2-Deoxy-D-glucose regulates dedifferentiation through β-catenin pathway in rabbit articular chondrocytes

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Abstract

2-deoxy-D-glucose (2DG) is known as a synthetic inhibitor of glucose. 2DG regulates various cellular responses including proliferation, apoptosis and differentiation by regulation of glucose metabolism in cancer cells. However, the effects of 2DG in normal cells, including chondrocytes, are not clear yet. We examined the effects of 2DG on dedifferentiation with a focus on the β-catenin pathway in rabbit articular chondrocytes. The rabbit articular chondrocytes were treated with 5 mM 2DG for the indicated time periods or with various concentrations of 2DG for 24 h, and the expression of type II collagen, c-jun and β-catenin was determined by Western blot, RT-PCR, immunofluorescence staining and immunohistochemical staining and reduction of sulfated proteoglycan synthesis detected by Alcain blue staining. Luciferase assay using a TCF (T cell factor)/LEF (lymphoid enhancer factor) reporter construct was used to demonstrate the transcriptional activity of β-catenin. We found that 2DG treatment caused a decrease of type II collagen expression. 2DG induced dedifferentiation was dependent on activation of β-catenin, as the 2DG stimulated accumulation of β-catenin, which is characterized by translocation of β-catenin into the nucleus determined by immunofluorescence staining and luciferase assay. Inhibition of β-catenin degradation by inhibition of glycogen synthase kinase 3-β with lithium chloride (LiCl) or inhibition of proteasome with z-Leu-Leu-Leu-CHO (MG132) accelerated the decrease of type II collagen expression in the chondrocytes. 2DG regulated the post-translational level of β-catenin whereas the transcriptional level of β-catenin was not altered. These results collectively showed that 2DG regulates dedifferentiation via β-catenin pathway in rabbit articular chondrocytes.

Keywords: cartilage, articular; cell dedifferentiation; chondrocytes; collagen type II; deoxyglucose; β-catenin

Introduction

Chondrocytes of articular cartilage develop through differentiation of mesenchymal cells during embryonic development (Barth *et al.*, 1997; Sandell and Adler, 1999; DeLise *et al.*, 2000). The differentiated chondrocytes can then proliferate and undergo hypertrophic maturation. Chondrocytes are composed of a dense extracellular matrix (ECM), such as collagen, fibronectin and sulfated proteoglycan (Eyre, 2002). The phenotype of the differentiated chondrocyte is distinguished by type II collagen expression and synthesis of sulfated proteoglycan including aggrecan. A sufficient number of cartilage-specific matrix molecules were required for the maintenance of homeostasis in normal articular cartilage (Poole, 1999; Sandell and Aigner, 2001). Destruction of cartilage-specific matrix molecules such as type II collagen and proteoglycan leads to arthritis by biochemical changes in chondrocytes (Charni-Ben Tabassi and Garnero, 2007; Rousseau and Delmas, 2007; Dayer *et al.*, 2007; Henrotin *et al.*, 2007). Therefore, synthesis and maintenance of type II collagen and proteoglycan are important for proper function of articular chondrocytes.

