Original Article

Involvement of multiple CCN family members in platelets that support regeneration of joint tissues

Chikako Hara¹,², Satoshi Kubota¹,³, Takashi Nishida¹, Miki Hiasa⁴, Takako Hattori¹, Eriko Aoyama³, Yoshinori Moriyama⁴, Hiroshi Kamioka², Masaharu Takigawa¹,³

¹Department of Biochemistry and Molecular Dentistry, ²Department of Orthodontics, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, ³Advanced Research Center for Oral and Craniofacial Sciences, Okayama University Dental School, ⁴Department of Membrane Biochemistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

Number of text pages and figure legends: 29
Number of tables: 1
Number of figures: 8

Corresponding authors: Satoshi Kubota and Masaharu Takigawa

Department of Biochemistry and Molecular Dentistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences
2-5-1 Shikata-cho, Kita-ku, Okayama 700-8525, Japan
E-mail: kubota1@md.okayama-u.ac.jp (S.K.), takigawa@md.okayama-u.ac.jp (M.T.)

Key words: cartilage · CCN family · megakaryocyte · platelet · regeneration
Abstract

**Objectives** Platelet-rich plasma (PRP) has been widely used to enhance the regeneration of damaged joint tissues, such as osteoarthritic and rheumatoid arthritic cartilage. In this study, we aimed to clarify the involvement of all of the CCN family proteins that are crucially associated with joint tissue regeneration.

**Methods** CCN family proteins in human platelets and megakaryocytic cells were comprehensively analyzed by Western blotting analysis. Production of CCN family proteins in megakaryocytes *in vivo* was confirmed by immunofluorescence analysis of mouse bone marrow cells. Effects of CCN family proteins found in platelets on chondrocytes were evaluated by using human chondrocytic HCS-2/8 cells.

**Results** Inclusion of CCN2, a mesenchymal tissue regenerator, was confirmed. Of note, CCN3, which counteracts CCN2, was newly found encapsulated in platelets. Interestingly, these 2 family members were not detectable in megakaryocytic cells, but their external origins were suggested. Furthermore, we for the first time found CCN5 and CCN1 that inhibits ADAMTS4 both in platelets and megakaryocytes. Finally, application of a CCN family cocktail mimicking platelets onto HCS-2/8 cells enhanced their chondrocytic phenotype.

**Conclusions** Multiple inclusion of CCN1, 2 and 3 in platelets was clarified, which supports the harmonized regenerative potential of PRP in joint therapeutics.
**Introduction**

Autologous platelets are widely utilized in regenerative medicine in a form of platelet-rich plasma (PRP), or other derivatives. These anuclear cells contain a number of growth factors and cytokines that are mostly encapsulated in $\alpha$-granules, which collectively promotes tissue regeneration through inflammation, extracellular matrix synthesis and angiogenesis. PRP is individually prepared platelets concentrated in the plasma for autologous implantation. The clinical application of PRP has been examined for the treatment of damaged soft and hard tissues including bone, joint, skin, eye and even neuron [1-4]. Especially, utility of PRP in joint tissue repair has been suggested by a number of clinical studies, and thus are actually employed in orthopaedic clinics [5]. Indeed, several recent reports showed that PRP effectively ameliorated the symptom of osteroarthritis (OA) and even rheumatoid arthritis (RA) [6,7]. Nevertheless, the effect of PRP treatment on OA and other joint disorders are occasionally variable, as typically represented in anterior cruciate ligament surgery [8]. Therefore, further investigation on the biologically active factors involved in platelets, which profoundly affects the clinical outcome, is now required, in order to establish a clinical protocol that would yield stable results.

Collagen synthesis is a critical step in the process of connective tissue regeneration. In this point of view, the involvement of the Cyr61-CTGF-NOV (CCN) family protein 2, formally designated as connective tissue growth factor, in platelets is of particular note [9]. CCN family is a unique family of secretory proteins that are involved in a number of physiological and pathological events in human body. This family of proteins consists of six members, and 5 out of 6 members have four conserved modules, whereas
only CCN5 has three modules [10-13]. In addition to the classical 3 members; cysteine-rich 61(Cyr61), connective tissue growth factor (CTGF) and nephroblastoma-overexpressed (NOV), 3 members with the initial names of Wnt-inducible signaling protein (WISP)-3, 4 and 4 belong to this family. The CCN proteins are mainly present in the extracellular milieu and interact with a vast number of biomolecules, including cell-surface receptors, extra-cellular matrix (ECM) components, growth factors, and proteases to modulate cellular function. Owing to such functional characteristics, these proteins are recently categorized to matricellular proteins [12].

