terminal domain (CT) that contains a cystine-knot motif. Hinge domain is hypothesized between VWC and TSP1 domains in CCN1, but its corresponding region is not yet fully confirmed in other CCN.

inactivates its angiogenic properties (Inoki *et al.* 2002). This masking effect on VEGF is cancelled by CCN2 degradation by MMP-2 mediated proteolysis (Hashimoto *et al.* 2002). CCN2 may be involved in silent storage of VEGF in tissues and help release it when MMP-2 is activated in an emergency like blessing injury, which may result in rapid proliferation of blood vessels for wound repair.

Reported CCN-interacting proteins are summarized in Figure 1, but still other binding proteins are under investigation and await publication.

Xenopus CCN1 and gastrulation

In *Xenopus*, maternal *CCN1* mRNA is ubiquitously distributed in the cytoplasm of fertilized eggs, but it seems to be specifically translated in the blastocoel roof (dorsal side) by some effect just before gastrulation. Translated CCN1 regulates gastrulation by modulating Wnt signals (Latinkic *et al.* 2003) (Fig. 2). Injection of *CCN1* antisense morpholino oligonucleotides causes defects of gastrulation: the arrest of epiblast movement disrupting the epiboly structure in the dorsal marginal zone with severe delay of blastopore closure. This fact demonstrates that secondary axis formation induced by *Xenopus* wnt8 (Xwnt8) is inhibited by *CCN1* antisense morpholino oligonucleotides. The change in moving cell morphology is accompanied by the disassembly of the actin fibers. It is proposed that the action of CCN1 is mediated by the Wnt canonical pathway (β catenin nuclear translocation), but this should be reconfirmed because now it is known that Wnt planar activity is principally mediated by non-canonical pathways such as Rho-JNK or the Ca-phospholipaseC pathway (Witze *et al.* 2008). In fact, CCN2 can cause actin disassembly with concurrent downregulation of small GTPase Rho A (Crean *et al.* 2006). On the other hand, overexpression of *CCN1* also results in disruption of epiboly in the dorsal marginal zone, which is similar to the *CCN1* downregulation phenotype. It has been shown that gastrulation is inhibited either by downregulation or upregulation of *Xwnt11* (Tada & Smith 2000). Therefore, the optimal intensity of Wnt signaling exists in *Xenopus* gastrulation, and CCN1

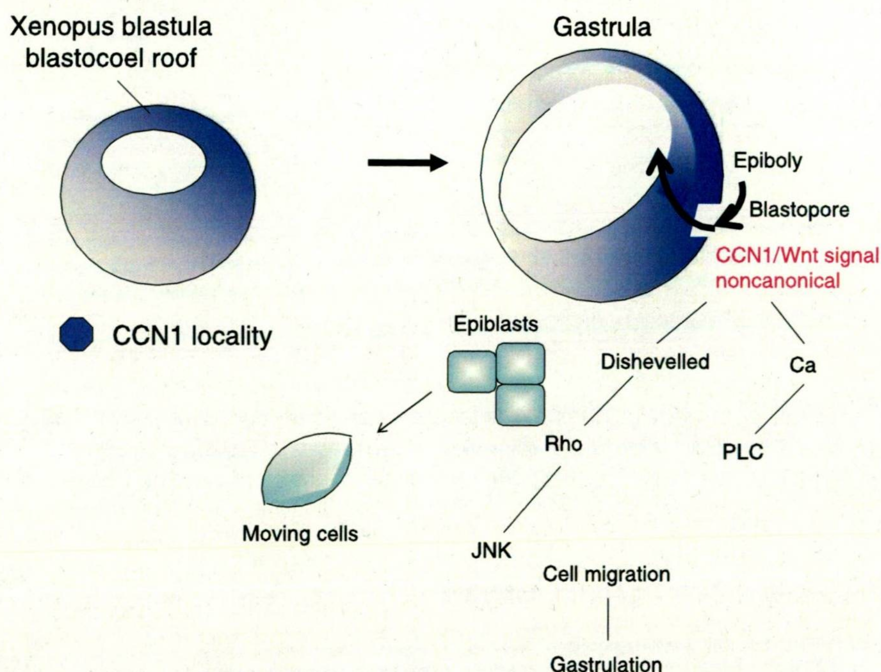

Fig. 2. Maternal effect of CCN1 in Xenopus. Maternal CCN1 mRNA distributed ubiquitously in unfertilized egg, but its translation seemed to occur specifically in the blastocoel roof before gastrulation. This verification is done by artificially injected mouse CCN1 mRNA injection and tracing with anti-mouse CCN1 antibody (Latinkic *et al.* 2003). CCN1 interacts with Wnt (probably non-canonical) signals and promotes the cell movement in involution. Downregulation or overexpression of CCN1 caused the severe delay of involution.

