ID	5	6	7	8	9	10	11
検査項目	2009.07.07	2009.08.18	2010.03.02	2010.06.22	2010.08.10	2011.03.29	2011.09.20
HBV							
HIV-1	–	–	–	–	–	–	–
HTLV-1	–	–	–	–	–	–	–
Parvo.B19NS1	–	–	–	–	–	–	–
Parvo.B19VP2	–	–	–	–	–	–	–
HSV	–	–	–	–	–	–	–
VZV	–	–	–	–	–	–	–
CMV	–	–	–	–	–	–	–
EBV	–	–	–	–	–	–	–
HHV6	–	–	–	–	–	–	–
HHV7	–	–	–	–	–	–	–
HHV8	–	–	–	–	–	–	–
HCV	–	–	–	–	–	–	–
HIV-1(RNA)	–	–	–	–	–	–	–
HTLV-1(RNA)	–	–	–	–	–	–	–
結果受け取り日	–	–	–	–	–	–	–
エンドトキシン	2009.07.08	2009.08.19	2010.03.08	2010.06.24	2010.08.11	2011.04.30	2011.09.26
結果受け取り日	0.00319EU/ml 未満	0.002291EU/ml 未満	0.004366EU/ml 未満	0.004822EU/ml 未満	0.004562EU/ml 未満	0.006737EU/ml 未満	0.007411EU/ml 未満
マイコプラズマ（RT-PCR 法）	2009.07.08	2009.08.18	2010.03.02	2010.06.22	2010.08.10	2010.03.29	2011.09.20
結果受け取り日							
マイコプラズマ(nested)	–	–	–	–	–	–	–
結果受け取り日	2009.07.08	2009.08.19	2010.03.03	2010.06.24	2010.08.11	201.04.11	2011.09.26
マイコプラズマ（培養法）							
結果受け取り日	–	–	–	–	–	–	–
細菌培養(好気性)	2009.07.08	2009.08.21	2010.03.05	2010.06.23	2010.08.11	2011.03.30	2011.09.22
細菌培養(嫌気性)							
結果受け取り日	–	–	–	–	–	–	–

厚生労働科学研究費補助金（再生医療実用化研究事業）
分担研究報告書

「自家骨髄間葉系幹細胞により活性化された椎間板髄核細胞を用いた椎間板再生研究における細胞、組織の安全性、品質確保に関する技術開発」

分担研究課題：活性化ヒト髄核細胞の腫瘍原性に関する研究

研究分担者　　中村　雅登　　東海大学医学部基盤診療学系再生医療科学・教授

研究要旨：
平成21～23年度に再生医療に用いられた活性化髄核細胞については最高感度in vivo移植実験、腫瘍原性否定―安全性確認システムによって実地再生医療用活性化髄核細胞の安全性が確認でき、今後の移植治療の臨床応用例における標準的な検査項目になると考えられる。

A．研究目的
活性化髄核細胞の腫瘍原性を否定するための超免疫不全NOGマウスを用いたin vivo安全性試験系により移植活性化髄核細胞の非腫瘍原性を確認する。

B．研究方法
超免疫不全NOGマウスの皮下に移植再生医療に用いる活性化髄核細胞を移植し、６週間以上できるだけ長期間観察し腫瘍形成の有無を組織学的に確認する。本年度は前年度に継続して、再生治療に供された10症例の内7症例についてNOGマウス皮下への移植実験を行った。

C．研究結果
ヒト脊索腫細胞株を$5x10^5$個/頭で皮下に移植した場合、6週間で腫瘍形成が確認できる。再生治療実施に供した活性化髄核細胞については更に感度をあげて(腫瘍形成能が高くなる)10^6個/頭の細胞を皮下移植し６週間以上の観察を行った。結果：移植７症例についてはいずれも腫瘍形成を認め無かった。

D．考察
低悪性度のヒト脊索腫細胞株でも腫瘍形成の確認できる感度の高いNOGマウス皮下移植、6週間以上の観察でも、ヒト髄核再生治療実施用-活性化髄核細胞では腫瘍形成が確認できなかった。現時点ではこの条件で腫瘍形成能を判定するのが、最も高感度の腫瘍形成能確認試験と考えられるが、それによってもヒト活性化髄核細胞の腫瘍原性にかかる安全性が確認できたと考えられる。

E．結論
平成21～23年度に再生医療に用いられた活性化髄核細胞については最高感度in vivo移植実験、腫瘍原性否定―安全性確認システムによって実地再生医療用活性化髄核細胞の安全性が確認できた。

G．研究発表
1.　論文発表

1.Masami Hasegawa, Kenji Kawai , Tetsuya Mitsui, Kenji Taniguchi, Makoto Monnai,, Masatoshi Wakui, Mamoru Ito, Makoto Suematsu, Gary Peltz, Masato Nakamura, Hiroshi Suemizu. The reconstituted ‘humanized liver’ in TK-NOG mice is mature and functional. Biochemical and Biophysical Research Communications 2011; 405: 405- 410

2. Hiroshi Yamazaki, Hiroshi Suemizu, Sho Igaya, Makiko Shimizu, Norio Shibata, Masato Nakamura, Goutam Chowdhury, and F. Peter Guengerich . In Vivo Formation of a Glutathione Conjugate Derived from Thalidomide in Humanized uPA-NOG Mice. Chemical Research in Toxicology 2011; 24: 287- 289

3. Akiko Kubo, Mitsuyo Ohmura, Masatoshi Wakui, Takahiro Harada, Shigeki Kajihara, Kiyoshi Ogawa, Hiroshi Suemizu, Masato Nakamura, Mitsutoshi Setou, Makoto Suematsu. Semi-quantitative analysis of metabolic systems of human colon cancer metastatic xenografts in livers of superimmunodeficient NOG mice. Analytical and Bioanalytical Chemistry; 2011: 1895-1904

2. 学会発表

無し

厚生労働科学研究費補助金(再生医療実用化研究事業)
分担研究報告書

「自家骨髄間葉系幹細胞により活性化された椎間板髄核細胞を用いた椎間板再生研究における細胞、組織の安全性、品質確保に関する技術開発」

分担研究課題：活性化椎間板髄核細胞の変性椎間板への移植術に関する研究

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山本至宏　東海大学医学部外科学系整形外科学　講師
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研究要旨：
20歳以上30歳未満の腰椎椎間板ヘルニア、分離症あるいは椎間板症で椎体間固定術を行う症例の内、その頭側あるいは尾側の隣接椎間板に中等度の変性を持つ症例に対して、2010年度までの9例に加え、2011年度は1例で活性化髄核細胞の移植術を施行した。椎間板組織からの髄核組織の分離、腸骨からの骨髄液採取の手技、培養用末梢血採取、cell processing centerへの移送は安全かつ確実に行われた。活性化が終了した髄核細胞の移植用キット（注射針セット）への注入、手術場への搬送も通常通り安全に実施された。骨髄間葉系幹細胞との共培養によって活性化された髄核細胞の中等度変性隣接椎間板内への経皮的移植は、安全、確実に実施できた。骨髄液採取部や活性化髄核細胞移植部に新たな愁訴は認められなかった。

A. 研究目的

自家骨髄間葉系幹細胞との細胞間接着を伴う共培養で活性化された自家椎間板髄核細胞移植術の全過程を検証し、安全、確実な移植術が実施可能かについて検討する。

B. 研究方法

椎体間固定時に採取された椎間板髄核組織と腸骨より採取された骨髄液の状態を術中に評価しcell processing centerへの移送の可否を検討する。最終製品として得られた活性化椎間板髄核細胞を、細胞判定委員会の議に従って受け取り、移植術が基準通りに実施されることを検証する。

C. 研究結果ならびにD. 考察

Cell processing centerに移送する椎間板髄核組織ならびに骨髄液は、研究実施計画通りの方法で採取され、その過程に一切の問題点はなかった。髄核組織、骨髄液の質、量ともに研究計画の基準を満たしていた。最終製品として得られた活性化髄核細胞の変性椎間板移植キットへの注入も、当該手術室内で安全に実施された。局所麻酔下で実施された活性化椎間板髄核細胞の移植術は、平均30分間で実施され、当該移植部の疼痛、下肢痛発生などの合併症は一切認められなかった。2010年度までに実施された9例と2011年度実施の1例を合わせて、細胞、組織採取から活性化椎間板髄核細胞移植術実施の手術過程は、基準通りに実施可能な定型手術であると評価できた。