The profound involvement of CCN2 in the wound healing process and the development of fibrosis is widely recognized [14-19]. In the context of rheumatology, it should be noted that CCN2 promotes regeneration of cartilaginous and bony tissues [20,21]. As PRP does, intra-articular application of CCN2 regenerates OA cartilage in a rat model [21]. Also, overexpression of CCN2 provides resistance to OA development in mice [22]. Platelets indeed contain CCN2 higher in amount than other growth factors in platelets, which again suggests a critical role of CCN2 in tissue repair [23,24].

However, recent research on CCN family is uncovering that, not only CCN2, but also other CCN family members may play certain roles in wound healing and tissue repair. Although less is known about the function of CCN4, this CCN family member was reported to enhance ECM deposition in a similar manner to CCN2 [25,26]. On the other hand, CCN1 and CCN3 counteracts fibrogenesis. CCN3 downregulates fibrogenesis principally by repressing the gene expression of profibrotic CCN2 and CCN4 [27-29]. Unlike the mechanism of CCN3 action, CCN1 controls fibrogenesis by invoking the induction of cellular senescence [12,30]. Recently, a novel function of
CCN1 as an inhibitor of ADAMTS4 was unveiled as well [31]. Since ADAMTS4 is known as a major enzyme to degrade the major cartilaginous proteoglycan, aggrecan, CCN1 is expected to counteract OA development. Nevertheless, no information has been available concerning the CCN family members other than CCN2 in platelets.

In contrast to OA, the role of CCN2 in RA is of significant controversy. A previous report suggested CCN2 as a mediator of RA development [32], whereas application of PRP containing abundant CCN2 reduced the inflammation in an RA model [7]. Here, the opposing functionalities of CCN2 and other family members should be noted. Suspecting the inclusion of the CCN family members other than CCN2, here we comprehensively analyzed all of the CCN family members in platelets. As a result, we found involvement of multiple CCN family members, which could collaboratively support the effect of PRP on OA and RA. Possible molecular origin of each CCN family member is also suggested.

**Materials and Methods**

*Cell culture*

Human megakaryocytic cell lines, MEG-01 [33] and CMK [34], were maintained in RPMI1640 containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in air containing 5% CO₂.

MEG-01 cells (5.0 × 10⁶ cells) were seeded in four 10 cm dishes and were treated with 0.4 μg/ml aphidicolin to induce the differentiation for 0 to 3 days at 37 °C. Alternatively, cytodifferentiation of MEG-01 cells (6.0 × 10⁵ cells) were mildly induced by treating either with valploic acid (VPA), or with all trans retinoic acid (ATRA) under the concentration of 2 × 10⁻³ M or 10⁻¹¹ M for 20 days, respectively. Thereafter, the
MEG-01 cells and platelet-like particles were collected at 5, 10, 15, 20 days by centrifugation (400 RCF or 1500 RCF) for 5 min at room temperature, respectively.

CMK cells (5.0 × 10^6 cells) were seeded in 10 cm dishes and cultured until reaching confluent. The cells were collected by centrifugation for 5 min at room temperature.

Human chondrocytic HCS-2/8 cells [35] cells were cultured in Dulbecco's modification of Eagle's minimum essential medium (D-MEM) containing 10% FBS until reaching 80-90% confluence. For the evaluation of the effects of CCN family proteins, the medium was changed with D-MEM containing 0.5% FBS 12 h before the addition of CCN family proteins. Thereafter, CCN2 alone (final concentration, 50 ng/ml with 150 ng/ml of BSA), a combination of CCN1/2/3/5 (final concentration, 50 ng/ml each), or bovine serum albumin (BSA)(final concentration, 200 ng/ml) was added. For the evaluation of the effects of the conditioned media, the medium was replaced with D-MEM containing 20% of a medium conditioned by MEG-01. Twelve hours after these treatments, cells were harvested for RNA analysis.

*Antibodies and recombinant proteins*

For the specific detection of CCN family proteins and β-actin by Western blotting, we employed the antibodies described below: SC-8560 (lot. C2509) goat polyclonal antibody against human CCN1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); SC-18678 (lot. G0108) goat polyclonal antibody against human CCN3 (Santa Cruz Biotechnology); SC-25441 (lot. B2806) rabbit polyclonal antibody against human CCN4 (Santa Cruz Biotechnology); ab38317 (lot. GR11842-7) rabbit polyclonal antibody against human CCN5 (Abcam, Cambridge, MA, USA); ab103263 (lot. GR72997-1) rabbit polyclonal
antibody against human CCN6 (Abcam); A2228 (lot. 112M4762V) mouse monoclonal antibody against human β-actin (SIGMA, St. Louis, MO, USA). A rabbit polyclonal antibody against human CCN2, α-CTGFw, was produced and prepared as described previously [23].