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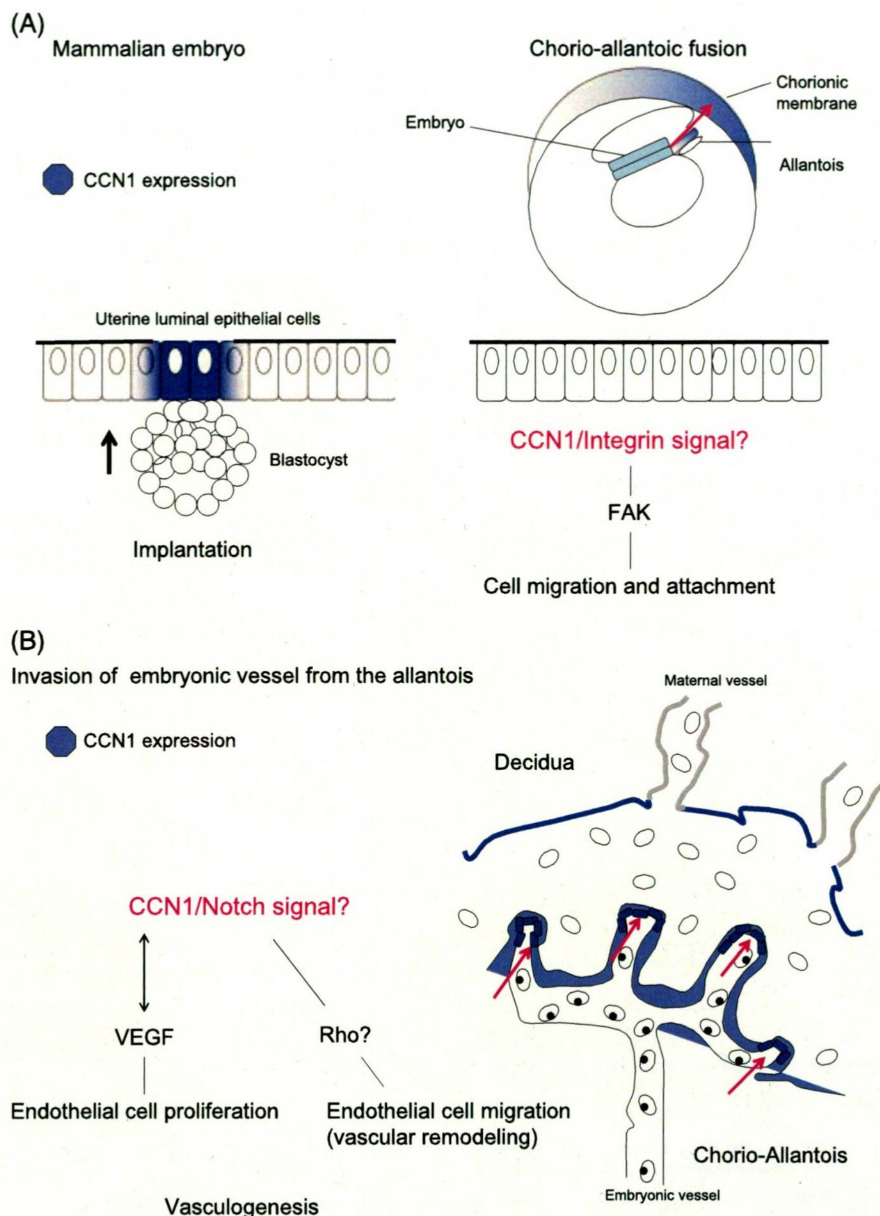

