The *in vitro* study of CCN2 on dentin regeneration

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Summary

Direct pulp capping has been used for the treatment of pulp exposed by deep dental caries or traumatic injury. Under conditions of severe damage, newly differentiated odontoblast-like cells may migrate to sites of injury, where they are thought to form reparative dentin that protects pulp tissue from stimuli.

Connective tissue growth factor/**C**ysteine-rich 61/**N**ephroblastoma overexpressed family 2 (CCN2) plays complex roles in various biological processes such as embryogenesis, angiogenesis, chondrogenesis, wound healing, tissue regeneration, etc. However, its effects in dentin regeneration remain unclear.

This study aimed to investigate the *in vitro* effects of CCN2 on odontoblast-like cells and human dental pulp stem cells (hDPSCs) in proliferation, mineralization and differentiation, as well as its potential application in dental treatment.

This study was divided into three independent experiments. In experiment 1, soluble CCN2 (100 ng/mL) was added into culture media; in experiments 2 and 3, CCN2 (1000 ng/mL) was coated onto culture polystyrene plates, addition or coating of distilled water (dH_2O) served as a control. Cell proliferation was assessed using the CCK-8 assay. The cytoskeleton was stained using Alexa Fluor 568-labeled phalloidin and visualized by fluorescence microscopy. Cell differentiation and mineralization were analyzed by ALPase activity assay, real time RT-PCR, and alizarin red staining. Data were analyzed using the post hoc Tukey honest significance difference test.

In experiment 1, cell proliferation of MDPC-23 cells was promoted by the addition of soluble CCN2. Gene expression of rBSP, rOCN and rOPN were promoted after 48h exposure to soluble CCN2. Mineralization of MDPC-23 cells was accelerated by the addition of soluble CCN2.

In experiment 2, CCN2 promoted MDPC-23 cells proliferation in a dose-dependent

manner, with the greatest effect observed at 1000 ng/mL. ALPase activity of cells cultured on CCN2-modified surfaces continuously strengthened from day six (0.83±0.02 units/μg protein *versus* 0.56±0.01 units/μg protein of control) until day eight (1.04±0.14 units/μg protein *versus* 0.70±0.06 units/μg protein of control). Moreover, gene expression of rBSP, rOCN, rOPN, rALP, rCOL1A1, rRunx-2, rDSPP, and rDMP-1 was significantly enhanced by the presence of immobilized CCN2. Finally, mineralization of MDPC-23 cells was markedly enhanced by CCN2.

In experiment 3, hDPSCs exhibited robust proliferative activity upon exposure to immobilized CCN2. Gene expression of hBSP, hCol1a1, and hALP in the CCN2-coated group was significantly higher than that of the control. The relative ALPase activity was also increased in the CCN2-coated group. Mineral nodule formation by hDPSCs grown on CCN2-coated surface was remarkably increased as compared to those grown on noncoated surfaces.

Recently, ongoing studies have identified CCN2 protein as a growth and signaling factor that interacts with cell surface molecules and intracellular signaling pathways. Through its interaction with cell surface receptors, integrins, extracellular counterparts and extracellular matrixes, CCN2 governs inter- and intracellular communications to either directly or indirectly regulate cell proliferation and differentiation. The present study also indicated that the addition of 100 ng/mL CCN2 directly into culture media in a short culture period of 48h primarily promoted mineralization rather than odontogenic differentiation on MDPC-23 cells.

The findings indicate that CCN2 promotes proliferation, differentiation and mineralization of MDPC-23 and hDPSCs. It is proposed that CCN2 may be a promising adjunctive formula for dentin regeneration.

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1. Introduction

In 1930, Hermann discovered that calcium hydroxide is effective in repairing an pulp exposure site (Dammaschke, 2008). Since then, it has been used in clinical treatment for decades in the form of powder, paste, and cement. The formation of complete dentin bridge has been believed to be the key to the clinical success of direct pulp capping. However, it has been reported that 89% of dentin bridges formed by calcium hydroxide contained tunnel defects. These tunnel defects not only fail to provide a permanent barrier, but also fail to provide a long-term biological seal against bacterial infection (Cox et al., 1996). Another disadvantage is dissolution, which may lead to the formation of dead space and microleakage (Cox et al., 1994).

Mineral trioxide aggregate (MTA) is presently one of the most commonly used pulp capping material. The mechanism of MTA is similar to that of calcium hydroxide. Although it is superior to calcium hydroxide in terms of sealing ability, biocompatibility, and bioactivity, MTA has some disadvantages including long setting times, poor handling, and coronal tooth discoloration (Komabayashi et al., 2016). As for the clinical outcome of direct pulp capping, a recent study showed that MTA and calcium hydroxide had a successful outcome rate of 78% and 60%, respectively (Valles et al., 2013).

Therefore, there is a need for better direct pulp capping materials. Some researchers have evaluated the use of biological molecules such as growth factors and extracellular matrix (ECM). Animal studies showed that growth factors such as bone morphogenetic proteins (BMP) and transforming growth factor (TGF) induced reparative dentin formation (Goldberg & Smith, 2004; Goldberg et al., 2008). Thus,

capping with these molecules is extremely promising.

The CCN family of proteins is a complex gene family consisting six distinguishing members, acronym of which was introduced from the first letter of **C**TGF (connective tissue growth factor), **C**YR61 (cysteine-rich protein 61) and **N**OV (nephroblastoma overexpressed) (Bork, 1993). Since the six members were discovered independently by different researchers, they were given different names historically. Therefore, to obviate misunderstanding, we now use a unifying nomenclature numbering system from CCN1 to CCN6 (Brigstock et al., 2003). Structurally, a prototypical CCN protein is encoded by five exons which correspond to an N-terminal secretory signal peptide and four distinct modules, i.e. insulin-like growth factor binding protein-like module (IGFBP), von Willebrand factor type C repeat (VWC), thrombospondin type 1 repeat (TSP) and C-terminal cysteine knot (CT) modules. Each of the four modules works both independently and interdependently. Primary translation products of most CCN family members comprise 343–381 residues and produce 35–40 kDa secreted proteins, which contain 38 conserved cysteine residues, except for CCN5 lacks the CT module and contains only 28 conserved cysteine residues, and CCN6 contains only six cysteine residues in the VWC module (Brigstock, 1999).

The CCN family is now known to play essential functions that is involved in various important biological processes such as growth and development, embryogenesis, angiogenesis, chondrogenesis, skeletogenesis, inflammation, tumorigenesis, wound healing, tissue regeneration, etc. CCN proteins also participate in cell adhesion, migration, proliferation, differentiation, and ECM formation (Brigstock, 2003; Katsube

et al., 2009; Kubota & Takigawa, 2013; Perbal, 2018).

Among the CCN family, CCN2 has been standing out from other members by virtue of its multifunctional properties. It was incipiently isolated from serum-stimulated NIH3T3 cells as an immediate early gene whose cDNA encoding "fibroblast-inducible secreted protein-12" (*fisp*-12) (Ryseck et al., 1991). The human ortholog of CCN2 was originally discovered as a single polypeptide (molecular weight: 38 kDa) secreted by cultured human vein endothelial cells, and was termed as connective tissue growth factor because it was found to be both mitogenic and chemotactic for fibroblast-like cells *in vitro* (Bradham et al., 1991). CCN2 was also identified in transforming growth factor-beta (TGF-β) stimulated mouse ARK-2B cells as βIG-M2 (Brunner et al., 1991). Moreover, a gene named hypertrophic chondrocyte-specific gene 24 isolated from human chondrocytic cell line was found to be identical to CTGF (Nakanishi et al., 1997). All these genes are now considered as the same and their proteins generally called CCN2.

Previous studies had revealed the diverse functionality of CCN2. It promotes adhesion, migration, and proliferation of endothelial cells *in vitro* and angiogenesis *in vivo* (Kubota & Takigawa, 2007). It facilitates the proliferation and differentiation of chondrocytes and osteoblasts *in vitro* (Takigawa, 2013). CCN2 was also found to play a key role in the development of orofacial tissues due to its functional property as a critical mediator of endochondral ossification, intramembranous ossification, cartilage formation, tooth development, periodontal fibrogenesis, and tissue remodeling (Kubota, 2012).

Recent studies have shown that CCN2 is highly expressed during odontogenesis in developing mouse tooth germs. CCN2 was detected in dental lamina, dental mesenchyme and primary enamel knot from the initiation stages, differentiating pre-ameloblasts and odontoblasts at the crown stage, and continued to be present in the outer dental epithelium and dental follicle, as well as involuting dental lamina and alveolar bony crypts (Kanyama et al., 2013; Shimo et al., 2002). Moreover, the expression of CCN2 increased during reparative dentinogenesis (Muromachi et al., 2012).

Despite the above findings, little information is available concerning the involvement of CCN2 in dentin regeneration. Therefore, the present study was carried out to investigate the potential effects of CCN2 on proliferation, mineralization and differentiation of MDPC-23 cells, a rat odontoblast-like cell line, and human dental pulp stem cells.

