Jiadifenolide induces the expression of cellular communication network factor (*CCN*) genes, and CCN2 exhibits neurotrophic activity in neuronal precursor cells derived from human induced pluripotent stem cells

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A R T I C L E   I N F O

A B S T R A C T

Jiadifenolide has been reported to have neurotrophin-like activity in primary rat cortical neurons, and also possesses neurotrophic effects in neuronal precursor cells derived from human induced pluripotent stem cells (hiPSCs), as we have previously reported. However, the molecular mechanisms by which jiadifenolide exerts its neurotrophic effects in rat and human neurons are unknown. Thus, we aimed to investigate the molecular mechanisms and pathways by which jiadifenolide promotes neurotrophic effects. Here, we found that jiadifenolide activated cellular communication network factor (CCN) signaling pathways by up-regulating mRNA level expression of CCN genes in human neuronal cells. We also found that CCN2 (also known as connective tissue growth factor, CTGF) protein promotes neurotrophic effects through activation of the p44/42 mitogen-activated protein kinase signaling pathway. This is the first discovery which links neurotrophic activity with CCN signaling.

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1. Introduction

Neurotrophic factors (neurotrophins) such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and glial cell line-derived neurotrophic factor (GDNF) have been reported to prevent neuronal degeneration, promote neurite regeneration and neuronal survival, and enhance synaptic plasticity [1,2]. Thus, neurotrophins have been expected to gain approval for therapeutic applications in neurological diseases that show neurodegenerative, behavioral, and psychiatric symptoms [3,4]. However, clinical trials of various neurotrophic factors have been failed by which neurotrophic factors possesses high molecular weights, the limited serum-stability, and fail to achieve adequate blood-brain-barrier (BBB) penetration [2,5–9]. W. M. Pardridge suggested in 2012 that small-molecules having a molecular weight (MW) of less than 400 Da and capable of forming eight or fewer hydrogen bonds may cross the BBB via lipid-mediated diffusion [10]. Therefore, novel small BBB-penetrating molecules with neurotrophin-like activity are needed to overcome the pharmacological disadvantages of peptide neurotrophic factors.

Jiadifenolide and its derivatives have been reported to promote neurite outgrowth in primary cultured rat cortical neurons [11], and enhance NGF activity, thereby facilitating PC12 rat adrenal pheochromocytoma cell differentiation [12]. In addition, we previously reported that jiadifenolide promotes neurite outgrowth, and

promotes cell growth or prevents cell death of neuronal precursor cells derived from human pluripotent stem cells (hiPSCs) [13]. Jiadifenolide has a MW of 310.11 Da, and two hydrogen bond donors in its chemical structure, thus possessing the properties of a small molecule that are expected to give it high BBB permeability [13]. It can also be synthesized in gram quantities using as few as eight steps [14]. Therefore, jiadifenolide is a strong potential drug candidate for clinical evaluation for treatment of human neurological disorders. However, the molecular mechanisms by which it exerts its neurotrophic effects in rat and human neurons are unknown.

The cellular communication network factor (CCN) family consists of the CCN proteins, CCN1, CCN2, CCN3, CCN4, CCN5, and CCN6. These matricellular proteins play roles in regulating the environment between cells [15–18]. All CCN proteins, except for CCN5, are composed of four peptide motifs, i.e. insulin-like growth factor-binding protein (IGFBP), von Willebrand factor type C repeat (VWC), thrombospondin type 1 repeat (TSP1), and C-terminal cystine-knot (CT) modules, which can bind a variety of key molecules, such as IGFs, transforming growth factor -β (TGF-β), bone morphogenetic proteins (BMPs), integrins, vascular endothelial growth factor (VEGF), and Notch1, and play roles in regulating many physiological processes [15,16,18]. CCN2 in particular mediates multiple functions in a variety of tissues and organs by interacting with multiple macromolecules. Its gene transcription is upregulated by mothers against decapentaplegic homolog (SMAD) mediated TGF-β signaling, in conjunction with signal transducer and activator of transcription 3 (STAT3) [19,20]. In addition, CCN2 interacts with tropomyosin receptor kinase A (TRKA) and p75[NTR] receptors, which are both neurotrophin responsive, and is itself a ligand of NGF receptor [19,21,22].

In this study, we aimed to investigate molecular mechanisms and pathways underlying the neurotrophic effects of jiadifenolide in neuronal precursor cells derived from hiPSCs. We found that CCN signaling pathways, especially that of CCN2, contributed to the neurotrophic effects of jiadifenolide in human neuronal precursor cells.

2. Materials and methods

2.1. Preparation of jiadifenolide

Jiadifenolide was chemically synthesized and purified as previously reported [11,13]. We dissolved synthetic jiadifenolide in dimethyl sulfoxide (DMSO).