E. 結論

活性化椎間板髄核細胞移植術に関わる手術室におけるすべての工程は、安全、確実に実施できることが確認された。

G. 研究発表　なし

H. 知的財産権の出願・登録状況　なし

厚生労働科学研究費補助金（再生医療実用化研究事業）
分担研究報告書

「自家骨髄間葉系幹細胞により活性化された椎間板髄核細胞を用いた椎間板再生研究における細胞、組織の安全性、品質確保に関する技術開発」

分担研究課題：適応患者選択ならびに細胞の均一化に関わる外部評価に関する研究

研究分担者　　波呂　浩孝　山梨大学大学院医学工学総合研究部整形外科学・教授

研究要旨：
2009 年以降、東海大学医学部附属病院における活性化椎間板髄核細胞移植症例の外部評価を行った。臨床研究参加者（患者）のデーター（年齢、性別、既往歴、単純 X 線画像と MRI の画像所見等）は、症例ごとに東海大学における適応決定前の段階で送付された。2010 年度までの 9 例に加え、2011 年度に 1 症例について評価を行い、本臨床研究の適応基準に合致していることを評価決定し、書面にて報告を行った。なお、移植された活性化椎間板髄核細胞の細胞処理の均一化については、全 10 例の臨床研究が終了し、3 年間の経過観察を経た時点で、全例のデーターを比較検討し、外部評価者としての見解を報告する。

A. 研究目的

同一患者の骨髄間葉系幹細胞による細胞間接着を伴う共培養で椎間板髄核細胞の活性化を行う本プロジェクトに関して、研究計画の順守、安全性と有効性に関して科学的に評価する。

B. 研究方法

東海大学医学部外科学系整形外科学では椎間板の再生に関する基礎的研究を行い、その結果を踏まえて、活性化椎間板髄核細胞を 7 日後に椎体間固定術を施行した部分の隣接椎間で、中等度の変性を持ち、年齢や臨床症状、および画像上の基準に合致する症例に移植するプロジェクトを継続している。

外部評価者としての研究方法は、ヒト幹細胞臨床研究に関する審査委員会で承認された適応基準に患者が合致していることを事前に検討し、また細胞処理が的確に実施されているかについての評価を実施することである。

C. 研究結果

2009 年以降、東海大学医学部附属病院における活性化髄核細胞移植症例の外部評価を行った。2011 年度は最終移植適応患者である 1 症例の評価を実施し、術前に患者さんの年齢と性別、症状、神経所見、既往歴、レントゲンと MRI の画像所見について適応を検討した。症例基準は、20 才代腰椎椎間板ヘルニア症例で後方椎体間固定術が適応であり、これに隣接した椎間が椎間板変性を有するが不安定性がみられず活性化髄核細胞移植部位として適当であるかについて、検討を行った。全て臨床研究の適応基準に合致していることを術前に評価を行い、書面にて報告を行った。

なお、活性化椎間板髄核の細胞処理の均一化については、全 10 例の臨床研究が終了後 3 年間の経過観察を経た時点で、全例のデーターを比較検討し、外部評価者としての見解を報告する

E. 結論

2009 年度以来 2011 年度までに東海大学において選択された 10 症例はすべて適応基準に合致しており、外部評価者として適応可との判定を行った。

G. 研究発表　なし

H. 知的財産権の出願・登録状況　なし

III.　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Arai F, Hiyama A, Sakai D, Yokoyama K, Mochida J.	The expression and role of non-canonical (PKC) signaling in nucleus pulposus cell metabolism.	J Orthop Res.			2012 in press
Sakai D	Stem cell regeneration of the intervertebral disk.	Orthop Clin North Am.	42	555-562.	2011
Hiyama A, Sakai D, Arai F, Nakajima D, Yokoyama K, Mochida J.	Effects of a glycogen synthase Kinase-3β inhibitor (LiCl) on c-myc protein in intervertebral disc cells.	J Cell Biochem.	112	2974-2986	2011
Hiyama A, Skubutyte R, Markova D, Anderson DG, Yadla S, Sakai D, Mochida J, Albert TJ, Shapiro IM, Risbud MV.	Hypoxia activates the notch signaling pathway in cells of the intervertebral disc: implications in degenerative disc disease.	Arthritis Rheum.	63	1355-1364	2011
持田譲治	特集　幹細胞治療　臨床応用の進歩　椎間板再生	日本臨床	69	2220-2224	2011
持田譲治	椎間板代謝とバイオロジー椎間板再生への細胞移植法も含めて	医学の歩み	236	540-544	2011

酒井大輔, 持田譲治	【運動器傷害における治療法の新しい試み】 脊椎　変性椎間板に対する治療の試み　変性椎間板に対する細胞移植療法	整形外科	62	744-748	2011

IV.　研究成果の刊行物・別刷

The Expression and Role of Non-Canonical (PKC) Signaling in Nucleus Pulposus Cell Metabolism

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ABSTRACT: Canonical Wnt/β-catenin (hereafter Wnt) signaling regulates the proliferation and differentiation of various cell types. However, the role of non-canonical signaling including protein kinase C (PKC) signaling has not been investigated in intervertebral disc (IVD) cells. The aim of this study was to elucidate whether the activation of PKC signaling act to modulate Wnt signaling in IVD cells. We performed several reporter assays, real-time reverse transcription polymerase chain reaction (RT-PCR), immunohistochemical and immunofluorescence analyses, and western blot analyses using rat nucleus pulposus (NP) cells. We also examined the cell proliferation and cell cycle distribution under phorbol 12-myristate 13-acetate (PMA) stimulation, a known activator of PKC signaling. We found that NP cells exhibited decreased β-catenin mRNA and protein levels upon stimulation with PMA. PMA treatment promoted proliferation and cell cycle progression in a time- and dose-dependent manner. In addition, activation of the PKC signaling also regulated the expression of aggrecan. Finally, activation by PMA induced the expression of several PKC isoforms in NP cells. It is concluded that activation of PKC signaling might lead to an increase in matrix synthesis and cell proliferation, thereby inhibiting IVD degeneration. Crosstalk in these signaling pathways plays an important role in the regulation of IVD homeostasis.

Keywords: intervertebral disc (IVD); PKC signal; Wnt signal; disc degeneration

Degenerative changes in the intervertebral disc (IVD) contribute to the development of low back pain. Although the phenotypic character of nucleus pulposus (NP) cells is still not defined, it has been reported that the NP cells synthesize extracellular matrix molecules, including type II collagen and proteoglycans (PG) to maintain IVD homeostasis. This homeostasis is lost in patients with disc diseases, eventually leading to low back pain. The destruction of the NP cells involves a loss of differentiated phenotypes (i.e., dedifferentiation), which is characterized by the cessation of type II collagen expression, and onset of fibroblastic type I collagen expression. A variety of soluble factors are known to cause NP dedifferentiation. One of the best studies is the matrix metalloproteinases (MMPs), which play key factors of IVD degeneration.[1–3] We previously analyzed that the Wnt/β-catenin (hereafter Wnt) signaling in the NP cells, and reported that activation of the Wnt signaling suppresses proliferation of NP cells and induces cell senescence, referring to the possibility of it triggering the process of degeneration of the IVDs.[4,5] In addition, we have also shown that MMPs, which are up-regulated by Wnt signaling, cause dedifferentiation of NP cells.[4,5] Corr[6] also demonstrated that Wnt signaling has also been associated with degenerative joint disease and matrix degeneration.

Abbreviations: IVD, intervertebral disc; NP, nucleus pulposus cells; PG, proteoglycans: MMP, matrix metalloproteinases: PKC, protein kinase C; DAG, diacylglycerol; PCP, planar cell polarity: JNK, c-Jun N-terminal kinase; GSK3β, glycogen synthase kinase-3; PMA, phorbol 12-myristate 13-acetate; GAG, glycosaminoglycan.

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Canonical Wnt signaling involves stabilization of cytoplasmic β-catenin and its translocation into the nucleus, where it acts as a transcriptional coactivator.[7–10] However, what remains unclear is how Wnt signaling is regulated during IVD homeostasis. Non-canonical Wnt signaling are independent of β-catenin signaling, and involve the activation of protein kinase C (PKC), calmodulin-dependent kinase II and planar cell polarity (PCP)/c-Jun N-terminal kinase (JNK). The PKC family consists of at least 12 isoforms that have distinct, and in some cases opposing, roles in cell growth and differentiation.[11,12] The PKC isoforms are subdivided into three subfamilies: The classical, novel, and atypical PKCs (cPKC, nPKC, and aPKC, respectively). The cPKCs are activated by Ca^{2+} and diacylglycerol (DAG), nPKCs are activated by DAG but not by Ca^{2+}, and the aPKC group is not activated by either of these molecules.[13,14] These different isoforms play pivotal roles in several signal transduction pathways that regulate cellular growth, transformation, and differentiation. Although much evidence suggests that PKC is involved in Wnt signaling,[15–17] the molecular roles of PKC in this process are not well understood in IVDs. Therefore, we hypothesized that PKC signaling might play important roles in IVDs, and we focused the present study on this points. We examined the interaction between Wnt and PKC signaling in NP cells and report for the first time that non-canonical (PKC) signaling inhibits canonical (Wnt) signaling in NP cells and promotes cell proliferation and matrix synthesis.

MATERIALS AND METHODS

Reagents and Plasmids

Plasmids were kindly provided by Dr. Michael C. Naski (University of Texas Health Science Center at San Antonio, San Antonio, TX) (plasmids aggrecan-Luc and type II

collagen-Luc).[18,19] Topflash (optimal Tcf-binding site) was purchased from Upstate Biotechnology Inc (Lake Placid, NY). As an internal transfection control, we used vector pGL4.74 (Promega, Madison, WI) containing the Renilla reniformis gene for luciferase. Phorbol 12-myristate 13-acetate (PMA) is the most commonly used phorbol ester.[20] It binds to and activates PKC causing a wide range of effects in cells and tissues. Ionomycin (Sigma-Aldrich, St. Louis, MO) increases intracellular Ca^{2+} and activates PKC indirectly.