2. Materials and methods

1) Materials

Recombinant human CTGF was purchased from PeproTech (Rocky Hill, NJ, USA) and stored at –30°C until use. Tissue culture treated polystyrene plate (TCPP) and non-tissue culture treated polystyrene plate (Non-TCPP) were purchased from Falcon (Corning, NY, USA). Dulbecco's modified eagle media (DMEM), dexamethasone tablets, cetylpyridinium chloride (CPC), 4,6-diamidino-2-phenylindole (DAPI) and Triton-X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), TrypLE™ Express, 1×phosphate buffered saline (PBS, pH 7.4) were all purchased from Gibco (Grand Island, NY, USA). PBS tablets were purchased from TaKaRa (Shiga, Japan). Glycerol-2-phosphate disodium salt n-hydrate (β-GP), L-Ascorbic acid phosphate magnesium salt n-hydrate (AA), 10% formalin neutral buffer solution, alizarin red S powder and ALP assay kit were all purchased from Wako (Osaka, Japan). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Rockville, MD, USA). Pierce BCA protein assay kit and methanol-free formaldehyde (16%, w/v) were purchased from Thermo Fisher Scientific (Rockford, IL, USA). TRIzol and phalloidin were purchased from Invitrogen (Carlsbad, CA, USA). Chloroform, 2-propanol and ethanol were purchased from Nacalai Tesque (Kyoto, Japan). FastStart Essential DNA Green Master for real-time reverse transcriptase-polymerase chain reaction (real time RT-PCR) analysis was purchased from Roche (Basel, Switzerland).

2) Recombinant CCN2 protein treatment

The present study was divided into three independent experiments which were not

related, in order to avert confusion when discussing the results, these three experiments were named as experiment 1, 2, and 3. In experiment 1, the effect of the direct addition of soluble CCN2 protein into culture media was analyzed using TCPP for culture. In experiments 2 and 3, Non-TCPP surface was physically coated with CCN2 protein. Briefly, CCN2 protein was dissolved in dH_2O and spread onto Non-TCPP, then dried at room temperature for two days until only CCN2 protein remained on the surface. Addition or coating of dH_2O was used as a negative control.

3) Cell culture

MDPC-23 cells (generated by the late CT Hanks at the University of Michigan, Ann Arbor, Michigan, USA) were generously provided by Professor Jacques E. Nör from the University of Michigan. These cells were used in cell culture in experiments 1 and 2. Cells were cultured in DMEM supplemented with 5% FBS and cell passages from 26 to 33 were used in this study.

Human dental pulp stem cells (hDPSCs) (catalog number PT-5025; Lot number 0000361427) used in experiment 3 were purchased from LONZA (Walkersville, MD, USA). Use of hDPSCs was approved and carried out under the guidelines set by the ethical committee of the Health Sciences University of Hokkaido. The characterization of its stem cell phenotype was carried out using flow cytometry including testing for surface antigens (CD105+, CD166+, CD29+, CD90+, CD73+, CD133−, CD34−, and CD45−) by the manufacturer (see the certificate of analysis in supplementary file available online at https://doi.org/10.1155/2017/2546261). Cells were cultured in DMEM supplemented with 10% FBS, 50 units/mL penicillin and 50 μg/mL streptomycin

(catalog number 15070063, Gibco). Cell passages from 3 to 5 were used in this study.

Cells were inoculated at a density of 1×10^3 cells/well in 96-well plates, 1×10^4 cells/well in 24-well plates, 2×10^4 cells/well in 12-well plates, and 3×10^4 cells/well in 6-well plates. All cells were cultured in a 37°C humidified incubator with an atmosphere of 5% CO_2 and 95% air.

4) Cell proliferation assay

The cell proliferation was measured using the CCK-8 assay, various concentrations of CCN2 were tested (1, 10, 100, 1000, and 2000 ng/mL). For experiment 1, CCN2 was added to the experimental group on day one and the cell proliferation assay was performed on day four. For experiment 2 and 3, the cell proliferation assay was carried out on day four. The absorbance of lysates was measured at 450 nm.

5) Cell morphology observation

In this study, cells grown on CCN2-coated plates (1000 ng/mL; experimental group) were compared with cells grown on dH_2O-coated plates (control group). In experiment 2, cells were visualized under phase contrast microscopy (Olympus, Shinjuku, Tokyo, Japan) at 48h, 72h and 96h respectively. In experiment 3, cells were observed after 24, 48, and 72h of culture respectively,

6) Fluorescence staining of actin cytoskeleton

In experiments 2 and 3, cells were inoculated into 24-well plates (Non-TCPP, surface area: 2 cm^2, coated with 1000 ng/mL CCN2 or dH_2O). On day three, culture media were aspirated and cell monolayers were rinsed twice with PBS before fixation. Fixation of cells was carried out using freshly made methanol-free formaldehyde at a

concentration of 4% (v/v) in PBS (200 μL/well) for 15 min at room temperature. Cells were briefly rinsed three times with PBS before the addition of permeabilisation reagent Triton X-100 (0.1%, v/v, in PBS, 400 μL/well). Five minutes later, 1% (w/v, in PBS) of BSA was added into each well (400 μL/well) for 30 min at room temperature to block any nonspecific binding. Phalloidin (working concentration: 2 U/200 μL, 200 μL/well) was used to localize F-actin. The nucleus was counterstained by DAPI (working concentration: 300 nM, 200 μL/well) for 5 min. Finally, cells were submerged in PBS and photographed using the EVOS FL Cell Imaging Station System (Thermo Fisher Scientific, Rockford, IL, USA).

7) Alkaline phosphatase (ALPase) activity assay

For determination of ALPase activity, MDPC-23 cells in experiment 2 and hDPSCs in experiment 3 were used. After incubation for the desired number of days, cells were collected and lysed with 0.1% Triton-X-100 in dH_2O. The lysates were then sonicated for 10 min on ice, centrifuged at 12,000 rpm at 4°C for 15 min and then the supernatants were collected. ALPase activity and protein quantification were determined according to the manufacturers' instructions. One unit of the enzyme activity was defined as the release of 1 nmol *p*-nitrophenol per minute at pH 9.8 and 37°C. The relative activity was determined as follows: units/μg protein = activity (units/μL) / protein concentration (μg/μL). Absorbance was read using a microplate reader at 405 nm and 570 nm for ALPase activity and protein quantification, respectively.

8) Conventional & real-time quantitative RT-PCR

Total RNA was isolated from cultured cells by using TRIzol reagent at the

designated time. Isolated RNA was pelleted, washed with 75% ethanol and re-suspended in nuclease-free water. RNA concentration of each sample was measured using NanoDrop ND-1000 (Thermo Fisher Scientific, Rockford, IL, USA) and 1 μg of isolated RNA was then reverse-transcribed into complementary DNA (cDNA) using M-MLV reverse transcriptase in a 20-μL reaction system according to the manufacturer's instructions. The resulting cDNA was used for both conventional RT-PCR and real time quantitative RT-PCR.

To investigate CCN protein family gene expressions in MDPC-23 cells, conventional RT-PCR was performed after seven days of culture, the PCR products were run on 2% agarose gels and stained with ethidium bromide. Primer sequences are illustrated in Table 1.

For evaluation of cell differentiation, odontogenic gene mRNA expression was quantified using primers described in Tables 2 and 3 by LightCycler® Nano (Roche, Basel, Switzerland) according to the manufacturer's instructions. In experiment 1, MDPC-23 cells were cultured in DMEM containing 5% FBS. On day five, the culture media were changed to FBS-free DMEM, and CCN2 was added to the cells one day after serum starvation. RNA was then collected after 12, 24, 36, and 48h, respectively. In experiment 2, RNA was collected on day eight. In experiment 3, RNA was collected on days 8, 15, and 22 respectively.

9) Alizarin red staining

In experiments 1 and 2, MDPC-23 cells were grown onto 12-well plates, cultured in DMEM containing 5% FBS for five days after they reached confluency, and then 10

mM β-GP, 50 μg/mL AA and 100 nM dexamethasone were added as a mineralizing media up to three days. In experiment 3, hDPSCs were grown on 6-well plates, cultured in DMEM containing 10% FBS, 50 units/mL penicillin, and 50 μg/mL streptomycin, β-GP (10 mM) and AA (50 μg/mL) were incorporated in the media after cells reached confluency. The culture media was then aspirated and the cell monolayer was washed with 1× PBS twice. Cells were fixed with 10% formalin neutral buffer solution for 20 min and washed again with 1× PBS. Then, alizarin red solution (ARS, 1%, pH 4.0) was added gently. After 10 min of incubation at 37°C, the staining solution was removed and the cell monolayer was washed several times until the solution turned clear and transparent. Photographs were taken using a digital imaging system (Funakoshi, Tokyo, Japan) incorporating an inverted digital camera (Canon, Tokyo, Japan). To quantify the calcific staining intensity, the CPC method was used. Briefly, after staining with ARS, CPC (10%, w/v, in distilled water) was added to each well and incubated for one hour at 37°C. After incubation, the solution was transferred to a 96-well plate and read at an absorbance of 570 nm.

