

PKC η Regulates the TGF β 3-induced Chondrogenic Differentiation of Human Mesenchymal Stem Cell

*Bo Mi Ku¹, *Young Phil Yune¹, Eun Shin Lee², Young-Sool Hah³, Jae Yong Park⁴, Joo Yeon Jeong¹, Dong Hoon Lee¹, Gyeong Jae Cho¹, Wan Sung Choi¹ and †Sang Soo Kang¹

¹Department of Anatomy & Neurobiology, Institute of Health Sciences, School of Medicine, Gyeongsang National University, Jinju 660-290, Republic of Korea

²Department of Rehabilitation Medicine, School of Medicine, Gyeongsang National University, Jinju 660-290, Republic of Korea

³Clinical Research Institute, Gyeongsang National University Hospital, Jinju 660-702, Republic of Korea

⁴Dept. of Physiology, School of Medicine, Gyeongsang National University, Jinju 660-751, Republic of Korea

ABSTRACT : Transforming growth factor (TGF) family is well known to induce the chondrogenic differentiation of mesenchymal stem cells (MSC). However, the precise signal transduction pathways and underlying factors are not well known. Thus the present study aims to evaluate the possible role of C2 domain in the chondrogenic differentiation of human mesenchymal stem cells. To this end, 145 C2 domains in the adenovirus were individually transfected to hMSC, and morphological changes were examined. Among 145 C2 domains, C2 domain of protein kinase C eta (PKC η) was selected as a possible chondrogenic differentiation factor for hMSC. To confirm this possibility, we treated TGF β 3, a well known chondrogenic differentiation factor of hMSC, and examined the increased-expression of glycosaminoglycan (GAG), collagen type II (COL II) as well as PKC η using RT-PCR, immunocytochemistry and Western blot analysis. To further evaluation of C2 domain of PKC η , we examined morphological changes, expressions of GAG and COL II after transfection of PKC η -C2 domain in hMSC. Overexpression of PKC η -C2 domain induced morphological change and increased GAG and COL II expressions. The present results demonstrate that PKC η involves in the TGF- β 3-induced chondrogenic differentiation of hMSC, and C2 domain of PKC η has important role in this process.

Key words : Human mesenchymal stem cell, Chondrogenesis, TGF- β 3, PKC η , C2-domain

INTRODUCTION

Chondrocytes are the cellular component of cartilage, responsible for generating and maintaining its extracellular environment (Muir, 1995). Cartilage has multiple functions such as providing cushion on the articular surfaces of joints, providing a template for the formation of endochondral

bone, and contributing to fracture repair (Sandell & Aigner, 2001; Karsenty & Wagner, 2002). However, cartilage defects have only a limited intrinsic healing capacity (Hellingman et al., 2011). Thus, the cell-based regeneration of (osteo) chondral defects presents a major challenge (Pelttari et al., 2008; Fong et al. 2010; Huang et al., 2010).

Human mesenchymal stem cells (MSCs) with multiple

* Both authors are equally contributed.

Manuscript received 18 October 2013, Received in revised form 7 November 2013, Accepted 11 November 2013

† Corresponding Author : Sang Soo Kang, Dept. of Anatomy & Neurobiology, Institute of Health Sciences, School of Medicine, Gyeongsang National University, 816 Beon-gil 15, Jinju-daero, Jinju 660-290, Korea. Tel. : +82-55-772-8033, E-mail : kangss@gnu.ac.kr

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License(<http://creativecommons.org/licenses/by-nc/3.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

differentiation potentials are a promising alternative cell source for cartilage regeneration (Csaki et al., 2008; Chen et al., 2009). The differentiation of mesenchymal cells into chondrocytes takes place along a multistep pathway (Shum & Nuckolls, 2002). The steps in this pathway include the recruitment of mesenchymal chondroprogenitor cells, the subsequent condensation of these mesenchymal cells, followed by the frank differentiation of the condensed mesenchymal cells into chondrocytes. Cells within these condensations commit to the chondrogenic lineage, acquire spherical cell morphology and induce expression of the essential chondrogenic transcription factor Sox9. Sox 5 and Sox6 cooperate with Sox9 to control chondrogenesis and are themselves under the transcriptional control of Sox9 (de Crombrughe et al., 2000; Kawakami et al., 2006). Together, these transcription factors activate transcription of the major chondrogenic matrix genes, collagen type II (COL II) and aggrecan. Furthermore, increased glycosaminoglycan (GAG) content is another marker of the chondrogenic extracellular matrix (ECM) (Bobick et al., 2009; Chen et al., 2009). In the growth plate of skeletal elements that undergo endochondral ossification, several layers of chondrocytes then become flattened and the cells proliferate mainly unidirectionally (Shum et al., 2002; Woods et al., 2007). These cells then stop proliferating, change their genetic program and become hypertrophic (Pelttari et al., 2008). The ECM of the most advanced hypertrophic chondrocytes becomes mineralized before these cells undergo apoptosis and are replaced by the cells that will become the constituent cells of bones (Fong et al., 2010).