Isolation of Intervertebral Disc Cells

Animal experiments were approved by the ethics review board of Tokai University and were performed in accordance with the guidelines on animal use of Tokai University. NP cells were isolated from 12-week-old Sprague Dawley rats lumbar discs ($n = 64$) using methods reported by Hiyama et al.[5] Briefly, the rats were euthanized by injection of an excess of pentobarbital sodium (100 mg/kg) (Nembutal®; Abbott Laboratories). The spinal column was removed under aseptic conditions, and the lumbar IVDs were separated under microscopy. The obtained NP tissue was digested in a mixture of 0.01% trypsin and allowed to digest at 37°C for 15 min. The isolated cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen) and 10% fetal bovine serum (FBS) supplemented with antibiotics at 37°C in a humidified atmosphere of 5% CO_2. When confluent, the NP cells were harvested and subcultured in 10-cm dishes. We used the low-passage (<3) cells cultured in monolayers before it changes character for all experiments.[21]

Immunofluorescence Staining

NP cells were plated in flat-bottom 96-well plates (5,000 cells/well) and treated with PMA (200 nM) for 24 h. The cells were then fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS for 10 min, blocked with PBS containing 5% FBS, and incubated with antibodies against β-catenin (1:200; Cell Signaling, Inc, Danvers, MA), Aggrecan (1:200; Thermo Scientific, Fremont, CA) or PKC-γ (1:200; Thermo Scientific) at 4°C overnight. After washing, cells were incubated with an anti-rabbit Alexa Fluor-488-labeled or an unlabeled anti-rabbit secondary antibody (Invitrogen), each at a dilution of 1:50, and with 10 μm DAPI for 1 h at room temperature. The cells were imaged using a laser-scanning confocal microscope.

Immunohistological Studies

Freshly isolated spinal tissue specimens from 12-week-old rats were fixed immediately in 4% paraformaldehyde in PBS, decalcified, and embedded in paraffin wax. Sagittal sections were deparaffinized in xylene, rehydrated through a graded ethanol series, and stained with hematoxylin. To localize PKC-γ, sections were incubated with the anti-PKC-γ antibody (Thermo Scientific) in 2% bovine serum albumin in PBS at a dilution of 1:200 at 4°C overnight. After thoroughly washing the sections, the bound primary antibody was incubated with a biotinylated universal secondary antibody at a dilution of 1:20 (Vector Laboratories, Burlingame, CA) for 10 min at room temperature. Sections were incubated with a streptavidin/peroxidase complex for 5 min, washed with PBS, and color was developed using 3′-3-diaminobenzidine (Vector Stain Universal Quick Kit, Vector Laboratories) and peroxidase substrate DAB kit (Vector) according to the manufacturer's protocol. Negative controls without the first antibody (anti -PKC-γ) were prepared.

Cell Proliferation Assay

To measure disc cell proliferation, the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out as described previously.[5] Briefly, exponentially growing NP cells were seeded into a 24-well plate at 1.5×10^4 cells/well. After PMA stimulation (200 nM) for 24–48 h, MTT diluted in serum-free DMEM was added to the culture medium to a final concentration of 0.5 mg/ml. At the end of the incubation period (2 h at 37°C), the medium was removed, and the precipitated formazan crystals were solubilized in dimethyl sufoxide. Product formation was measured by reading the absorbance at 590 nm using a microplate reader (Pharmacia).

Cell Cycle Analysis by Fluorescence-Activated Cell Sorting (FACS)

Following treatment, the cell cycle distribution of the NP cells was analyzed by flow cytometry after DNA staining with propidium iodide using the CycleTEST™ PLUS kit (BD PharMingen). CELLQuest (BD PharMingen) and ModFit LT (BD PharMingen) software packages were used for cell acquisition and analysis. Each plot represents the analysis of 10,000 events.

Measurement of PG Content

PG content was measured by the dimethylmethylene blue assay (DMMB, Polysciences, Warrington, PA). After PMA stimulation (10–200 nM) for 6–24 h, NP cells were digested with 20 μg/ml papain at 55°C for 18 h. The digested sample solution (75 μl) was mixed with 25 μl of 2.88 M GuHCl solution and 200 μl of DMMB reagent in a 96-well plate, and absorbance at 530 nm and 595 nm were measured immediately using a plate reader (SPECTRA MAX250, Molecular Devices, MDS, Toronto, Canada). Purified bovine nasal septum-D1 PG (Sigma-Aldrich) was used as a standard, and the 530/595 nm absorbance ratio was calculated. The total amount of PG per well was normalized to the total amount of DNA per well (GAG/DNA).

Real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

NP cells were cultured in 6-cm plates (5×10^5 cells/plate) with or without exposure to different times and doses of PMA, and total RNA was extracted using the Trizol RNA isolation protocol (Invitrogen). Before elution from the column, RNA was treated with RNase-free DNAse I. Total RNA (100 ng) was used as a template for the real-time PCR analyses. The mRNA was quantified using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA), and cDNA was synthesized by the reverse transcription of mRNA as described.[4,5] The real-time PCR analyses were performed in triplicate using 96-well plates with the Fast SYBR Green Master Mix (Applied Biosystems). Two microliters of cDNA per sample were used as the template for real-time PCR; 1 μl forward primer and 1 μl reverse primer were added to 20 μl SYBR green master mix. PCR reactions were performed in an Applied Biosystems 7500 Fast system (Applied Biosystems) according to the manufacturer's instructions. All primers (β-catenin, PKC-α, PKC-ε, PKC-γ, PKC-ι, PKC-ζ, aggrecan, col2a1, and GAPDH) were designed based on coding sequences from Genbank. Forward and reverse primer sequences are listed in Table 1 and synthesized by Takara Bio Inc. (Tokyo, Japan). To normalize each sample, a control gene (GAPDH) was used, and the arbitrary

Table 1. Primers for Real-Time PCR

Target	NCBI Number	Forward Primer, 5′-3′	Reverse Primer, 5′-3′
β-catenin	AF_121265.1	GCCAGTGGATTCCGTACTGT	GAGCTTGCTTTCCTGATTGC
PKC-α	NM001105713.1	GCCGCAGTGTCGTTTATGAAAGTA	GCTCCATGTGTGCCATTCAATTAG
PKC-ε	NM_017171.1	TGGCGTGACAACTACCACCTTC	CCGGCCATCATCTCGTACATC
PKC-γ	NM_012628.1	TGGAGTCCTGCTGTATGAGATGTTG	CAGTTTGTTCCATGATGGCTTGA
PKC-ι	NM_032059.1	TTCCGAGCCATGCCAAATC	ATCACTGCCCGTCCACACTG
PKC-ζ	NM_022507.1	CTGGGTGTCCTTATGTTTGAGATGA	GACGTGTGAGGCCTTGACAGA
Aggrecan	NM_022190.1	TCCGCTGGTCTGATGGACAC	CCAGATCATCACTACGCAGTCCTC
Col2al	NM 012929.1	GAGGGCAACAGCAGGTTCAC	TGTGATCGGTACTCGATGATGG

intensity threshold (C_t) of amplification was computed. The expression scores were obtained by the $\Delta\Delta C_t$ calculation method.

Western Blot Analysis

After treatment, NP cells were immediately placed on ice and washed with cold PBS. Proteins were prepared using the

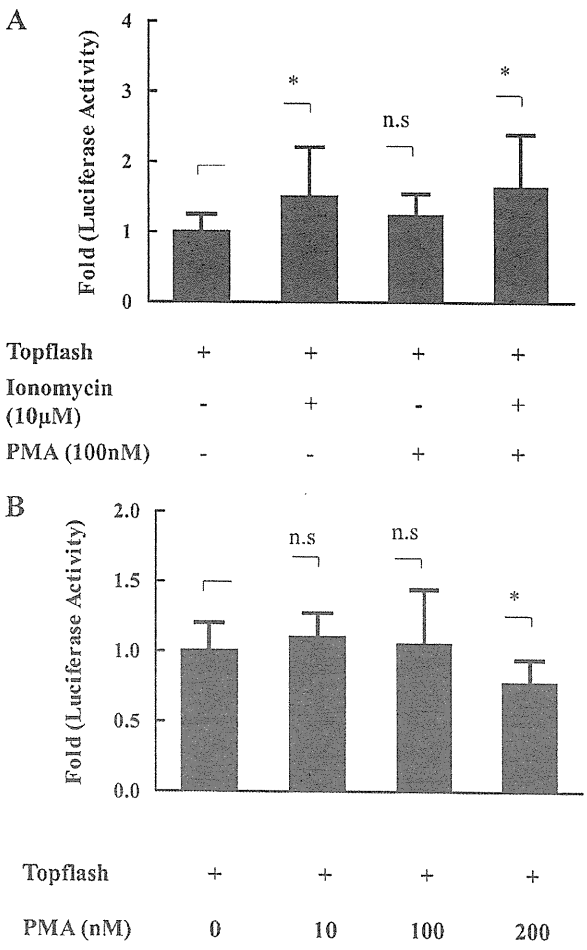


Figure 1. A: NP cells were co-transfected with the Topflash reporter plasmid and the pGL4.74 vector, and were treated with ionomycin (10 μM) or PMA (100 nM). The luciferase activity was measured 24 h after transfection. B: NP cells were co-transfected with the Topflash reporter plasmid and the pGL4.74 vector, and were treated with different concentrations of PMA (10–200 nM). The luciferase activity was measured 24 h after transfection. Values in (A) and (B) are the mean ± S.D results from three independent experiments. $^*p < 0.05$; n.s = not significant.