Glucose is the main energy source and also known as a precursor of sulfated proteoglycan of chondrocytes (Kim and Conrad, 1976; Sweeney *et al.*, 1993). Destruction of articular cartilage is a hall marker of arthritis (Sandell and Aigner, 2001) and is associated with abnormal glucose metabolism (Dunham *et al.*, 1989, 1992; Nahir *et al.*, 1990). 2-deoxy-D-glucose (2DG) is a synthetic analogue of glucose that is capable of inhibiting glycolysis and glycosylation (Wick *et al.*, 1957; Jain *et al.*, 1985;
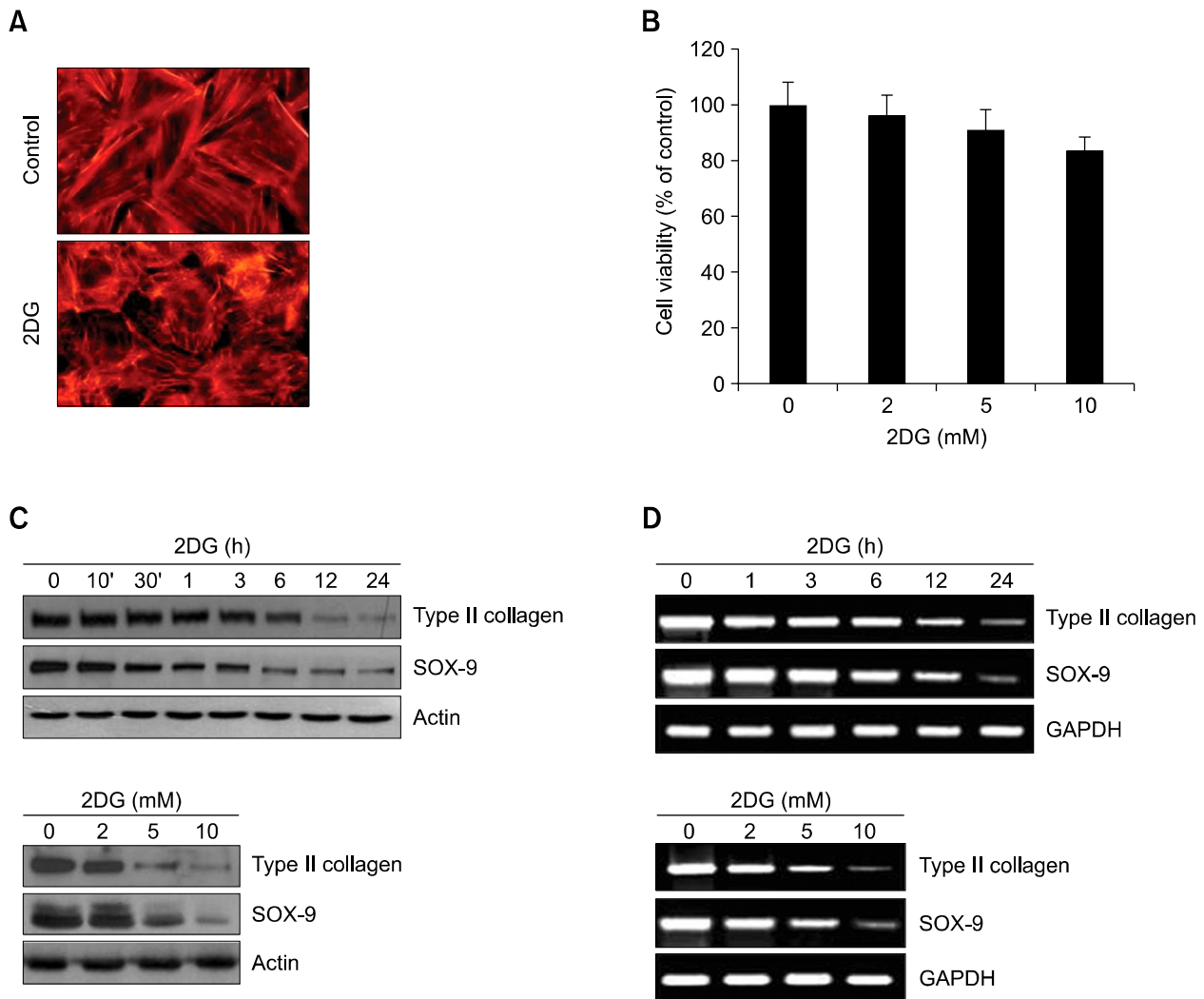
Figure 1. 2DG causes dedifferentiation in rabbit articular chondrocytes. (A) Rabbit articular chondrocytes were untreated or treated with 5 mM 2DG for 24 h. Cells were stained for F-actin with rhodamine-conjugated phalloidin. (B) Cells were untreated or treated with the 5 mM 2DG for the indicated time periods. Cell viability was determined by MTT assay. (C) Rabbit articular chondrocytes were untreated or treated with 5 mM 2DG for the indicated time periods (upper panel) or with the specific concentrations of 2DG for 24 h (lower panel). Expressions of type II collagen and SOX-9 were analyzed by Western blot analysis. Expressions of β-actin were used as loading controls. (D) Articular chondrocytes were untreated or treated with 5 mM 2DG for the indicated time periods (upper panel) or with the specific concentrations of 2DG for 24 h (lower panel). Expressions of type II collagen and SOX-9 were detected by RT-PCR. Expressions of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as loading controls. The data represent a typical experiment, whereby similar results were obtained from four independent experiments.