As secondary antibodies, we employed SC-2020 (lot. B0912) donkey anti-goat IgG-HRP (Santa Cruz Biotechnology), ab97085 (lot. GR66988-2) donkey anti-rabbit IgG-HRP (Abcam) and 18-8817-31 (lot. 28959) rat anti-mouse IgG-HRP (ROCKLAND, Gilbertsville, PA, USA) for the detection of corresponding primary antibodies.

The recombinant human CCN1, CCN4, CCN5, CCN6 were purchased from Pepro Tech (Rocky Hill, NJ, USA). These proteins were diluted with 0.1 % bovine serum albumin (BSA)-phosphate buffered saline (PBS) to a concentration of 0.1 mg/ml. The recombinant human CCN2 (500 μg/ml) was purchased from BioVendor Laboratory Medicine (Karasek, Brno, Czech). The recombinant human CCN3 (200 μg/ml) was a gift from Takako Sasaki (Department of Matrix Medicine, Faculty of Medicine, Oita Univ., Oita, Japan).

In flow cytometric analysis, a PE mouse anti-human CD41a; 555467 (lot. 17016; BD Biosciences, Franklin Lakes, NJ, USA) was employed to monitor the differentiation along megakaryocytic lineage. As a control staining, we used a mouse IgG K Isotype Control PE; 12-4714-73 (lot. E033380; eBioscience, San Diego, CA, USA).

For the immunofluorescence analysis of CCN1, the same antibody was employed as that was used in Western blotting. However, since the antibody used for the detection of human CCN5 did not cross-react to murine CCN5, we used another antibody; SC-25442 (lot. E2214) rabbit polyclonal antibody against human CCN5 (Santa Cruz Biotechnology) for the immunofluorescence analysis. As secondary antibodies, we used Life
Technologies A11055 (lot. 1605893) donkey anti-goat IgG, Alexa Fluor® 488 conjugate or A11034 (lot. 1386400) goat anti-rabbit IgG, Alexa Fluor® 488 conjugate (Thermofisher Scientific, Waltham, MA, USA).

**Protein extraction and Western blotting analysis**

Human platelets from a healthy donor were provided by AllCells LLC (Berkley, CA, USA). Protein extraction from the cells was performed with RIPA buffer (50 mM Tris-HCl, 0.15M NaCl, 4 mM EDTA, 1 % Nonidet P-40, 0.1 % sodium deoxycholate). Extracted total proteins (10 μg) were mixed with equal volumes of 2 x sodium dodecyl sulfate (SDS) sample buffer containing 5 % 2-mercaptoethanol and heated at 100 °C for 5 min. The proteins were separated by 10 % Bis-Tris gel electrophoresis and transferred onto a polyvinylidenedifluoride (PVDF) membrane (GE Healthcare, Waukesha, WI, USA). After being transferred, the membrane was blocked with 5 % skim milk, incubated at 4 °C over night with 500-fold-diluted antibody against human CCN1, 1000-fold-diluted antibody against human CCN2, 100-fold-diluted antibody against human CCN3, 100-fold-diluted antibody against human CCN4, 100-fold-diluted antibody against human CCN5, 500-fold-diluted antibody against human CCN6 or 2000-fold-diluted antibody against β-actin. After wash, the membrane was incubated with each secondary antibody for 1 h at 4 °C. The dilutions of secondary antibodies applied were as follows: an anti-goat IgG-HRP was used at a 3000-fold dilution, an anti-mouse IgG-HRP was used at a 2000-fold dilution, and an anti-rabbit IgG-HRP was used at a 3000-fold or 4000-fold dilution. The blot was visualized by use of an enhanced chemiluminescence (ECL) Western blotting detection system.
RNA extraction and real-time reverse transcription PCR (RT-PCR) analysis

Total RNA was extracted from the cells and purified with ISOGEN (NIPPON GENE CO., Tokyo, Japan), following the manufacture’s protocol. Reverse transcription was carried out by using avian myeloblastosis virus (AMV) reverse transcriptase (Takara) at 42 °C for 30 min. Amplification reactions were performed with a SYBR® Green Real-time PCR Master Mix (TOYOBO, Tokyo, Japan) by using StepOne™ software v2.1 (Applied Biosystems, Foster City, CA, USA).