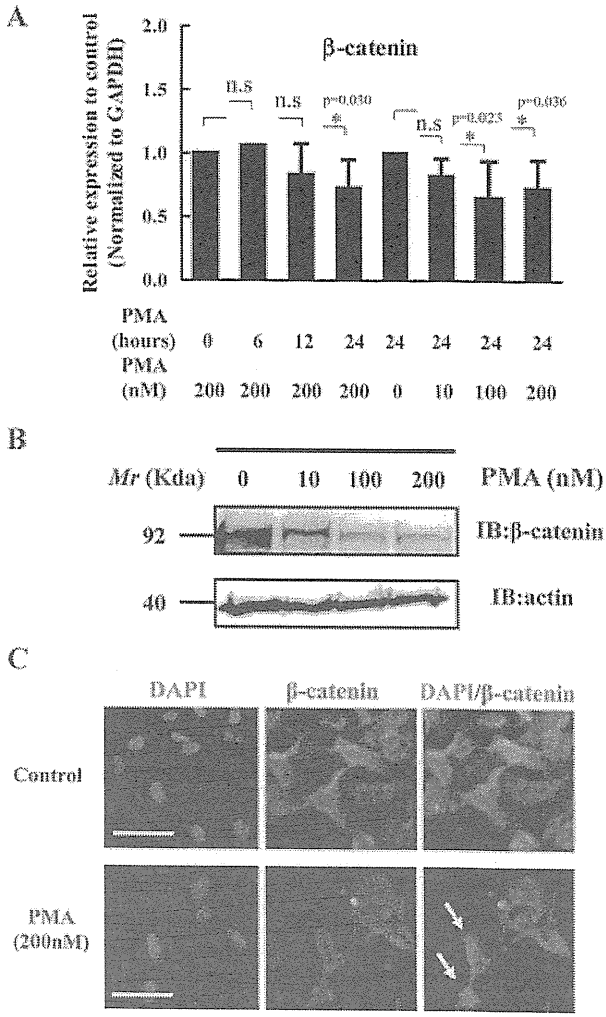


Figure 2. (Original magnification) The effects of PMA-mediated down-regulation on the expression of β-catenin mRNA and protein were determined by real-time reverse transcription-polymerase chain reaction analysis (A) and western blots (B). NP cells were treated with various dose of PMA for different times. Anti-actin was used as a positive control in western blots. Values in (A) are the mean ± S.D results from three independent experiments. $^*p < 0.05$; n.s = not significant. C: NP cells were grown in 96-well plates and exposed to PMA (200 nM) for 24 h. Representative results of the immunocytochemical analyses using anti-β-catenin (red) and DAPI (blue) are shown. The arrows indicate that over-expression of PMA significantly decreased the nuclear staining for β-catenin in NP cells. Left: Cells stained with DAPI to identify healthy nuclei. Middle: Cells stained with an antibody to β-catenin. Right: Cells immunostained for β-catenin and DAPI. Scale bars = 50 μm; (×20).

CellLytic NuCLEAR extraction kit (Sigma-Aldrich). All wash buffers and the final re-suspension buffer included 1X protease inhibitor cocktail (Pierce, Rockford, IL), NaF (5 mM), and Na_3VO_4 (200 mM). Nuclear or total cell proteins were resolved on a SDS polyacrylamide gel and were electro-transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% BSA in TBST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20) and were incubated overnight at 4°C in 5% BSA in TBST with an anti-β-catenin antibody (1:1,000, Cell Signaling). Immunolabeling was detected using enhanced chemiluminescence (ECL) reagents (Amersham Biosciences, Roosendaal, Netherlands).

Transfections and Dual Luciferase Assay

NP cells were transferred to 24-well plates at a density of 6×10^4 cells/well one day prior to transfection. The next day, NP cells were transfected with 900 ng of Topflash and 100 ng of the pGL4.74 plasmid. In addition, to evaluate the effect of PKC signaling on the transcriptional activity of aggrecan and collagen II, NP cells were cotransfected with 900 ng of aggrecan reporter plasmid (Agg-luc) or the collagen II reporter plasmid (Col2-luc) along with the pGL4.74 plasmid; in some experiments, cells were treated with PKC activator ionomycin (10 μM) or PMA (10–200 nM). Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used as the transfection reagent. Forty-eight hours after the initial transfection, the cells were harvested and a Dual-Luciferase reporter assay system (Promega) was used for the sequential measurements of the firefly and renilla luciferase activities. To check the transfection efficiency, rat NP cells were transfected with a plasmid encoding GFP. The obtained results showed that transfection efficiency for rat NP cells was about 60–70%. Quantification of luciferase activities and calculation of relative ratios were carried out using a Turner Designs Luminometer Model TD-20/20 instrument (Promega).

Statistical Analysis

Typically, data were compiled from at least three independent triplicate experiments, each performed on separate cultures and on separate occasions. Data are presented as mean ± standard deviation (SD). Differences between groups were analyzed by Student's *t*-test and ANOVA. Statistical significance ($p < 0.05$) is denoted with an asterisk.

RESULTS

Non-Canonical (PKC) Signaling Inhibited Canonical (Wnt) Signaling in NP Cells

To investigate the role of PKC signaling in the IVD, we first measured the activity of Topflash in NP cells

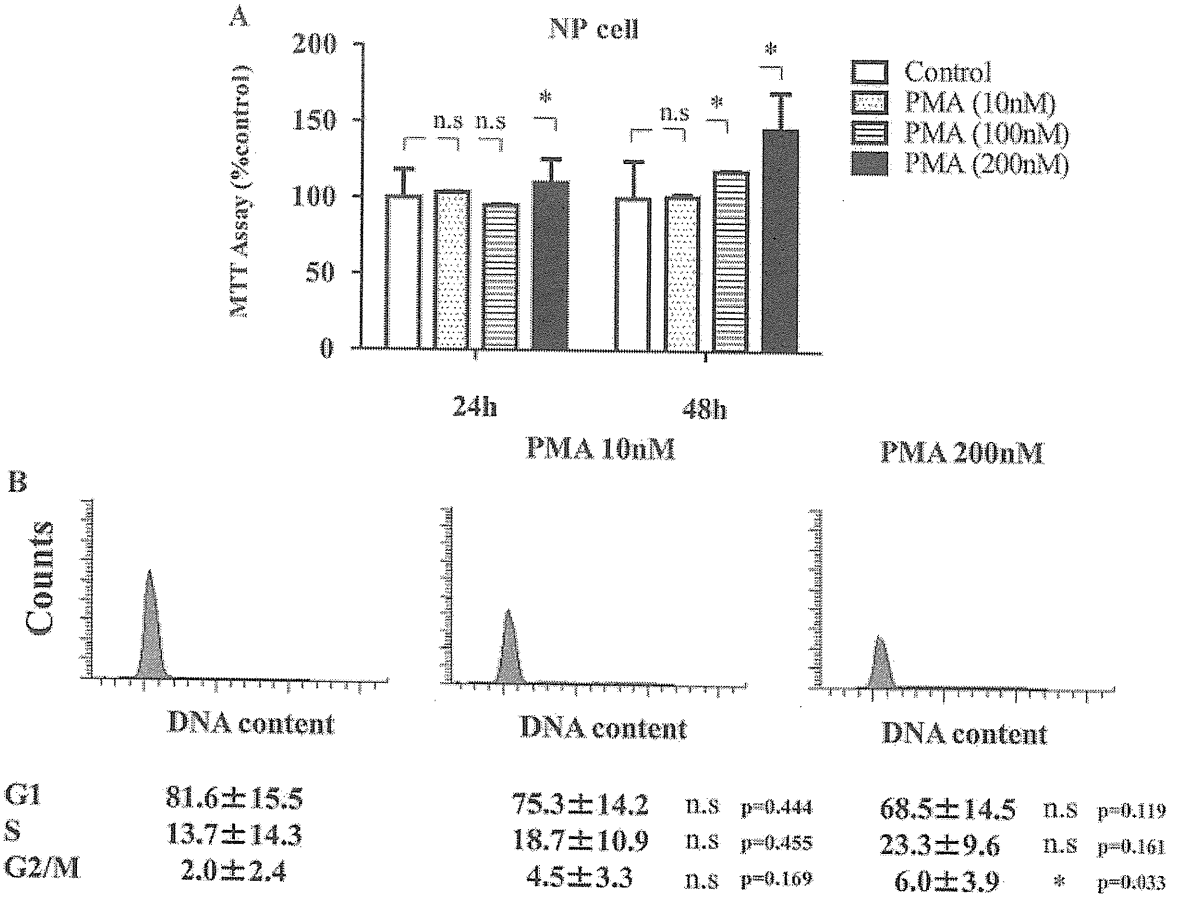


Figure 3. A: NP cells were treated with PMA (0–200 nM). Cell proliferation was evaluated using the MTT viability assay at 24–48 h after treatment. Data are presented as mean ± S.D. $^*p < 0.05$. B: NP cells were cultured for 24 h, then the cells were treated with or without different concentrations of PMA for 24 h, harvested, and the nuclei were stained with propidium iodide. DNA histograms were generated using flow cytometry. Data are presented as mean ± S.D. $^*p < 0.05$; n.s = not significant.