Fig. 3. (A) Maternal and extra-embryonic tissue expression of CCN1. Mouse CCN1 mRNA expression started in the uterine epithelial cells (Chen *et al.* 2006) induced by implantation of blastocyst. This expression may be considered as a maternal 'effecter' in mouse embryogenesis. Shortly after, embryonic CCN1 expression is induced in the chorioallantoic fusion area. Direct binding molecule to CCN1 is not yet confirmed, but possibly integrins may interact. CCN1 knockout mice showed abnormality in some individuals in the chorio-allantoic fusion stage. (B) CCN1 expression in placenta formation. After chorioallantoic fusion, CCN1 regulates the embryonic blood vessel bifurcation in villous formation. CCN1 expression is observed in choiro-allantois tissue and surface cells of the decidua. Both of them are derived from the extraembryonic tissue. Also in this stage, direct binding molecule to CCN1 is not yet confirmed, but Notch signal genes may be involved. CCN1 knockout mice showed the failure of blood vessel bifurcation, which resulted in a poor vasculogenesis.

may exert on this optimization. A recent investigation shows the importance of laminin/integrin association in gastrulation (Nakaya *et al.* 2008). CCN1 may be also involved in this interaction, orchestrating the organization of the basement membrane structure and epithelial mesenchymal transition. CCN activity has been studied from the aspect of cell differentiation and proliferation, but involvement of CCN in cell migration seems interesting as well since the role of CCN in tumor malignancy may be related to this property.

Mammalian CCN1 and implantation

CCN1 expression in early stage embryogenesis of mouse starts in the implantation stage, but is not in the embryonic tissue (Chen *et al.* 2006). *CCN1* is expressed in uterine epithelial cells where the blastocyst attaches (Fig. 3A). Several experiments demonstrate that this expression is embryo (blastocyst) dependent, and independent of the maternal hormonal condition. Following the embryo (blastocyst) invagination into the uterine wall (at E8.5), the chorionic tissue (embryonic tissue) extends from the dorsal part of the blastocyst. Then, allantois develops from the posterior-visceral side of the embryo and invades the chorionic tissue, forming the chorioallantoic fusion (Fig. 3A). This is the initial step of the labyrinth, a villous blood vasculature in the placenta. The chorion starts to branch, forming a complicated structure receiving the invasion of fetal vessels from the allantois (Fig. 3B). These chorioallantoic tissues enhance the expression

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of *CCN1* in the embryo before the chorioallantoic fusion stage. Most of the *CCN1* knockout embryos die due to the failure of chorioallantoic fusion or to the disruption of placental vessel sprouting in the embryonic side, which results in poor development of the placenta and systemic lethal edema of the embryo (Mo *et al.* 2002). Some surviving embryos after this stage exhibit lethal hemorrhagic rupture of the aorta and AVSD caused by precocious apoptosis in the atrial junction of the cushion tissue and impaired gelatinase activities in the muscular component of the interventricular septum, when fusion between the endocardial cushion tissue and the atrial and ventricular septa occurs (Mo & Lau 2006). Although these abnormalities may be partially interpreted as the impaired activity of VEGF, they are not fully explained by its proliferative activity for endothelial cells. *VEGF* haploinsufficiency (*VEGF* +/–) causes vast defects in vasculogenesis in extraembryonic tissues, but its effect is much stronger in the yolk sac tissue (Carmeliet *et al.* 1996). Additionally, it is noteworthy that knockout mice of Notch signal genes show close resemblance with CCN1 knockout mice, in which they show the failure of placenta blood vessel bifurcation (Gasperowicz & Otto 2008) or cardiovascular deformities (Watanabe *et al.* 2006; Fischer *et al.* 2007). VEGF has been considered to be an upstream mediator of the Notch signal in vasculogenesis (Weinstein & Lawson 2002), but recent studies have demonstrated that the Notch signal is sometimes upstream of the VEGF signal (Ota *et al.* 2007; Suchting *et al.* 2007), which indicates an interactive feedback loop for mutual gene expression between VEGF and Notch. It may be possible that CCN1 regulates this Notch/VEGF signal interaction by direct binding.

The effect of CCN1 in implantation is still in question since a conditional knockout of CCN1 in uterine epithelial cells has not yet been created. Its expression pattern in the uterus strongly indicates that CCN1 plays a role either in retraction of luminal epithelial cells to receive embryos or development of extraembryonic tissues. Considering the maternal effect of CCN1 in *Xenopus*, CCN1 induction in uterine epithelial cells may have some 'maternal effect' in mammals.