The nucleotide sequences of the primer sets used were as follows: **CCN2** (sense, 5’-TGC GAG GAG TGG GTG TGT GAC-3’; anti-sense, 5’-TGG ACC AGG CAG TTG CCT CTA ATC-3’); **CCN3** (sense, 5’-GAG CAG TGC TGC CAA TCT ACA GCG AAG-3’; anti-sense, 5’-AGA TGG AGA AGC AGG AAG GTC AGG-3’); aggrecan (**ACAN**) (sense, 5’-TCT TGG AGA AGG GAG TCC AAC TCT-3’; anti-sense, 5’-ACA GCT GCA GTG ATG ACC CTC AGA-3’); type II collagen (**COL2A1**) (sense, 5’-TGC TGT TCT TGC AGT GGT AGG TGA-3’; anti-sense, 5’-AGA AGA ACT GGT GGA GCA GCA AGA-3’); glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**) (sense, 5’-GCC AAA AGG GTC ATC ATC TC-3’; anti-sense, 5’-GTC TTC TGG GTG GCA GTG AT-3’).

Flow cytometry

Flow cytometric analysis of megakaryocytes was performed using a BD ACCURI C6 (BD Biosciences, Franklin Lakes, NJ, USA). The cells were stained with the anti-CD41a antibody or an isotype control for 30 minutes and were washed three times with PBS containing 0.5% BSA. The 3 x 10⁴ events were acquired and the list mode files were analyzed with FLOW JO (FLOW JO, LLC, OR, USA).
**Immunofluorescence analysis of mouse bone marrow cells**

Normal mouse bone marrow cell suspension was obtained from femur and tibia of 8–12 w male Balb/cJ mice. After the bone marrow cells were flushed out with PBS and filtered through 100 μm cell strainer (CORNING, Wiesbaden, Germany), the blood corpuscles were removed with the Ammonium-Chloride-Potassium (ACK) buffer (0.15M NH₄Cl, 10mM KHCO₃, 0.1mM Na₂ EDTA, pH 7.2- 7.4). Then, one drop of a sample on the glass microscopic slide was spread by using cover glass as a wedge smear technique. The cells were fixed in a 4% formaldehyde solution for 10 minutes at room temperature and washed with PBS three times. After permeabilization with 0.5% Tween-PBS for 10 minutes at room temperature, they were blocked with blocking buffer (10% normal serum/ 0.3M glycine/ 0.1% BSA, 0.1% Tween-PBS) and stained by the primary antibodies at 4 °C over night. Next day, the slides were washed with PBS three times and stained by the secondary antibody 1000-fold-diluted with blocking buffer containing DAPI (1 µg/ml) for 1 h at room temperature. The smears were viewed, and the images were captured by using a Keyence BZ-X710 all-in-one fluorescence microscope (Osaka, Japan).

**Statistical analysis**

Unless otherwise specified, the experiments were performed at least 3 times, yielding comparable results. Statistical comparisons between the groups were performed by using Student’s t-test.

**Results**
CCN1 and CCN5 found both in platelets and megakaryocytic cells

Upon the analysis of the involvement of CCN family proteins in platelets, we also investigated whether these molecules were produced by megakaryocytes per se. However, it is quite difficult to induce terminal differentiation of megakaryocytes in a pure culture of human haematopoietic progenitors without feeder cells. Thus, we employed a human megakaryocytic cell line, MEG-01, which can be differentiated to form platelet-like particles in vitro without feeders. Following an established protocol, differentiation was rapidly induced in MEG-01 cells by aphidicolin, which was confirmed by the expression of megakaryocytic marker genes, such as GATA1, NFE2 and TUBB (data not shown). Utilizing this system, we comparatively investigated the CCN family members in human platelets and their producers.

The first CCN family member, CCN1, and CCN5 are both produced in the proliferative zone of growth plate cartilage [16], suggesting their roles in endochondral bone formation. In addition, CCN1 is reported to terminate fibrotic post-inflammatory response by inducing cellular senescence [30], whereas CCN5 was once reported to counteract CCN2 during cardiac hypertrophy [36]. These previous findings suggest possible involvement of these 2 CCN family members in tissue remodeling as well. However, there still is no report describing whether these CCN family members are present in platelets, or not. In order to clarify this point, we initially examined if CCN1 and CCN5 were included in human platelets. As shown in Fig. 1, a distinct band around the expected apparent molecular weight was observed for each member, indicating the inclusion of CCN1 and CCN5 in human platelets. The involvement of CCN1 in platelets is of particular note in the context of arthritis, since a recent report revealed a novel functional aspect of CCN1 as a specific inhibitor of ADAMTS4 [31].
Next, we estimated whether these proteins are endogenously supplied from the platelet producer, or not, using the in vitro models for megakaryocytes; one is the MEG-01 system, and the other is the CMK cell stably maintaining mature megakaryocytic phenotype. Western blotting analysis of CCN1 and CCN5 of the cell lysates revealed that both CCN1 and CCN5 are present in these megakaryocytic cells, regardless of differentiation stages (Fig. 1). Expression of CCN1 and CCN5 in those cells was also confirmed by RT-PCR analysis (data not shown). These results suggest that the source of CCN1 and CCN5 in platelets is their producer, megakaryocyte, itself.

