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Journal

The Journal of Biochemistry, 157(2)

ISSN

0021-924X

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Publication Date

2015-02-01

DOI

10.1093/jb/mvu056

Peer reviewed

OXFORD JOURNALS Journal of Biochemistry

J Biochem. 2015 Feb; 157(2): 91–100.

Published online 2014 Sep 26. doi: 10.1093/jb/mvu056

PMCID: PMC6281389

PMID: 25261584

CCN family protein 2 (CCN2) promotes the early differentiation, but inhibits the terminal differentiation of skeletal myoblasts

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Abstract

Muscle development during embryogenesis is conducted by myogenic stem cells (1). These cells initially proliferate to produce myogenic precursor cells, called myoblasts, to regenerate the muscle (1, 2). These myoblasts then proliferate, increasing their number; and then, these cells undergo terminal differentiation and form multinucleate myotubes by cell fusion (1, 2). Eventually, new muscle fibers are produced, which finally mature into contracting muscle fibers (1, 2). The entire process is guided by various extracellular environments and is regulated by distinct intracellular signaling pathways, resulting in the activation of myogenic regulatory factors (MRFs) such as MyoD, Myf5, myogenin, and MRF4 (1, 2). MyoD and Myf5 are expressed in myoblasts; whereas myogenin and MRF4 induce terminal differentiation of myoblasts into the myotubes, which express important genes for muscle cell function, such as myosin heavy chain (MHC; *1*, *2*).

CCN2 (also known as connective tissue growth factor: CTGF) is a protein secreted from various cell types including chondrocytes, osteoblasts and vascular endothelial cells and its structure is characterized by four distinct modules, *i.e.* insulin-like growth factor binding protein-like, von Willebrand factor type C, thrombospondin type 1 repeat and a carboxyl terminal cystine knot (CT; *3–6*). CCN2 belongs to the CCN family, which consists of six distinct proteins; *i.e.* Cysteine-rich 61 (Cyr61/CCN1), CTGF/CCN2, Nephroblastoma overexpressed (Nov/CCN3), from which the family name is derived; and Wnt-inducible secreted proteins 1, 2 and 3 (Wisp1-3/CCN4-6), having structures similar to those described above, except that Wisp2/CCN5 lacks the CT module (3–6). It has been reported that CCN family members are involved in a number of biological processes in development, including chondrogenesis, osteogenesis and angiogenesis (3–6). Therefore, it is suspected that CCN family members are potentially involved in musuclogenesis. It has been reported that excess CCN2 induces damage and fibrosis in skeletal muscle tissues (7–9) and that both CCN1 (10) and CCN3 (11) impair skeletal muscle differentiation. However, these findings only suggest the effects of overproduced CCN family members in skeletal muscle under the pathological condition. In fact, although overexpression of CCN2 causes fibrotic disorders in various

tissues and organs, regulated expression of CCN2 is observed during the development of the same tissues and organs (12–14). Therefore, we hypothesized that CCN2 plays certain roles in skeletal myogenesis. Adult skeletal muscle possesses remarkable regeneration capabilities (1), and tissue regeneration is a recurrence of tissue development (2). In this study, to examine the validity of our hypothesis, we investigated the functions of CCN2 in skeletal muscle regeneration in the physiological state.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan) and Cancera International (Rexcalale, ON, Canada), respectively. Plastic dishes and multiwell plates were obtained from Greiner Bio-One (Frickenhausen, Germany). Anti-integrin α5 and anti-CCN2 antibodies were purchased from Chemicon International, Inc. (Billerica, MA, USA) and Abcam (Cambridge, UK), respectively. Anti-β-actin and anti-proliferating cell nuclear antigen (PCNA) were from Sigma (St. Louis, MO, USA), anti-hemagglutinin (HA) was from Covance (Princeton, NJ, USA), anti-desmin was from Nichirei (Tokyo, Japan) and both anti-MyoD and anti-myogenin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA,USA). Recombinant tumor necrosis factor-α (TNFα) was purchased from Promega (Madison, WI, USA), and recombinant CCN2 (rCCN2) was purified as described previously (15).

Cell culture

Mouse myoblast cell line C2C12 was purchased from the European collection of cell cultures (ECACCs, Salisbury, UK). The cells were inoculated at a density of 1.5×10^4 cells/cm^2 in DMEM supplemented with 10% FBS and were cultured at 37°C under 5% CO_2 in air. Primary mouse myoblasts were isolated from the gastrocnemius muscle of *Ccn2*-deficient mice at embryonic day (E) 18.5 (16). The isolated tissues were digested with 0.2% collagenase (Wako Co., Osaka, Japan) for 40 min at 37°C, and then filtered through a 40 μm cell strainer (BD Biosciences; Bedford, MA, USA; *17*, *18*). After genotyping, these cells were passaged, inoculated at a density of 1.5×10^4 cells/cm^2, and cultured in DMEM containing 10% FBS at 37°C under 5% CO_2 until they reached 70–80% confluence. For induction of differentiation, the culture medium for both C2C12 cells and primary myoblasts was changed to serum-free DMEM containing 1 mg/ml bovine serum albumin (BSA; Sigma), 10 μg/ml bovine insulin (I; Wako Co.), 5 μg/ml human transferrin (T; Sigma) and 1×10^{-8} M sodium selenite (S; Sigma); and the cells were then cultured at 37°C under 5% CO_2 for 4–7 days (19). The Animal Committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences had approved all of the procedures.

Quantitative RT–PCR analysis

Total RNA was isolated from C2C12 cells and primary myoblasts by using ISOGEN reagent (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized by use of a Takara RNA PCR kit (AMV) Version 3.0 (Takara Shuzo, Tokyo, Japan), and amplification reactions were performed with a SYBR® Green Real-time PCR Master Mix (Toyobo, Tokyo, Japan) and a StepOnePlus™ Real-time PCR System (Applied Biosystems, Basel, Switzerland; *20*, *21*). The primer sequences are indicated in Table I.

Table I.

Sense (S) and antisense (AS) primers used for PCR.