Recently, conditional knockout mice of CCN1 in chondroblasts using Sox9 promoter are created (K. Lyons, pers. comm., 2008). These cartilage-specific conditional knockout mice of CCN1 died in the perinatal stage although skeletogenesis is not so much drastically impaired. However, double knockout mice of CCN1 and CCN2 in cartilage demonstrated a severely delayed osteogenesis in long bones accompanied by an expansion of the hypertrophic chondrocyte zone (K. Lyons, pers. comm., 2008). These facts indicate that CCN1 is also important in osteo/chondrogenesis cooperating with CCN2.

Mammalian CCN2 and skeletogenesis

CCN2 is the most extensively investigated among the CCN family of genes, as over 50% of published CCN papers mainly concern CCN2 (B. Perbal, pers. comm., 2006). Most papers discuss its role in fibrosis (skin, lung, kidney, etc.) or in osteo/chondrogenesis. CCN2 is strongly expressed in a variety of tissues in midgestation embryos, with the highest levels in vascular tissues (endothelial cells and smooth muscles) and maturing chondrocytes. CCN2 knockout mice die just after birth due to respiratory failure (Ivkovic *et al.* 2003). This failure is attributed to hypoplasia of the thoracic skeleton (Fig. 4) and deformity of the oral cavity (palatal cleft and shortened mandible). CCN2 knockout mice show skeletal dysmorphisms as a result of impaired chondrocyte proliferation and reduced extracellular matrix composition within the hypertrophic chondrocytic zone in the growth plate. The lengths of individual ribs are not significantly reduced, but the extent of ossification is reduced in mutants, and the zone of mineralizing cartilage is expanded, suggesting defective replacement of cartilage by bone during endochondral ossification. Histologically, angiogenesis in the growth plate is impaired accompanied with defective replacement of cartilage by bone during endochondral ossification. These defects may be linked to decreased expression of *VEGF*. However, the effect of CCN2 is not simple because it has a masking effect on VEGF protein, which results in the suppression of angiogenesis (Hashimoto *et al.* 2002). This direct inhibition of VEGF is found in the TSP1 domain, but the CT domain acts as an angiogenic factor without any interaction with VEGF. These facts indicate its dual role of CCN and the mechanism of dysregulated skeletogenesis in CCN2 knockout mice should be further investigated. Recently, upregulation of a Notch downstream gene (*Hes1*) by *in vitro* induction of CCN2 was demonstrated in an osteoblast cell line (Smerdel-Ramoya *et al.* 2008a), which indicates the possibility of Notch signal involvement in skeletogenesis. CCN2 overexpressing mice were created driven by an osteoblast-specific (osteocalcin) promoter (Smerdel-Ramoya *et al.* 2008b) and the mice turned out to have decreased bone mineral density (decreased trabecular bone volume due to impaired osteoblastic activity). This skeletogenic phenotype is possibly due to the antagonizing effect of CCN2 against BMP, Wnt, and IGF-I signals. Controversial results of CCN2 knockout and transgenic mice may partially come from differential regulation of CCN2 expression depending on cell status, but may also indicate the complicated signal crosstalk related to CCN2.

The phenotype exhibited by the introduction of CCN2 transgenes is subtle but discrete in zebrafish. Osteogenic

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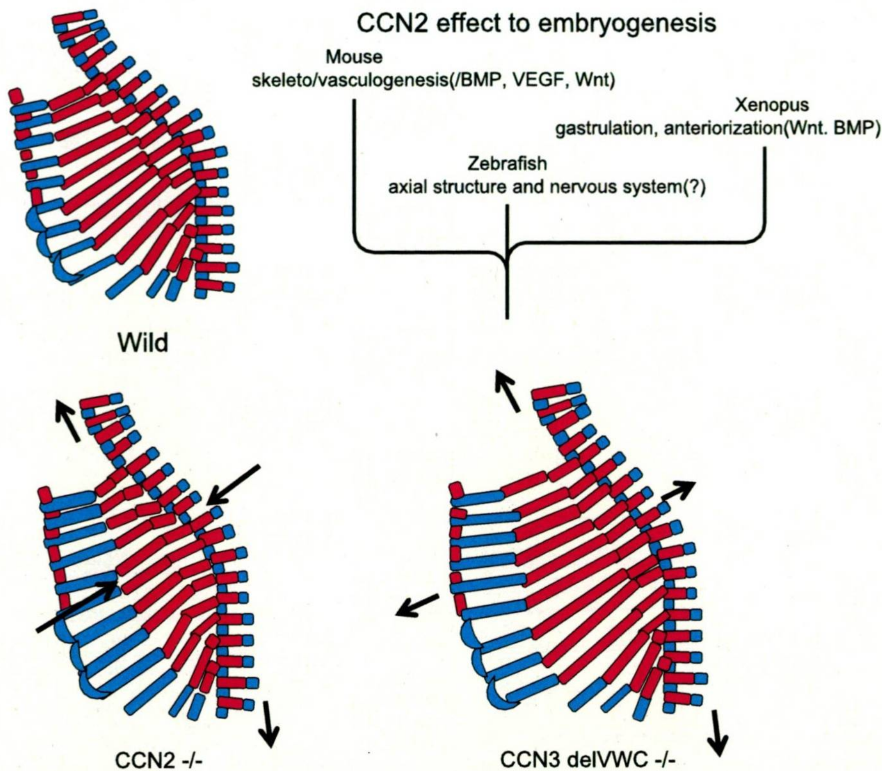