**Co-persistence of CCN2 and CCN3 in platelets, which are both absent in megakaryocytic cells**

CCN2 is strongly expressed in the prehypertrophic chondrocytes and promote the entire process of endochondral ossification, while CCN3 is known to functionally counteract CCN2 in a variety of mesenchymal tissues [27-29]. Inclusion of CCN2 in platelets is already known [23,24], and both CCN2 and PRP are shown to improve damaged joint tissues [1,2,20,21,23]. On the other hand, a recent report suggested that CCN2 might mediate the development of rheumatoid arthritis (RA) [32]. Suspecting the involvement of a factor that regulate CCN2 action, we next analyzed whether CCN3 as well as CCN2 is encapsulated in platelets, or not.

Western blotting of the lysates from these cells confirmed that CCN2 was encapsulated in platelets, but was not in megakaryocytic cells. Interestingly, comparable findings were observed with CCN3. Indeed, Western blotting analysis to detect CCN3 revealed that this protein was also present in platelets, but was absent in both megakaryocytic cell lines examined (Fig. 2). These data suggest collaborative
roles of CCN2 and CCN3 in platelet function and the external origin of CCN3 in platelets, as indicated in the case of CCN2.

**CCN family members undetectable in platelets**

Osteogenic function of CCN4 is recently reported [37], and CCN6 is known as a causative gene of pseudorheumatoid dysplasia [38], indicating their positive role in mineralized tissues. In addition, since both family members are indicated to be related to fibrotic tissue remodeling [25,26,39], these 2 family members may be expected to be present in platelets, considering their possible ability to enhance ECM deposition. However, our examination revealed that these proteins are below the level of the detection threshold either in platelets or megakaryocytes throughout the cell culture period by Western blotting analysis. On the other hand, we observed distinct signals from the recombinant CCN4, CCN6 proteins, indicating that the employed antibodies were potent, and that the analysis was performed properly (Fig. 3).

**No CCN2 or CCN3 production by MEG-01 even under mild differentiation inducing conditions**

To further confirm that CCN2 and CCN3 may not be produced by megakaryocytes, an alternative differentiation protocol, which was recently established, was employed to induce terminal differentiation in MEG-01 cells. In this strategy, differentiation is induced by long-term treatment with all trans retinoic acid (ATRA) or valproic acid (VPA) [40]. As shown in Fig. 4, VPA-induced culture produced more CD41 cells, showing terminally-differentiated cell morphology with particle deposition. Nevertheless, even in those cells, no signals indicating CCN2 and CCN3 production
were observed, confirming the results with aphidicolin. Additionally, although its biological significance is unclear, it is interesting to note that β-actin appears to be partially processed during long-term cell culture.

**Presence of CCN1 and CCN5 in primary megakaryocytes in vivo**

Finally, in order to verify if CCN1 and CCN5 are actually produced in megakaryocytes *in vivo*, smear samples of bone marrow cells were prepared from normal mice and were subjected to immunohistological analyses. A significant number of megakaryocytes were observed in these samples by regular May-Gruenwald-Giemsa staining. Immunofluorescence analysis of these cells with specific antibodies against murine CCN1 or CCN5 revealed specific signals in megakaryocytes, whereas no significant staining was observed in other cells from the bone marrow (Fig. 5a and 5b). We found >70% of megakaryocytes were positive for CCN1 and CCN5, both were found co-localized in the same cell (Fig. 5c). The same analysis with anti-CCN2 and anti-CCN3 antibodies revealed no signals in megakaryocytes, which serve as negative controls. Of note, positive signals for CCN3 were observed in small spherical cells, which are supposed to be haematopoietic stem cells [12]. Several megakaryocytes were observed as a large nuclear complex forming a platelet-ribbon (proplatelet) like structure (Fig. 5). These findings solidly indicate the production of CCN1 and CCN5 in megakaryocytes, which are eventually encapsulated in platelets.