Genes	Accession Number	Primer sequence
MyoD	NM_010866	(S) 5′-CGCTCCAACTGCTCTGAT-3′
		(AS) 5′-AGATGCGCTCCACTATGC-3′
Desmin	NM_010043	(S) 5′-CTTGACTCAGGCAGCCAATAA-3′
		(AS) 5′-AGCTCCCTCATCTGCCTCAT-3′
MHC	NM_080728	(S) 5′-CGCAATGCAGAGTCAGTGAA-3′
		(AS) 5′-TTGCGGAACTTGGACAGGTT-3′
Myostatin	NM_ 010834	(S) 5′-AATCCACCACGGTGCTAATG-3′
		(AS) 5′-CTCCCGCTGTGCTAGAGTTG-3′
Myogenin	NM_ 031189	(S) 5′-CCTTGCTCAGCTCCCTCAA-3′
		(AS) 5′-CTGGGTTGGGACCGAACT-3′
Gapdh	NM_008084	(S) 5′-GCCAAAAGGGTCATCATCTC -3′
		(AS) 5′-GTCTTCTGGGTGGCAGTGAT-3′

Transfection with CCN2 expression plasmid and western blot analysis

C2C12 cells were transfected with a CCN2 expression plasmid bearing an HA-tag (22) by using Fugene6 reagent (Roche Applied Science, Mannheim, Germany). After 4 days, the cell lysate was collected; and then western blot analysis was performed as described previously (23).

Cell proliferation assay

For measurement of cell proliferation, C2C12 cells were inoculated into each well of a 96-well multiwell plate at a density of 1×10^4 cells/well and cultured in DMEM containing 10% FBS. Then, the next day, the medium was replaced with DMEM without FBS; and the cells were treated with rCCN2 for 16 h. The effect of CCN2 on the proliferation of C2C12 cells was determined by use of a Cell proliferation ELISA, BrdU (5-bromo-2′-deoxyuridine) colorimetric immunoassay (Roche Applied Science; *24*).

Immunohistochemistry

Ccn2-deficient mice at E 18.5 were euthanized to obtain hind limbs, which were fixed in 10% formalin overnight at 4°C before being embedded in paraffin. Five-micrometre sections were mounted on glass slides and deparaffinized with xylene, rehydrated and treated with hyaluronidase (25 mg/ml; Sigma) for 30 min at room temperature for antigen retrieval. Immunofluorescence staining of these sections was performed with both anti-PCNA and anti-MyoD antibodies, or anti-desmin (Nichirei) alone as described previously (25).

Indirect immunofluorescence analysis

Myoblasts from WT and *Ccn2*-deficient mice were cultured until they had reached 70–80% confluence. Then, the culture medium was exchanged for serum-free DMEM containing 1 mg/ml BSA and insulin–transferrin–selenite (ITS). After 7 days, the cultures were fixed with 3.5% paraformaldehyde for 30 min at room temperature, and made permeable with 0.1% NP-40 in PBS. Indirect immunofluorescence analysis was performed as described previously (26).

Statistical analysis

Unless otherwise specified, all experiments were repeated at least twice, and similar results were obtained. Statistics were calculated using Student's *t* test or one-way ANOVA.

Results

CCN2 promotes the proliferation of C2C12 cells

Firstly, we performed a BrdU incorporation assay on C2C12 cells stimulated by rCCN2. As shown in Fig. 1A, rCCN2 significantly stimulated the cell proliferation of C2C12 cells in a dose-dependent manner. Next, we performed western blot analysis of cell lysates of C2C12 cells treated with rCCN2 at a concentration of 50 and 100 ng/ml by using antibody against PCNA, which is a marker of cell proliferation. As shown in Fig. 1B, the signal for PCNA was increased by the treatment with rCCN2 in a dose-dependent manner. To support the data of Fig. 1B, we transfected C2C12 cells with an HA epitope-tagged full-length *Ccn2* (HA-*Ccn2*) expression plasmid (22). After 4 days we collected the cell lysates and performed western blot analysis with anti-HA and PCNA antibodies. As a result, an HA-specific signal was clearly detected in C2C12 cells transfected with HA-*Ccn2* expression plasmid, and the intensity of the band of PCNA was increased in the cells transfected with the HA-*Ccn2* expression plasmid (Fig. 1C). These findings indicate that CCN2 promotes the proliferation of C2C12 cells.