Chondrogenic induction *in vitro* stands as a special culture system achieved by forcing aggregation for mesenchymal cells or chondroprogenitor cells to generate a 'micromass' or 'pellet' culture and treating this with transforming growth factor- β (TGF- β) superfamily members (Boeuf & Richter, 2010; Vater et al., 2011). TGF- β promotes cartilage-specific gene expression through intracellular signaling cascades involving SMAD proteins, and the mitogen activated protein (MAP) kinases (Augello & De Bari, 2010; Li et al., 2010; Arita et al., 2011; Hellingman et

al., 2011). The therapeutic potential of MSCs for cartilage repair is clear (Csaki et al., 2008; Pelttari et al., 2008; Chen et al., 2009). However, the requirements and conditions for effective induction of chondrogenesis in MSCs and for the production of a stable cartilaginous tissue by these cells are far from being understood. Thus, gaining a better understanding of signaling pathways that regulate these conditions is essential.

A C2 domain is a protein structure domain involved in targeting protein to cell membrane. The C2 domain is found in many cellular proteins involved in signal transduction or membrane trafficking. C2 domains are unique among membrane targeting domains in that they show wide range of lipid selectivity for the major components of cell membranes, including phosphatidylserine and phosphatidylcholine (DiNitto et al., 2003). This C2 domain is about 116 amino-acid residues and is located between the two copies of the C1 domain in Protein Kinase C (PKC) and the protein kinase catalytic domain. Regions with significant homology to the C2 domain have been found in many proteins (Corbalán-García & Gómez-Fernández, 2010).

Although the function of C2 domain in chondrogenesis is unknown, C2 domain may play a role in signaling pathways that regulate chondrocyte differentiation. The present study was undertaken to reveal whether the C2 domain is involved in signaling processes of chondrogenesis.

MATERIALS AND METHODS

1. Cell culture

Human MSCs were purchased from Lonza (Walkersville, MD). The cells were expanded in low-glucose DMEM containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μ g/ml) in a 5% CO₂ incubator at 37°C. Normal human fibroblast (NHFB) were obtained from Chungnam National University and cultured in DMEM containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μ g/ml). All culture media and supplements were obtained from Gibco (Carlsbad, CA).

2. Screening of hMSC differentiation-related C2-domains

The C2 domain library containing 145 kinds was manufactured using the gateway adenovirus system (Nochi et al., 2004; Park et al., 2007). That adenovirus library was then infected to hMSCs individually. Final candidates were classified and selected by the degree of their effects on morphological changes.

3. *In vitro* chondrogenic induction

Chondrogenic differentiation of the hMSCs was initiated in a micromass culture system (Zhang et al., 2010; Vater et al., 2011). Cells were dissociated for single-cell suspension stating at density of 2.0×10^7 cells/ml, and a 10- μ l drop of this cell suspension was placed in the center of a culture dish. The cells were allowed to adhere at 37°C for 2 h, followed by the addition of chondrogenic medium (high-glucose DMEM containing 100 nM dexamethasone (Sigma, St. Louis, MO), 50 μ g/ml ascorbic acid-2-phosphate (Sigma), 1% penicillin streptomycin, and ITS-Premix (6.25 μ g/ml insulin, 6.25 μ g/ml transferrin, 6.25 μ g/ml selenous acid, 5.33 μ g/ml linoleic acid and 1.25 mg/ml bovine serum albumin; BD Biosciences, Bedford, MA) with or without 10 ng/ml TGF- β 3 (R&D systems, Minneapolis, MN). After 24 h, the cell droplets coalesced and became

spherical. The medium was changed every 2 days.

4. Construction of recombinant adenovirus and Adv-PKC η -C2 -domain transduced culture

Human MSCs at 80% confluence were transfected with 100 MOI of Adv-GFP or Adv- PKC η -C2 domain for 2 h, and media were changed with fresh DMEM + 10% FBS. Seven day after infection, cell morphology was examined using an inverted fluorescence microscope (IX81, Olympus, Japan) coupled to a CCD camera (Olympus DP71). For micromass culture, transfected cells were seeded 24 h after infection.

5. Reverse transcription-PCR (RT-PCR)

Total RNA was isolated with the TRIzol reagent (Invitrogen, Carlsbad, CA) and 1 μ g RNA was reverse transcribed for synthesis of first cDNA strand. Synthesized cDNA was used as a template in PCR using *Taq* polymerase (Takara, Japan) with gene specific primers (Table 2). PCR products were separated on 1.5 % agarose gel and visualized by ethidium bromide staining.

6. Western blot analysis

Cells were lysed in lysis buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.02% sodium azide, 0.5% sodium deoxycholate, 0.1% SDS, 100 μ g/ml phenylmethylsulphonyl fluoride, 0.5 μ g/ml leupeptin, 1 μ g/ml aprotinin. After a brief sonication, the lysates were clarified by centrifugation at 12,000 \times g for 30 min at 4°C. Protein concentrations were determined with a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL). Equal amounts of protein were loaded on 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked with 5% skim milk in TBS containing 0.1% Tween-20 for 2 h at room temperature and incubated with the appropriate primary and secondary antibodies. Labeled proteins were detected using ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ). Antibodies used were: COL1A1, COL2A1, and PKC η (Santa Cruz Biotechnology, Santa Cruz, CA),