Fig. 4. Schematic presentation of skeletal deformity of CCN knockout mice fetus (~E19. Alizarin Red-Alcian Blue stains). CCN2 knockout mice (CCN2 -/-) exhibit no significant difference in the lengths of individual ribs, but the extent of ossification is reduced in knockout mice, and the zone of mineralizing cartilage is expanded, suggesting defective replacement of cartilage by bone during endochondral ossification. On the contrary, practical CCN3 knockout mice (CCN3 delVWC -/-) exhibit the barrel-shaped thorax due to the elongation of each rib. Vertebrae also showed overgrowth and enlargement. This change of the bones is formed by the elongation of both the bone and mineralizing cartilage. CCN2 interactions are more or less conserved among species, but the phenotype of downregulation or overexpression is rather different.

dysmorphisms by antisense morpholino oligonucleotides of CCN2 are represented principally by a distorted notochord and shortened mandibles (Chiou *et al.* 2006), which are similar to the phenotype of CCN2 knockout mice. This morphant zebrafish also shows deformity in nervous system development such as a lack of eyes and reduced development of the cerebrum, which are not observed in CCN2 knockout mice. In *Xenopus*, downregulation of CCN2 by antisense morpholino oligonucleotides has an anti-dorsalizing effect, which is mediated by the inhibition of Wnt signals similar to CCN1 (Mercurio *et al.* 2004). This effect in *Xenopus* is mediated by direct binding to LRP6, a coreceptor of Wnt and inhibited non-canonical pathway. On the other hand, overexpression of CCN2 in *Xenopus* induces a secondary axis formation antagonizing ventralization (Abreu *et al.* 2002).

Mammalian and avian CCN3

A report of a genuine CCN3 knockout has not yet been published, but practical *CCN3* knockout mice were created by homozygous deletion of the VWC domain (*CCN3 delVWC*). These VWC-targeted knockout mice not only lost the original full length CCN3, but almost lost the VWC deleted form itself. These knockout mice exhibit abnormal skeletal (Fig. 5) and cardiac development, which are probably responsible for the high ratio of fetal and neonatal death of the mice. They showed hyperproliferative long bones, accompanied by enhanced differentiation of osteocytes and chondrocytes. However, the interpretation of these phenotypes is problematic because the molecular mechanism of the VWC targeting effect is unclear (Perbal 2008).

In early stages of chicken embryo, we showed that CCN3 is expressed in a left–right asymmetrical pattern in Hensen's node area (Fig. 5) (Katsube *et al.* 2001). Little is understood about how CCN3 expression is related to a left–right asymmetrical pattern during involution, but it is noteworthy that Sonic Hedgehog expression is almost identical to that of CCN3 in the node area. Genes involved in left–right asymmetry are classified into several different categories: secreted, cell surface, cytosolic, and nuclear. Given that CCN3 is a secreted protein, the BMP superfamily (Nodal, Lefty1, GDF1) is the most plausible candidate for interaction. CCN3 may regulate cell movement like CCN1 in *Xenopus* gastrulation, but this remains to be demonstrated. Preliminary experiments with CCN3 overexpression in *Xenopus* show that CCN3 does not share the activities of CCN1 (Latinkic, commented in [Perbal & Takigawa 2005]).