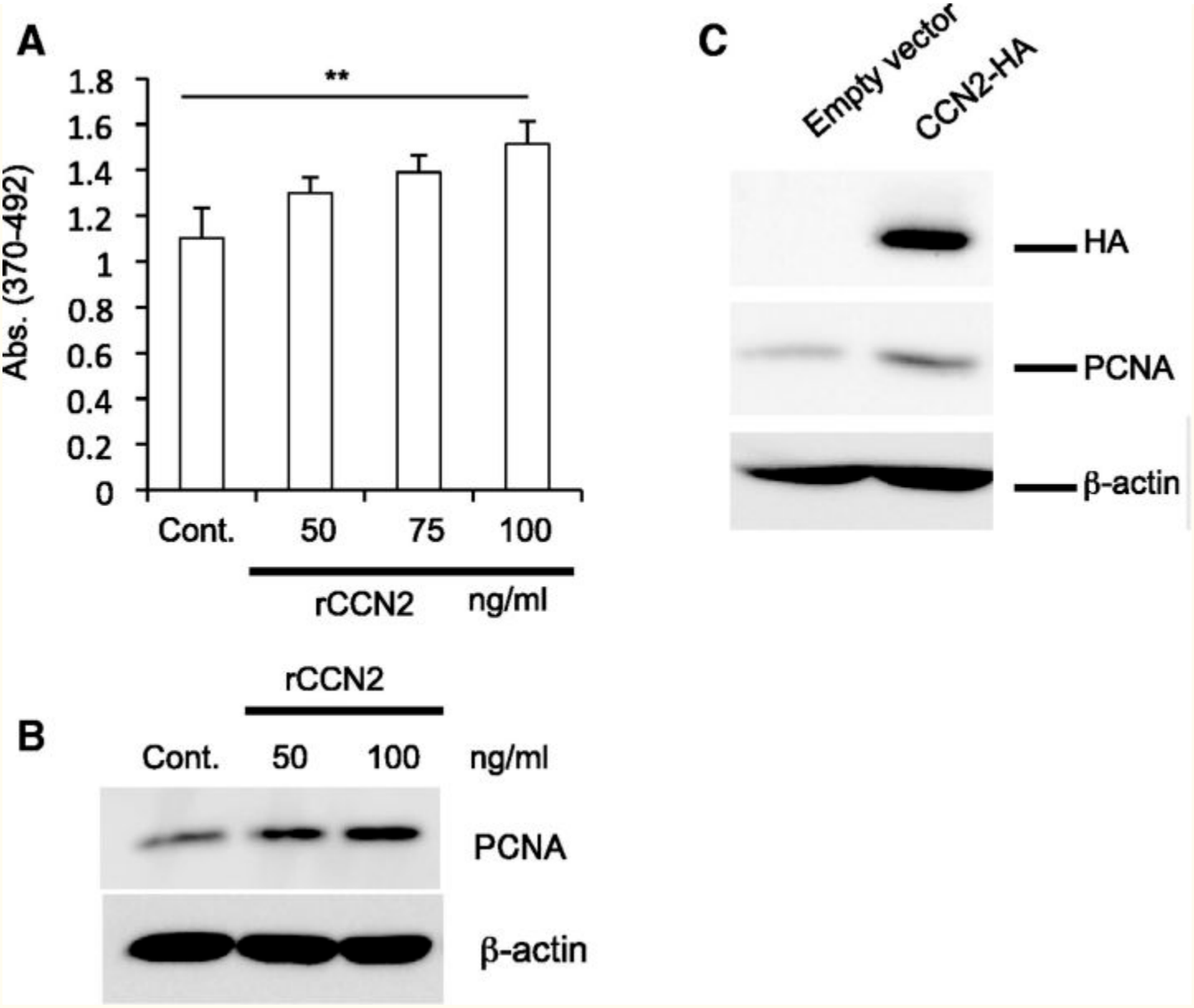


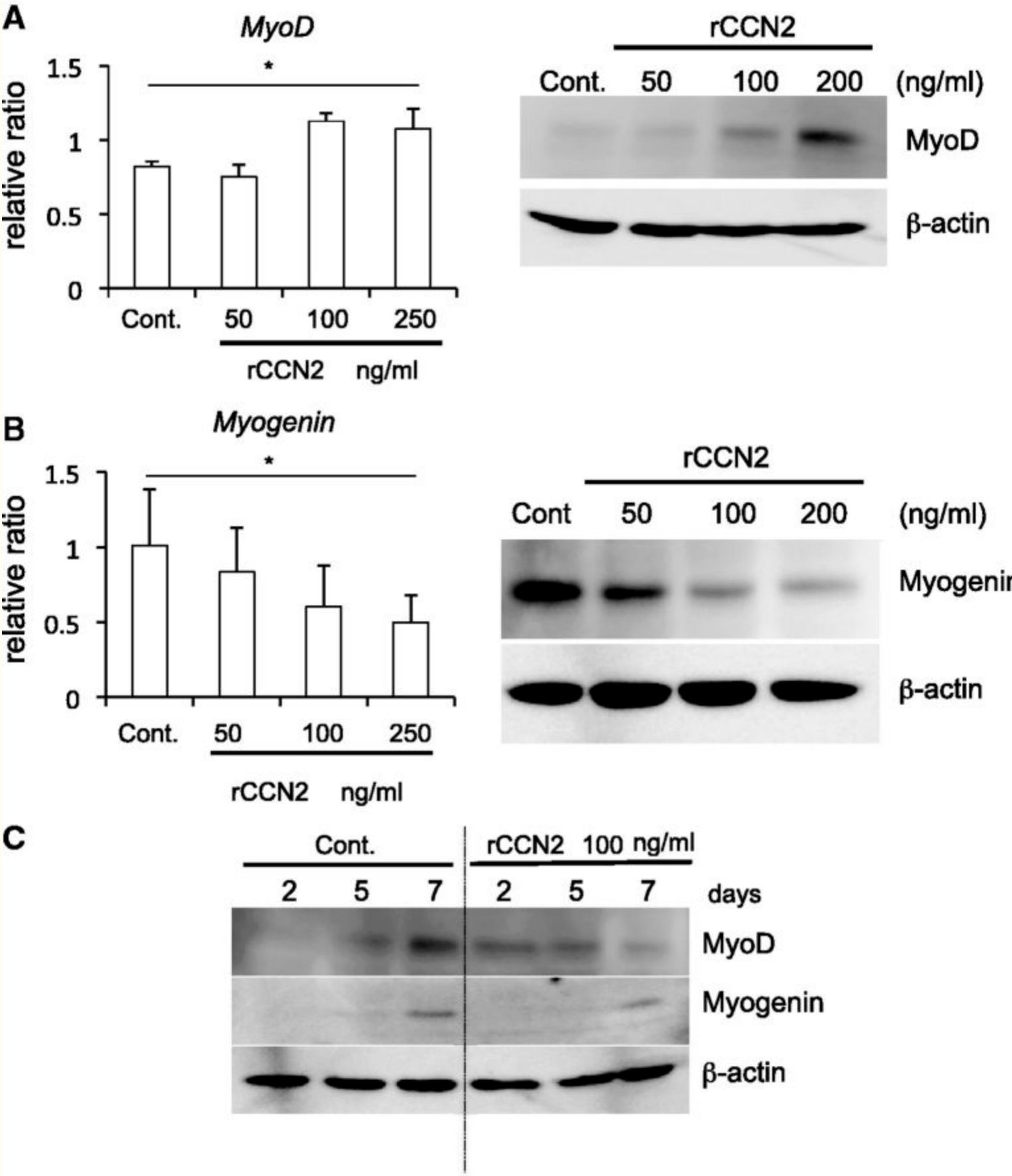
Fig. 1

CCN2 promotes the cell proliferation of C2C12 cells. (**A**) C2C12 cells were stimulated for 16 h with rCCN2 at the indicated concentrations in differentiation medium. Then, cell proliferation assay was performed. Each bar shows the mean value and standard deviation of the results from nine wells. $^{**}P < 0.01$ indicates significant difference from control by one-way ANOVA. (**B**) C2C12 cells were treated with rCCN2 at the indicated concentrations for 24 h. Western blot analysis was then performed by using anti-PCNA and anti-β-actin antibodies. (**C**) C2C12 cells were transiently transfected with CCN2 expression plasmid containing an HA tag, and the cells were cultured for 4 days. Western blot analysis was performed by using antibodies against the indicated antigens.