Table 1. List of selected C2-domain containing genes

Gene	Symbol
Dysferlin	DYSF
HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2	HECW 2
Myoferlin	MYOF
Phospholipase C-like 2	PLCL 2
Protein kinase C eta	PKC η
Ras GTPase-activating protein 2	RASAL 2
Regulating synaptic membrane exocytosis 1	RIMS 1
Synaptotagmin III	SYT 3
Synaptotagmin IV	SYT 4
Synaptotagmin XII	SYT 12
Synaptotagmin XVII	SYT 17
Synaptotagmin-like 2	SYTL 2

Table 2. List of primers used for PCR

Gene	Primer sequence: sense/antisense	Product size(bp)
Collagen I	5'-ATCCAGCTGACCTTCCTGCG-3'/5'-TCGAAGCCGAATTCCTGGTCT-3'	322
Collagen II	5'-GAAACCATCAATGGTGGCTTCC-3'/5'-CGATAACAGTCTTGCCCCACT-3'	301
Collagen X	5'-AGCTGGCATAGCAACTAAGG-3'/5'-CTTGGGTCATAATGCTGTTG-3'	324
Sox-9	5'-TCTGGAGACTTCTGAACGAG-3'/5'-GTCTCGATGTTGGAGATGAC-3'	439
GAPDH	5'-AGCTGAACGGGAAGCTCACT-3'/5'-TGCTGTAGCCAAATTCGTTG-3'	300
DYSF	5'-GACTATGACCTCCTCTCCAA-3'/5'-CTGAAACAATTACACGGTCTG-3'	228
HECW 2	5'-CACGTAAACAGAACCACGAC-3'/5'-TAGAGTGAGGCAGGATGTTC-3'	241
MYOF	5'-ATCACAGGAGAGGAAATGAG-3'/5'-GAAACCCAAGTAGTCATCCA-3'	285
PLCL 2	5'-TTGTAGTGTGGATGATGAC-3'/5'-CCACAGTGTCTCAAAGATG-3'	251
PKC η	5'-GCCATACTGAATGATGAGGT-3'/5'-TTTGATTCTGGGTCTGAAAAG-3'	216
RASAL 2	5'-GAACCCTGTCTATCACCTCA-3'/5'-GCAAAGCAAGAGGTATGTGT-3'	233
RIMS 1	5'-TCCTCGAAATCCCTATGTAA-3'/5'-AGTTTATACCAATGCGGGTTC-3'	273
SYT 3	5'-CTCCTACTTGGACATGGACT-3'/5'-TAATCTGCCCAATGAGTTTG-3'	335
SYT 4	5'-ATCAGTCCACCACAAACT-3'/5'-ACTAACTGCCCGATTACCTC-3'	286
SYT 12	5'-TCCAGAGAAATGCCTACTCC-3'/5'-TAAATAGAGCCAGCCACTGA-3'	200
SYT 17	5'-ACCAGAAGAACTCAAAGCAG-3'/5'-GCACTTGGGAGATAATTCAG-3'	292
SYTL 2	5'-GGATGGGATAACAAACAGA-3'/5'-TAAGCTCTACACAGGCTTCC-3'	274

α -tubulin (Sigma).

7. Histology

Micromasses were fixed in 4% paraformaldehyde for 3 h, then dehydrated with ethanol, cleared with xylene and embedded in paraffin. Sections at 5 μ m were cut and mounted on glass slides. Sections were deparaffinized and hydrated to distilled water, followed by staining with hematoxylin and eosin or Alcian blue and fast red. Finally sections were brought to xylene solution with several dehydration steps and mounted.

8. Alcian blue staining

Chondrogenic differentiation was measured by Alcian blue staining of sulfated cartilage glycosaminoglycans. Cells were fixed in 4% paraformaldehyde for 10 min and then incubated with 0.1% HCl-Alcian blue for 1 h. Excess stain was washed off with distilled water, and pictures were taken. To evaluate staining intensity, Alcian-blue-bound sulfated glycosaminoglycans were extracted with 6 M guanidine-HCl, and quantified by measuring

the absorbance of the extracts at 620 nm.

9. Lectin peanut agglutinin (PNA) staining

Binding of peanut agglutinin (PNA) was used as a specific marker for precartilaginous condensation. Cells were fixed in 4% paraformaldehyde for 10 min and then incubated with 100 μ g/ml FITC conjugated PNA (Sigma) for 1 h. PNA binding was visualized using FITC fluorescence.

10. Statistical analysis

The data were presented as the mean \pm SE. Student's *t* test was used for all comparisons. A *P* value of <0.05 was considered statistically significant.

RESULTS

1. Screening of hMSC differentiation-related C2-domains

To screen whether C2 domain can differentiate hMSCs to chondrocyte, 145 C2-domain containing adenovirus were infected to hMSCs individually. Seven days after