Kaplan *et al.*, 1990). In comparison with glucose, the 2-hydroxyl group at the second carbon is replaced by a hydrogen group in 2DG, and this change leads to inhibition of glycolysis and glycosylation. 2DG also induces stress in the endoplasmic reticulum (ER) by disturbance of cell homeostasis (Kishi *et al.*, 2010), which causes accumulation of unfolded protein in the ER. Up to now, the effect of 2DG on the cell response was mainly investigated in a variety of cancer cells. In cancer cells, 2DG regulates cell responses such as proliferation (Halicka *et al.*, 1995; Zhang *et al.*,

2006) by inhibiting glycolysis and glycosylation. However, the effects of 2DG on normal cells including chondrocytes are not clear yet.

β-catenin is composed of a 130 amino acid amino-terminal domain, 12 imperfect repeats of 42 amino acids, and a carboxy-terminal domain of amino acids (Willert and Nusse, 1998). The amino terminus of β-catenin is important for stability of β-catenin, and recognized and degradaded by a ubiquitin-proteasome pathway (Munemitsu *et al.*, 1996; Yost *et al.*, 1996; Barth *et al.*, 1997). The β-catenin directly binds to the adenomatous

polyposis coli (APC) protein in association with axin/axil, protein phosphatase 2A (PP2A) and glycogen synthase kinase 3 β (GSK3 β). This complex results in the phosphorylation of ser 29, ser 33, ser 37, thr41 and ser 45 at the N-terminal of β-catenin (Orford *et al.*, 1997).

β-catenin plays a crucial role in cell to cell adhesion through both cadherin and Wnt signaling and is expressed during the chondrogenesis or cartilage destruction. β-catenin plays a pivotal role in the developmental process through regulating the Wnt signaling pathway (Tufan and Tuan, 2001; Tufan *et al.*, 2002). Wnt signaling leads to the inactivation of GSK-β kinase, which leads to the accumulation of β-catenin in the cytoplasm. β-catenin then translocates into the nucleus to increase transcription of target genes, such as those controlled by the T cell factor (TCF)/lymphoid enhancer factor (LEF) promoter. Previous data showed increased β-catenin by treatment with interleukin (IL)-1 β (Goldring *et al.*, 1994; Demoor-Fossard *et al.*, 2001), retinoic acid (RA) (Cash *et al.*, 1997; Weston *et al.*, 2000), or by monolayer cultured (Lefebvre *et al.*, 1990; Yoon *et al.*, 2002) and this led to dedifferentiation in chondrocytes, which could be redifferentiated by a three-dimentional cultured condition. Therefore, β-catenin is believed to be an essential component of the biological processes and maintenance of chondrocytes phenotypes. Previous studies also indicated that a variety of cytokines induce β-catenin, and that β-catenin inhibits differentiation in chondrocytes. However, the relative roles of β-catenin and glucose deprivation have not been determined. In this study, we investigated the effect of β-catenin on 2DG-regulated dedifferentiation of primary rabbit articular chondrocytes.

Results

2DG induces dedifferentiation in rabbit articular chondrocytes

Because glucose serves as a precursor of glycosaminoglycan in chondrocytes, the supply of glucose is important for the formation of cartilage. The differentiated phenotype of chondrocytes is determined by the expression levels of the extracellular matrix, such as glucosaminoglycan and type II collagen. We first investigated the effects of 2DG on the morphology of chondrocytes. Chondrocytes were treated with 5 mM 2DG for 24 h or treated with 5 mM 2DG for the indicated time periods (Figures 1A and 1B). Treatment of 2DG was accompanied by significant changes in the actin cytoskeletal architecture. However, this reor-