after PMA treatment. Figure 1A,B shows that there was a decrease in the activity of Topflash upon stimulation with a high concentration (200 nM) of PMA. However, the Topflash activity was not affected when cells were treated with 10–100 nM PMA. Fopflash promoter (mutated TCF binding site promoter) activity was unresponsive to PMA treatment (data not shown). In addition, there were different effects of ionomycin and PMA on Topflash activity. The probable explanation for the differences in the effects is that ionomycin might render activated NP cells unresponsive to Ca^{2+}-induced PKC activation, or it is possible that the intracellular Ca^{2+} increase induced by ionomycin has multiple effects on NP cell signaling, in addition to PKC signaling.

To further examine the expression of β-catenin after the PMA treatment, we analyzed β-catenin mRNA expression after the exposure of the cells to PMA for different times or at different concentrations by real-time PCR. Figure 2A shows that the addition of PMA resulted in decreased β-catenin mRNA levels in NP cells in a dose- and time-dependent manner. In addition, as shown in Figure 2B, western blot analysis with an anti-β-catenin antibody demonstrated that PMA treatment decreased the expression of β-catenin protein in a dose-dependent manner. To validate the results of the western blot analysis, we also showed that PMA treatment markedly decreased the expression of β-catenin as detected by immunofluorescence (Fig. 2C).

PKC Signaling Induced Cell Proliferation in Nucleus Pulposus Cells

After PMA stimulation for 24–48 h, cell proliferation was measured using the MTT assay. Proliferation of NP cells was significantly increased after the treatment with PMA (200 nM) (24 h: $p = 0.043$, $p < 0.05$; 48 h: $p = 0.0000174$, $p < 0.05$), but not 10–100 nm of PMA concentrations (24 h) not significantly increased cell proliferation (Fig. 3A). We then used flow cytometry to determine cell cycle progression by quantifying DNA. The effects of activation of PKC signaling in the presence of different concentrations of PMA were determined. Treatment with PMA (200 nM) for 24 h significantly increased the percentage of cells in the G2/M phase to 6.0 ± 3.9% compared with untreated cells ($p = 0.033$, $p < 0.05$). From MTT assay and cell cycle analysis, these results showed that activation of PKC signaling by PMA could promote the proliferation of NP cells (Fig. 3B).

PKC Signaling Enhanced the Transcription and mRNA Expression of Aggrecan in NP Cells

We next examined the effect of ionomycinc or PMA treatment on aggrecan and collagen type II promoter activity. When transfected cells were treated with different concentrations of PMA, there was a statistically significant increase in aggrecan reporter activity (Fig. 4A,B), whereas the collagen type II reporter

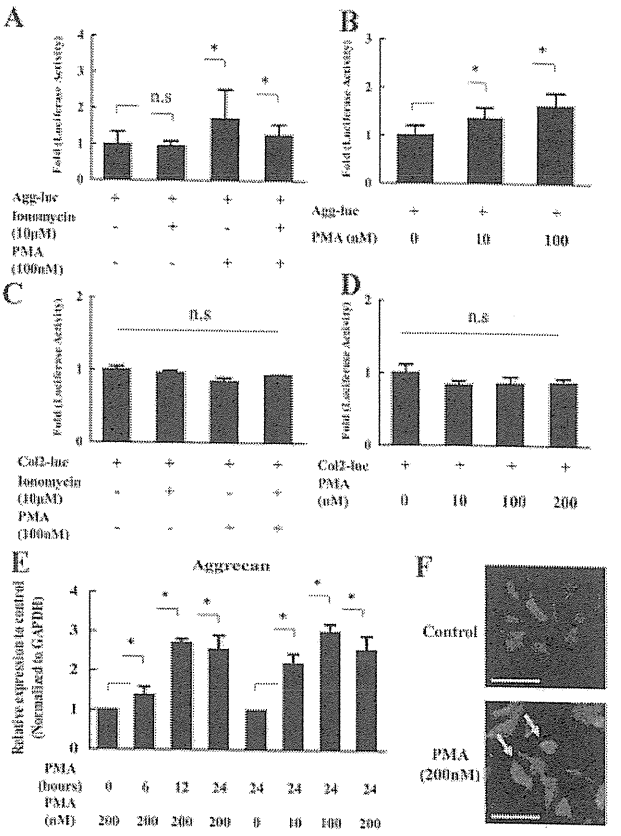
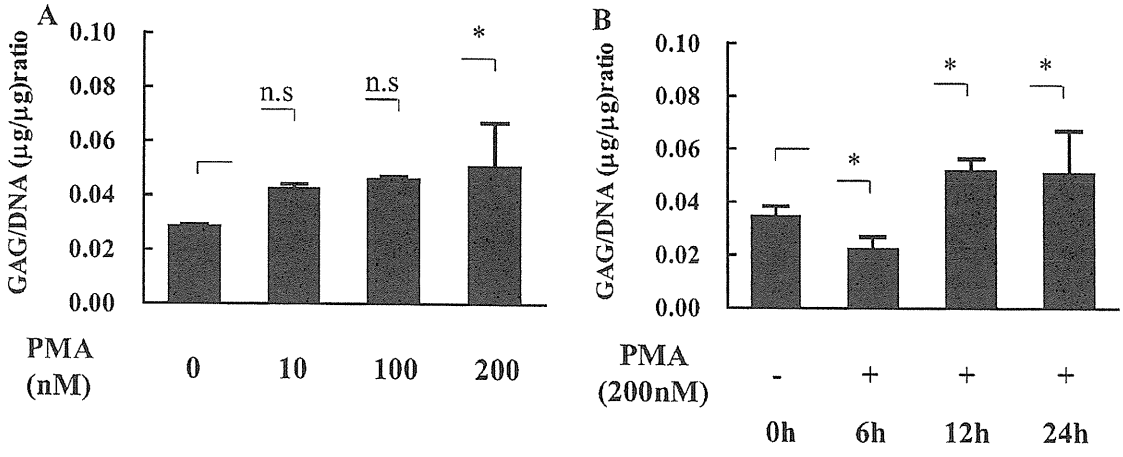


Figure 4. (Original magnification) A, B: NP cells were transfected with the aggrecan reporter plasmid (900 ng) along with the pGL4.74 vector (100 ng) and treated with ionomycin or PMA. C, D: The collagen II (Col2-luc) reporter plasmid was transfected into rat NP cells along with the pGL4.74 vector and transfected cells were treated with ionomycin or PMA, and the activities of the reporter were measured by a dual luciferase assay (A–D). E: NP cells were treated with various dose (10–200 nM) of PMA for different times (6–24 h) and the response to PMA treatment was assessed by real-time reverse transcription–polymerase chain reaction analysis of aggrecan mRNA expression. F: Representative results of the immunocytochemical analyses using anti-aggrecan (red) are shown. Upper: Control, Bottom: PMA (200 nM), Scale bars = 50 μm; (×20). Values in (A–E) are the mean ± S.D results from three independent experiments. $^*p < 0.05$; n.s = not significant.

activity did not change (Fig. 4C,D). In contrast, treatment with a PKC inhibitor (Bisindolylmaleimide I: 10 nM, Calphostin C: 50 nM, Ro-32-0432: 100 nM) did not result in an increase in aggrecan reporter activity (data not shown). To investigate the effects of PKC signaling on the expression of the aggrecan gene and collagen type II mRNA (Col2a1), we analyzed aggrecan and Col2a1 mRNA expression by real-time PCR. Treatment with PMA induced a significant increase in aggrecan mRNA levels in a time- and dose-dependent manner (Fig. 4E), whereas Col2a1 mRNA was not affected by PMA treatment (data not shown). These data were similar to the reporter assay analyses. Immunofluorescence microscopy also demonstrates that overexpression of PMA significantly increased the nuclear staining for aggrecan in NP cells (arrows in Fig. 4F).



		p value	
PMA0	PMA10	0.1475	
PMA0	PMA100	0.0767	
PMA0	PMA200	0.0155	*
PMA10	PMA100	0.7002	
PMA10	PMA200	0.3109	
PMA100	PMA200	0.5569	

		p value	
0h	6h	0.004863	*
0h	12h	0.000661	*
0h	24h	0.000010	*
6h	12h	0.000015	*
6h	24h	0.000001	*
12h	24h	0.002390	*

Figure 5. A, B: The PG content was measured by the dimethylmethylene blue (DMMB) assay. The NP cells were seeded into 24-well plate at 1.5×10^4 cells/well. After PMA stimulation (10–200 nM) for 6–24 h, cells were digested and the GAG content was measured using a plate reader. The total amount of PG per well was normalized to the total amount of DNA per well (GAG/DNA). Values in (A) and (B) are the mean ± S.D results from three independent experiments. $^*p < 0.05$; n.s = not significant.