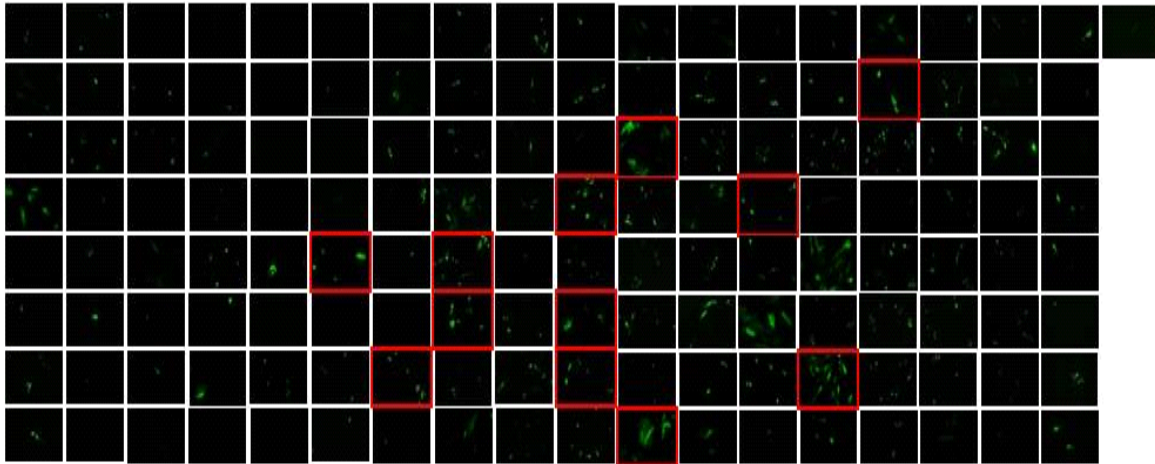


Fig. 1. Morphological change of hMSCs 7 days after 145 C2 domain containing adenovirus library transfection. The origin of 12 red boxed C2 domain was listed in Table 1. Human MSCs at 80% confluence were transfected with 100 MOI of Adv-GFP or Adv-C2 domain for 2 h, and media were changed with fresh DMEM+10% FBS. Seven day after infection, cell morphology was examined using an inverted fluorescence microscope (IX81, Olympus, Japan) coupled to a CCD camera (Olympus DP71).

transfection, primary candidates were selected by the degree of their effects on morphological changes (Fig. 1). As shown in Table 1, 12 C2 domains were selected for further study.

2. TGF- β 3 induced chondrogenesis of hMSCs in a micromass culture

To induce chondrogenesis of hMSCs, micromasses were cultured in chondrogenic medium and stimulated with

TGF- β 3 (10 ng/ml). In most MSC chondrogenesis studies, differentiation is induced under serum-free media conditions in the presence of TGF- β 3, a known inhibitor of matrix mineralization (Huang et al., 2010). In this micromass culture system, cellular condensation occurred at 1 day after TGF- β 3 treatment and a spherical formation was confirmed at 2 days after (Fig. 2A). Consistent with this observation, the degree of precartilage condensation as assessed by PNA binding was significantly increased in

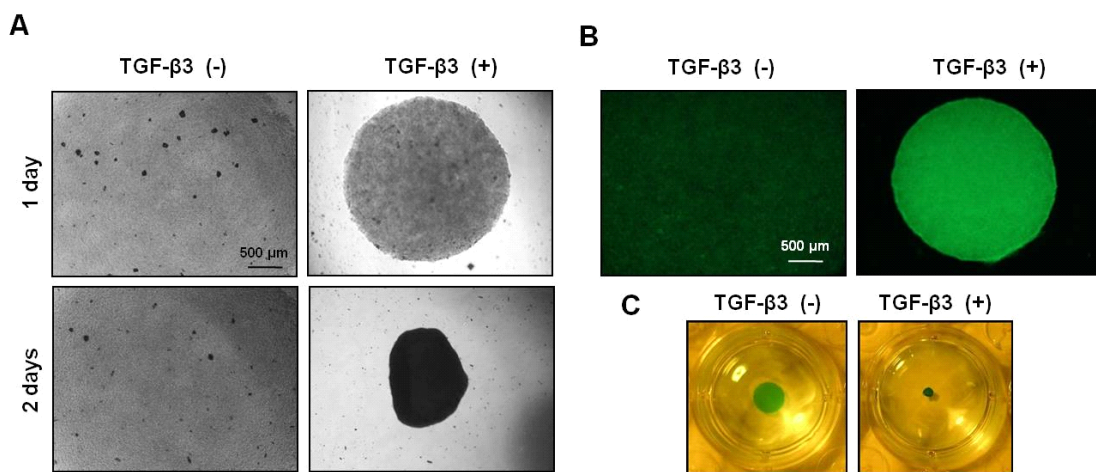


Fig. 2. Chondrogenic differentiation of hMSCs in a micromass culture. (A) Pictures of differentiating hMSC aggregates from micromass culture. Bar: 500 μ m. (B) PNA staining of cultures treated with TGF- β 3 demonstrates a change in cellular condensation. Bar: 500 μ m. (C) MSCs were cultured as micromass and treated with TGF- β 3 (10 ng/ml). Cultures were stained with Alcian blue after two days.

TGF- β 3 treated micromass (Fig. 2B). After 2 days, micromass cultures were stained with Alcian blue to visualize GAG content (Fig. 2C).