ganization of the actin cytoskeleton by 2DG did not induce cytotoxicity at concentrations up to 10 mM in the chondrocytes, as determined by the immunofluorescence staining and MTT assay (Figures 1A and 1B). Next, we tested the effect of 2DG on dedifferentiation of chondrocytes. The primary chondrocytes were treated with 5 mM 2DG for the indicated time periods or treated with specific concentrations of 2DG for the 24 h (Figures 1C and 1D). 2DG significantly decreased the expression of type II collagen (a marker for differentiation of chondrocytes) and SOX-9 (a potent activator of the chondrocyte-specific enhancer of the proalpha 1 (II) collagen gene) in a time- and dose- dependent manner, as determined by Western blot analysis and RT-PCR (Figures 1C and 1D). Consistent with the expression patterns of type II collagen, 2DG reduced production of sulfated proteoglycan in a dose- and time- dependent manner, as determined by immunohistochemical staining (Figure 2A) and Alcian blue staining (Figure 2B). Cells treated with 2DG resulted in a 40% decrease in sulfated proteoglycan production compared to the control cells (Figure 2B). As expected, 2DG treatment of cartilage explants caused a decrease of type II collagen at a dose of from 2 mM to 10 mM, as detected by immunohistochemical staining (Figure 2C) and immunofluorescence staining (Figure 2D), respectively. These results demonstrated that 2DG causes dedifferentiation of articular chondrocytes in both primary cultured cells and cartilage explants.

2DG causes β-catenin accumulation in rabbit articular chondrocytes

Previous data showed that chondrocytes were dedifferentiated by IL-1 β (Goldring *et al.*, 1994; Demoor-Fossard *et al.*, 2001), retinoic acid (RA) (Cash *et al.*, 1997; Weston *et al.*, 2000; Ryu *et al.*, 2002) and specific culture conditions, such as a monolayer culture (Lefebvre *et al.*, 1990; Yoon *et al.*, 2002). On the other hand, β-catenin is up-regulated by IL-1 β, RA, and monolayer cultured cells in both primary cultured cells and explants cartilage, whereas when the dediffered cells re-differentiated by 3-dimensional culture in alginate gel, the levels of β-catenin dramatically decreased (Ryu *et al.*, 2002). Thus, β-catenin is a crucial modulator of the phenotypes of chondrocytes during the differentiation and dedifferentiation of chondocytes.

We next investigated whether changes in β-catenin expression with 2DG-induced dedifferentiation in chondrocytes. The β-catenin expression levels were significantly increased by treatment
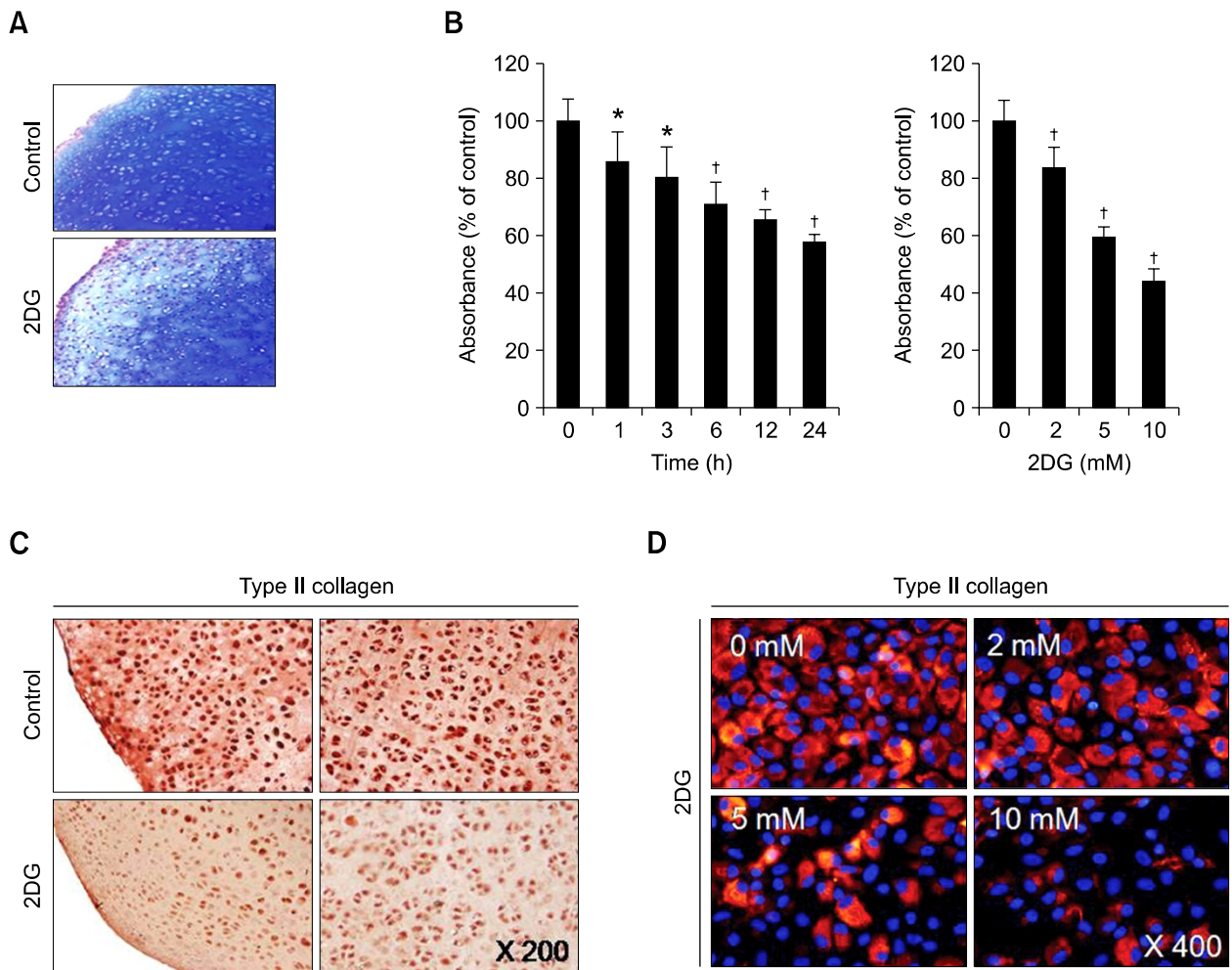
A