Since the expression pattern of CCN3 is reminiscent of Notch1 or Delta1 in the presomitic mesoderm, we suspected that CCN3 may be related to Notch signaling. We carried out co-immunoprecipitation assays and

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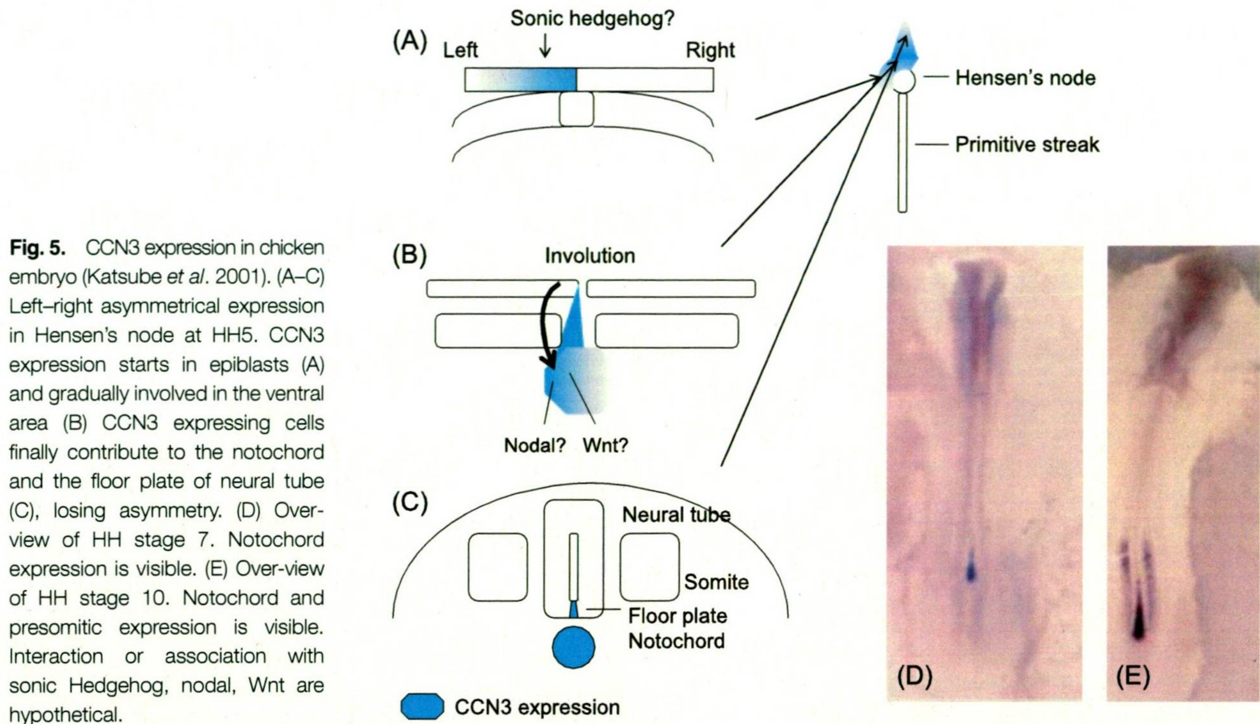

Fig. 5. CCN3 expression in chicken embryo (Katsube *et al.* 2001). (A–C) Left–right asymmetrical expression in Hensen's node at HH5. CCN3 expression starts in epiblasts (A) and gradually involved in the ventral area (B) CCN3 expressing cells finally contribute to the notochord and the floor plate of neural tube (C), losing asymmetry. (D) Over-view of HH stage 7. Notochord expression is visible. (E) Over-view of HH stage 10. Notochord and presomitic expression is visible. Interaction or association with sonic Hedgehog, nodal, Wnt are hypothetical.

showed an association between CCN3 and Notch1 (Sakamoto *et al.* 2002) and verified that the CT domain of CCN3 specifically recognizes the several different EGF motifs of Notch1 (Sakamoto *et al.* 2002). CCN3 association stimulates the Notch signal, and suppresses the differentiation of mesenchymal stem cells. We demonstrated the effect of CCN3 on Notch signaling in a myoblastic cell line (C2/4, a sub-line of C2C12) and later in osteoblastic cell lines, which resulted in the suppression of osteogenesis (MC3T3 E1 and KusaA-1. Notch authentic ligands like Delta or Jagged recognize a specific motif in Notch extracellular EGF repeats (EGF34–36) while CCN3 binds to several different sites of EGF motifs. This manner of interaction indicates that the CCN3/Notch1 association is not usual ligand-receptor interaction, but rather a supporting effect (coactivator) to Notch authentic ligand.