10) Statistical analysis

All experiments were repeated three times, and results are expressed as the mean ± standard deviation. Data were subjected to post-hoc Tukey HSD (Honestly Significant Difference) test. Statistical significance level was set at $p<0.05$.

3. Results

1) CCN protein family expression in MDPC-23 cells

To investigate whether CCN protein family members are expressed during odontogenic differentiation of MDPC-23 cells, conventional RT-PCR on cell cultured for seven days and real-time quantitative RT-PCR on cells cultured for six and eight days were performed. The results showed that among the six CCN protein family members, only CCN1, CCN2, CCN4 and CCN5 were expressed (Fig. 1A). Moreover, only CCN2 mRNA expression was markedly enhanced during odontogenic differentiation of MDPC-23 cells (Fig. 1B). Therefore, up regulation of CCN2 mRNA expression might be related to the development of MDPC-23 cells.

2) Cell proliferation

Cell proliferation changes in response to different concentrations of CCN2 protein were evaluated by the CCK-8 assay. First, the direct addition effect of CCN2 in experiment 1 was explored. As shown in Figure 2A, CCN2 stimulated the proliferation of MDPC-23 cells at concentrations ranging from 100 to 2000 ng/mL, but the effect was not dose-dependent. The effect of CCN2 immobilized on culture surfaces was also investigated. In experiment 2, CCN2 promoted MDPC-23 cells proliferation in a dose-dependent manner and observably reached a maximum at the concentration of 1000 ng/mL (Fig. 2B). Similarly, in experiment 3, significant differences were observed between control and CCN2-coated groups. The promotion of hDPSCs proliferation was maximized at 2000 ng/mL among all the tested concentrations (Fig. 2C). For convenience, the concentration of 100 ng/mL for addition and 1000 ng/mL for coating

of CCN2 were chose during subsequent evaluations.

3) Cell morphology observation and cytoskeleton visualization

It can be clearly observed that MDPC-23 cells grown on CCN2 coated surface (experiment 2, 1000 ng/mL) displayed an extended morphology and formed well-developed actin stress fibers, while most cells in the control group showed a spherical morphology and extended incompletely (Fig. 3 and 4).

As for hDPSCs, it is clearly shown in Figure 5 that cells started to form protrusions on CCN2-treated surfaces at 24 h. After 72h culture, hDPSCs were well spread. Fluorescence staining of the actin cytoskeleton showed that hDPSCs in the noncoated group maintained a tight shape without spreading. However, cells spread widely and well-developed actin stress fibers were formed and clearly observed in the CCN2-coated group (Fig. 6).

4) ALPase activity

ALPase activity is considered to be essential for biomineralization. Figures 7 and 8 illustrate that the relative ALPase activity of the CCN2-coated group was significantly increased compared to that of the control group on all days evaluated. The ratios of ALPase activity of the CCN2-coated group divided by that of the control group were 1.17 on day seven and 1.47 on days six and eight in experiment 2; 3.94 on day 14, 4 on day 21, and 1.45 on day 28 in experiment 3, respectively.

5) Real time RT-PCR

To investigate the effects of CCN2 on the mRNA expression of odontogenic differentiation markers genes, real-time quantitative RT-PCR was performed using

specific primers for dentin matrix acidic phosphoprotein 1 (DMP-1), dentin sialophosphoprotein (DSPP), alkaline phosphatase (ALP), bone sialoprotein (BSP), osteopontin (OPN), osteocalcin (OCN), runt-related transcription factor-2 (Runx-2), and type I collagen (Col1a1). In experiment 1, data shows the addition of soluble CCN2 protein into culture media for 48h led to significant upregulation of the expression of target genes compared to that of the control group, including rBSP, rOCN, and rOPN (Fig. 9 A−C). In experiment 2, the relative mRNA levels of rBSP, rOCN, rOPN, rDSPP, rDMP-1, rRunx-2, rALP, and rCol1a1 in the CCN2-coated group were significantly higher than those in the dH_2O-treated control group on day eight (Fig. 9D). In experiment 3, the immobilized form of CCN2 significantly promoted the expression of hard tissue-forming markers including hBSP, hCol1a1 and hALP after 22 days of culture (Fig. 9E−G).

6) Alizarin red staining

To explore whether CCN2 induces mineral nodules formation, alizarin red staining was performed on day eight in experiment 1, days seven and eight in experiment 2, and days 29 and 32 in experiment 3, respectively. The results showed that experiment group had promoted the calcium nodules formation in both process methods of CCN2 protein (Fig. 10A, C, and E). The CPC quantification of alizarin red staining further revealed significant differences between CCN2 and control groups in experiment 1 (1.38 ± 0.06 versus 1.14 ± 0.08), experiment 2 (day 7: 0.68 ± 0.08 versus 0.40 ± 0.01, day 8: 1.29 ± 0.06 versus 0.74 ± 0.04) and experiment 3 (day 29: 1.23 ± 0.06 versus 0.24 ± 0.01, day 32: 2.37 ± 0.65 versus 0.52 ± 0.03), respectively (Fig. 10B, D, and F).

4. Discussion

The present study demonstrated first that CCN family members, excluding CCN3 and CCN6, are stably expressed to different extent during *in vitro* induction of odontogenic differentiation of MDPC-23 cells. On this basis, only CCN2 gene expression was markedly increased. Given that the CCN2 expression was strongly discernible in odontoblast-like cells in carious human teeth (Muromachi et al., 2012), furthermore, CCN2 might play some role in the regulation of MDPC-23 cells and hDPSCs.

Second, the results showed that the proliferation potential of MDPC-23 cells and hDPSCs was markedly strengthened in the presence of both the soluble and coated form of CCN2. Moreover, it was found that the enhancing effect of CCN2 on cell proliferation was dose-dependent in its immobilized form. In contrast, its soluble form showed less efficient to promote cell proliferation.

Odontoblast, a highly specialized cell line originated from dental papilla, which forms the dentin by secreting collagenous and noncollagenous organic matrix constituents and controlling the mineralization process throughout life (Arana-Chavez & Massa, 2004). It goes through multiple stages including pre-odontoblast, secretory, polarization, and mineralization during tooth development. Such a unique differentiation pattern of odontoblast results from the spatiotemporal regulation of a series of genes. In the past 20 years, many specific highly expressed genes in the process of odontoblast differentiation had been reported. For example, BSP is involved in regulating hydroxyapatite crystal formation in teeth, Col1a1 is the major organic

component of the dentin matrix, DSPP is highly expressed in dentin compared to other tissues, DMP-1 is related to hard tissue formation especially dentin, and Runx2 is essential for odontoblast differentiation which regulates the expression of numerous tooth-related genes (Balic et al., 2010; Chen et al., 2008; Chen et al., 2009; Sasaguri et al., 2000). Thereinto, BSP, OPN, DMP-1, and DSPP which belong to the small integrin-binding ligand N-linked glycoproteins family (SIBLINGs family) are expressed in dentin, and being received to control dentinogenesis (Chen et al., 2008).

Shimo et al. revealed that exogenous recombinant CCN2 stimulated the proliferation of dental epithelial and mesenchymal cells, which are crucial for differentiation and maturation of odontoblast, and odontoblast differentiation is inhibited by CCN2 neutralizing antibody (Shimo et al., 2002). From the present experiments, it can be discovered that the addition of CCN2 to culture media upregulated the gene expression of rBSP, rOCN, and rOPN in experiment 1. Interestingly, the gene expression of rCol1a1 and rRunx-2 was downregulated after 48h culture, and other odontogenic differentiation markers including DSPP did not show significant changes (data not shown). In contrast, when CCN2 was immobilized onto the culture plate surface, it significantly enhanced the mRNA expression of tooth-related genes as described above, including rBSP, rDMP-1, rDSPP, rOPN, rCol1a1, and rOCN, while slightly increasing the expression of rRunx-2 in experiment 2. The promotion of rDSPP and rDMP-1 mRNA expression level indicates the possible involvement of CCN2 in MDPC-23 cells odontogenic differentiation. CCN2 also significantly upregulated the mRNA expression of hBSP and hCol1a1 in experiment 3, denoting that hDPSCs were

able to differentiate into hard tissue-forming cells *in vitro*. Furthermore, CCN2 stimulated ALP gene expression and ALPase activity, which is an early marker of odontogenic/osteogenic differentiation and a membrane-bound enzyme needed for mineralization. The results showed a comparably higher ALPase activity of MDPC-23 cells and hDPSCs cultured on CCN2-treated surfaces. The present study also revealed that MDPC-23 cells and hDPSCs produced calcific deposits when cultured in the presence of mineralization reagent, and CCN2 significantly enhanced cell mineralization compared to that of control. These findings have verified the hypothesis that CCN2 promotes the proliferation, differentiation and mineralization of odontoblast-like cells and human dental pulp stem cells, but also have indicated that the addition of 100 ng/mL CCN2 into culture media in a short culture period of 48h was confined to promotes mineralization rather than odontogenic differentiation.