3. PKC η gene expression is increased during chondrogenesis

To evaluate which selected gene is closely related to the chondrogenesis of hMSC, gene expressions were analyzed by RT-PCR. Among 12 C2 domains, domain of PKC η showed similar expression pattern to COL II during TGF- β 3 treatment (Fig. 3). The gene expression of Sox9,

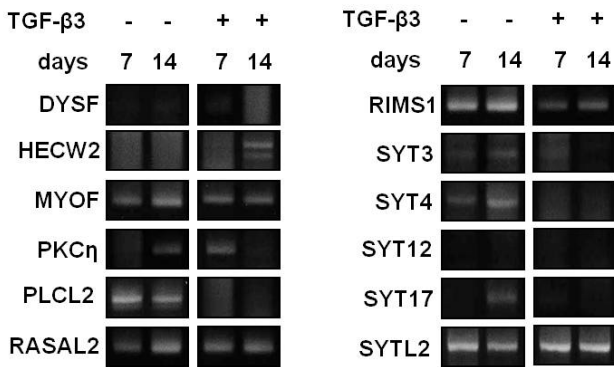


Fig. 3. C2 domain containing gene expressions during chondrogenic differentiation. During chondrogenic differentiation of hMSC, C2 domain containing gene expressions were changed by TGF- β 3.

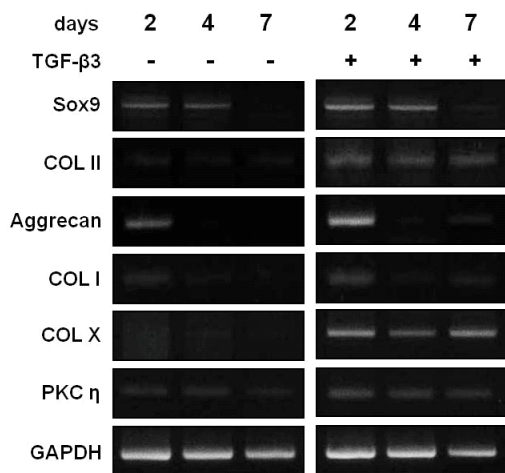


Fig. 4. PKC η gene expression during chondrogenic differentiation. Addition of TGF- β 3 led to rapid expression of PKC η , which increased at day 2, and was sustained until day 7.

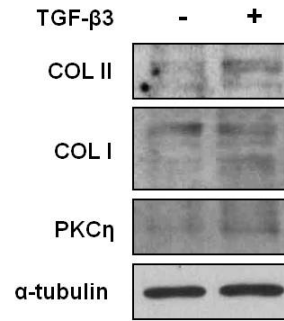


Fig. 5. PKC η protein expression during chondrogenic differentiation. Chondrogenesis was initiated by micromass culture, supplemented with TGF- β 3 (10 ng/ml), the COL II, COL I, and PKC η expression was determined by Western blot analysis at day 3.

COL II, aggrecan, and PKC η strongly increased by day 2 of culture with TGF- β 3, and expression remained until day 7 (Fig. 4). The levels of COL II and PKC η protein were also increased by TGF- β 3 (Fig. 5). COL I, which signifies inadequate differentiation, did not show a significant increase in protein level (Fig. 5).

4. PKC η -C2 domain induces chondrogenic differentiation in hMSCs

The C2 domain of PKC η is thought not to affect substrate-specificity of the kinase but instead aid in its localization to membranous systems (Littler et al., 2006). PKC η overexpression induces G1 arrest and differentiation in keratinocytes. In addition to epithelial cells, recent studies revealed that PKC η acts as a key regulator in early B-cell development (Kashiwagi et al., 2002). To test whether PKC η -C2 domain functions in chondrogenesis, hMSCs were infected with Adv-vec or Adv-PKC η -C2 domain and cultured in monolayer for 7 days. Although high-density cell culture environment is pivotal for the chondrogenic differentiation of hMSCs, PKC η -C2 domain induced morphological change of hMSCs from a characteristic fibroblast-like morphology to a large round shape (Fig. 6A). In addition PKC η -C2 domain markedly induces chondrocyte matrix formation after 7 days of monolayer culture, as shown by Alcian blue staining of

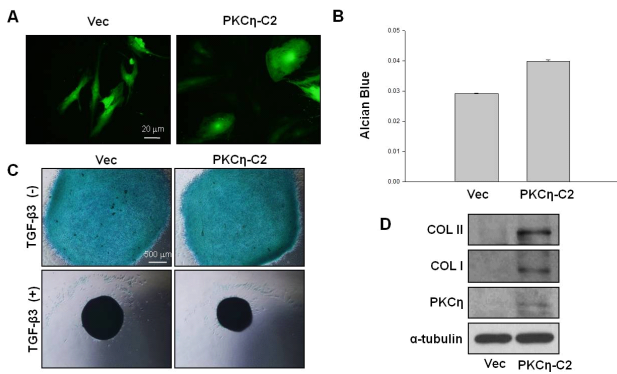


Fig. 6. Effect of PKC η -C2 domain on differentiation of hMSCs.

(A) In a monolayer culture, Adv-PKC η -C2 domain infection led to morphological change at day 7 after transfection. Transfected cells were confirmed by GFP expression. Bar: 20 μ m. (B) Chondrogenesis of hMSCs after PKC η -C2 domain infection. Transfected cells were cultured for 7 days in monolayer, chondrogenesis was quantified by measuring the absorbance of bound Alcian blue at 620 nm. (C) Effect of PKC η -C2 domain on chondrogenic differentiation. After PKC η -C2 domain transfection, chondrogenesis was initiated by micromass culture. Chondrogenesis was measured by Alcian blue staining at 4 days after induction. Bar: 500 μ m. (D) Effect of PKC η -C2 domain on COL II expression. Transfected cells were cultured for 7 days in monolayer, the COL II, COL I, and PKC η expression was determined by Western blot analysis.

the cartilaginous matrix (Fig. 6B). Chondrogenic effect of PKC η -C2 domain was further confirmed in adenovirus infected MSCs micromass model by assessing spherical size and chondrocyte matrix formation (Fig. 6C). The level of a chondrogenic protein, COL II was also increased by PKC η -C2 domain (Fig. 6D). PKC η -C2 domain also increased chondrogenic differentiation in micromass model.