CCN2 promotes the myoblast differentiation at an early stage but inhibits differentiation at late stage

We next investigated the effect of CCN2 on the differentiation of myoblasts. C2C12 cells were inoculated into 35-mm dishes, and cultured until they reached 70–80% confluence. Then, the medium was changed to serum-free DMEM containing ITS (differentiation medium) and rCCN2. After 24 h, total RNA was collected; and quantitative RT–PCR analysis was then performed by using specific primers for MyoD, which is a marker of early-stage myoblast differentiation. As shown in Fig. 2A left panel, the gene expression level of MyoD was increased in the cells treated with rCCN2 at the concentration of 100 and

250 ng/ml. To confirm this result at the protein level, cell lysates were collected after 2 days of stimulation with rCCN2; and western blot analysis was then performed by using anti-MyoD antibody. As shown in Fig. 2A right panel, MyoD production was increased by rCCN2 in a dose-dependent manner. Next, to investigate the effect of CCN2 on the myoblast differentiation at the late-stage, we performed quantitative RT–PCR analysis and western blot analysis after 4 and 5 days of stimulation with rCCN2, respectively. As shown in Fig. 2B, the gene expression and protein production levels of myogenin, which is a marker of terminally differentiated myoblasts (1, 2), were oppositely decreased by rCCN2 in a dose-dependent manner. Because it was reported that MyoD up-regulates the gene expression level of *Myogenin* (27), we examined how CCN2 regulated the MyoD and myogenin production during myoblast differentiation. Cell lysate of C2C12 cells at days 2, 5 and 7 after treatment with rCCN2 was collected; and western blot analysis was performed by using anti-MyoD and myogenin antibodies. In the early stage of differentiation, CCN2 induced MyoD production, which was, however, rather repressed by CCN2 in the late stage. Consistent with these results, myogenin production was repressed in the late stage by CCN2 under the support of less MyoD (Fig. 2C). To further investigate CCN2 functions during myoblast differentiation, we performed quantitative RT–PCR analysis by using specific primers to evaluate late-stage myoblast differentiation. As shown in Fig. 3A and B, the gene expression levels of *Desmin* and *MHC*, which are markers of differentiated myoblasts at the late stage (1, 2), was significantly decreased by the addition of rCCN2. Interestingly, the gene expression level of *Myostatin*, which is an important regulator of muscle growth (28, 29), was oppositely increased in C2C12 cells treated with rCCN2 in a dose-dependent manner (Fig. 3C). Taken together, these findings indicate that CCN2 up-regulates the MyoD production at the early-stage of differentiation, but down-regulates the MyoD and myogenin production at the late-stage of differentiation. Consequently, CCN2 down-regulated the gene expression of late stage markers, thus suggesting that CCN2 promotes the early-stage differentiation but inhibits the late-stage differentiation of myoblasts.


A
MyoD
relative ratio
1.5
1
0.5
0
*
Cont.
50
100
250
rCCN2 ng/ml
rCCN2
Cont.
50
100
200
(ng/ml)
MyoD
β-actin
B
Myogenin
relative ratio
1.5
1
0.5
0
*
Cont.
50
100
250
rCCN2 ng/ml
rCCN2
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50
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(ng/ml)
Myogenin
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C
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rCCN2 100 ng/ml
2
5
7
2
5
7
days
MyoD
Myogenin
β-actin

Fig. 2

CCN2 regulates the gene expression and protein production of MyoD and myogenin in C2C12 cells. (**A**) C2C12 cells were stimulated with rCCN2 at the indicated concentrations in differentiation medium. After 1 and 2 days, the gene expression and protein production of MyoD were examined, respectively. *$P < 0.05$ indicates significant difference by one-way ANOVA. (**B**) C2C12 cells were treated with rCCN2 in differentiation medium. After 4 and 5 days, the gene expression and protein production of myogenin were evaluated, respectively. *$P < 0.05$ indicates significant difference by one-way ANOVA. (**C**) C2C12 cells were stimulated with rCCN2 in differentiation medium. At the indicated time after stimulation, cell lysate was collected and western blot analysis was performed.

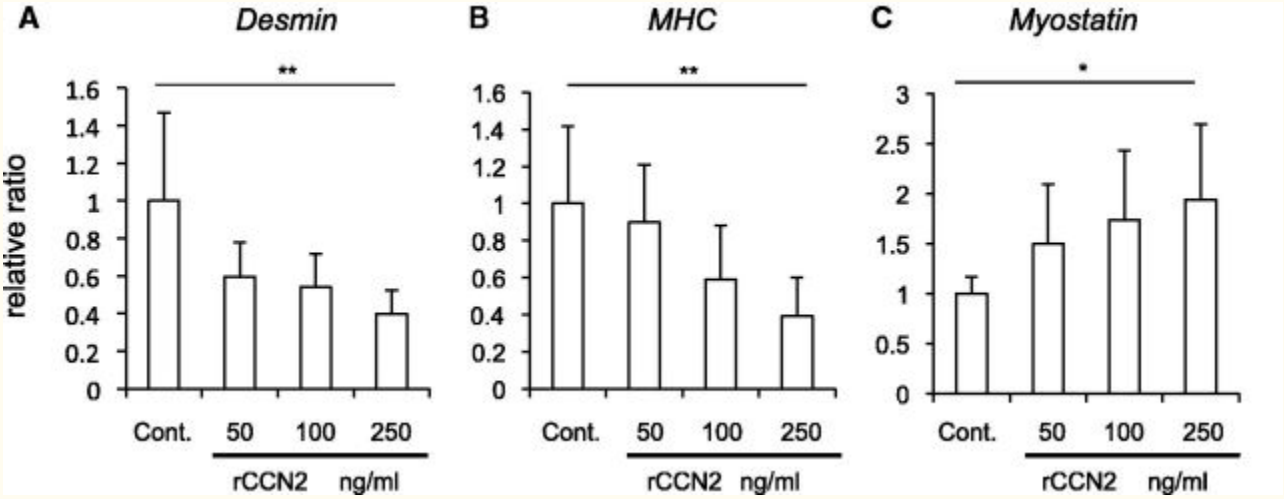

Fig. 3

CCN2 regulates the gene expression of *Desmin* (A), *MHC* (B), and *Myostatin* (C) in C2C12 cells. C2C12 cells were stimulated with rCCN2 at the concentrations indicated for 4 days, and real-time PCR analysis was performed. The amounts of these gene products were normalized to that of *Gapdh* with respect to the control sample (ratio = 1.0). Bars represent means and standard deviations of results obtained from eight to nine different cultures for each sample. *$P < 0.05$ and **$P < 0.01$ indicate significant differences from control by one-way ANOVA.