Recently, ongoing studies have identified CCN2 protein as a growth and signaling factor that interacts with cell surface molecules and intracellular signaling pathways. Through the interaction with low-density lipoprotein receptor-related proteins (LRPs) leads to the modulation of Wnt, extracellular regulated kinases (ERK) and protein kinase C (PKC) signaling (Mercurio et al., 2004; Yosimichi et al., 2006). By interacting with integrins such as $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_V\beta_3$, and $\alpha_{IIb}\beta_3$, CCN2 triggers adhesion and migration of various cells (Lau, 2016). Similarly, CCN2 has been found to bind fibroblast growth factor receptor 2 (FGFR2) and dose-dependently enhance the binding of FGFR2 to FGFs (Aoyama et al., 2012). According to a recent review, the differentiation of odontoblast is induced by FGFs from enamel knots (Du et al., 2018). CCN2 protein is also highly

interactive with extracellular counterparts such as growth factors, which modify signal transduction. It has been reported that the combination of VWC module of CCN2 with TGF-β enhances the ligand-receptor interaction as well as modifies TGF-β signaling (Abreu et al., 2002). As a matricellular protein, the most important biochemical function of CCN2 may be the promotion of and collaboration with ECM molecules, forasmuch as adheres to ECM exert its function in cell survival. For example, interacting with fibronectin and perlecan, CCN2 serving as a co-receptor to induce sustained ERK activation which promote cell adhesion (Nishida et al., 2003). In this regard, the interactions with multiple counterparts indicate that CCN2 governs inter- and intracellular communications to either directly or indirectly regulate cell proliferation and differentiation.

To date, it has been confirmed that the gene expression of CCN2 is greatly influenced by different factors such as hormones, cytokines, growth factors, and even CCN2 itself. As an immediate early gene, it is well-established that upstream regulators such as TGF-β, bone morphogenetic protein-4 (BMP-4), FGFs, and vascular endothelial growth factor (VEGF) stimulate CCN2 gene expression (Yeger & Perbal, 2007). These regulators also induce cells to deposit ECM components which are considered as extracellular counterparts of CCN2. It is noteworthy that not only these molecules, but also external stimuli are involved in CCN2 gene expression. In particular, injury, exposure to oxygen deprivation, inflammation, and mechanical forces are critically involved (Chen & Lau, 2009; Kular et al., 2011; Yamashiro et al., 2001).

Odontoblasts are aligned along the periphery of the dental pulp in a single layer

and possess the ability to secrete dentine matrix and further form the tertiary dentine in response to stimuli such as attrition, caries, trauma and dental procedures (Arana-Chavez & Massa, 2004; Ruch et al., 1995). Differing from primary and secondary dentine, which are formed during dentinogenesis process of tooth development, tertiary dentin is the only regenerative form capable of preventing dental pulp from the damage afterwards. In particular, odontoblasts secrete and form the reactionary dentine under mild stimuli, whilst when irreversible damage causes the death of original odontoblasts, a new generation of odontoblast-like cells will form the reparative dentine for tissue repair (Smith et al., 1995). As far as reparative dentinogenesis is concerned, it has been demonstrated that BMP-1 and matrix metalloprotease-3 (MMP-3) are implicated in reparative dentinogenesis via CCN2 expression (Muromachi et al., 2012; Muromachi et al., 2015). These metalloproteases are upregulated in response to caries and restorative procedures in dental pulp which exert anti-inflammatory and regenerative effects on human dental pulp cells. It is obvious that interactions with extracellular components mentioned above result in stimulatory effects of CCN2 on the activities of odontoblasts and odontoblast-like cells in tertiary dentin formation.

5. Conclusion

This is the first *in vitro* study to investigate the effects of CCN2 on dentin regeneration. The results of present study provide evidence that CCN2 plays a stimulatory role in the processes of dentinogenesis on odontoblast-like cells and human dental pulp cells. In this respect, the findings suggest that CCN2 might have the potential as an adjunctive formula for dentin regeneration. Further studies should focus on the detailed molecular mechanisms of CCN2, and for a possible clinical application, *in vivo* study should also be required for analysis (Qiu et al., 2018).

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Figure legends

Figure 1 Expressions of CCN protein family and β-actin in MDPC-23 cells. Cells were cultured in DMEM containing 5% FBS for five days until they reached confluency. Then media were changed to DMEM containing 5% FBS, 10 mM β-GP and 50 μg/mL AA for up to three days. (A) Conventional RT-PCR results. (B) Real time quantitative RT-PCR results. Only CCN2 mRNA expression was markedly increased during the odontogenic differentiation of MDPC-23 cells. rβ-actin was used as an internal standard. $p < 0.05$.

Figure 2 Effects of CCN2 on the proliferation of cells. Absorbance was measured using microplate reader, OD=450 nm. (A) Soluble CCN2 protein was directly added into culture media. MDPC-23 cells were treated with indicated concentrations of CCN2 for three days. Concentrations from 100 to 2000 ng/mL stimulated the proliferation of MDPC-23 cells. (B) CCN2 group coated with soluble CCN2 protein versus control group coated with dH_2O. After four days of culture, significant differences were observed between control and CCN2-treated groups. CCN2 at the concentration of 1000 ng/mL maximize the promotion of MDPC-23 cells proliferation. (C) CCN2 group coated with soluble CCN2 protein versus control group coated with dH_2O. After two days of culture, concentration of 2000ng/mL maximize the promotion of hDPSCs proliferation. $p < 0.05$.

Figure 3 Cell morphology observation. MDPC-23 cells morphology was observed at 48h, 72h, and 96h respectively. The scale bar in the control group image of day two applies to all panels (bar = 200 μm).

Figure 4 Fluorescence microscopy photographs. MDPC-23 cells adhered to and spread onto Non-TCPP coated with CCN2 (1000 ng/mL) or dH_2O (control). Actin stress fibers were visualized with Alexa Fluor 568-conjugated phalloidin (red-orange), and nuclei were visualized with DAPI (blue). The scale bar in the merge image of the control group applies to all panels (bar = 200 μm).

Figure 5 Cell morphology observation. hDPSCs morphology was observed after 24h, 48h, and 72h of culture respectively. The scale bar in the control group image of day two applies to all panels (bar = 200 μm).

Figure 6 Fluorescence microscopy photographs. hDPSCs adhered to and spread onto Non-TCPP coated with CCN2 (1000 ng/mL) or dH_2O (control). Actin stress fibers were visualized with Alexa Fluor 568-conjugated phalloidin (red-orange), and nuclei were visualized with DAPI (blue). The scale bar in the merge image of the control group applies to all panels (bar = 200 μm).

Figure 7 Relative ALPase activity. MDPC-23 cells adhered to and spread on Non-TCPP coated with CCN2 (1000 ng/mL) or dH_2O (control). ALPase activity was then assessed on day six, seven, and eight respectively. The CCN2-coated group exhibited significantly higher ALPase activity than the control group. $p < 0.05$.

Figure 8 Relative ALPase activity. hDPSCs adhered to and spread onto Non-TCPP coated with CCN2 (1000 ng/mL) or dH_2O (control). ALPase activity was then assessed on day 14, 21, and 28 respectively. The relative ALPase activity of the CCN2-coated group was maintained at a higher level during differentiation of hDPSCs as compared to that of the control group. $p < 0.05$.

Figure 9 Effects of CCN2 on the mRNA expression of odontogenic differentiation markers. In direct addition of CCN2 (experiment 1), the mRNA expression of rBSP (A), rOCN (B) and rOPN (C) were significantly upregulated after 48h administration of CCN2 (100 ng/mL) on MDPC-23 cells compared to that of the control group. In the CCN2-coated group (1000 ng/mL) of experiment 2, all tested genes had significantly higher mRNA expression levels than those in the control group after eight days of MDPC-23 cells culture (D). The immobilized form of CCN2 also significantly promoted the expression of hard tissue-forming markers during the differentiation of hDPSCs, including hBSP (E), hCol1a1 (F), and hALP (G) on day 22. $p < 0.05$.

Figure 10 CCN2 induces cell mineralization. (A) Photographs of mineral nodules formed after eight days of culture in experiment 1. (B) CPC quantification of mineralized products staining intensity of (A). The CCN2-treated group (100 ng/mL) showed a slightly higher result compared to the control group. (C) Photographs of mineral nodules formed after six and seven days of culture in experiment 2. The CCN2-coated group (1000 ng/mL) exhibited a significant promotion of calcific deposition as compared to the control group. (D) CPC quantification of mineralized products staining intensity of (C). On both day six and seven, the absorbance of released ARS in the CCN2-coated group was comparatively higher than that in the control group. (E) Photographs of mineral nodules formed after 29 and 32 days of culture in experiment 3. (F) CPC quantification of mineralized products staining intensity of (E). In 1000 ng/mL CCN2 coated group, the mineralization of hDPSCs

was strongly promoted on both day 29 and 32. $p < 0.05$.