5. PKC η -C2 domain induces collagen type II expression

As shown in Fig. 6D, COL II gene expression was affected by PKC η -C2 domain. To investigate further the roles of PKC η -C2 domain in COL II gene expression, PKC η -C2 domain was overexpressed in NHFB cells. The COL II protein expression was increased by PKC η -C2 domain and this effect was more evident according to time (Fig. 7).

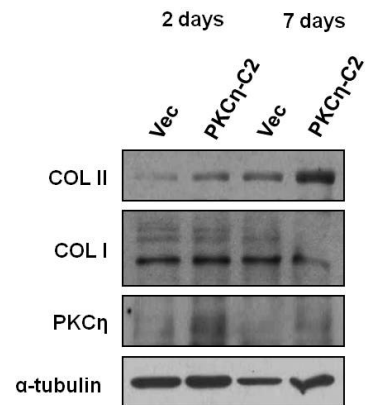


Fig. 7. Effect of PKC η -C2 domain on NHFB. In a monolayer culture, Adv-PKC η -C2 domain transfection increased COL II expression.

DISCUSSION

Natural chondrogenesis is a well-coordinated developmental differentiation program that leads to permanent articular cartilage in the joint or to transient cartilage during endochondral bone formation (Muir, 1995; Shum & Nuckolls, 2002; Csaki et al., 2008; Chen et al., 2009). Generation of stable hyaline cartilage from MSCs is currently still a challenge. Although chondrogenesis and cartilage formation are achieved, it eventually leads to terminal differentiation of chondrocytes instead of the production of stable hyaline cartilage (Pelttari et al., 2008; Huang et al., 2010). Further, the tissue-engineered cartilage construct is not stable when it is implanted *in vivo* but mineralizes (Pelttari et al., 2006). A better knowledge of mechanisms determining chondrocyte differentiation and terminal differentiation is therefore crucial to control the chondrogenic differentiation of MSCs. In the present study, it was demonstrated that PKC η -C2 domain regulates COL II gene expression and TGF- β 3-induced chondrogenic differentiation.

Chondrogenic differentiation was achieved following the direct chondrogenic differentiation *in vitro* micromass protocol (Wang et al., 2005; Woods et al., 2007; Jin et al., 2010). The production of specific chondrogenic markers were measured at 2, 4, 7, and 14 days of culture in chondrogenic medium. The quantitative RT-PCR analysis

measured the expression of COL I, COL II, and COL X in the spheroid formed during the chondrogenic process. Because spheroids first appear in this study after 2 days in culture in differentiation medium, the measurement of the expression of COL I, COL II, and COL X in the spheroids was began at 2 days. The expression of COL I, COL II, and COL X was found as early as 2 days of chondrogenic differentiation. These results are coincident with previous reports (Wang et al., 2005; Woods et al., 2007; Jin et al., 2010). COL II and COL X both increased their expression over time.

It is known that during the differentiation process of MSCs, one or several intracellular chemical cascades are modified influencing the ultimate commitment of the cell (de Crombrughe et al., 2000). However, it remains unclear how each individual pathway affects the differentiation program of the cells and how manipulation of these pathways could lead to more efficient differentiation protocols.

PKC is a family of related protein kinase, which includes at least 10 different isoforms in mammalian cells (Newton, 1997). They play important roles in the transduction of signals coupled to receptor-mediated hydrolysis of membrane phospholipids. The mammalian isoenzymes can be classified into three groups according to their structure and cofactor regulation. The first group includes the classical isoforms (α , β I, β II, and γ), which function is regulated by calcium, acidic phospholipids, and diacylglycerol. The second group corresponds to the novel PKCs (δ , ϵ , η , and θ), which are activated by acidic phospholipids and diacylglycerol in a calcium-independent manner. These two groups contain in their regulatory regions both conserved C1 domains responsible for sensing diacylglycerol, and C2 domains responsible for sensing Ca^{2+} and/or acidic phospholipids at different subcellular compartments. The third group comprises the atypical PKC isoforms (ξ , τ / λ), which are not regulated by diacylglycerol or by calcium (Nishizuka, 1992; Rosse et al., 2010).

The C2 domains of classical and novel PKC play a important role in decoding signals, which trigger the

translocation of these enzymes to the plasma membrane and/or other membrane (Littler et al., 2006; Corbalán-García & Gómez-Fernández, 2010). Although the C2 domains of novel PKCs were supposed to play only a secondary role with respect to the C1 domain in the activation process of these enzymes, new insights reveal that these C2 domains may also receive regulatory inputs and play an important role in the localization and activation of these enzymes (Nishizuka, 1992; Newton, 1997; Corbalán-García & Gómez-Fernández, 2010; Rosse et al., 2010).