In *Xenopus*, a CCN2-BMP interaction was reported (Abreu *et al.* 2002), and we also examined CCN3-BMP binding in mouse osteoblastic cell lines (Minamizato *et al.* 2007). BMP signal inhibition causes a low phosphorylated state of SMAD 1/5/8 proteins. CCN3 blocks the BMP signal like chordin or noggin, which indicates a complicated but coordinated signal crosstalk in osteogenesis (Fig. 6). Notch stimulation upregulates Hey1, a member of the basic HLH family, which results in the inhibition of stem cell (here, osteoblasts) differentiation, which results in the suppression of osteoblast differentiation. Thus, enhancement of the Notch signal

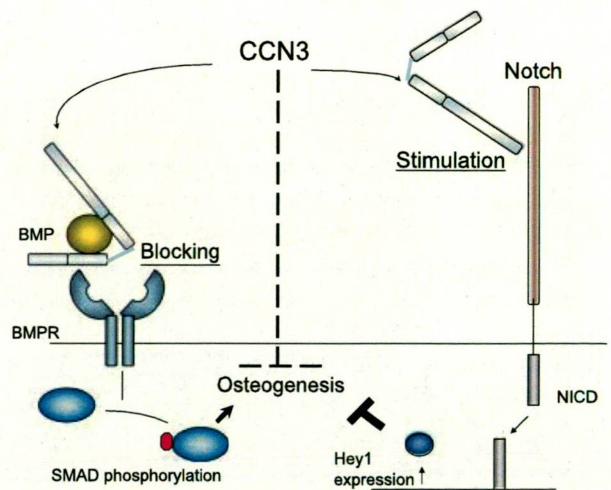

Fig. 6. Coordinated inhibitory effect of CCN3 on osteogenesis (Sakamoto *et al.* 2002; Minamizato *et al.* 2007; Katsuki *et al.* 2008). CCN3 stimulates Notch signal and blocks the bone morphogenetic protein (BMP) binding to the receptor. Notch upregulated several Notch specific basic HLH transcriptional factors and Hey1 (Hes1) is one of the principal factors in osteogenic stem cells (Sakamoto *et al.* 2002). BMP stimulation upregulated the phosphorylation of SMAD proteins, which resulted in the osteogenic factors expression such as RUNX2 (Minamizato *et al.* 2007; Katsuki *et al.* 2008). Low density lipoprotein receptor-related protein (LRP) interaction is also interpreted as the same with BMP. LRP function is suppressed, which resulted in the low reception of frizzled in the Wnt signal pathways. Wnt signal is also important for osteogenesis. BMP signal block and Notch signal activation simultaneously inhibit osteogenesis.

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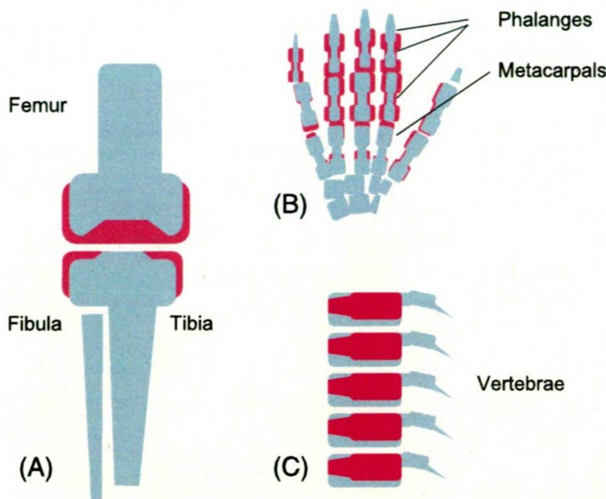

Fig. 7. Schematic view of progressive pseudorheumatoid dysplasia (PPD) in human. (A) Pale green zone shows the normal bone structure and red zone shows the deformity caused by PPD. Knee joint demonstrates enlargement of the femoral and tibial epiphyses and a reduction in cartilage thickness causing narrowing of the joint cavity space. (B) Hand bones demonstrate enlargement of the epiphyseal and metaphyseal portions of the metacarpals and phalanges. (C) Vertebral corpus shows the flattening structure and the beaking of the anterior part.