Tables

Table 1 Rat primer sets used for conventional RT-PCR

Target gene	Primer sequence	Product size (bp)
rCCN1	Forward: 5'- GGGTTTCTAGTGTGGGTCGG -3' Backward: 5' - AACCCGGGCTCCAGTACTAT -3'	194
rCCN2	Forward: 5'- CACCCGGGTTACCAATGACA -3' Backward: 5'- TTCATGATCTCGCCATCGGG -3'	288
rCCN3	Forward: 5'- TCTGAGATGAGACCCTGCGA -3' Backward: 5'- GACCCCATCGAACACACAGT -3'	120
rCCN4	Forward: 5'- AGGTGTGTGGCTTGGAGAAC -3' Backward: 5'- GAGGGACCCATTGTCTGAGC -3'	198
rCCN5	Forward: 5'- GTAGAGCCACTGAGCGATCC -3' Backward: 5'- GCAATCTGCTGTCTGGTCCT -3'	223
rCCN6	Forward: 5'- ATCAGCGTAAAGAGGTCTGCC -3' Backward: 5'- GTACAGGCCTTTGTGTGGGT -3'	173
rβ-actin	Forward: 5'- AACCCTAAGGCCAACAGTGAAAAG -3' Backward: 5'- TCATGAGGTAGTCTGTGAGGT -3'	240

Table 2 Rat primer sets used for real time quantitative RT-PCR

Target gene	Primer sequence	Product size (bp)
rDMP-1	Forward: 5'- CGTTCCTCTGGGGGCTGTCC -3' Backward: 5' - CCGGGATCATCGCTCTGCATC -3'	577
rALP	Forward: 5'- GGAAGGAGGCAGGATTGACCAC -3' Backward: 5'- GGGCCTGGTAGTTGTTGTGAGC -3'	388
rBSP	Forward: 5'- CTGCTTTAATCTTGCTCTG -3' Backward: 5'- CCATCTCCATTTTCTTCC -3'	211
rOCN	Forward: 5'- AGCTCAACCCCAATTGTGAC -3' Backward: 5'- AGCTGTGCCGTCCATACTTT -3'	190
rOPN	Forward: 5'- TTTCCCTGTTTCTGATGAACAGTAT -3' Backward: 5'- CTCTGCTTATACTCCTTGGACTGCT -3'	228
rDSPP	Forward: 5'- TCAATGGCGGGTGCTTTAGA -3' Backward: 5'- TGCTCACTGCACAACATGAAGA -3'	111
rRunx-2	Forward: 5'- CCACAGAGCTATTAAAGTGACAGTG -3' Backward: 5'- AACAAACTAGGTTTAGAGTCATCAAGC -3'	87
rCol1a1	Forward: 5'- ATCAGCGTAAAGAGGTCTGCC -3' Backward: 5'- GTACAGGCCTTTGTGTGGGT -3'	173
rβ-actin	Forward: 5'- AACCCTAAGGCCAACAGTGAAAAG -3' Backward: 5'- TCATGAGGTAGTCTGTGAGGT -3'	240

Table 3 Human primer sets used for real time quantitative RT-PCR

Target gene	Primer sequence	Product size (bp)
hALP	Forward: 5'- ATGGGATGGGTGTCTCCACA -3' Backward: 5'- CCACGAAGGGGAACTTGTC -3'	108
hBSP	Forward: 5'- AAGGGCACCTCGAAGACAAC -3' Backward: 5'- CCCTCGTATTCAACGGTGGT -3'	119
hCol1a1	Forward: 5'- CCAGACCAGGAATTCGGCTT -3' Backward: 5'- GCAGAAAGGGACTTACCCCC -3'	371
hGAPDH	Forward: 5'- CACTAGGCGCTCACTGTTCTCT -3' Backward: 5'- CGTTCTCAGCCTTGACGGT -3'	250