The novel PKC η isoform has a unique tissue distribution and is primarily expressed in epithelial tissue and cells undergoing high turnover (Kashiwagi et al., 2002). It is implicated in diverse cellular functions, including a role in terminal differentiation, proliferation, and secretion (Shtutman et al., 2003; Lampasso et al., 2006; Adhikary et al., 2010; Lee et al., 2010). Recent studies suggest that PKC η has a special role in response to stress and regulation of apoptosis (Rotem-Dai et al., 2009). However the function of the PKC η in chondrogenesis is not well understood.

In this study, PKC η gene expression increased during TGF- β 3-induced chondrogenic differentiation. In addition, PKC η -C2 domain induced the gene expression of COL II in monolayer and micromass culture. The molecular mechanisms activated in MSCs, leading to the increased expression of PKC η , are currently not understood. Chondrogenesis might be a product of orchestration of related genes as shown in the TGF- β 3/bone morphogenic protein 2-induced chondrogenesis of MSC (Sang et al., 2014). However, introduction of one protein into human amniotic fluid-derived stem cells changed the pluripotency (Jo et al., 2010). Furthermore, PKC δ gene silencing with shRNA caused a severe reduction in cartilage formation (Matta et al., 2011). Although it seems not to enough, only PKC η -C2 domain can induce chondrogenesis via influencing the Ca^{2+} -related signaling.

In summary, the present study demonstrates interaction between PKC η -C2 domain and the COL II gene expression, providing new insights into the possible links

of PKC η to the chondrogenesis. Further studies are needed for the elucidation of the molecular mechanisms involved.

ACKNOWLEDGEMENTS

This work was carried out with the support of the Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ00900001) Rural Development Administration, Republic of Korea.

REFERENCES

- Adhikary G, Chew YC, Reece EA, Eckert RL (2010) PKC-delta and-eta, MEKK-1, MEK-6, MEK-3, and p38-delta are essential mediators of the response of normal human epidermal keratinocytes to differentiating agents. *J Invest Dermatol* 130:2017-2030.
- Arita NA, Pelaez D, Cheung HS (2011) Activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) is needed for the TGF β -induced chondrogenic and osteogenic differentiation of mesenchymal stem cells. *Biochem Biophys Res Commun* 405:564-569.
- Augello A, De Bari C (2010) The regulation of differentiation in mesenchymal stem cells. *Hum Genet Ther* 21:1226-1238.
- Bobick BE, Chen FH, Le AM, Tuan RS (2009) Regulation of the chondrogenic phenotype in culture. *Brith Defects Res C Embryo Today* 87:351-371.
- Boeuf S, Richter W (2010) Chondrogenesis of mesenchymal stem cells: role of tissue source and inducing factors. *Stem Cell Res Ther* 1:31.
- Chen WH, Lai MT, Wu AT, Wu CC, Gelovani JG, Lin CT, Hung SC, Chiu WT, Deng WP (2009) *In vitro* stage-specific chondrogenesis of mesenchymal stem cells committed to chondrocytes. *Arthritis Rheum* 60: 450-459.
- Corbalán-García S, Gómez-Fernández JC (2010) The C2 domains of classical and novel PKCs as versatile decoders of membrane signals. *Biofactors* 36:1-7.
- Csaki C, Schneider PR, Shakibaei M (2008) Mesenchymal stem cells as a potential pool for cartilage tissue engineering. *Ann Anat* 190:395-412.
- de Crombrughe B, Lefebvre V, Behringer RR, Bi W, Murakami S, Huang W (2000) Transcriptional mechanisms of chondrocyte differentiation. *Matrix Biol* 19:389-394.
- DiNitto JP, Cronin TC, Lambright DG (2003) Membrane recognition and targeting by lipid-binding domains. *Sci STKE* 2003 (213):re16.
- Fong EL, Chan CK, Goodman SB (2010) Stem cell homing in musculoskeletal injury. *Biomaterials* 32:395-409.
- Hellingman CA, Davidson EN, Koevoet W, Vitters EL, van den Berg WB, van Osch GJ, van der Kraan PM (2011) Smad signaling determines chondrogenic differentiation of bone-marrow-derived mesenchymal stem cells: inhibition of Smad1/5/8P prevents terminal differentiation and calcification. *Tissue Eng Part A* 17: 1157-1167.
- Huang AH, Farrell MJ, Mauck RL (2010) Mechanics and mechanobiology of mesenchymal stem cell-based engineered cartilage. *J Biomech* 43:128-136.
- Jin EJ, Park KS, Kim D, Lee YS, Sonn JK, Jung JC, Bang OS, Kang SS (2010) TGF-beta3 inhibits chondrogenesis by suppressing precartilaginous condensation through stimulation of N-cadherin shedding and reduction of cRREB-1 expression. *Mol Cells* 29:425-432.
- Jo J, Lee Y, Oh MH, Ko JJ, Cheon Y-P, Lee DR (2010) Up-regulation of pluripotency-related genes in human amniotic fluid-derived stem cells by ESRRB conjugated with cell-penetrating peptide. *Dev Reprod* 14:243-251.
- Karsenty G, Wagner EF (2002) Reaching a genetic and molecular understanding of skeletal development. *Dev Cell* 2:389-406.
- Kashiwagi M, Ohba M, Chida K, Kuroki T (2002) Protein kinase C eta (PKC eta): its involvement in keratinocyte differentiation. *J Biochem* 132:853-857.
- Kawakami Y, Rodríguez-León J, Izpisua Belmonte JC (2006) The role of TGFbetas and Sox9 during limb chondrogenesis. *Curr Opin Cell Biol* 18:723-729.
- Lampasso JD, Chen W, Marzec N (2006) The expression