B

C

Type II collagen

D

Type II collagen

Figure 2. 2DG reduces sulfated proteoglycan accumulation and type II collagen expression in cartilage explants and chondrocytes. (A) Articular cartilage was untreated or treated with 5 mM 2DG for 24 h. Accumulation of sulfated proteoglycan was determined by Alcian blue staining of cross-sections of cartilage. (B) Rabbit articular chondrocytes were untreated or treated with 5 mM 2DG for the indicated time periods (left panel) or with the specific concentrations of 2DG for 24 h (right panel). (C) Cartilage explants were untreated or treated with 5 mM 2DG for 24 h or with the specific concentrations of 2DG for 24 h. (D) Primary rabbit articular chondrocytes were untreated or treated with 5 mM 2DG for 24 h or with the specific concentrations of 2DG for 24 h. Cells were stained with DAPI. Type II collagen was determined by immunohistochemical staining (C) and immunofluorescence staining (D). Data are presented as results of a typical experiment (A, C, D) and as mean values with standard deviation (B) ($n = 4$). *, $P < 0.05$; †, $P < 0.01$ compared with untreated cells.

with 5 mM 2DG for the indicated time periods as determined by Western blot analysis (Figure 3A). On the other hand, the transcriptional levels of β-catenin in 2DG treated cells did not alter, as indicated by RT-PCR (Figure 3B). As mentioned above, RA treated cells showed increased β-catenin (Figure 3A), so RA was used as a positive control for our experiments. Because inactivation of GSK-3β is required for the activation of β-catenin, we examined the protein levels of phospho-GSK-3β in 2DG treated cells (Figure 3C). These results indicated that 2DG regulates β-catenin expression through the inactivation of

GSK-3 in rabbit articular chondrocytes.