2.2. Culture of human induced pluripotent stem cells (hiPSCs)

Human induced pluripotent stem cell (hiPSC) line 201B7 (Cell No. HPS0063) [23] was provided by Cell bank (Riken Bioresource Center, Ibaraki, Japan). Cells were maintained on a mitomycin-C (Kyowa Hakko Kirin, Tokyo, Japan)-treated mouse feeder cell layer. For more details, please see Supplementary materials.

2.3. Induction and maturation of neuronal precursor cells from hiPSC line 201B7

We differentiated neuronal precursor cells from hiPSC line 201B7 according to a method we have previously described [13]. Neuronal precursor cells were matured depending on treatment conditions. Depending on treatment conditions, media was supplemented with one of the following: 0.01% DMSO, neurotrophins (a protein mixture of 10 ng/mL recombinant human BDNF (rhBDNF; Wako Pure Chemical Industries, Osaka, Japan), 10 ng/mL recombinant human glial-cell derived neurotrophic factor (rhGDNF; Wako Pure Chemical Industries) and 10 ng/mL recombinant human NT-3 (rhNT-3; Wako) [24]), 50 ng/mL recombinant human NGF (rhNGF; Wako Pure Chemical Industries), 1 μM jiadifenolide, or 1, 10, 100 ng/ mL recombinant human CCN2 (rhCCN2; BioVendor, Brno, Czech Republic). In cultures incubated for 23 days, media in wells was changed every three days after plating. In cultures incubated for five days, media in wells was changed at 24 and 72 h after plating. For more details, please see Supplementary materials.

2.4. Other methods

We exhibited full methods and any associated references in Supplementary materials.

3. Results

We have included the complete results and any relevant references in the Supplementary materials.

3.1. Jiadifenolide activates CCN signaling in human neuronal cells

To investigate host factors in human neuronal precursor cells that mediate jiadifenolide's neurotrophic effects, we performed transcriptome analysis by comprehensive RNA sequencing of the transcriptome of neuronal precursor cells derived from hiPSC line 201B7 incubated for 23 days with jiadifenolide [Table S1, deposited in the DNA Data Bank of Japan (accession number DRA008385)]. As shown in Table S1 and Table 1, Comprehensive RNA sequencing

Table 1
Analysis of mRNA expressions between DMSO and jiadifenolide-treated neuronal precursor cells from hiPSCs line 201B7 by next-generation sequencing.

Gene	DMSO	jiadifenolide	Ratio (jiadifenolide per DMSO RPKM averages)
	RPKM		
TAGLN/SM22a (transgelin)	13.01 ± 1.39	28.78 ± 1.37	2.21
ANXA2 (annexin A2)	5.82 ± 0.80	11.93 ± 0.28	2.05
CTGF/CCN2 (connective tissue growth factor)	5.7 ± 0.22	11.53 ± 0.45	2.02
IGFBP3 (insulin-like growth factor-binding protein 3)	4 ± 0.37	7.4 ± 0.18	1.87
CYR61/CCN1 (cysteine-rich angiogenic inducer 61)	4.07 ± 0.45	6.98 ± 0.50	1.72
SULF1 (sulfatase 1)	14.96 ± 1.44	24.95 ± 2.62	1.67
MIR3620	12.48 ± 1.03	18.69 ± 0.3	1.50
TP53	21 ± 0.56	17.29 ± 0.35	0.82
ZIC3 (zinc finger protein of the cerebellum 3)	8.96 ± 0.43	7.1 ± 0.42	0.79
MIR939	15.52 ± 1.42	10.04 ± 0.86	0.65
ATP5EP2 (ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit pseudogene 2)	7.55 ± 0.15	4.19 ± 0.63	0.56
GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	332.75 ± 3.26	320.34 ± 4.86	0.96

showed that mRNA level expression of smooth muscle protein 22-alpha (SM22a), annexin A2 (ANXA2), CCN2, IGFBP3, and CCN1 were up-regulated greater than 1.5 fold in human neuronal cells, whereas those of the TP53 was down-regulated to less than 0.85 fold in human neuronal cells by treatment with jiadifenolide relative to levels in DMSO treated cells.