PKC Signaling Enhanced GAG Content in NP Cells

After PMA stimulation (10–200 nM) for 6–24 h, NP cells were digested and their viability was measured using the MTT assay. Samples treated with PMA showed a significant increase in GAG content in a time- and dose-dependent manner relative to untreated controls (Fig. 5A,B).

The Effects of PMA on the mRNA and Protein Expression of PKC Isoforms in NP Cells

We also investigated the effects of PMA on the expression of the mRNAs of major PKC isoforms in NP cells. Real-time PCR showed that cultured NP cells expressed five PKC isoforms (PKC-α, PKC-ε, PKC-γ, PKC-ι, and PKC-ζ) and that the expression levels of the various PKC isoforms changed after the treatment with PMA (200 nM, 24 h). Treatment with PMA resulted in the up-regulation of PKC-ε, -γ and −ι but not −α or -ζ mRNA. PKC-γ mRNA was highly elevated (>threefold) after the PMA treatment (Fig. 6A). In addition, we determined the expression levels of PKC-γ protein in NP cells following treatment with PMA (200 nM). As shown in Figure 6B, immunofluorescence microscopy analysis with an anti-PKC-γ antibody demonstrated that PMA treatment induced this protein expression in NP cells. To determine whether rat IVDs would express PKC-γ in vivo, we performed immunohistochemical staining. Figure 6C showed the expression of PKC-γ in the 12 week-old rat IVDs. Immunohistological analyses revealed that PKC-γ was expressed in the NP cells. The majority of the staining was intranuclear. However, some staining was present in the cytosol. On the other hand, weak staining for PKC-γ was observed in the inner AF cells. Immunostaining for PKC-γ in the spindle-shaped annulus cells was from the inner to the outer annulus of the IVD.

DISCUSSION

It has been shown that in some cases, treatment of cultured cells with different PMA concentrations results in different responses.[22] Our results show that a high PMA concentration was able to stimulate β-catenin degradation, demonstrating that PMA-mediated β-catenin degradation is GSK-3β-dependent. Thus, PKC might be involved in PMA-induced β-catenin degradation in NP cells. Based on our previous data, Wnt signaling is known to suppress NP cell proliferation,[5] and the present experiments suggest that NP cells may be being suppressed via down-regulated Wnt signaling mediated by PKC signaling. Gwak et al. suggest that the PKC signaling negatively regulates the β-catenin level outside of the Wnt signaling. They demonstrated that the small molecule A23187 was found to inhibit the Wnt signaling. A23187 increased the intracellular Ca^{2+} level and subsequently stimulated PKC, which phosphorylated the N-terminal Ser residues of β-catenin, leading to the promotion of β-catenin degradation.[23]

The PKC family has long been known to play pivotal roles in controlling cell proliferation and differentiation by regulating the activity of transcription factors

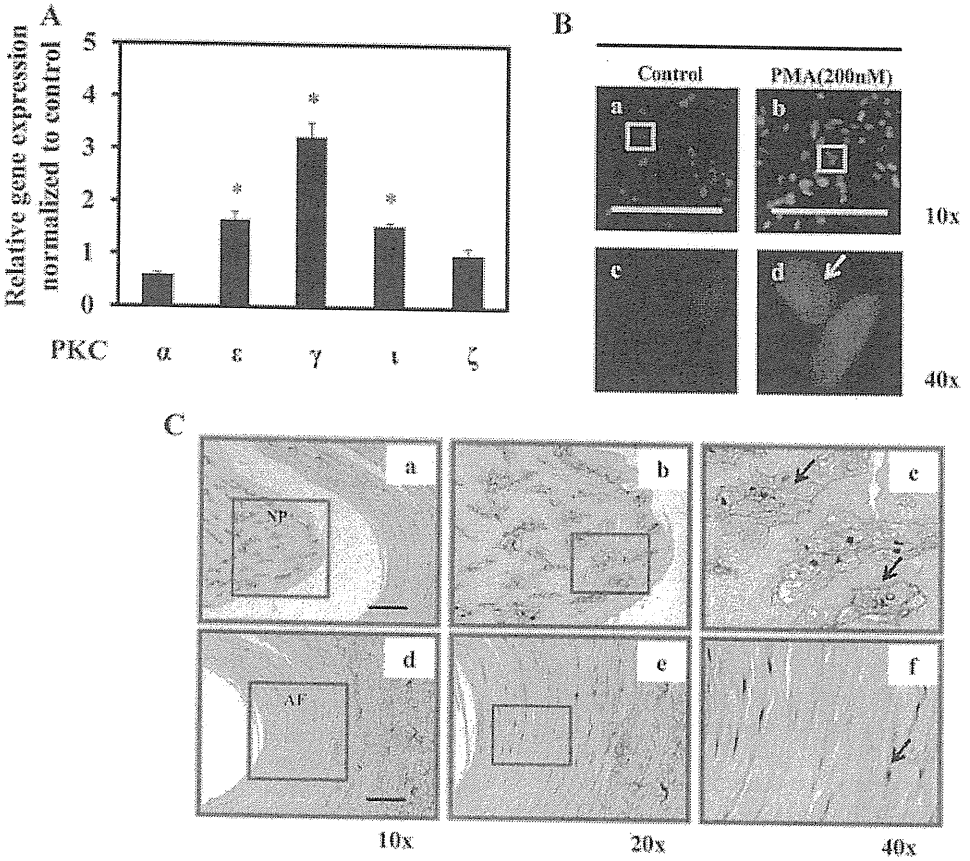


Figure 6. (Original magnification) A: The real-time reverse transcription–polymerase chain reaction analysis of PKC isoforms (PKC-α, PKC-ε, PKC-γ, PKC-ι, and PKC-ζ) quantified the mRNA expression in the presence or absence of PMA in NP cells. The values were quantified and normalized to untreated cells, the value of which was set at 1.0. Values were then normalized to the levels of glyceraldehydes 3-phosphate dehydrogenase (GAPDH) mRNA. The relative expression levels of PKC isoforms and GAPDH were determined. Data are presented as mean ± S.D. $^{*}p < 0.05$. B: NP cells were grown in 96-well plates and exposed to PMA (200 nM) for 24 h. NP cells were assessed by immunofluorescence analysis for the PKC-γ. Representative results of immunocytochemistry using anti- PKC-γ are shown. The arrows indicate that overexpression of PMA significantly increased the nuclear staining for PKC-γ in NP cells. The cells stained with an antibody to PKC-γ. A magnified view of the areas [panel (c) and (d)] is marked by the square in panel (a) and (b). Scale bar: 250 μm; (×10) or (×40). C: Sagittal sections of the intervertebral discs of 12- week-old rats were treated with an anti- PKC-γ antibody, and counterstained with hematoxylin. Upper (a, b, or c): NP, Bottom (d, e, or f): AF. Note that the NP cells expressed PKC-γ protein [arrows in (c) and (f)]. Scale bars = 200 μm, (×10, ×20, or ×40).

such as AP-1 and NF-κβ, and the biological effects exerted by the activation of PKC with phorbol esters could be cell- or tissue-specific.[24] In addition, some groups have reported that PKC appears to upregulate the chondrogenesis of mesenchymal cells.[25,26] Here, we found differential expression of specific PKC isoforms in PMA-treated NP cells compared with non-treated NP cells. One of the isoforms that was significantly upregulated in PMA-treated NP cells was PKC-γ. Generally, this is a member of the classical PKC (cPKC) subfamily, which is activated by both Ca^{2+} and DAG in the presence of phosphatidylserine. Physiologically, PKC-γ is activated by a mechanism coupled with receptor-mediated breakdown of inositol phospholipid, similar to other cPKC isotypes, such as PKC-α and PKC-β. There have been few reported studies on PKC-γ, and how it is involved in generation, differentiation, and degeneration in cartilage, bones, and nerves, including the IVD, is still not well understood. In this study, we reported for the first time that PKC-γ is expressed to a considerable extent in IVD, especially within the NP. This finding extends the current knowledge about how degeneration of the IVD occurs and might help clarify the cellular characteristics of the IVD, especially the NP.