CCN2 production is increased in C2C12 cells treated with TNFα

Then, to clarify whether or not the CCN2 production was up-regulated when an inflammatory response that damages tissue occurred in skeletal muscle, we investigated the CCN2 production in C2C12 cells stimulated by the proinflammatory cytokine TNFα in a time course. As shown in Fig. 4A and C, CCN2 production was increased at 48 h after the treatment with TNFα at a concentration of 20 ng/ml. Next, we evaluated whether the production level of CCN2 was increased by treatment with TNFα in a dose-dependent manner. As shown in Fig. 4B, the promotion of CCN2 production was confirmed at the protein level in C2C12 cells treated with TNFα, which increase occurred at a concentration of 5 ng/ml. These results indicate that CCN2 production is increased after 48 h of stimulation with TNFα, also suggesting that CCN2 is induced after inflammation to be engaged in muscle regeneration.

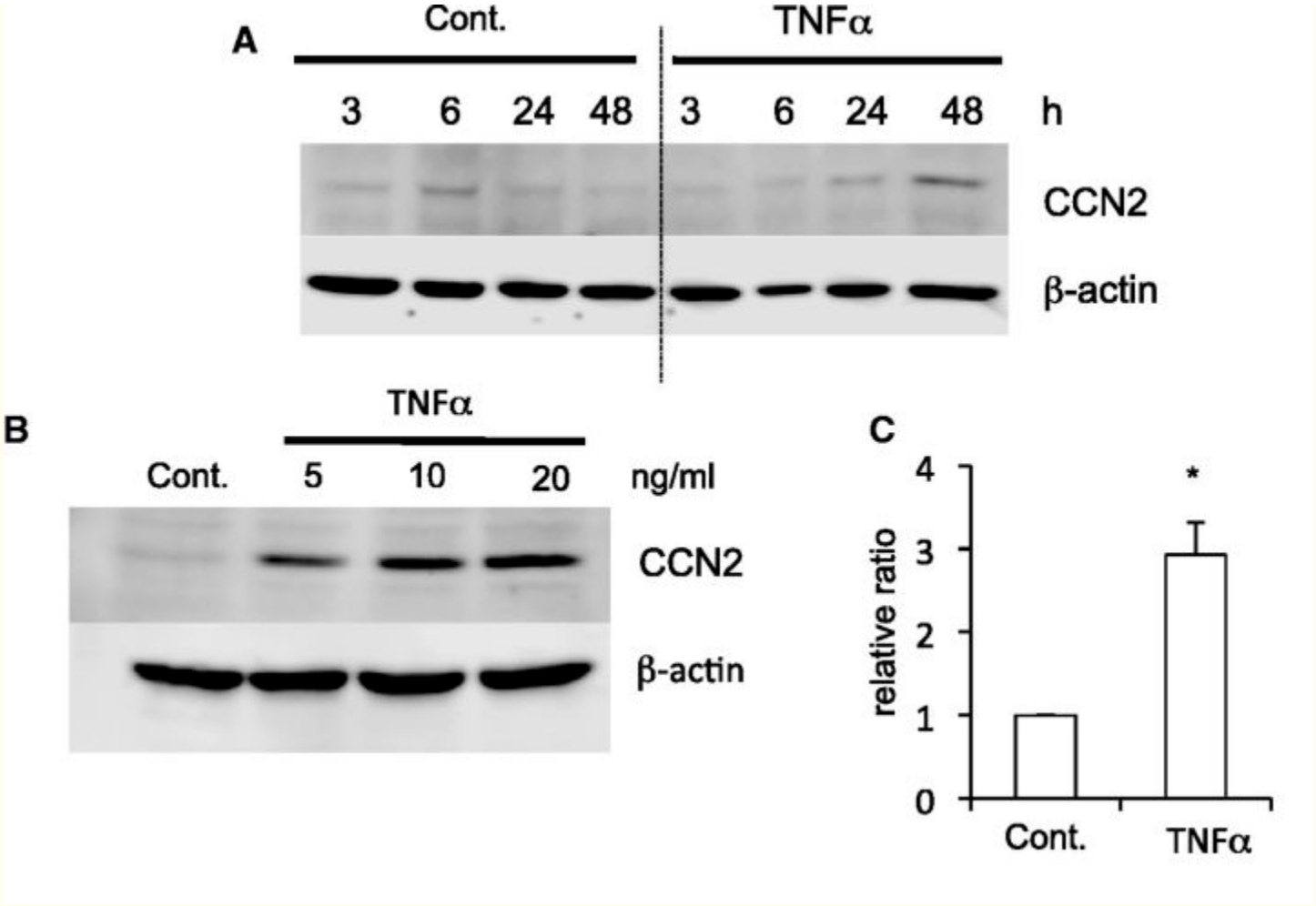

Fig. 4

CCN2 production is increased in C2C12 cells treated with TNFα. (**A**) C2C12 cells were stimulated by 20 ng/ml TNFα in differentiation medium. At the indicated time the cell lysates were collected and western blot analysis was performed by using anti-CCN2 and β-actin antibodies. (**B**) C2C12 cells were treated with TNFα at the indicated concentrations for 2 days. Then, western blot analysis was similarly performed. (**C**) Quantification of the results obtained from the experiments for panel A at 48 h in triplicates. *$P < 0.05$ indicates significant difference from control by Student's *t*-test.