Next, transcriptional regulators were predicted by *in silico* molecular network analysis of the comprehensive RNA sequencing results using KeyMolnet software (Tables S1–3) to identify molecular pathways affected by jiadifenolide in human neuronal precursor cells. This analysis showed that the up-regulated mRNA level expression of CCN, CCN1, CCN2, SM22a, ANXA2, and IGFBP3 genes
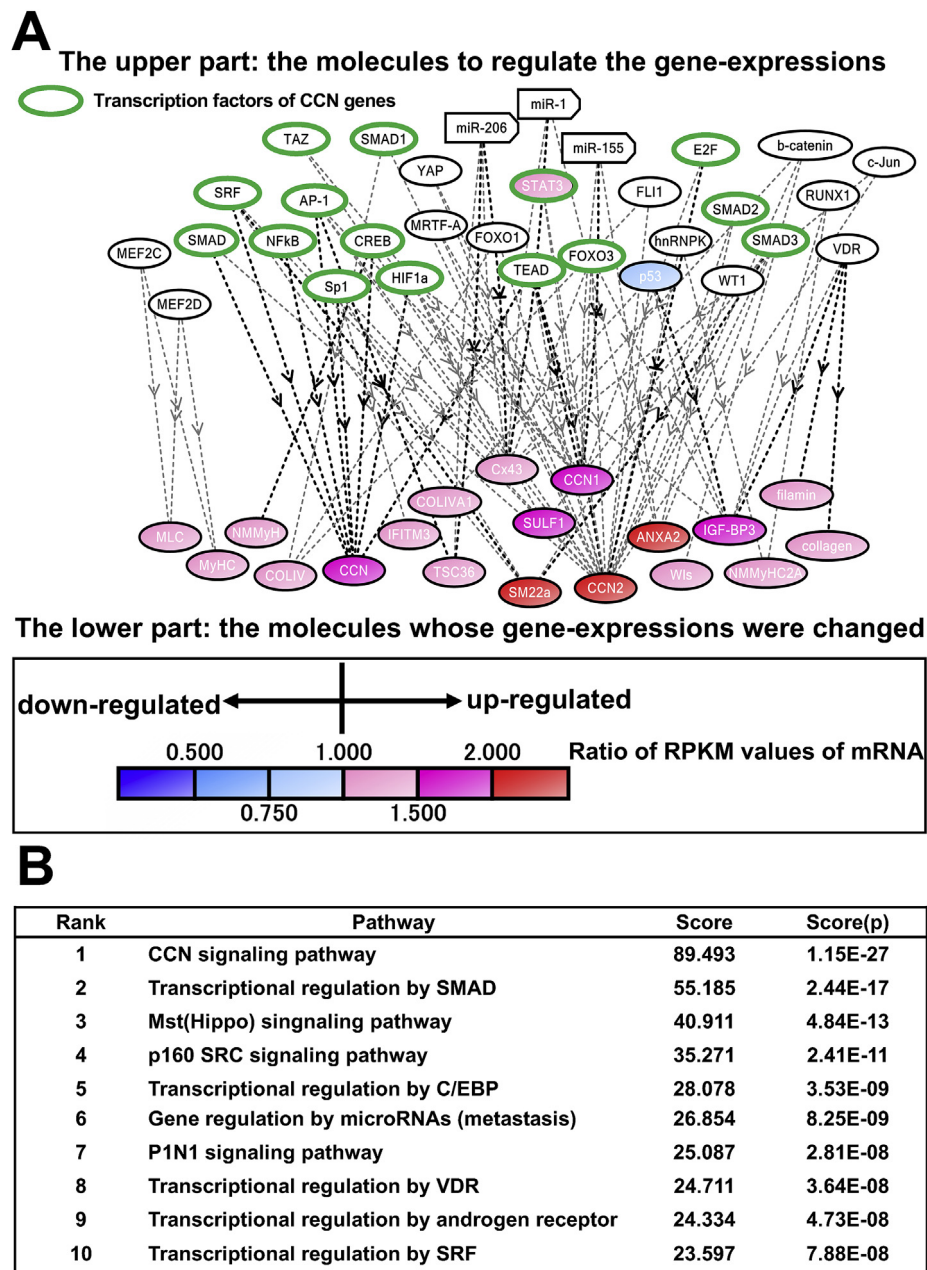

Fig. 1. Molecular network analysis using KeyMolnet of comprehensive RNA sequencing data from DMSO- and jiadifenolide-treated neuronal precursor cells from the hiPSC line 201B7. Using our comprehensive RNA sequencing data [deposited in the DNA Data Bank of Japan database (accession number DRA008385) and summarized in Table 1] from 0.01% DMSO- and 1 μM jiadifenolide-treated neuronal precursor cells derived from 201B7 cells after 23 days of incubation (n = 3 each), the molecular networks, pathways, and transcriptional regulators of altered gene-expression were analyzed using KeyMolnet. (A) Molecular network of transcription factors influenced by jiadifenolide, and their regulated genes. The upper part of the panel shows the molecules that regulate gene expression. Green-framed circles in the upper part indicate transcription factors belonging to the CCN gene family. The lower part shows those molecules whose gene expression was altered. The ratios of RPKM values of mRNAs expressed in jiadifenolide-treated cells, to those of mRNAs expressed in DMSO-treated cells, as revealed by comprehensive RNA analysis, are shown in red and blue to indicate up- and down-regulated molecules, respectively. (B) The predicted molecular pathways regulating altered gene expression in 1 μM jiadifenolide-treated cells relative to 0.01% DMSO treated cells, and their calculated scores. The extracted molecular network shown in panel (A) was compared with distinct canonical pathways in the KeyMolnet library. This makes it possible to identify canonical pathway making the most significant contribution to the extracted network. Scores and *p* values were calculated as described in experimental procedures. Abbreviations used in the list in (B) are indicated below. SMAD: mothers against decapentaplegic homolog, SRC: steroid receptor coactivator, C/EBP: CCAAT-enhancer-binding protein, VDR: vitamin D receptor, SRF: serum response factor.