Previous studies showed that most β-catenins are localized in cell-to-cell contacts and RA remarkably increased levels of β-catenin in the nuclear fraction. Therefore, we examined the localization of β-catenin in chondrocytes. As shown in Figure 4, consistent with the treatment of RA, 2DG-treated cells revealed β-catenin distribution mainly distributed in the nucleus, which was demonstrated by immunocytochemical staining (Figure 4A). To establish whether the β-catenin-TCF/LEF complex induces transcriptional activity via translocation of β-catenin, we
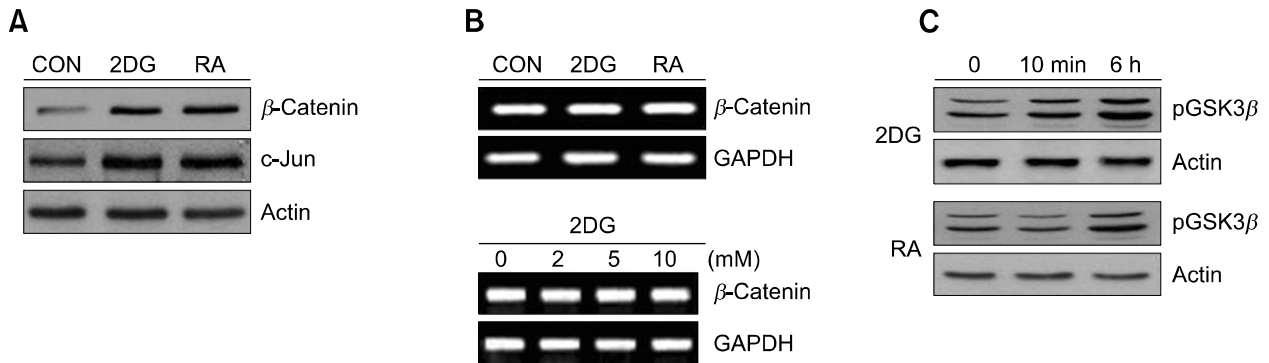
Figure 3. 2DG stimulates β-catenin expression in rabbit articular chondrocytes. (A) Articular chondrocytes were untreated or treated with 5 mM 2DG or with 1 μM RA for 24 h. Expressions of β-catenin, c-jun and actin were determined by Western blot analysis. (B) Primary chondrocytes were untreated or treated with 5 mM 2DG or with 1 μM RA for 24 h or with the specific concentrations of 2DG for 24 h. Expressions of β-catenin and GAPDH were detected by RT-PCR. GAPDH was used as a loading control. (C) Cells were untreated or treated with 5 mM 2DG or with 1 μM RA for the indicated time periods. Expressions of phosphorylated GSK-3β and actin were analyzed by Western blot analysis. (A, C) Expressions of Actin were used as loading controls of blots. The data represent a typical experiment, whereby similar results were obtained from four independent experiments.

next proceeded to examine TCF/LEF reporter gene activity using TOPflash (optimal TCF/LEF-binding site) and FOPflash (mutanted TCF/LEF-binding site) (Figure 4A). This reporter gene assay showed a transcriptional activity of β-catenin-TCF/LEF transcriptional activity. Immunofluorescence staining and luciferase activity assay showed significantly increased levels of β-catenin and TCF/LEF promoter activity in 2DG-treated cells (Figures 4A and 4B).

We hypothesized that β-catenin might play a crucial role in 2DG-induced dedifferentiation in chondrocytes. To confirm this possibility, we examined the expression level of β-catenin in cells treated with MG132. As shown in Figure 5. The β-catenin expression by 2DG caused increase in a dose- and time- dependent manner, as determined by Western blot analysis (Figure 5A). As expected, there was inhibition of β-catenin degradation via inhibition of ubiquitin proteasome with MG132-induced accumulation of β-catenin in chondrocytes (Figure 5B).
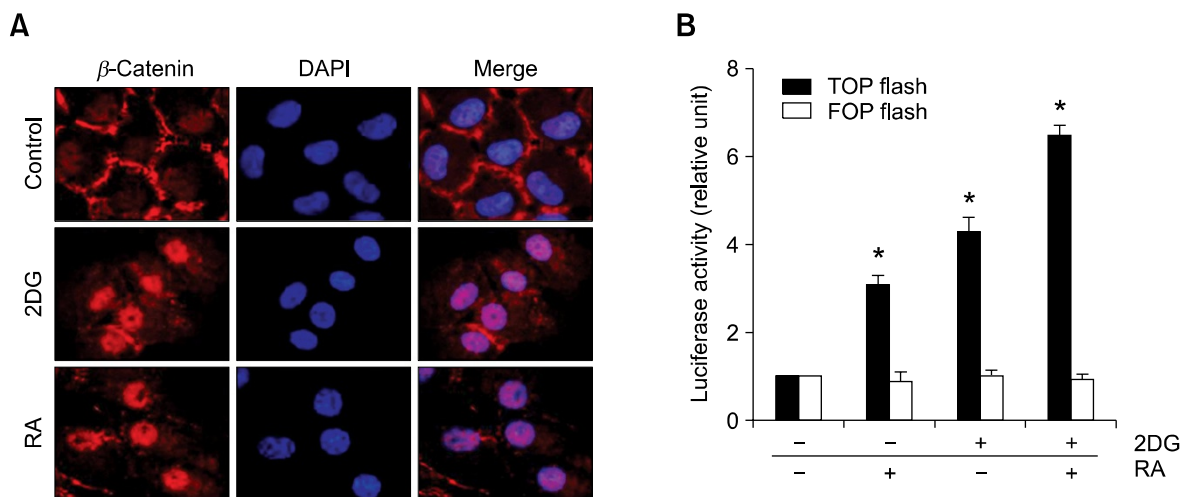