Cell proliferation and myotube formation are impaired in Ccn2-deficient myoblasts

Next, to investigate CCN2 functions in primary myoblasts, we isolated from wild-type (WT) and *Ccn2*-deficient myoblasts. Firstly, we examined the role of CCN2 in the production of MyoD. As shown in Fig. 5A, although the production of MyoD was increased in WT myoblasts treated with rCCN2 in a dose-dependent manner, MyoD production in *Ccn2*-deficient myoblasts was decreased compared with that in WT myoblasts, and the reduced MyoD production in *Ccn2*-deficient cells was not increased by rCCN2 treatment (Fig. 5A). To investigate why MyoD production level was not increased by treatment with rCCN2 in *Ccn2*-deficient cells, we examined the production of integrin α5, which has an important role in myogenesis (30, 31) and interacts with CCN2, in WT and *Ccn2*-deficient myoblasts. As shown in Fig. 5B, the production level of integrin α5 in *Ccn2*-deficient cells was lower than that in WT cells. These findings suggest that the impaired response of *Ccn2*-deficient cells by rCCN2 may be due to decreased integrin α5. Moreover, we investigated the effect of CCN2 on the myotube formation using WT and *Ccn2*-deficient myoblasts. Immunofluorescence analysis by using anti-desmin antibody was performed to detect the formation of myotubes. As shown in Fig. 5C, immunoreactivity for desmin was detected in both WT and *Ccn2*-deficient myotubes, but the number of myotube formation derived from *Ccn2*-deficient cells was

decreased compared with that derived from WT cells. These findings suggest that myoblast differentiation is impaired in *Ccn2*-deficient cells. Next, to clarify the direct effect of CCN2 on the myoblast differentiation, we examined the gene expression levels of myoblast differentiation markers such as *MyoD* and *MHC* by determining mRNA expression levels in WT and *Ccn2*-deficient myoblasts. As shown in Fig. 5D, the expression levels of these genes were decreased in *Ccn2*-deficient myoblasts compared with those in the WT cells. Interestingly, gene expression of *Myostatin* in *Ccn2*-deficient cells was increased at a marginally significant level ($P = 0.051$). Finally, to confirm the effect of CCN2 functions on the proliferation and myotube formation of myoblasts *in vivo*, we performed immunofluorescence double staining with anti-PCNA and anti-MyoD antibodies and single staining with an anti-desmin antibody of WT littermates and *Ccn2*-deficient skeletal muscle tissues at E18.5. In skeletal muscle, myoblasts, which are localized at the periphery of myofibers, proliferate, differentiate and then fuse with each other to form multinucleate myotubes during regeneration. Indeed, PCNA and MyoD double immunoreactivity was found in the nuclei of cells at the periphery of myofibers in both WT and *Ccn2*-deficient tissues, whereas the number of the immunoreaction was significantly reduced in the *Ccn2*-deficient muscle (Fig. 6A, graph), suggesting decreased regeneration potential in *Ccn2*-deficient muscle. Immunoreactivity for desmin, a cytoskeletal intermediate filament protein, was detected in muscle fibers of both WT and *Ccn2*-deficient skeletal muscle tissues, whereas the transversal area of muscle fibers was decreased in the *Ccn2*-deficient mice (Fig. 6B, graph). These findings indicate that the skeletal muscle from *Ccn2*-deficient mice showed reduced PCNA/MyoD staining and impaired muscle integrity, thus suggesting that both proliferation of myoblasts and muscle formation had been impaired in *Ccn2*-deficient skeletal muscle tissues *in vivo*.

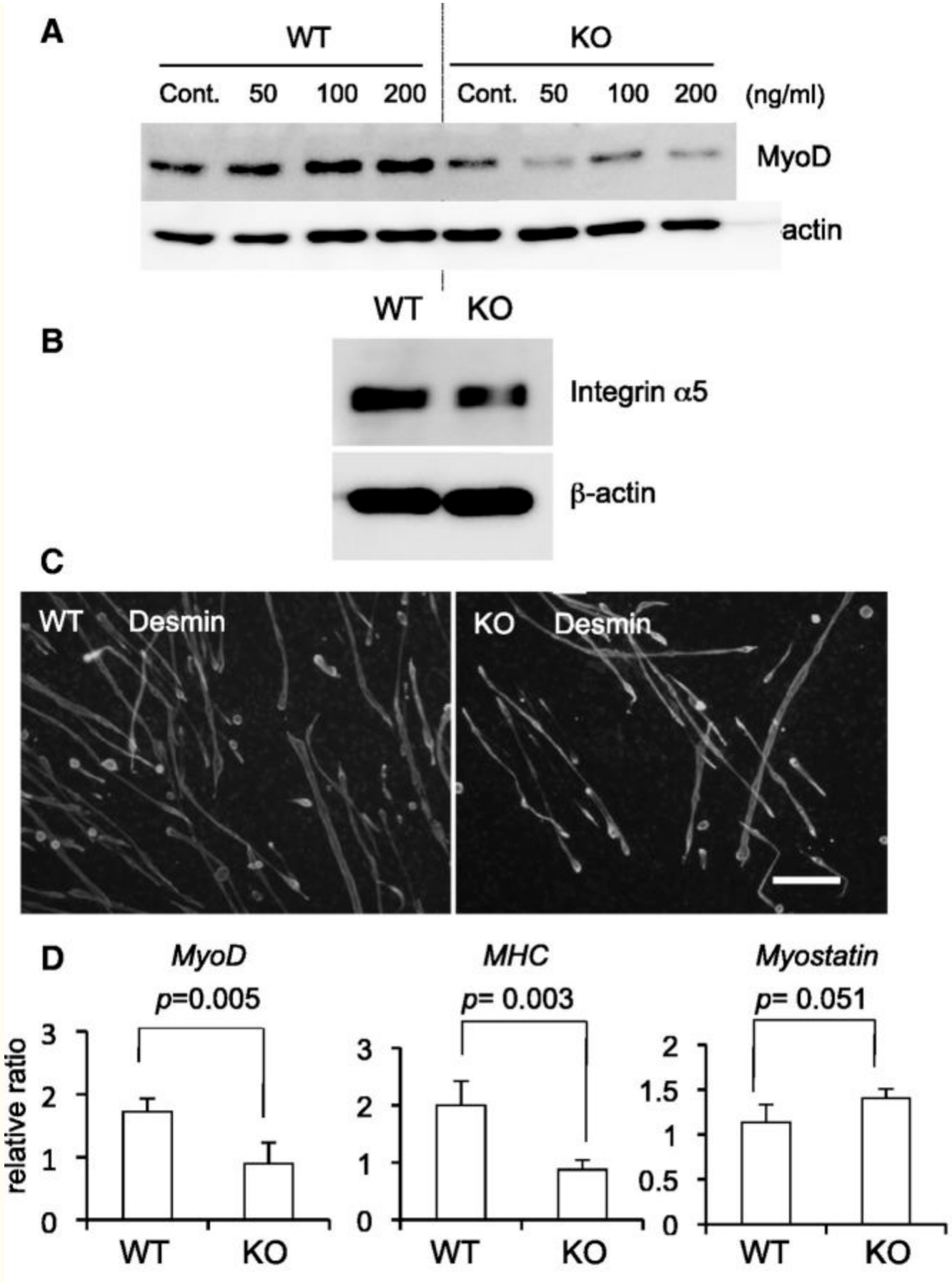
A
WT
KO
Cont. 50 100 200
Cont. 50 100 200
(ng/ml)
MyoD
actin
B
WT KO
Integrin α5
β-actin
C
WT Desmin
KO Desmin
D
MyoD
p=0.005
MHC
p= 0.003
Myostatin
p= 0.051
relative ratio
3
2
1
0
1.5
0.5
WT
KO