**Induction of CCN2 expression in mesenchymal cells by media conditioned by MEG-01 at earlier differentiation stages**
Platelets are active in endocytosis, and thus it is reasonable to consider the external origin of CCN2 and CCN3. Therefore, since neither CCN2 nor CCN3 was present in megakaryocytic cells, we sought other producers in the same environment and found CCN3-positive cells in bone marrow cell suspensions, whereas CCN2-positive cells were absent therein (Fig. 5). However, a previous study suggests that megakaryocyte progenitors at early differentiation stages release soluble factor(s) that induce strong CCN2 production by mesenchymal cells, which is subsequently taken up by platelets [41]. Using our experimental system with MEG-01 cells, we also examined whether the media conditioned by MEG-01 at earlier (day 1 and day 2) differentiation stages could induce the gene expression of CCN2 in mesenchymal HCS-2/8 cells. Indeed, we found the conditioned media remarkable increased the CCN2 gene expression in HCS-2/8, which also suggested successful induction of megakaryocytic differentiation under this condition. However in contrast, no significant effect on the CCN3 gene expression was observed (Fig. 6). The comparable findings obtained by 2 different experimental systems indicate that megakaryocyte are stimulating mesenchymal cells to overproduce CCN2, possibly for its inclusion into platelets.

**Effects of a CCN family cocktail mimicking the platelet lysate on chondrocytic cells**

In addition, to examine if the combination of the CCN family proteins included in platelets could drive chondrocytes towards tissue regeneration, we have employed an established *in vitro* model with human chondrocytic HCS-2/8 cells [35]. It is known that CCN2 alone is able to enhance the cartilaginous ECM synthesis at a concentration of 50 ng/ml in those cells, which was reproduced in this experiment. Moreover, a simple combination of the 4 CCN family members at the same concentration also
revealed significant, and possibly, stronger enhancement in the expression of aggrecan and type II collagen genes (Fig. 7) than CCN2 alone. It should be noted that this CCN family cocktail exerted effects comparable to CCN2 alone, in spite of the involvement of CCN3 and CCN5 at the same concentration, which might counteract CCN2.

**Discussion**

Tissue regeneration process usually mimics a series of biological events that occur during developmental process. Indeed, it is widely known that, upon bone fracture, endochondral ossification process recurs. Therefore, key molecules that play central roles in cartilage and bone regeneration are essentially the same as those working in cartilage and bone development. This notion is well represented by the function of CCN2, which enhance all of the steps of endochondral ossification and accelerate cartilage and bone regeneration. It is also highly suspected that, not only CCN2, but also the other factors are commonly involved both in endochondral ossification and cartilage/bone regeneration process, in order to construct, or reconstruct the same normal tissue at the end. In this point of view, what we observed in this study is of significant importance. Our present study for the first time revealed the involvement of CCN1, CCN3 and CCN5 in platelets, in addition to CCN2 that is already known to be present, whereas CCN4 and CCN6 were absent therein. Interestingly, this composition of 4 CCN family members in platelets is quite comparable to that of the growing zone in the growth plate cartilage (Table 1). Namely, platelets can supply the optimal composition of the CCN family members to damaged cartilage and bone for recurring normal growth of cartilage, which is required for reconstructing the damaged cartilage and bone in the arthritic joints. Furthermore, activated platelets provide a
fibrin matrix to the released CCN1, 2, 3 and 5, which may enable the storage and gradual release of these matricellular proteins. Although a few reports described certain roles of CCN4 in fibrosis and wound repair, it was not detectable in platelets, indicating relatively minor contribution of CCN4 to the initial stages of tissue repair conducted by platelets. Collectively, it is now clear that platelet contains an ideal cocktail of the CCN family members for joint regeneration. Our finding also presents a scientific basis to support the utility of PRP in the regenerative therapeutics of arthritic tissues. In the field of oral surgery, PRP has been used routinely in the treatment of bone defect after tooth extraction and other surgical intervention as safe and effective means for regenerating tissues in oral surgery [42]. Currently, the utility of PRP is being expanded to a variety of other systemic clinical fields [1-4]. Particularly, orthopedic application of PRP for joint tissue regeneration is attracting the interest of clinicians [43,44].

Based on the results obtained, possible collaboration system among these 4 CCN family members to promote cartilage and bone regeneration can be proposed. Immediately after tissue damage, these 4 members are supplied from activated platelets, and the regeneration process starts. Initially, CCN2 promotes proliferation and vigorous ECM production in chondrocytes under the collaboration with other growth factors. At the same time, CCN3 prevents the excess collagen production and counteract the pro inflammatory effects of CCN2, and CCN1 invokes the induction of cellular senescence to terminate regenerative response. Moreover, as an inhibitor of ADAMTS4, CCN1 counteracts cartilage damage and protect it during growing phase. This collaboration system among the 3 CCN family members is estimated to be critical in regenerating tissues without sustained inflammatory ECM deposition leading to
fibrosis, which is also effective in RA joints. Although less is known about the functions of CCN5 during wound healing, it is previously reported that over expression of CCN5 reduced cardiac hypertrophy and fibrosis [12,36], suggesting a regulatory role of CCN5 in ECM deposition. Of note, positive effect of CCN5 on chondrocytic differentiation was once reported [16]. Further detailed study on the function of CCN5 is needed to clarify the biological significance of this CCN family member in the growth plate and platelets.