- profile of PKC isoforms during MC3T3-E1 differentiation. *Int J Mol Med* 17:1125-1131.
- Lee HK, Yeo S, Kim JS, Lee JG, Bae YS, Lee C, Baek SH (2010) Protein kinase C- ϵ and phospholipase D2 pathway regulates foam cell formation via regulator of G protein signaling 2. *Mol Pharmacol* 78:478-485.
- Li J, Zhao Z, Liu J, Huang N, Long D, Wang J, Li X, Liu Y (2010) MEK/ERK and p38 MAPK regulate chondrogenesis of rat bone marrow mesenchymal stem cells through delicate interaction with TGF- β 1/Smads pathway. *Cell Prolif* 43:333-343.
- Littler DR, Walker JR, She YM, Finerty PJJr, Newman EM, Dhe-Paganon S (2006) Structure of human protein kinase C ϵ (PKC ϵ) C2 domain and identification of phosphorylation sites. *Biochem Biophys Res Commun* 349:1182-1189.
- Matta C, Juhász T, Sziógyártó Z, Kolozsvári B, Somogyi C, Nagy G, Gergely P, Zákány R (2011) PKC δ is a positive regulator of chondrogenesis in chicken high density micromass cell cultures. *Biochimie* 93:149-159.
- Muir H (1995) The chondrocyte, architect of cartilage. Biomechanics, structure, function and molecular biology of cartilage matrix macromolecules. *Bioessays* 17:1039-1048.
- Newton AC (1997) Regulation of protein kinase C. *Curr Opin Cell Biol* 9:161-167.
- Nishizuka Y (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 258:607-614.
- Nochi H, Sung JH, Lou J, Adkisson HD, Maloney WJ, Hruska KA (2004) Adenovirus mediated BMP-13 gene transfer induces chondrogenic differentiation of murine mesenchymal progenitor cells. *J Bone Miner Res* 19:111-122.
- Park JY, Hwang EM, Park N, Kim E, Kim DG, Kang D, Han J, Choi WS, Ryu PD, Hong SG (2007) Gateway RFP-fusion vectors for high throughput functional analysis of genes. *Mol Cells* 23:357-362.
- Pelttari K, Steck E, Richter W (2008) The use of mesenchymal stem cells for chondrogenesis. *Injury* 39(Suppl 1):S58-65.
- Pelttari K, Winter A, Steck E, Goetzke K, Hennig T, Ochs BG, Aigner T, Richter W (2006) Premature induction of hypertrophy during *in vitro* chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. *Arthritis Rheum* 54:3254-3266.
- Rosse C, Linch M, Kermorgant S, Cameron AJ, Boeckeler K, Parker PJ (2010) PKC and the control of localized signal dynamics. *Nat Rev Mol Cell Biol* 11:103-112.
- Rotem-Dai N, Oberkovitz G, Abu-Ghanem S, Livneh E (2009) PKC ϵ confers protection against apoptosis by inhibiting the pro-apoptotic JNK activity in MCF-7 cells. *Exp Cell Res* 315:2616-2623.
- Sandell LJ, Aigner T (2001) Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. *Arthritis Res* 3:107-113.
- Sang Y, Zang W, Yan Y, Liu Y, Fu Q, Wang K, Chen Y, Qi N (2014) Study of differential effects of TGF- β 3/BMP2 on chondrogenesis in MSC cells by gene microarray data analysis. *Mol Cell Biochem* 385:191-198.
- Shtutman M, Hershko T, Maissel A, Fima E, Livneh E (2003) PKC ϵ associates with cyclin E/Cdk2 complex in serum-starved MCF-7 and NIH-3T3 cells. *Exp Cell Res* 286:22-29.
- Shum L, Nuckolls G (2002) The life cycle of chondrocytes in the developing skeleton. *Arthritis Res* 4:94-106.
- Vater C, Kasten P, Stiehler M (2011) Culture media for the differentiation of mesenchymal stromal cells. *Acta Biomater* 7:463-377.
- Wang Y, Belflower RM, Dong YF, Schwarz EM, O'Keefe RJ, Drissi H (2005) Runx1/AML1/Cbfa2 mediates onset of mesenchymal cell differentiation toward chondrogenesis. *J Bone Miner Res* 20:1624-1636.
- Woods A, Wang G, Beier F (2007) Regulation of chondrocyte differentiation by the actin cytoskeleton and adhesive interactions. *J Cell Physiol* 213:1-8.
- Woods A, Wang G, Dupuis H, Shao Z, Beier F (2007)

Rac1 signaling stimulated N-cadherin expression, mesenchymal condensation, and chondrogenesis. *J Biol Chem* 282:23500-23508.

Zhang L, Su P, Xu C, Yang J, Yu W, Huang D (2010)

Chondrogenic differentiation of human mesenchymal stem cells: a comparison between micromass and pellet culture systems. *Biotechnol Lett* 32(9):1339-1346.