Fig. 5

Differentiation of myoblasts from *Ccn2*-deficient mice is impaired. (**A**) Primary myoblasts isolated from gastrocnemius muscle of WT and *Ccn2*-deficient (KO) mice at E18.5 were treated with rCCN2 at the concentration indicated for 7 days. Western blot analysis was performed by using anti-MyoD and anti-β-actin antibodies. (**B**) WT and KO myoblasts were induced to differentiate for 7 days in differentiation medium. Then, western blot analysis was performed by using anti-integrin α5 and anti-β-actin antibodies. (**C**) WT and KO myoblasts were induced to differentiate for 7 days. The cells were thereafter fixed, and immunofluorescence analysis by using anti-desmin antibody was performed. Bar represents 100 μm. (**D**) WT and KO myoblasts were induced to differentiate for 6 days, and real-time RT–PCR analysis was performed. The amounts of *MyoD*, *MHC*, and *Myostatin* mRNAs were normalized to that of *Gapdh* mRNA and the bars represent means and standard deviations of four sets of independent cultures. Exact *P*-values are determined by Student's *t* test.

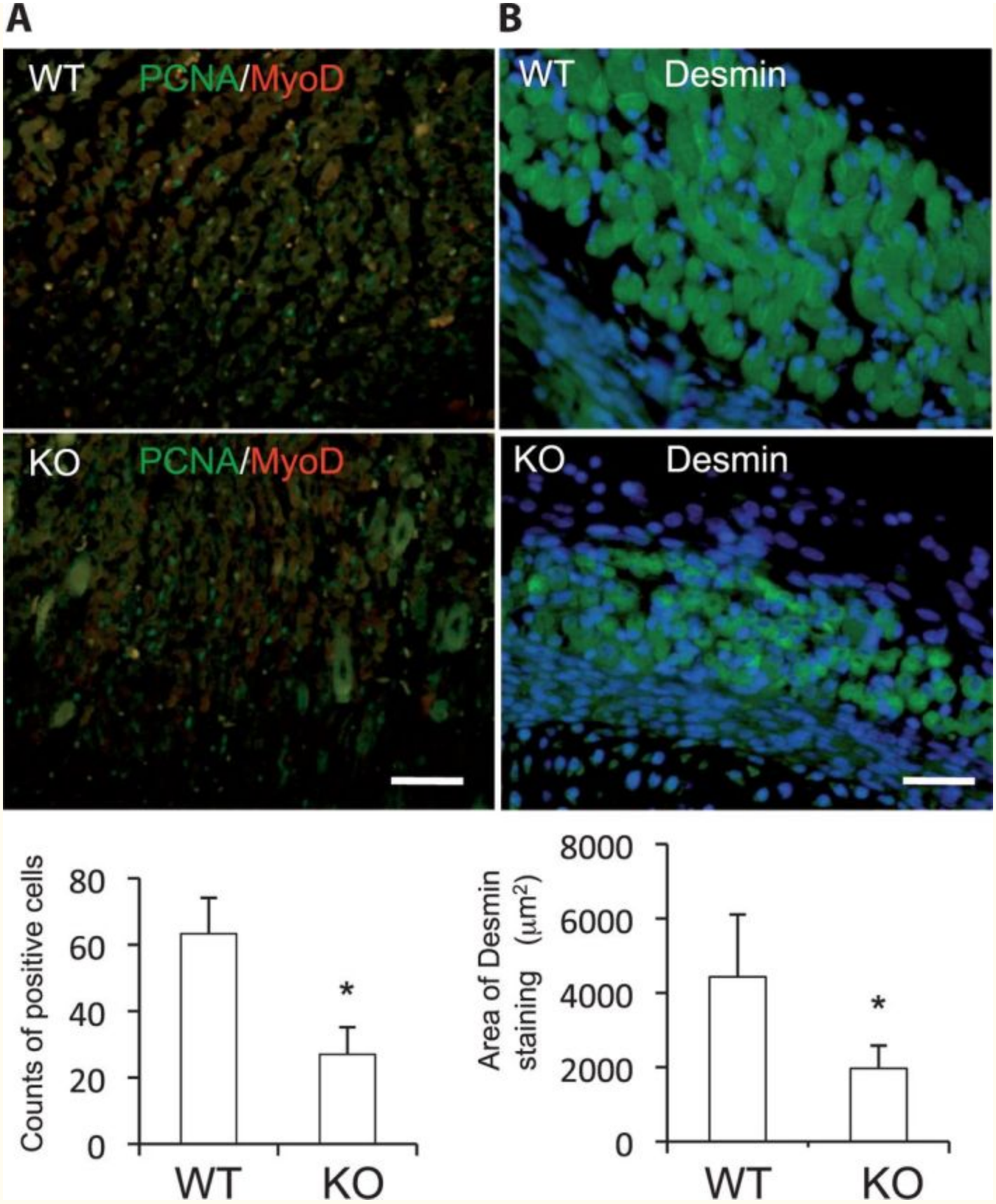

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Fig. 6

Both proliferation and differentiation of *Ccn2*-deficient (KO) myoblasts is impaired *in vivo*. (**A**) Double immunoreactivity for PCNA and MyoD was detected in the sections of the skeletal muscle from E18.5 WT and KO littermates by immunofluorescence analysis, and the bottom graph shows the means and standard deviations of the number of double positive cells from three different views. (**B**) Immunoreactivity for desmin was detected in the sections from WT and KO skeletal muscle tissues, and the bottom graph shows the means and standard deviations of areas of muscle fibers stained with anti-desmin antibody. *$P < 0.05$ indicates significant difference by Student's *t* test. Bar represents 50 μm.