Figures

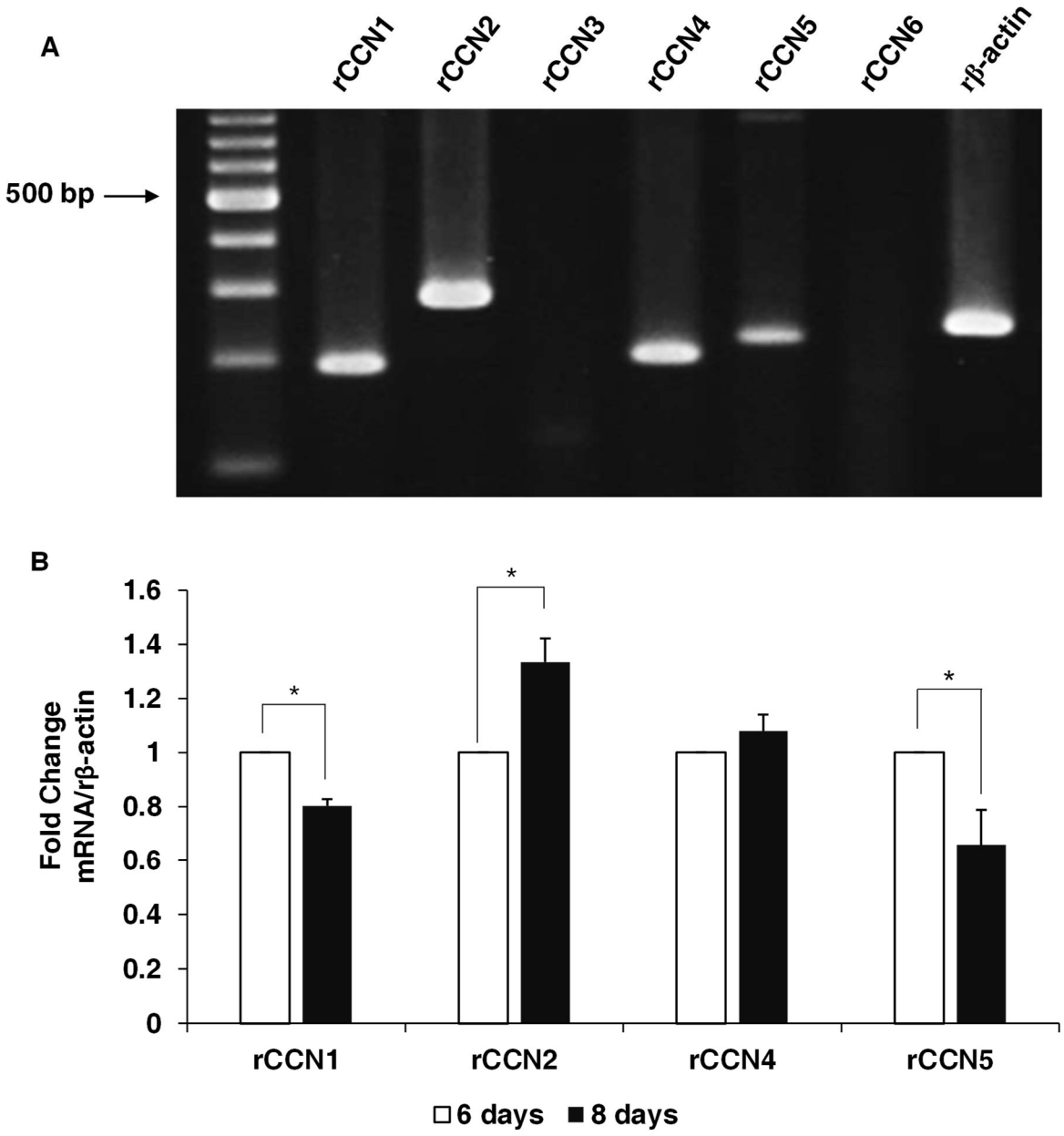

Figure 1

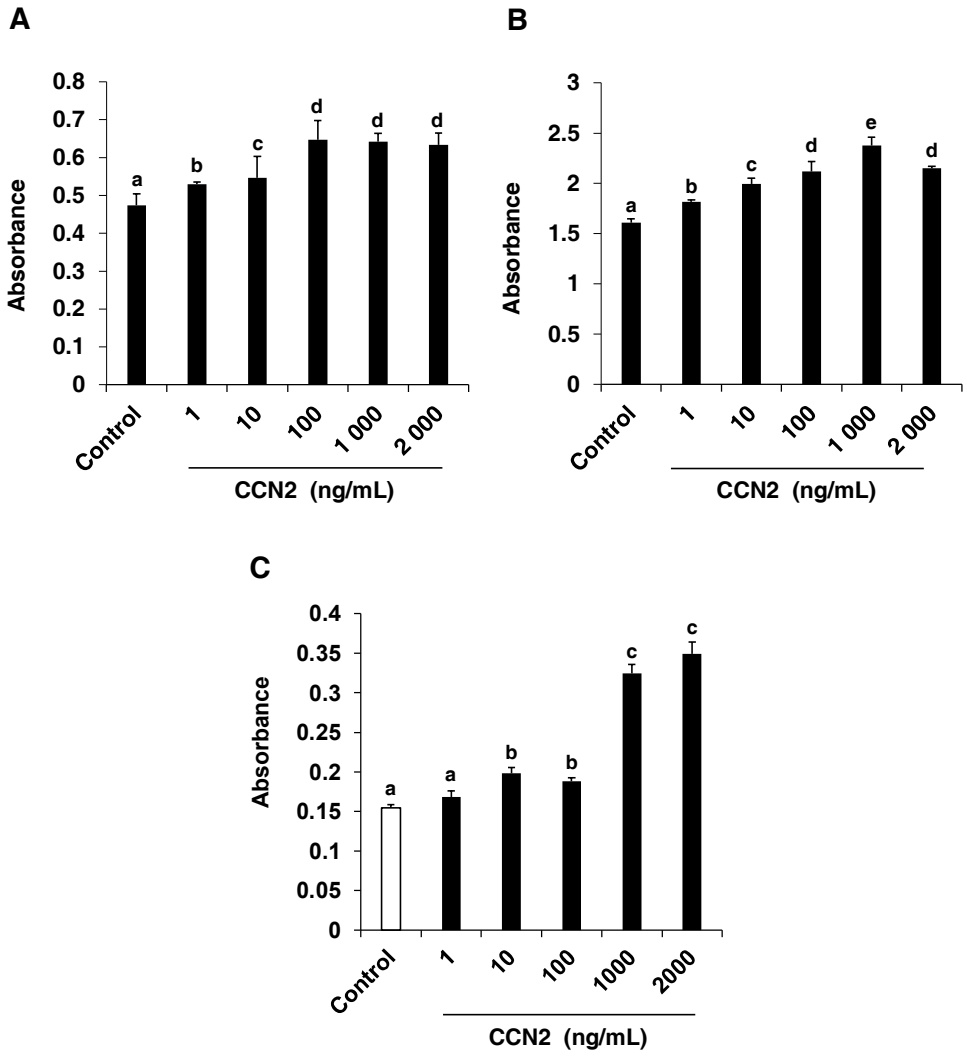
A
Absorbance
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
c
d
d
d
Control
1
10
100
1 000
2 000
CCN2 (ng/mL)
B
Absorbance
3
2.5
2
1.5
1
0.5
0
a
b
c
d
e
d
Control
1
10
100
1 000
2 000
CCN2 (ng/mL)
C
Absorbance
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a
a
b
b
c
c
Control
1
10
100
1000
2000
CCN2 (ng/mL)