in human neuronal cells treated with jiadifenolide were related to the regulation of SMAD, SMAD1, SMAD2, SMAD3, nuclear factor-κB (NF-κB), and STAT3 or p53 which were up- or down-regulated by jiadifenolide, respectively. In addition, KeyMolnet analysis indicated relatively effects of jiadifenolide on CCN signaling, transcriptional regulation by SMAD signaling pathway (Fig. 1B). These results suggest that these defined signaling pathways may be upstream of the gene expression changes caused by jiadifenolide in human neuronal precursor cells. The CCN signaling pathway may be the main molecular pathway by which human neuronal precursor cells are affected by jiadifenolide, because many CCN family

transcription factors [15,16,19], including CCN, CCN1, CCN2, SM22a, and ANXA2 were up-regulated by treatment with jiadifenolide (Table 1 and green-framed circles in Fig. 1A).

3.2. Jiadifenolide up-regulated CCN family members CCN1, CCN2, and CCN6

CCN2 gene transcription is regulated by SMADs which transduce TGF-β and STAT3 signals [19,20]. SM22a, also known as transgelin, is induced by TGF-β via SMAD3 protein [25]. To investigate whether jiadifenolide up-regulates the mRNA level expression of CCN family
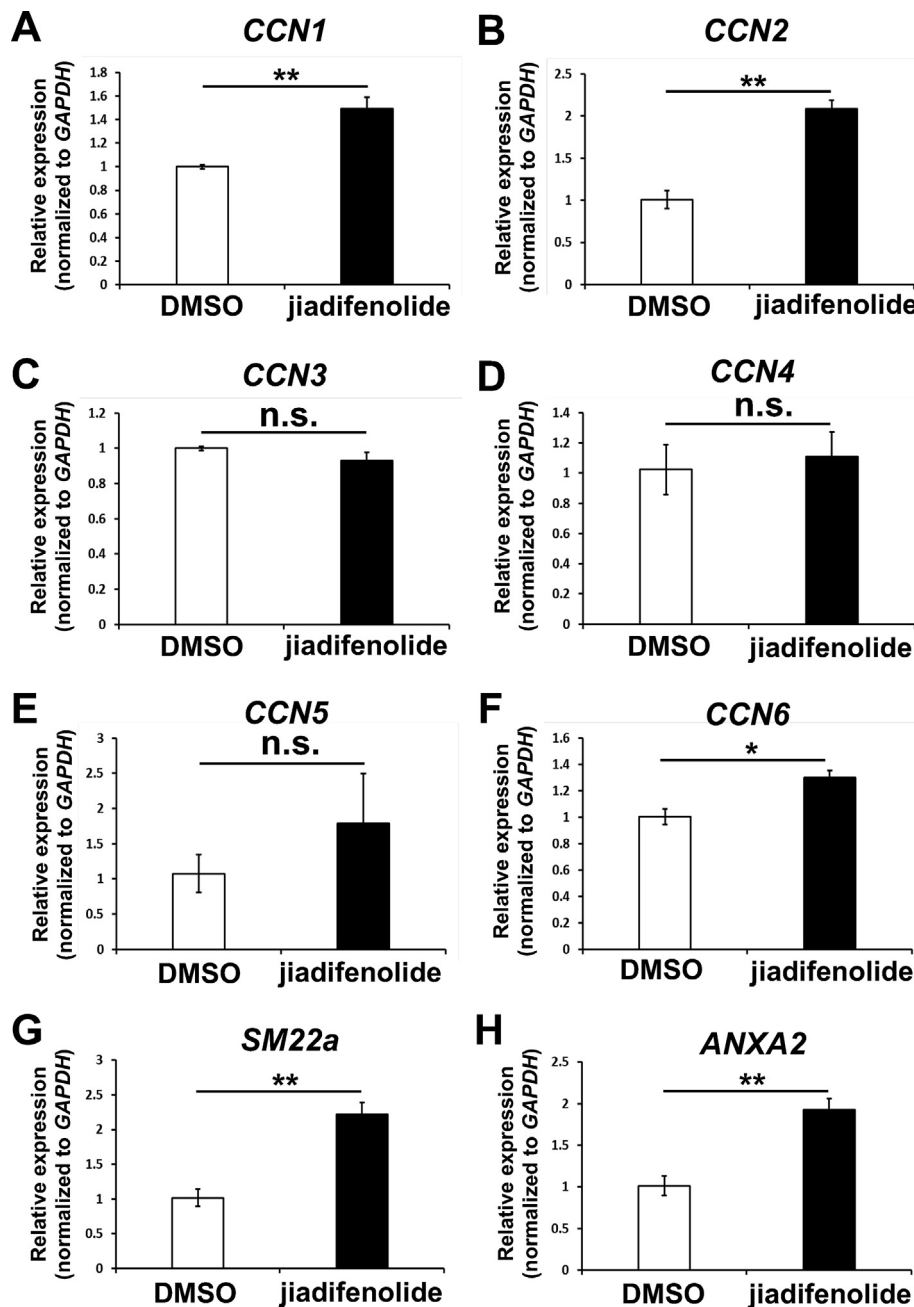
Fig. 2. Expression analysis by RT-qPCR of CCN family and SMAD-related genes in DMSO and jiadifenolide-treated neuronal precursor cells from 201B7 cells. (A–H) RT-qPCR analysis was used to evaluate transcription of CCN family and SMAD-related genes in neuronal precursor cells derived from 201B7 cells incubated with 0.01% DMSO or 1 μM jiadifenolide for 23 days. Samples of the total RNA obtained for comprehensive transcriptome analyses of cells treated with 0.01% DMSO- or 1 μM jiadifenolide for 23 days were used for cDNA synthesis. Relative mRNA expression levels of CCN family molecules *CCN1* (A), *CCN2* (B), *CCN3* (C), *CCN4* (D), *CCN5* (E), and *CCN6* (F) or SMAD-related genes *SM22a* (G) and *ANXA2* (H) (n = 3 each) were determined by RT-qPCR, normalized to levels of human *GAPDH*, and expressed in relation to levels found in DMSO-treated cells (which were set to 1). Data represent means ± SEM. *$p < 0.05$ or **$p < 0.01$, versus DMSO-treated cells. n.s.: not significant.