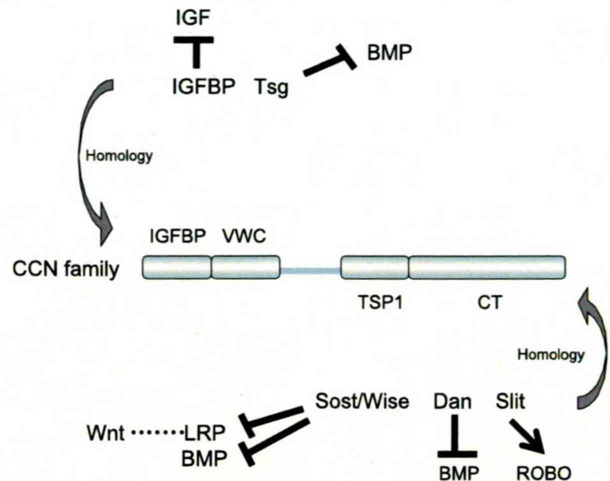

Fig. 8. Structural similarity of CCN and relative gene families. Insulin like growth factor binding protein (IGFBP) and Twisted gastrulation (TSG) have homology in the c-terminal part of CCN (IGFBP and von Willebrand factor type C [VWC]). Sost/Wise, Dan and Slit have homology in the N-terminal part of CCN (TSP1 and CT). These relative families have modulator effects on bone morphogenetic protein (BMP) and low density lipoprotein receptor-related protein (LRP). Only Slit is different and is a ligand protein of ROBO in axonal guidance.

and inhibition of the BMP signal synergistically suppress osteogenesis (Katsuki *et al.* 2008) (Fig. 6). The group of E. Canalis emphasizes the priority of BMP signal inhibition (Calhabeu *et al.* 2006; Parisi *et al.* 2006; Rydziel *et al.* 2007) rather than Notch, but our results demonstrate that Notch stimulation is equally important for osteogenesis. So far, the priority problem between Notch and BMP (occasionally, Wnt) could be attributed to the different cell status of the used cell line. Probably, CCN3 acts in a context-dependent manner in mesenchymal stem cells or in mature osteoblasts *in vivo*, changing the principal counterpart such as BMP and Notch (Katsuki *et al.* 2008).

CCN6 as a cause of human genetic skeletal disorder, PPD

Progressive pseudorheumatoid dysplasia is a rare human autosomal recessive skeletal disorder characterized by progressive disruption of articular cartilage. PPD patients show no overt phenotype at birth, and the disorder starts from early childhood. PPD shows clinical symptoms such as stiffness and swelling of joints, motor weakness, and joint contractures like rheumatoid arthritis, but histologically, there are no inflammatory signs (Zhou *et al.* 2007). Clinicopathological examination indicates pathologic exudation and enhanced bone metabolism accompanied by activated secondary osteoarthritis with no synovitis (Ehl *et al.* 2004). Radiographic examination depicts PPD as a loss of joint space, widened epiphyses, and vertebral flattening (Fig. 7) (Marik *et al.* 2004). Cell biological studies using patient articular chondrocytes demonstrated increased cell proliferation and a decreased apoptosis ratio accompanied by downregulation of MMPs (Zhou *et al.* 2007). From a mutation search by linkage analyses of PPD, point mutations and deletions of *CCN6* were detected in several independent cases (Hurvitz *et al.* 1999). It has not yet been confirmed whether the CCN6 mutation causes a loss or gain of function in PPD. To determine the biological aspect of the mutation, *CCN6* knockout mice were created, but unexpectedly, they showed no apparent phenotype (Kutz *et al.* 2005). Overexpression of CCN6 in mice also showed no specific phenotype (Kutz *et al.* 2005). Until now, CCN6 transcription is fairly detected in any mouse tissue although it is not a true pseudogene (L. Lau, pers. comm., 2008). This fact indicates that CCN6 may lose its original significance in mouse. In zebrafish, downregulation of CCN6 by antisense morpholino oligonucleotides caused developmental abnormalities such as reduced size of the mandibular cartilage and alteration of other pharyngeal cartilages (Nakamura *et al.* 2007). *CCN6* overexpression in zebrafish caused a decrease in ventral structure, and a concomitant expansion of dorsal cell fates. The pattern of disorders is different between zebrafish and humans, but these phenotypes seem to be attributed to a

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