Figure 2

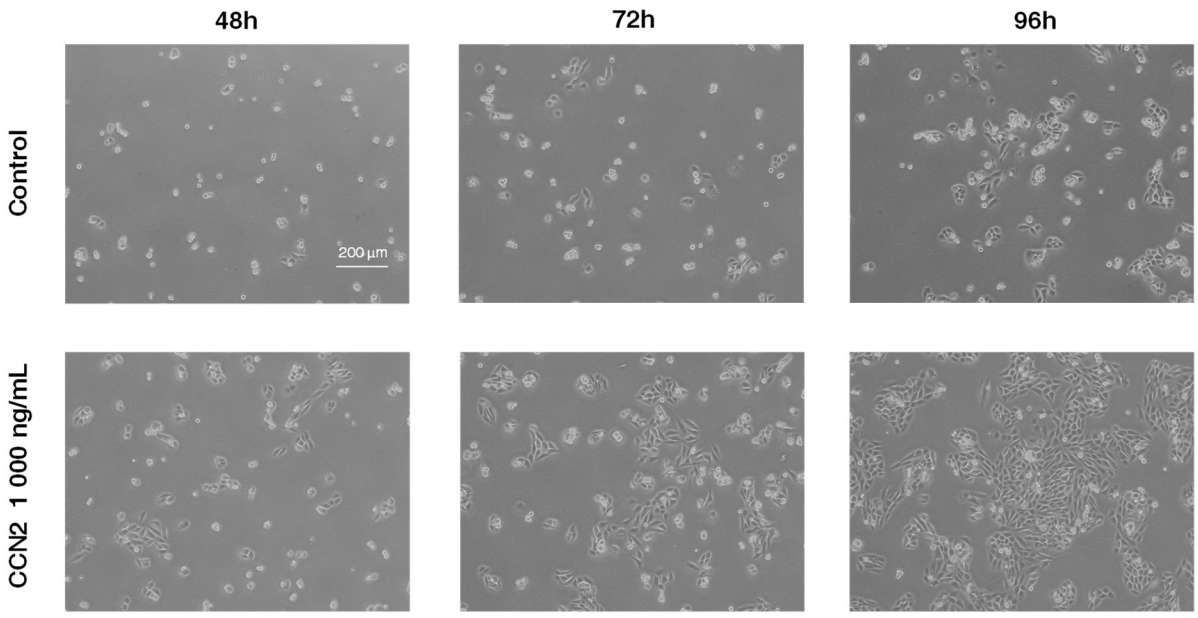

Figure 3

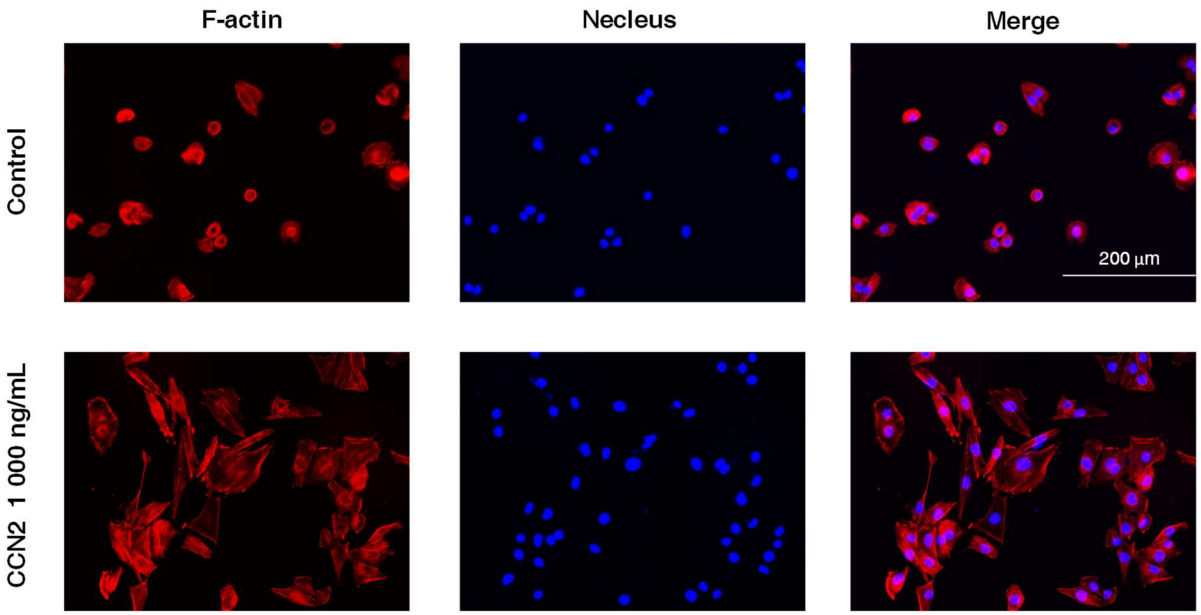

Figure 4

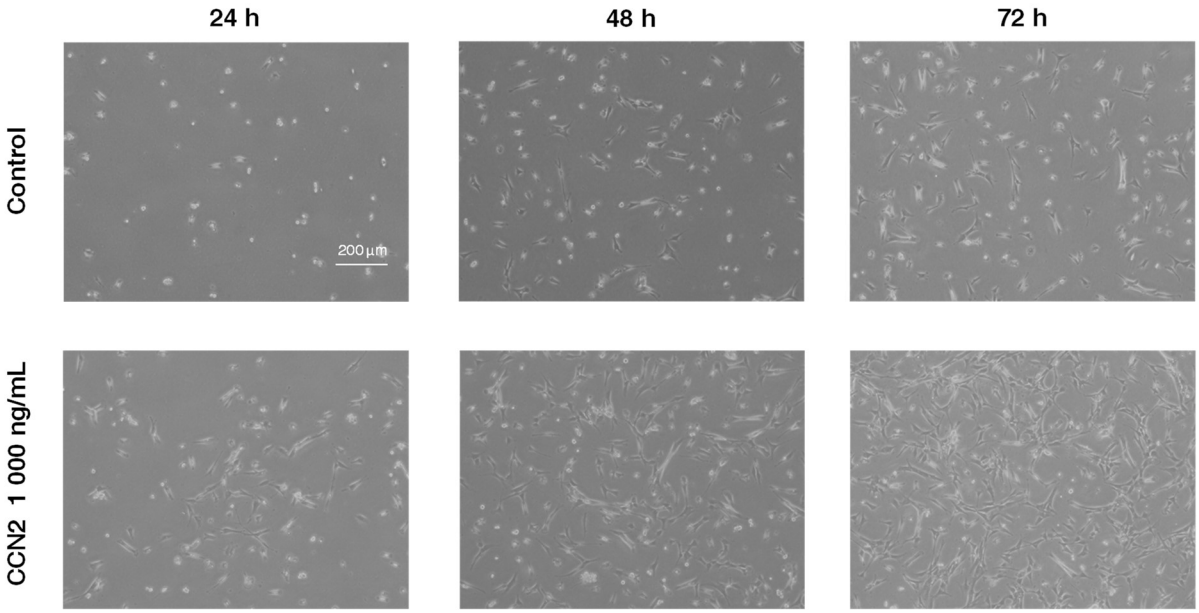

Figure 5

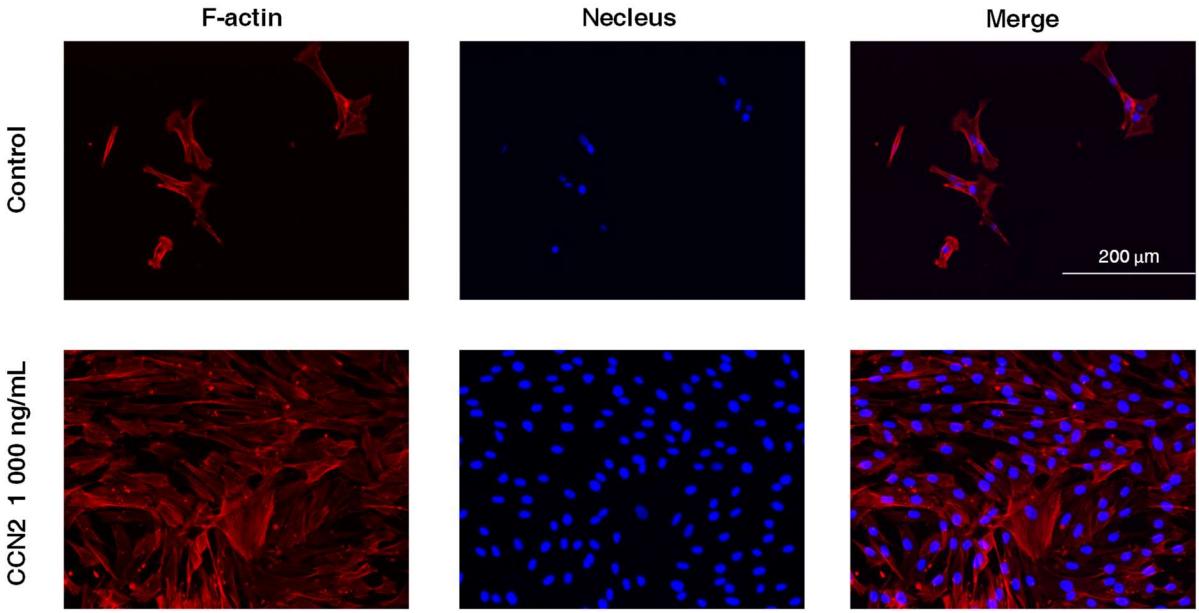

Figure 6

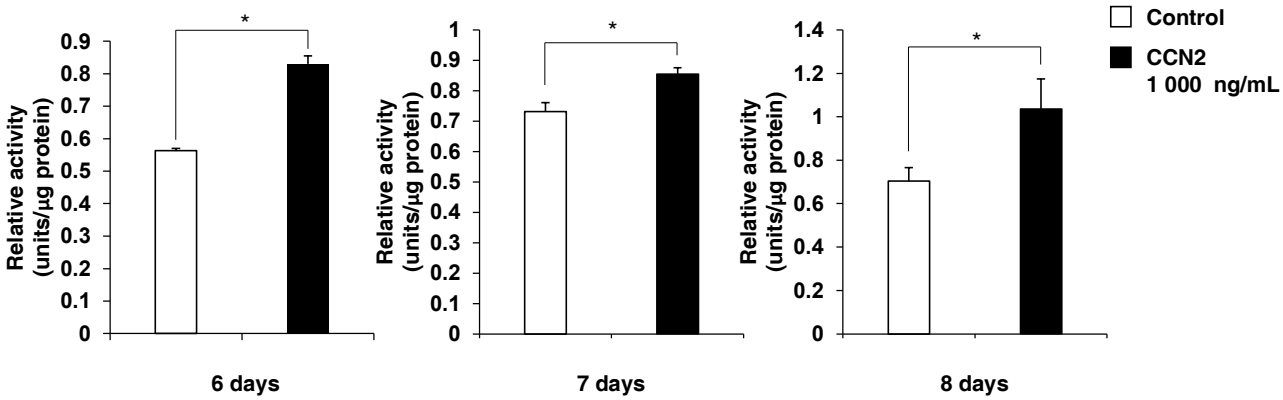
Relative activity (units/µg protein)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
1.2
1.4
*
6 days
7 days
8 days
Control
CCN2
1 000 ng/mL

Figure 7

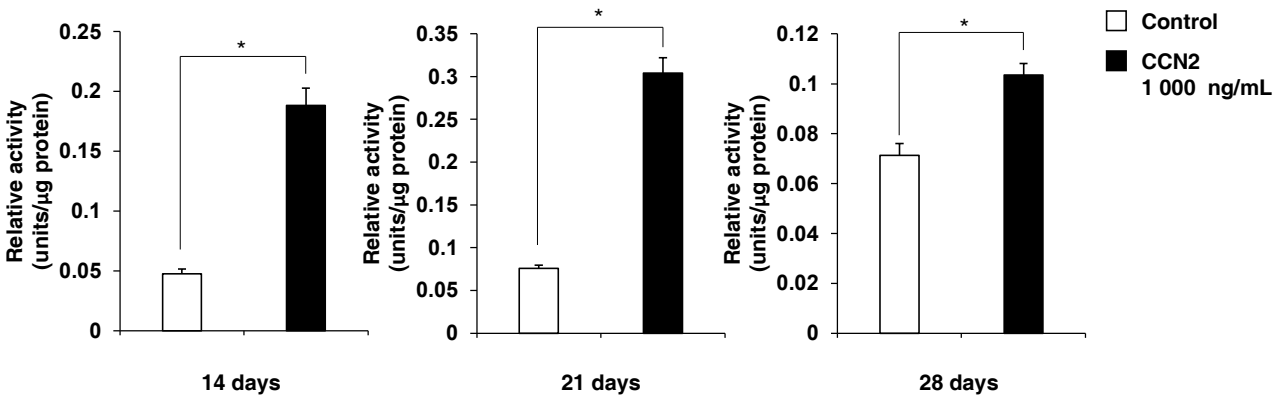
Relative activity (units/µg protein)
0.25
0.2
0.15
0.1
0.05
0
*
14 days
Relative activity (units/µg protein)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
21 days
Relative activity (units/µg protein)
0.12
0.1
0.08
0.06
0.04
0.02
0
*
28 days
Control
CCN2
1 000 ng/mL

Figure 8

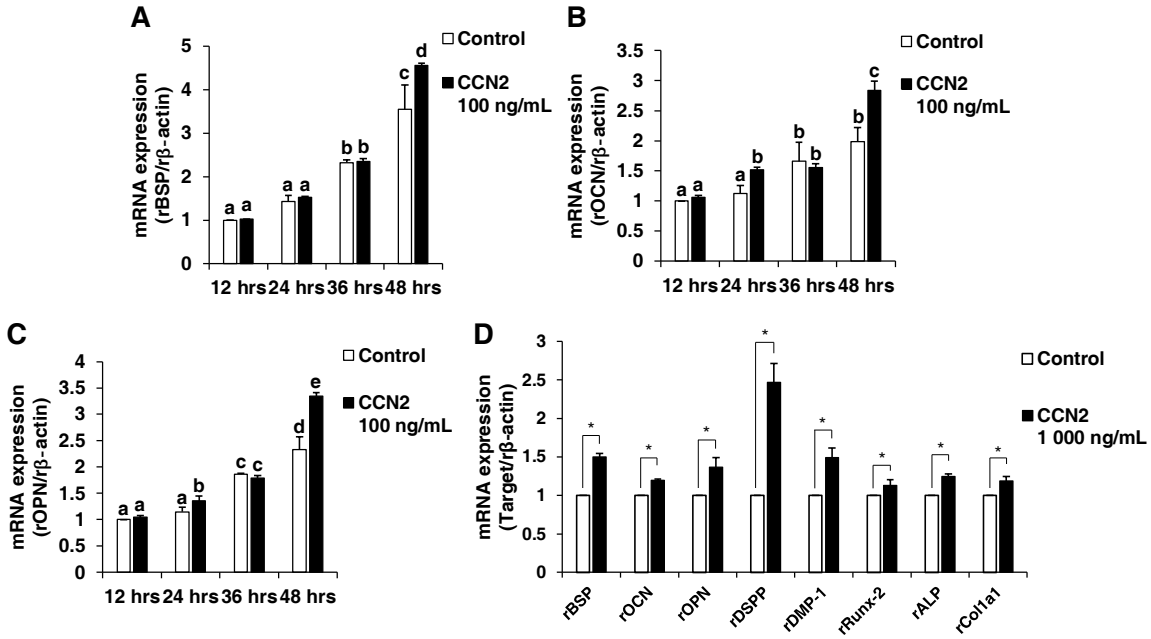
A
mRNA expression (rBSP/rβ-actin)
Control
CCN2 100 ng/mL
12 hrs 24 hrs 36 hrs 48 hrs
B
mRNA expression (rOCN/rβ-actin)
Control
CCN2 100 ng/mL
12 hrs 24 hrs 36 hrs 48 hrs
C
mRNA expression (rOPN/rβ-actin)
Control
CCN2 100 ng/mL
12 hrs 24 hrs 36 hrs 48 hrs
D
mRNA expression (Target/rβ-actin)
Control
CCN2 1 000 ng/mL
rBSP
rOCN
rOPN
rDSPP
rDMP-1
rRunx-2
rALP
rCol1a1