genes, we analyzed mRNA expression levels of the CCN genes *CCN1*, *CCN2*, *CCN3*, *CCN4*, *CCN5*, and *CCN6*, and *SM22a* and *AXNA2* in DMSO- or jiadifenolide-treated human neuronal precursor cells by RT-qPCR. Total RNA samples extracted for sequencing analysis (Fig. 1) were also used for this RT-qPCR analysis. Expression levels of *CCN1* (Fig. 2A), *CCN2* (Fig. 2B), *CCN6* (Fig. 2F), *SM22a* (Fig. 2G), and *AXNA2* (Fig. 2H) mRNA were significantly up-regulated in jiadifenolide-treated cells. Those of *CCN3* (Fig. 2C), *CCN4* (Fig. 2D), and *CCN5* (Fig. 2E) remained unchanged compared with DMSO-treated cells. Interestingly, mRNA expression levels of *CCN2* (Fig. 2B) and *SM22a* (Fig. 2G) in jiadifenolide-treated cells were increased greater than 2 fold relative to those of DMSO-treated cells

after 23 days of incubation.

Taken together, these results showed that jiadifenolide activates CCN signaling via up-regulating the mRNA level expression of the CCN family members CCN1, CCN2, and CCN6, and CCN2 gene expression may be the primary up-regulator of jiadifenolide mediated CCN signaling.

3.3. CCN2 protein promoted neuronal dendritic outgrowth and increased neuronal cell numbers in human neuronal precursor cells