One interesting question is how CCN3 can be incorporated into platelets. In case of CCN2, we indicated that megakaryocytes produce soluble factor(s), which stimulate mesenchymal cells to release CCN2, and then platelets incorporate CCN2 via endocytosis [41]. This idea is supported further by a recent study describing that CCN2 is incorporated into the cells via endocytosis by low-density lipoprotein receptor-relate protein 1 (LRP-1) [45]. There still is no report that CCN3 may interact with LRP-1. Nevertheless, endocytotic incorporation of CCN3 via LRP-1 is, at least theoretically, possible in the co-presence of CCN2, since CCN3 was proven to directly bind to CCN2 [46]. Next question is which cells in bone marrow produce CCN3 for platelets. Here, we observed CCN3 is present in spherical cells in bone marrow, which are suspected to be haematopoietic stem cells known to produce CCN3 (Fig. 5d). Therefore, CCN3 present in platelets can originate from those cells. These possible pathways for the uptake of CCN2 and CCN3 are also illustrated in Fig. 8.

In this study, we found 4 CCN family members in platelets, which are estimated to be incorporated through multiple pathways and collaborate with each other in platelet-induced cartilage and bone regeneration. We previously reported the utility of CCN2 in connective tissue repair and regeneration. However, considering the results
obtained here, combinatory use of 4 CCN family members, as a CCN family protein cocktail can be an ideal therapeutic tool to promote tissue regeneration, especially in the case of RA treatment. Indeed, the data in Fig. 7 supports this notion; however, 4 CCN family members may not be involved in equal amount in platelets. Thus, another cocktail with 4 CCN family members at the same proportion as that in platelets is expected to yield more striking outcome. Quantitative comparison of the 4 CCN family members in platelets, followed by the preparation of CCN family protein mixture mimicking platelets more precisely, is currently underway. Subsequent translational research in vitro and in vivo may lead to the establishment of a recipe for preparing best joint regeneration cocktail for OA and RA therapeutics.

Acknowledgement

We gratefully thank Drs. Toshio Yamamoto and Mika Ikegame for the instruction on microscopy, and Dr. Tarek Abd El Kader for critically reading the manuscript. This study was supported by grants from the program Grants-in-aid for Scientific Research (B) [#24390415 and #15H0514] to M.T. and (C) [#25462886] to S.K. from the Japan Society for the Promotion of Science and from Wesco Scientific Promotion Foundation.

Conflict of Interest

The authors have no conflict of interest to declare.
References


34. Yasui K, Furuta RA, Matsumoto K, Tani Y, Fujisawa J. HIV-1- derived


5-14.


Figure Legends

**Fig. 1. CCN1 and CCN5 in platelets and megakaryocytic cells.** Western blotting analysis of CCN1 (a) and CCN5 (b) in platelets (left panels) and megakaryocytic cells (right panels), respectively. Recombinant CCN1 (rCCN1: 15 ng) and CCN5 (rCCN5: 10 ng) were loaded as positive controls for CCN1 and CCN5 immunoreaction (upper panels). The same membrane was re-probed to show β-actin as an internal control (lower panels). CCN1 and CCN5 signals were detected both in platelets and megakaryocytic cells. Numbers at the top represent the periods of cell culture in days. Positions of molecular weight markers are depicted at the left in kilodaltons. Arrowheads indicate specific signals.

**Fig. 2. CCN2 and CCN3 in platelets.** Western blotting analysis of CCN2 (a) and CCN3 (b) in platelets (left panels) and megakaryocytic cells (right panels), respectively. Recombinant CCN2 (rCCN2: 20 ng) and CCN3 (rCCN3: 10 ng) were utilized as positive controls for CCN2 and CCN3 detection (upper panels). The same membrane was re-probed to show β-actin as an internal control (lower panels). Strong CCN2 and CCN3 signals were detected in platelets, but no signal was observed in megakaryocytic cells. Numbers at the top indicate the cell culture periods in days. Positions of molecular weight markers are indicated at the left in kilodaltons. Arrowheads indicate specific signals.