However, there were a few limitations with respect to the analyses and data that could affect the accuracy of these results. First, it will be necessary to confirm the effects of PKC-γ on Wnt signaling in additional models. It would also be useful to examine the expression and responses to activation of PKC isoforms in NP cells. Second, it is known that the main pathway, activated by PKC-, is the MEK-ERK pathway.[27,28] However, the effect of downstream events such as PKC isoforms-MEK-ERK pathway following PKC activation are not well characterized in this study. In addition, it is necessary to examine the effects of the other non-canonical Wnt signal such as Wnt/PCP and JNK signaling. Third, it is reported that a completely different cellular reaction and changes in the manifestation of

the transcription factor occur in accordance with changes in intracellular Ca^{2+} concentrations; however, many factors remain to be clarified for IVD cells.

Mechanical factors appear to regulate the responses of the IVD cells through mechanisms involving intracellular Ca^{2+} transients and cytoskeletal remodeling, which might regulate downstream effects, such as gene expression and posttranslational biosynthesis.[29,30] The mechanical compression could activate Wnt or PKC signaling. Therefore, it might be important for future studies of Wnt and PKC signaling to assess the interactions of these signaling in models designed to assess biomechanical loading, which may be critical in the regulation of IVD degeneration. In summary, the findings presented herein show the first documentation of regulation between the Wnt and PKC signalings, and the expression of PKC isoforms in the IVD. PMA-dependent activation of PKC signaling inhibited β-catenin responsive transcription by down-regulation of intracellular β-catenin, and induced cell proliferation and matrix synthesis in NP cells. These findings suggest that PKC signaling regulates NP cell proliferation via β-catenin phosphorylation/down-regulation and might facilitate the development of new strategies to treat IVD degeneration.

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REFERENCES

1. Kanemoto M, Hukuda S, Komiya Y, et al. 1996. Immunohistochemical study of matrix metalloproteinase-3 and tissue inhibitor of metalloproteinase-1 human intervertebral discs. Spine 21:1–8.
2. Rutges JP, Kummer JA, Oner FC, et al. 2008. Increased MMP-2 activity during intervertebral disc degeneration is correlated to MMP-14 levels. J Pathol 214:523–530.
3. Shen B, Melrose J, Ghosh P, et al. 2003. Induction of matrix metalloproteinase-2 and -3 activity in ovine nucleus pulposus cells grown in three-dimensional agarose gel culture by interleukin-1beta: a potential pathway of disc degeneration. Eur Spine J 12:66–75.
4. Hiyama A, Sakai D, Tanaka M, et al. 2011. The relationship between the Wnt/β-catenin and TGF-β/BMP signals in the intervertebral disc cell. J Cell Physiol 226:1139–1148.
5. Hiyama A, Sakai D, Risbud MV, et al. 2010. Enhancement of intervertebral disc cell senescence by WNT/β-catenin signaling-induced matrix metalloproteinase expression. Arthritis Rheum 62:3036–3047.
6. Corr M. 2008. Wnt-beta-catenin signaling in the pathogenesis of osteoarthritis. Nat Clin Pract Rheumatol 4:550–556.
7. Church VL, Francis-West P. 2002. Wnt signaling during limb development. Int J Dev Biol 46:927–936.
8. Giles RH, van Es JH, Clevers H. 2003. Caught up in a Wnt storm: Wnt signaling in cancer. Biochim Biophys Acta 1653: 1–24.
9. Lee E, Salic A, Krüger R, et al. 2003. The roles of APC and Axin derived from experimental and theoretical analysis of the Wnt pathway. PLoS Biol 1:E10.
10. He TC, Sparks AB, Rago C, et al. 1998. Identification of c-MYC as a target of the APC pathway. Science 281:1509–1512.
11. Blobe GC, Obeid LM, Hannun YA. 1994. Regulation of protein kinase C and role in cancer biology. Cancer Metastasis Rev 13:411–431.
12. Ron D, Kazanietz MG. 1999. New insights into the regulation of protein kinase C and novel phorbol esters. FASEB J 27:1658–1676.
13. Newton AC. 1997. Regulation of protein kinase C. Curr Opin Cell Biol 9:161–167.
14. Nishizuka Y. 1995. Protein kinase C and lipid signaling for sustained cellular responses. FASEB J 9:484–496.
15. Way KJ, Chou E, King GL. 2000. Identification of PKC-isoform specific biological actions using pharmacological approaches. Trends Pharmacol Sci 21:181–187.
16. Garrido JL, Godoy JA, Alvarez A, Bronfman M, Inestrosa NC, et al. 2002. Protein kinase C inhibits amyloid beta peptide neurotoxicity by acting on members of the Wnt pathway. FASEB J 16:1982–1984.
17. Cook D, Fry MJ, Hughes K, et al. 1996. Wingless inactivates glycogen synthase kinase-3 via an intracellular signaling pathway which involves a protein kinase C. EMBO J 15: 4526–4536.
18. Yamada Y, Miyashita T, Savagner P, et al. 1990. Regulation of the collagen II gene in vitro and in transgenic mice. Ann NY Acad Sci 580:81–87.
19. Reinhold MI, Kapadia RM, Liao Z, et al. 2006. The Wnt-inducible transcription factor Twist1 inhibits chondrogenesis. J Biol Chem 281:1381–1388.
20. Wolfe PC, Chang EY, Rivera J, et al. 1996. Fewtrell C: differential effects of the protein kinase C activator phorbol 12-myristate 13-acetate on calcium responses and secretion in adherent and suspended RBL-2H3 mucosal mast cells. J Biol Chem 271:6658–6665.
21. Purmessur D, Freemont AJ, Hoyland JA. 2008. Expression and regulation of neurotrophins in the nondegenerate and degenerate human intervertebral disc. Arthritis Res Ther 10:R99.
22. Clemens MJ, Trayner I, Menaya J. 1992. The role of protein kinase C isoenzymes in the regulation of cell proliferation and differentiation. J Cell Sci 103:881–887.
23. Gwak J, Cho M, Gong SJ, et al. 2006. Protein-kinase-C-mediated beta-catenin phosphorylation negatively regulates the Wnt/beta-catenin pathway. J Cell Sci 15:4702–4709.
24. Liu WS, Heckman CA. 1998. The sevenfold way of PKC regulation. Cell Signal 10:529–542.
25. Choi B, Chun JS, Lee YS, et al. 1995. Expression of protein kinase C isozymes that are required for chondrogenesis of chick limb bud mesenchymal cells. Biochem Biophys Res Commun 216:1034–1040.
26. Chang SH, Oh CD, Yang MS, et al. 1998. Protein kinase C regulates chondrogenesis of mesenchymes via mitogen-activated protein kinase signaling. J Biol Chem 273:19213–19219.
27. Kolch W, Heidecker G, Kochs G, et al. 1993. Protein kinase C alpha activates RAF-1 by direct phosphorylation. Nature 364:249–252.
28. Ueda Y, Hirai S, Osada S, et al. 1996. Protein kinase C activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf. J Biol Chem 271:23512–23519.
29. Blain EJ. 2007. Mechanical regulation of matrix metalloproteinases. Front Biosci. 12:507–527.
30. MacLean JJ, Lee CR, Grad S, et al. 2003. Effects of immobilization and dynamic compression on intervertebral disc cell gene expression in vivo. Spine 28:973–981.

Stem Cell Regeneration of the Intervertebral Disk

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KEYWORDS

• Intervertebral disk • Nucleus pulposus • Cell transplantation
• Stem cell • Regenerative medicine

The intervertebral disk (IVD) functions as an essential load absorber between all vertebrae by allowing bending, flexion, and torsion of the spine. IVD degeneration is a cell-mediated response to progressive structural failure and causes instability of the vertebral motion segments that are responsible for neural compressive manifestations and low back pain.[1] Prolonged segmental instability eventually leads to deformity of the spine and many clinical problems.[2] These manifestations have a high impact on society and the economy, including direct costs for medical treatment and insurance, lost productivity, and disability benefits. These direct and indirect costs are estimated at £12 billion per year in the United Kingdom and $50 billion in the United States.[1,2,3] Therefore, prevention and treatment of IVD degeneration should have significant effects on society and the economy.

CELLULAR MICROENVIRONMENT OF THE INTERVERTEBRAL DISK

The IVD comprises the central nucleus pulposus (NP), the surrounding annulus fibrosus (AF), and the vertebral end plate, which isolates the blood supply from penetrating the largest avascular organ in the human body.[4] Developmentally, the NP originates from the notochord, although in human and other animal species, such as the rat, chondrodystrophoid breed canines, and cattle, there is a marked change in cell and tissue morphology during the early stage of life.[5,6] In human adults, most NP cells resemble chondrocytes of other cartilaginous tissues. These cells are interspersed at low density (approximately 5000 cells/mm^3) and are sometimes arranged in clusters within the matrix.[7] In the surrounding AF, the cells are more typically fibroblastic with a density of approximately 9000 cells/mm^3 and display a fibrous matrix, comprising 15 to 25 lamellae of collagen fibers oriented alternately at approximately 60° to the vertical axis.[8] In-between the lamellae are the elastin and proteoglycan matrix, which reinforces the viscoelastic structure.[9] The vertebral end plate is characterized by a thin layer of chondrocytes and hyaline matrix, which resembles articular cartilage. A capillary network of blood vessels ending here, called the vascular buds, is found within the end plate and supplies approximately 80% of the nutrients needed to support the viability of IVD cells through diffusion. The microenvironment for these cells comprising the IVD is characterized by low oxygen tension and high lactic acid concentration and thus an acidic pH level, compared with the levels in the blood plasma.[10]

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STEM CELL APPLICATIONS IN INTERVERTEBRAL DISK RESEARCH

Decreased number and viability of the IVD cells, especially the NP cells, initiate disk degeneration, and maintaining the homeostasis and restoring the IVD tissue and function are important determinants of the cells' condition. Basic in vitro studies have shown that IVD cells have a low proliferative ability and that most cells in the adult human IVD are in a senescent state.[11] These facts have led researchers to focus on the idea of using stem cells to treat IVD degeneration.