Discussion

Because many studies concerning the role of CCN2 in skeletal muscle almost always focused on pathological states such as fibrosis, little is known concerning the physiological significance of CCN2 in skeletal muscle. In this study, we showed that CCN2 promoted the proliferation of C2C12 cells and increased the MyoD production in both C2C12 cells and primary myoblasts at an early-stage of differentiation (Figs 2, ,33 and 5A). Furthermore, we clarified that myogenin production and the gene expression levels of *Myogenin*, *Desmin* and *MHC*, which are the markers of terminally differentiated myoblasts, were decreased by the addition of rCCN2 to cultures of C2C12 cells at a later-stage of differentiation (Figs 2–4). These findings indicate that CCN2 promoted cell proliferation and early differentiation of myoblasts, whereas inhibited their terminal differentiation, suggesting that CCN2 plays an important role in early stages of myoblast differentiation. To confirm the physiological role of CCN2 in myoblast differentiation, using WT and *Ccn2*-deficient skeletal muscle, we performed immunofluorescence analysis with anti-PCNA/MyoD and anti-desmin antibodies. As a result, skeletal muscle fibers of *Ccn2*-deficient mice showed hypoplasia, and the number of PCNA and MyoD double positive cells was decreased in the skeletal muscle of these mice (Fig. 6). Moreover, the gene expression levels of MRFs, such as *MyoD*, and those of MRF-regulated *MHC*, were found to have decreased in the *Ccn2*-deficient myoblasts (Fig. 5D). Previously, it was reported that inhibition of CCN2 expression by small interfering RNA (siRNA) treatment decreases the myogenic differentiation of human rhabdomyosarcoma cells (32). Our results indicating impaired proliferation and differentiation of *Ccn2*-deficient myoblasts are consistent with those obtained with human rhabdomyosarcoma cells treated with siRNA against CCN2. Both results may have been due to the inhibition of the early stage of differentiation of myoblasts via decreased MyoD production caused by depletion or knockdown of CCN2. Taken together, these findings suggest that CCN2 plays an important role in both the proliferation and early differentiation of myoblasts in the physiological state.

It was reported that myostatin belonged to the TGF-β superfamily, and was a negative regulator of myoblast proliferation and differentiation (33, 34). However, it has been reported that myostatin stimulates the proliferation of C2C12 cells and promotes the terminal differentiation of embryonic muscle progenitors, thus suggesting that myostatin plays a context-dependent role in skeletal muscle to maintain its homeostasis (28, 29, 33, 34). In this study, we demonstrated that the gene expression level of *Myostatin* was increased in C2C12 cells by rCCN2 in a dose-dependent manner, whereas it was increased in *Ccn2*-deficient myoblasts compared with that in WT cells with a marginally significant difference ($P = 0.051$). Since skeletal muscle mass of *Ccn2*-deficient mice was decreased compared with that of WT mice, these findings suggest that CCN2 may play an important role in maintaining muscle homeostasis at least in part through regulating myostatin expression.

Unexpectedly, the suppressed MyoD production in *Ccn2*-deficient myoblasts was not recovered to normal by the addition of exogenous rCCN2 (Fig. 5A). To address this point, we performed western blot analysis by using anti-integrinα5 antibody on WT and *Ccn2*-deficient myoblasts. As shown in Fig. 5B, the production of integrin α5 was decreased in *Ccn2*-deficient cells. It was reported that integrin α5 acts to regulate myoblast proliferation and differentiation (30, 31), and we also showed that CCN2 regulated chondrocytes via binding with this molecule (35, 36). These findings indicate that, because the level of

integrin α5, which functions as a CCN2 receptor, is decreased, the effect of rCCN2 remains transient and not sufficient to redeem MyoD production in *Ccn2*-deficient cells.

It was previously reported that over-expression of CCN2 inhibited myoblast differentiation and induced a fibrotic response in skeletal muscle (7–9). Based on these findings, the authors suggest that CCN2 plays major roles in the pathogenesis of Duchenne muscular dystrophy. However, in this study, we showed that *Ccn2*-deficient skeletal muscle tissues were hypoplastic (Fig. 6). In addition, we revealed that CCN2 promoted the production of MyoD in both C2C12 cells and primary myoblasts, although CCN2 inhibited the late stage of myoblast differentiation (Fig. 2). These results indicate that proper myoblast differentiation under physiological condition requires CCN2. Therefore, we suspect that CCN2 may be cleaved at the late stage of myoblast differentiation to allow the completion of myoblast differentiation. Zimowska *et al.* (37) reported that matrix metalloproteinase (MMP)-9 activity was present during all stages of myoblast differentiation but MMP-2 activity reached its highest level during myoblast fusion. Furthermore, it was previously reported that MMP-2 cleaved CCN2 into approximately half-sized fragments (38). Taken together, these findings suggest that CCN2 promotes the myoblast proliferation and the production of MyoD at the early stage of myoblast differentiation and that harmonious skeletal muscle regeneration may progress via conditional CCN2 cleavage by MMP-2 at the late differentiation stage.

In summary, this study showed that CCN2 plays an important role in myoblast differentiation in the physiological state. However, further investigation is needed to entirely clarify CCN2 functions during myoblast differentiation, particularly at late stages.

Acknowledgements

This work was supported in part by grants from the programs Grants-in-Aid for Scientific Research (C) to T.N. (Nos. 23592732, 26462810) and to S.K. (No. 25462886), and (B) to M.T. (No. 24390415) from Japan Society for the Promotion of Sciences, Japan. D.J. is a recipient of a Postdoctoral Fellowship from Japan Society for the Promotion of Science. They thank Drs Takako Hattori and Mitsuhiro Hoshijima for helpful suggestions and Miss Yoshiko Miyake for secretarial assistance.

Glossary

Abbreviations

BrdU	5-bromo-2′-deoxyuridine
BSA	bovine serum albumin
DMEM	Dulbecco’s modified Eagle’s medium
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HA	hemagglutinin
ITS	insulin–transferrin–selenite
KO	*Ccn2*-deficience
MHC	myosin heavy chain
PCNA	proliferating cell nuclear antigen
rCCN2	recombinant CCN2 protein
TNFα	tumor necrosis factor-α

WT	wild-type