**Fig. 3. Absence of CCN4 and CCN6 in platelets.** Western blotting analysis of CCN4 (a) and CCN6 (b) in platelets (left panels) and megakaryocytic cells (right panels),
respectively. Recombinant CCN4 (rCCN4: 5 ng) and CCN6 (rCCN6: 20ng) were loaded as positive controls to ensure proper detection of these proteins (upper panels). The same membrane was re-probed to show β-actin as an internal control (lower panels). Clear signals were obtained with recombinant CCN4 and CCN6; however, CCN4 and CCN6 were undetectable both in platelets and megakaryocytic cells. Positions of molecular weight markers are indicated at the left.

**Fig. 4. No production of CCN2 and CCN3 along with slow and mild differentiation induction in MEG-01 cells.**  
**a.** Experimental time course of the mild induction of differentiation by VPA or ATRA. MEG-01 cells were treated with VPA or ATRA as described in Materials and Methods for 20 days, and sampling was carried out as indicated.  
**b.** Increasing population of CD41 positive cells along the time course, which was enhanced by VPA (solid lines with square dots) as evaluated by flow cytometric analysis. Data from control and ATRA-treated cells are represented by solid lines with circular dots and dotted lines with triangular dots, respectively.  
**c.** Phase contrast view of MEG-01 cells after 20 days of VPA treatment. Proplatelet formation from large cells with expanded nuclei is observed. Scale bar: 50 µm.  
**d** and **e** Western blotting analysis of the cell lysate from MEG-01 treated by VPA with an anti-CCN2 (d) and anti-CCN3 (e) antibodies. The rCCN2 and rCCN3 were used as positive controls, as described for Fig. 2. Numbers above the images denote the days of sampling. The same membrane was re-probed to show β-actin as an internal control (lower panels). Positions of molecular weight markers are indicated at the left.
**Fig. 5. CCN family members present in megakaryocytes from mouse bone marrow.** Smear samples from mouse bone marrow were prepared and were analyzed by immunofluorescence analysis with anti-CCN1 (a) and anti-CCN5 (b) antibodies. Nuclear counterstaining with DAPI is also shown. Distinct signal representing CCN1 is observed only in a large megakaryocyte with an expanded nucleus (a), whereas CCN5 signals are present in megakaryocytes and also in another population of bone marrow cells (b). Co-localization of CCN1 and CCN5 in the same cell was observed as well (c). In contrast, no specific signals were detected in megakaryocytes with anti-CCN2 and anti-CCN3 antibodies (negative controls: d). Also, note the presence of CCN3-positive spherical cells (arrowheads). Scale bars: 50 µm.

**Fig. 6. Effects of MEG-01 conditioned media on the expression of CCN2 and CCN3 in HCS-2/8 cells.** (a) Experimental strategy. RPMI conditioned by MEG-01 at an earlier differentiation stage (day 0, 1 or 2) was collected and was added to HCS-2/8 cells in culture. Twelve hours later, cells were collected and RNAs were analyzed by real-time RT-PCR. Mean values from 3 independent samples are shown in error bars. Asterisks (*) indicate statistical significance of difference vs. the control (RPMI) at p < 0.01.

**Fig. 7. Enhancement of the expression of cartilaginous matrix component genes by a CCN family cocktail mimicking platelets.** Human chondrocytic HCS-2/8 cells were treated with the indicated CCN family proteins or BSA (control) for 12 h, and the expression of aggrecan (a) and type II collagen (b) genes was evaluated. Mean values from the data obtained with 9 independent samples are shown in error bars (standard
deviations). Asterisks (*) indicate statistical significance of difference vs. the control (BSA) at p < 0.05.

**Fig. 8. Possible inclusion pathways and collaboration of CCN family members in platelets.** Based on the findings obtained, it is estimated that CCN1, CCN5 were produced by megakaryocytes and endogenously supplied to platelets during thrombopoiesis. In contrast, CCN3 as well as CCN2 were produced from other cells, possibly haematopoietic (H) cells and mesenchymal (M) cells under the stimulation by soluble factor(s) from megakaryocytes, respectively, and platelets take them up exogenously. These CCN family members in platelets collaboratively conduct the adequate cartilage regeneration and endochondral ossification after bone fracture without hypoplasia or hyperplasia (Harmonized regeneration). Namely, CCN2 encourages fibroblasts to produce ECM components, the major ones of which are collagens, while CCN3 genetically represses it to avoid the overcorrection. Mutual interaction between CCN2 and CCN3 may be also involved in this CCN2-CCN3 collaboration. Fibrotic remodeling is negatively regulated by CCN1 via induction of cellular senescence in fibrogenic myofibroblasts (Regulatory termination), while it protects cartilage from catabolic degradation by inhibiting ADAMTS4.