We showed that jiadifenolide strongly induced transcription of the CCN2 gene in human neuronal precursor cells (Table S1, Table 1,
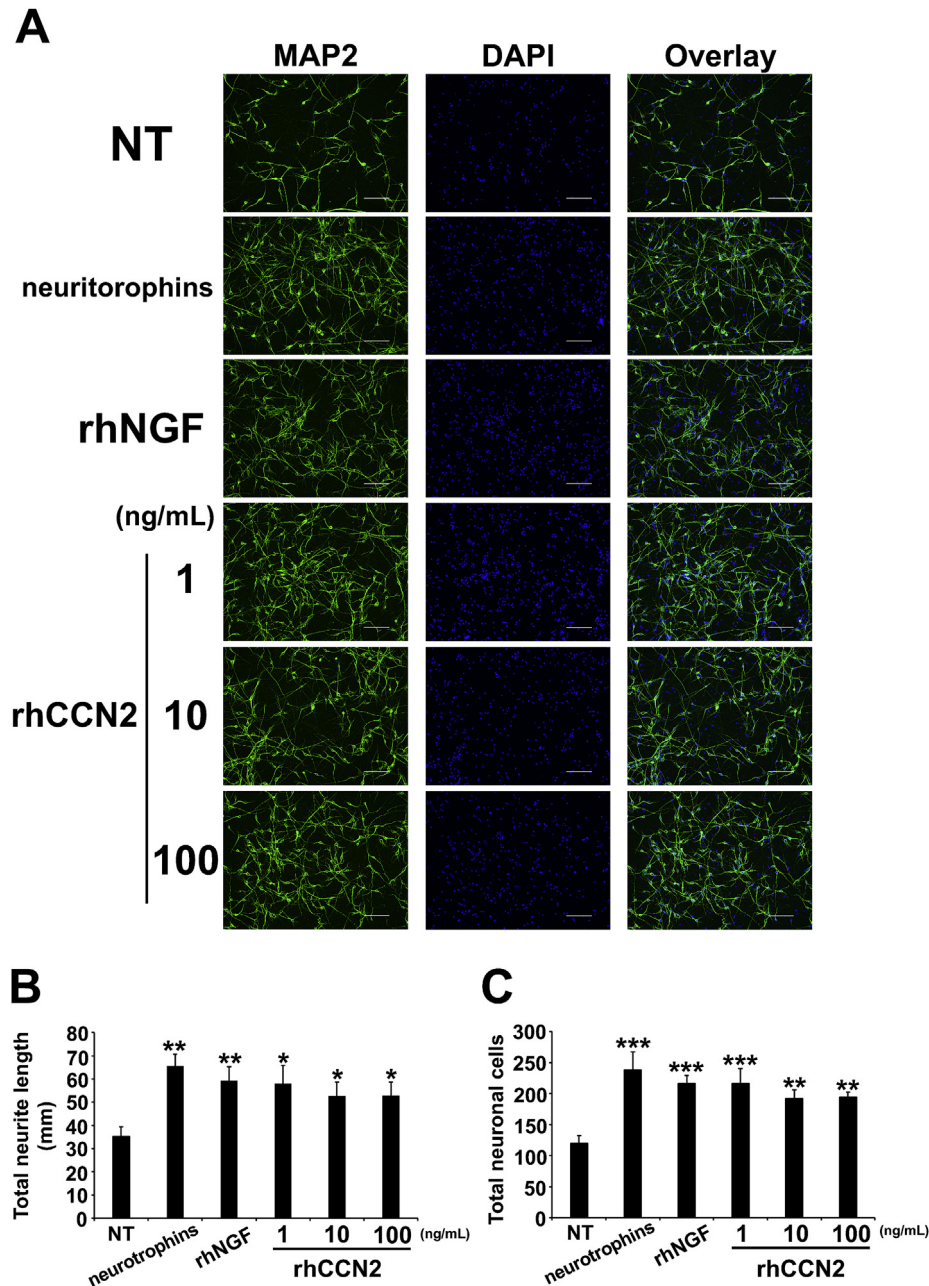
Fig. 3. Immunofluorescent staining of the dendrites of neuronal precursor cells from 201B7 cells incubated with recombinant human CCN2 protein. To visualize neuronal dendrites, we performed immunofluorescent staining of human neuronal cells for MAP2, a neurite marker, and the nuclear stain DAPI. Each well was incubated with 1, 10, or 100 ng/ mL rhCCN2 (n = 6 each) for 23 days. Non-treated (NT) or neurotrophin treated (10 ng/mL rhBDNF, 10 ng/mL rhGDNF, and 10 ng/mL rhNT-3) and 50 ng/mL rhNGF (n = 6 each) were used as negative or positive controls, respectively. Cells were subsequently photographed under a fluorescence microscope (A). The white scale bar in each image represents 100 μm. Images in (A) were analyzed by NeuriteTracer [30]. Total neurite length (mm) (B) and number of neurons (C) ((DAPI-positive cells) were measured. Data are presented as means ± SEM of three independent experiments. $^*p < 0.05$, $^*p < 0.01$, and $^{***}p < 0.001$, compared with NT, respectively.