Figure 9A-D

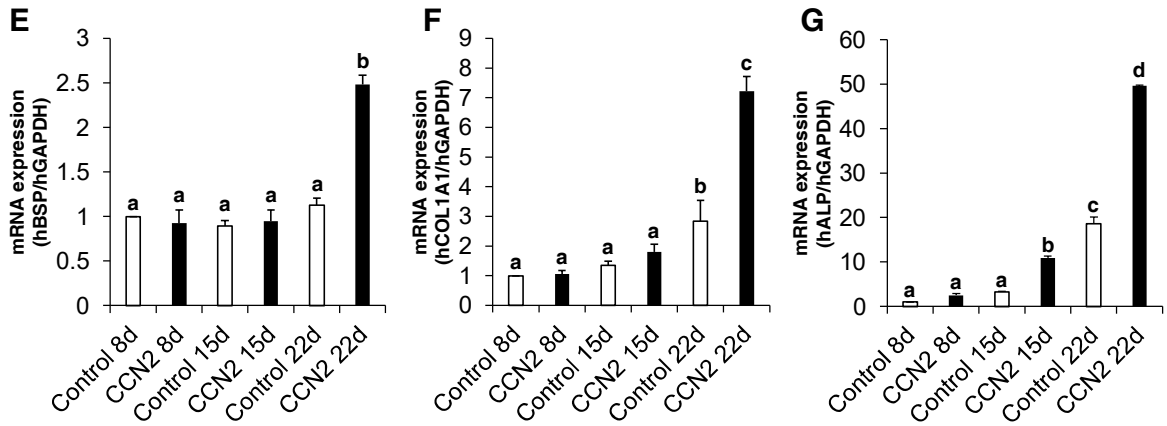
E
mRNA expression (hBSP/hGAPDH)
3
2.5
2
1.5
1
0.5
0
a
a
a
a
a
b
Control 8d
CCN2 8d
Control 15d
CCN2 15d
Control 22d
CCN2 22d
F
mRNA expression (hCOL1A1/hGAPDH)
9
8
7
6
5
4
3
2
1
0
a
a
a
a
b
c
Control 8d
CCN2 8d
Control 15d
CCN2 15d
Control 22d
CCN2 22d
G
mRNA expression (hALP/hGAPDH)
60
50
40
30
20
10
0
a
a
a
b
c
d
Control 8d
CCN2 8d
Control 15d
CCN2 15d
Control 22d
CCN2 22d

Figure 9E-F

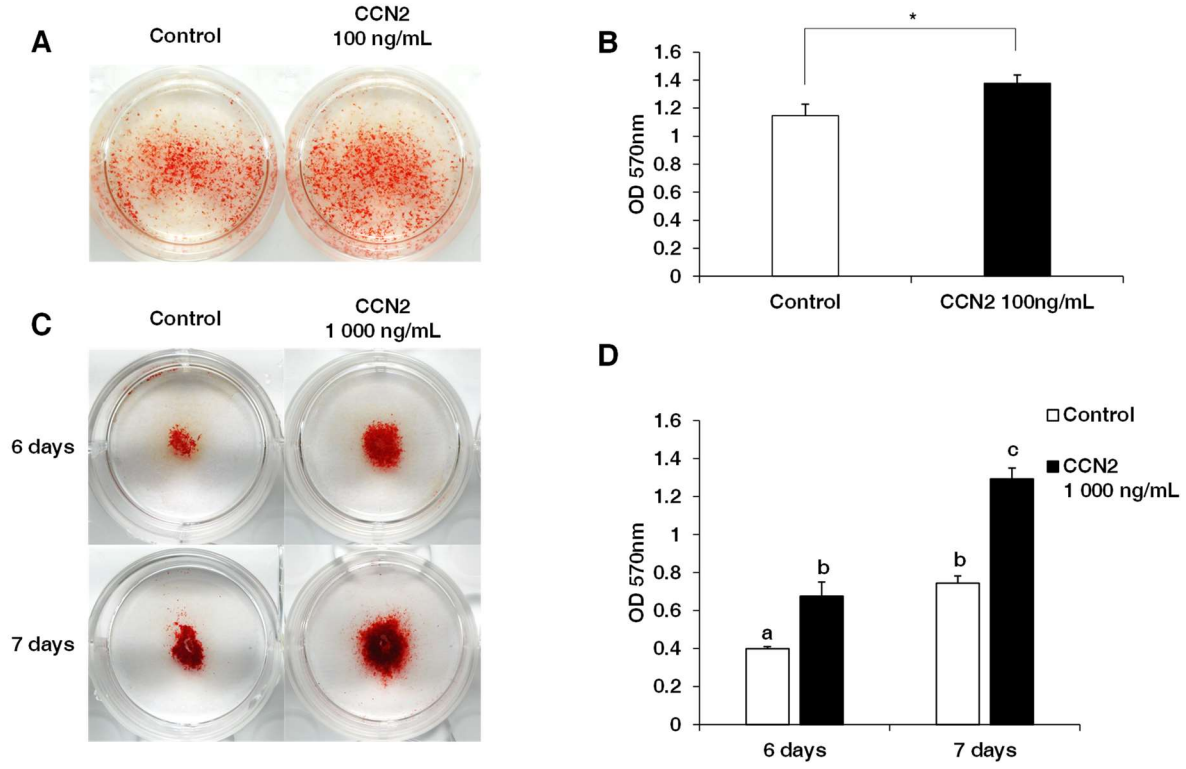
A
Control
CCN2
100 ng/mL
B
*
OD 570nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
CCN2 100ng/mL
C
Control
CCN2
1 000 ng/mL
6 days
7 days
D
Control
CCN2
1 000 ng/mL
OD 570nm
a
b
b
c
6 days
7 days

Figure 10A-D

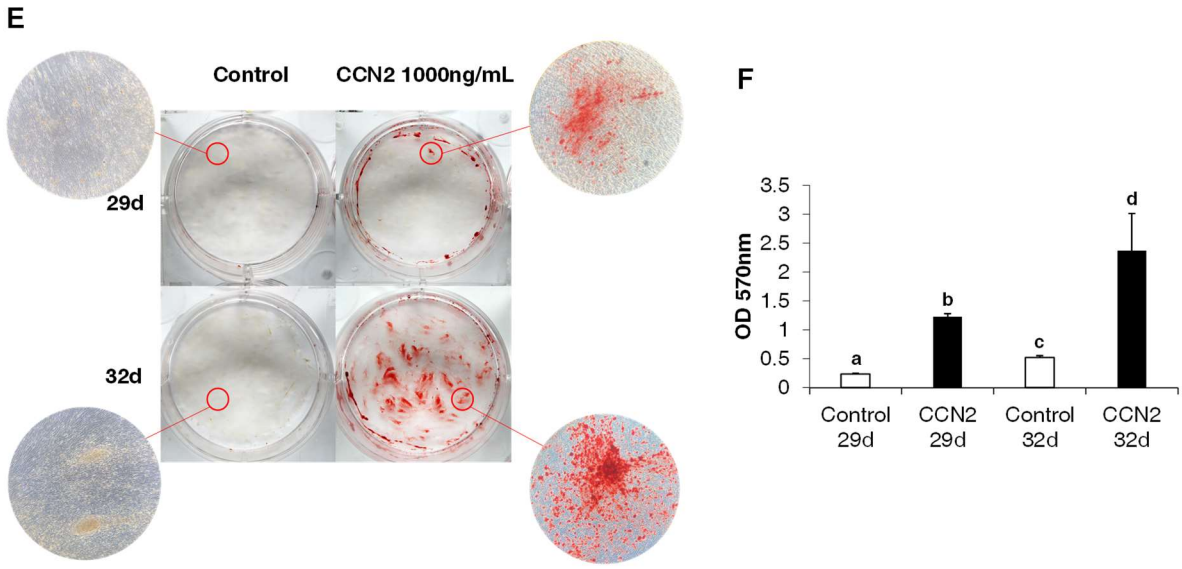
E
Control
CCN2 1000ng/mL
29d
32d
F
OD 570nm
3.5
3
2.5
2
1.5
1
0.5
0
a
b
c
d
Control 29d
CCN2 29d
Control 32d
CCN2 32d

Figure 10E-F