Stem cells are characterized by the ability to self-renew and multipotent capabilities. The application of stem cells and stem cell research techniques in IVD research has been investigated from several directions. Use of new stem cell sources, such as induced pluripotent stem cells or embryonic stem cells, may provide new insight into the field of IVD research.

DEFINING ENDOGENOUS STEM CELL POPULATIONS IN THE ADULT INTERVERTEBRAL DISK

Recent stem cell research has reported the presence of a stem/progenitor cell system as the key to maintaining normal homeostasis and self-renewal in various organs. Decreased number and altered function of stem/progenitor cells cause dysfunction of the composing organ. Activation of the endogenous stem/progenitor cells is one approach for maintaining cellular homeostasis of the IVD. Risbud and colleagues[12] reported that cells isolated from degenerate human tissues express CD105, CD166, CD63, CD49a, CD90, CD73, p75 low-affinity nerve growth factor receptor, and CD133/1, proteins that are characteristic of marrow mesenchymal stem cells (MSCs) and that represent the differentiation ability toward osteogenesis, adipogenesis, and chondrogenesis. A study by Blanco and colleagues[13] compared the differentiation capabilities of MSCs induced from the bone marrow or the NP from the same 16 individuals and found that MSCs similar to bone marrow MSCs are present in the human NP, with the exception that NP MSCs show poor adipogenic differentiation. Feng and colleagues[14] reported that AF cells express several of the cell surface antigens sometimes associated with MSCs, including CD29, CD49e, CD51, CD73, CD90, CD105, CD166, CD184, and Stro-1, and two neuronal stem cell markers, nestin and neuron-specific enolase. Varying the stimulants added to the induction media determined whether AF cells differentiated into adipocytes, osteoblasts, chondrocytes, neurons, or endothelial cells. These research data suggest that stimulation of endogenous stem cell populations may be effective for treating IVD degeneration or for providing cells for the allogeneic transplantation of somatic tissue-specific stem cells.

INDUCTION OF STEM CELLS FROM OTHER ORGANS OF THE BODY

Another scenario involves promoting the mobilization of stem cell populations from the stem cell pool, such as the bone marrow. In cerebral and cardiac infarctions, stem cells are recruited from the stem cell pool and mobilized by agents, such as stem cell growth factor or granulocyte colony-stimulating factor, to restore cells in the injured lesion.[15,16] This kind of system may not be applicable to IVD degeneration because there is no blood supply through which to mobilize the cells; however, there may be different pathways for stem cells to approach the IVD. Detailed research on this problem awaits investigation.

USING STEM CELLS AS FEEDER CELLS TO INTERVERTEBRAL DISK CELLS

Stem cells may serve as feeder cells to stimulate directly other cells in the environment by cell-to-cell contact or indirectly through the secretion of various factors. In a rabbit IVD cell culture, Yamamoto and colleagues[17] showed that direct cell-to-cell contact between NP cells and MSCs occurs across a membrane with 0.45-μm pores, which allowed only the processes to adhere to each other without more extensive contact between the cultured cells. The extent of cell adhesion was assessed by scanning electron microscopy, cell proliferation was evaluated by the WST-8 assay, and the syntheses of DNA and proteoglycans was evaluated by the uptake of ^{3}H and ^{35}S, respectively. The levels of various growth factors and the secretion of cytokines into the culture supernatant were measured using a cytokine protein array. The results were confirmed by electron microscopy and showed that MSCs and NP cells adhered to each other by extending processes across the membrane. The number of cells significantly increased in NP cells cocultured with MSCs and allowed cell-to-cell contact. In addition, the synthesis of DNA and proteoglycans increased significantly in the NP cells cocultured with MSCs when cell-to-cell contact was allowed. The analysis using the cytokine protein array revealed that the secretion of cytokines known to increase the activity of NP cells (transforming growth factor β1 [TGF-β1]), insulinlike growth factor 1, platelet-derived growth factor, and epidermal growth factor) was also significantly

higher in the media collected from NP cells cocultured, allowing cell-to-cell contact. Compared with the conventional NP cell-activation method, the coculture system allowing intercellular adhesion with MSCs led to a marked increase in NP cell proliferation, DNA synthesis, and proteoglycan synthesis. A possible explanation is the increased secretion of various cytokines into the culture medium because of the direct contact with MSCs, which act as feeder cells.

In a preliminary study at the author's laboratory, NP cells activated by coculture that allows intercellular contact (**Fig. 1**) were implanted in an in vivo rabbit model of IVD degeneration.[18] The severity of degeneration was determined over time according to Nishimura's histologic classification. The severity of degeneration was compared between cells treated with the new and conventional methods of activation. The Nishimura grade 24 weeks after transplant was 0 in the normal control group without degeneration induction, 2.8 (the most severe degeneration) in the control group with no treatment, 2.2 in the group receiving NP cells activated by conventional coculture with AF cells, 1.8 in the group receiving NP cells activated by conventional coculture with MSCs, and 1.2 in the group receiving NP cells activated by coculture involving contact with MSCs, the smaller value reflected a significantly less degree of degeneration.

The positive results of this coculture system have been extended to preclinical studies using human cells. Watanabe and colleagues[19] showed that human NP cells obtained from surgery and cocultured with MSCs of the same patient demonstrate up-regulated cellular proliferation and matrix synthesis, as described in animal models.

Strassburg and colleagues[20] demonstrated in the same coculture system using degenerate and nondegenerate NP cells that cellular interactions between MSCs and degenerate NP cells may stimulate both MSC differentiation to an NP-like phenotype and the endogenous NP cell population to regain a nondegenerate phenotype, which consequently increases matrix synthesis for self-repair.

INDUCING STEM CELLS TOWARD THE INTERVERTEBRAL DISK CELL PHENOTYPE

Using the multipotent differentiation capacity of stem cells, the author attempted to induce MSC differentiation in a mixed coculture system with NP or AF cells in alginate beads (**Fig. 2**). IVD tissue was retrieved during surgery for a burst fracture in a 19-year-old man. Under a microscope, the tissue was separated approximately into the NP and inner and outer AF. The separated tissue was digested with 0.02% pronase (Sigma) and 0.0125% collagenase P (Roche) for 8 hours to obtain cells for primary culture. The NP, inner AF, and outer AF cells were cultured and passaged twice and labeled with PKH26 red fluorescent dye (Sigma). Human MSCs were obtained commercially (Cambrex) and genetically labeled with green fluorescent protein (GFP) by infection with a retrovirus vector. The NP, inner AF, or outer AF cells were cocultured with MSCs in alginate beads in a 50:50 ratio at a density of 30,000 cells/bead. The cells were cocultured for 3 weeks in DMEM + 10% fetal bovine serum, and the cells were recovered. The recovered cells were analyzed, and GFP-positive MSCs were separated by flow cytometry (BD FACSVantage). Characterization of the recovered MSCs by flow cytometry showed that, in forward scatter analysis, the size of MSCs changed markedly after the coculture. MSCs cocultured with NP cells showed significantly greater average cell size, whereas cells cocultured with inner or outer AF cells had a smaller average cell size. The internal complexity analyzed by side scatter showed that MSCs cocultured with NP cells became more complex and that MSCs cocultured with inner or

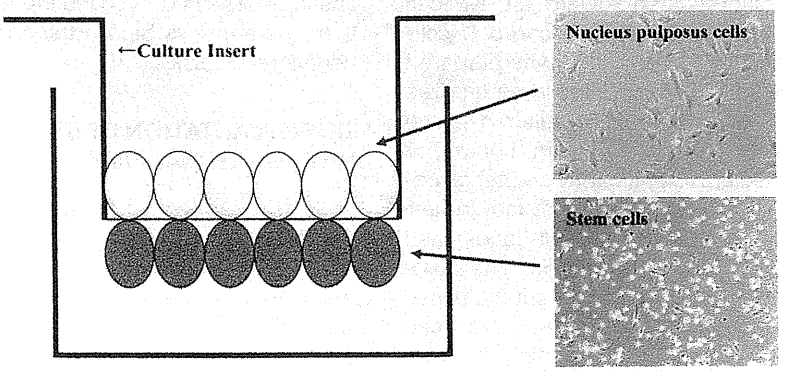

Fig. 1. Use of stem cells as feeder cells for up-regulation of NP cell metabolism. Coculture system allowing cell-to-cell contact.