Figs. 1 and 2B), suggesting that the induction of CCN2 protein expression by jiadifenolide will contribute to its neurotrophic effects in human neuronal precursor cells. However, it remains unclear whether CCN2 protein will exert neurotrophic effects in human neuronal precursor cells. To investigate whether CCN2 protein exhibits neurotrophic effects in human neuronal precursor cells, we co-cultured neuronal precursor cells derived from 201B7 cells with 1, 10, or 100 ng/mL rhCCN2 protein. Neurotrophins or recombinant human NGF (rhNGF) were used as positive controls, and no treatment (NT) was used as a negative control. After 23 days of co-culture, we performed immunofluorescent staining for microtubule-associated protein 2 (MAP2; a neurite marker) and used diamidino-2-phenylindole (DAPI) to stain nuclear DNA, to evaluate the effect of CCN2 on neurite outgrowth and cell numbers. Compared with NT, neurotrophins, rhNGF, and 1, 10, or 100 ng/mL rhCCN2 protein promoted neurite extension (Fig. 3A), significantly increasing total neurite length (Fig. 3B) in human neuronal cells. These results demonstrated that jiadifenolide promotes dendrite outgrowth in human neuronal cells. Additionally, treatment with neurotrophins, rhNGF, or 1, 10, or 100 ng/mL rhCCN2 protein significantly increased the number of human neuronal cells (DAPI-positive cells) (Fig. 3C), suggesting that rhCCN2 protein promoted cell growth or prevented cell death of human neuronal cells.

These results demonstrated that CCN2 protein exhibited similar neurotrophic effects on human neuronal precursor cells as did jiadifenolide.

3.4. CCN2 protein increased phosphorylation of p44/42 MAPK protein in human neuronal precursor cells

MAPK-ERK signaling is one of the pathways activated by interaction with TRK receptors and neurotrophins, such as NGF, BDNF, or NT3 [2]. The regulation of MAPK and CCN2 may be bidirectional, with MAPK acting as upstream regulators of CCN2 and with CCN2 mediating MAPK phosphorylation. Mendes et al. reported that CCN2 protein increases the phosphorylation level of p44/42 MAPK protein, but not p38 or JUN N-terminal kinase (JNK; p54/46) MAPK proteins in mouse neuronal progenitor cells after five days of culture *in vitro* [26]. Thus, we hypothesized that CCN2 protein may induce neurotrophic effects in human neuronal precursor cells via activation of p44/42 MAPK protein. To evaluate whether CCN2 protein activates p44/42 MAPK protein in neuronal precursor cells derived from 201B7 cells, we analyzed total and phosphorylated p44/42 MAPK levels after five days of co-culture with rhCCN2 protein by western blotting. Compared with NT controls, treatment with neurotrophins or 100 ng/mL rhCCN2 protein significantly increased the phosphorylation level of p44/42 MAPK protein in human neuronal precursor cells (Fig. 4A and B).

These results demonstrate that CCN2 protein promotes phosphorylation of p44/42 MAPK protein in human neuronal precursor cells, suggesting that CCN2 protein can induce neurotrophic effects via activation of p44/42 MAPK signaling pathways.

4. Discussion

Neurotrophins, NGF, BDNF, NT-3, and GDNF, exert their effects on neurons by each interacting with a different TRK or p75[NTR] receptor on the cell surface: NGF interacts with TRKA; BDNF interacts with TRKB; NT3 interacts with TRKC; GDNF interacts with GDNF family receptor α-1 (GFRα-1); p75[NTR] interacts with all neurotrophins extracellularly [2,9,27]. The binding of neurotrophins to their receptors activates intracellular signaling pathways, such as the MAPK-ERK pathway, the phosphoinositide 3-kinase-AKT pathway, the phospholipase Cγ1 (PLCγ1)-protein kinase C (PKC) pathway, the JUN pathway, and the HIF pathway, and thereby
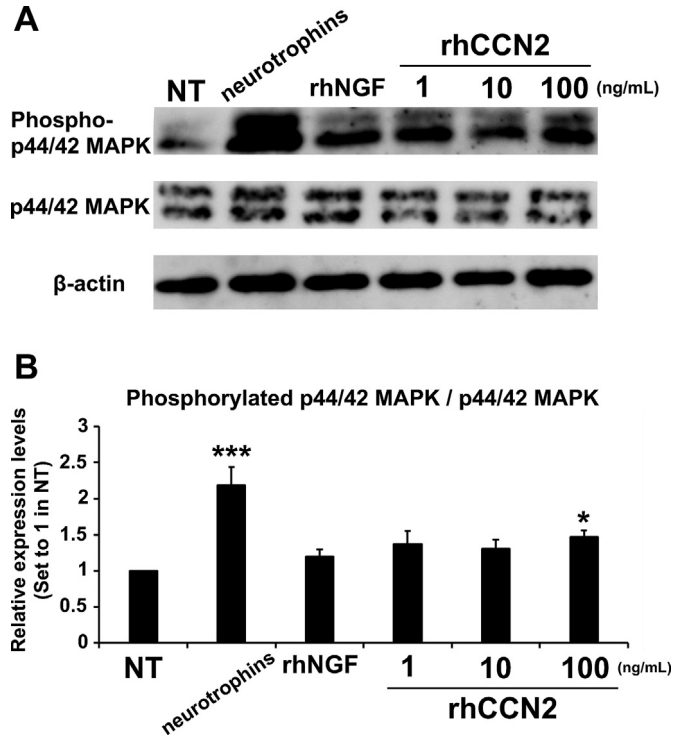
Fig. 4. Western blot analysis of p44/42 MAPK activation by recombinant human CCN2 protein in neuronal precursor cells from 201B7 cells. Neuronal precursor cells derived from 201B7 cells were treated with neurotrophins (10 ng/mL rhBDNF, 10 ng/mL rhGDNF, and 10 ng/mL rhNT-3), and 50 ng/mL rhNGF or 1, 10, or 100 ng/mL rhCCN2 protein (n = 3 each), respectively. NT (n = 3) were used as negative controls. After five days of incubation, protein expression and phosphorylation of p44/42 MAPK and p44/42 MAPK were analyzed in each cell lysate by western blot (A). β-actin was used as a protein loading control. Band intensities of phosphorylated and total p44/42 MAPK proteins were measured. Ratios of phosphorylated/total p44/42 MAPK in each lysate were determined relative to the ratio in NT-cells (set as 1) (B). Data are presented as means ± SEM of two independent experiments. *$p < 0.05$ or ***$p < 0.001$ for comparisons with NT-cells.

promotes neurite outgrowth and neuronal survival [2,9,27]. Activation of TRK receptors by binding to neurotrophins triggers phosphorylation of their intracellular domain tyrosine residues via autophosphorylation, which activates kinase activity. In the case of the TRKA receptor, phosphorylation of tyrosine 490 activates MAPK-ERK and PI3K-AKT signaling, whereas phosphorylation of tyrosine 785 activates PLCγ1-PKC signaling. In each case, adaptor proteins, such as MAPK, PI3K, and PKC, are also activated by phosphorylation [2]. By *in silico* molecular network analysis of our comprehensive RNA sequencing results on jiadifenolide-treated human neuronal cells using KeyMolnet software, we found that jiadifenolide highly activated CCN signaling and transcriptional regulation of SMAD (Fig. 1), and up-regulated mRNA expression of CCN2 (Fig. 2B). These pathways activated by jiadifenolide are expected to contribute to neurotrophic effects via TRK receptor-related signaling. The TRKA signaling pathway is especially strongly suggested.

CCN2 protein mediates multiple functions in a variety of tissues and organs by interacting with multiple molecules. It is induced by a variety of transcription factors, such as SMAD, serum response factor (SRF), NF-κB, activator protein-1 (AP-1), cAMP response element binding protein (CREB), transcriptional coactivator with PDZ-binding motif (TAZ), specific protein 1 (Sp1), hypoxia inducible factor 1 alpha (HIF1α), Forkhead box class O3 a (FOXO3a) and STAT3 [16,19]. CCN2 protein can interact with neurotrophin receptor TRKA and p75[NTR] receptors, and its ligand, NGF [19,21,22]. CCN2 protein

stimulates phosphorylation of tyrosine 490 in TRKA, and promotes phosphorylation of MAPK, JUK, and PKC in human mesangial cells [19,21,22,28]. Khodosevich et al. reported that CCN2 expressed in mouse olfactory bulb (OB) by adeno-associated virus (AAV) regulates survival of neurons in the mouse OB by apoptosis of immature neurons via TGF-β2–SMAD signaling by *in vivo* analysis at four and eight weeks [29]. In addition, Mendes et al. reported that CCN2 protein promotes phosphorylation of p44/42 MAPK protein in mouse neuronal progenitor cells, but not p38 or p54/46 MAPK proteins, and induces astrogenesis in mouse primary embryonic neural cells in five day cultures as assessed by *in vitro* analysis [26]. We showed that rhCCN2 protein exhibited neurotrophic effects and promoted phosphorylation of p44/42 MAPK protein in human neuronal precursor cells (Figs. 3 and 4). Therefore, CCN2 protein can induce neurotrophic effects via activation of p44/42 MAPK signaling.

We found that jiadifenolide up-regulated mRNA level expression of not only CCN2 but also CCN1 (Table 1 and Fig. 2A) and CCN6 genes (Fig. 2F). In Supplementary materials, we discussed the relationships of CCN1 or CCN6 against neurotrophic effects in human neuron.

Taken together, jiadifenolide promotes transcription of the CCN2 gene via various transcription factors, and the expressed CCN2 protein induces neurotrophic effects through TRKA and/or p75^{NTR}–MAPK signaling pathways. In conclusion, the findings in this study reveal the molecular mechanisms and signaling pathways underlying jiadifenolide's neurotrophic effects in human neurons for the first time. This is the first discovery to connect neurotrophic effects with CCN signaling.

Conflicts of interest

None.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.09.003.

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