Figure 4. 2DG increases nuclear localization of β-catenin in rabbit articular chondrocytes. (A) Articular chondrocytes were untreated or treated with 5 mM 2DG, 1 μM RA, and 5 mM 2DG in the presence of 1 μM RA for 24 h. Expressions of β-catenin and 4,6-diamidini-2-phenylindole (DAPI) were double stained and analyzed by immunofluorescence microscopy. (B) Chondrocytes were transfected with active (TOPflash) or inactive (FOPFlash) TCF/LEF reporter gene for β-catenin and untreated or treated with 5 mM 2DG or with 1 μM RA or with 5 mM 2DG in the presence of 1 μM RA for 24 h. The TCF/LEF reporter activity was detected using luminometer. Data are presented as results of a typical experiment (A) and as mean values with standard deviation (B) (*n* = 4). *, *P* < 0.01 compared with untreated cells.
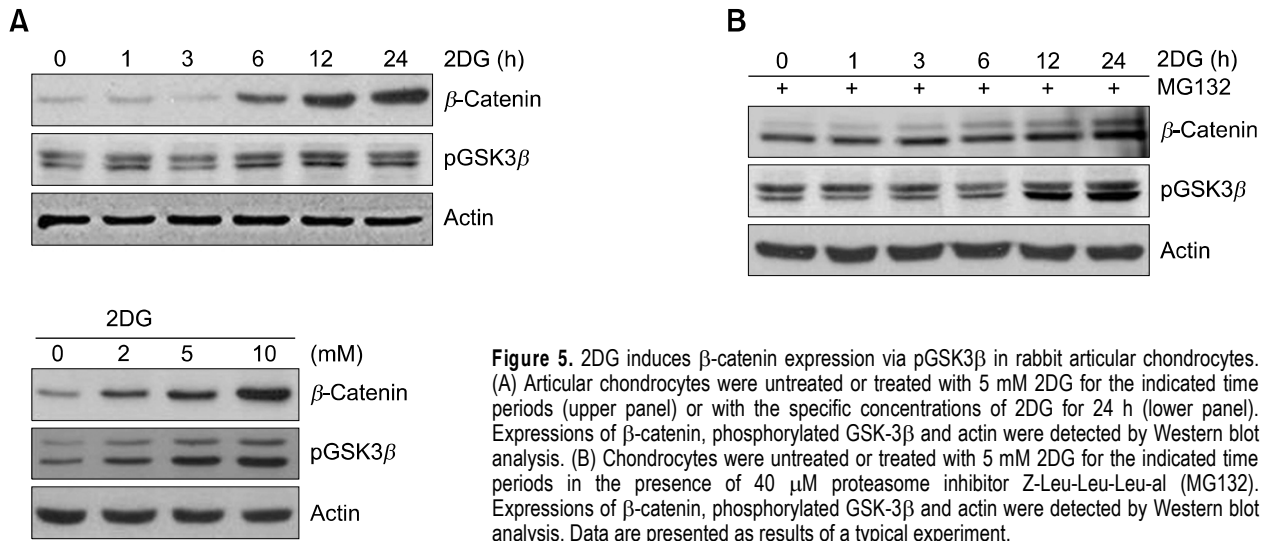
A

B

Figure 5. 2DG induces β-catenin expression via pGSK3β in rabbit articular chondrocytes. (A) Articular chondrocytes were untreated or treated with 5 mM 2DG for the indicated time periods (upper panel) or with the specific concentrations of 2DG for 24 h (lower panel). Expressions of β-catenin, phosphorylated GSK-3β and actin were detected by Western blot analysis. (B) Chondrocytes were untreated or treated with 5 mM 2DG for the indicated time periods in the presence of 40 μM proteasome inhibitor Z-Leu-Leu-Leu-al (MG132). Expressions of β-catenin, phosphorylated GSK-3β and actin were detected by Western blot analysis. Data are presented as results of a typical experiment.

2DG regulates dedifferentiation through β-catenin accumulation in rabbit articular chondrocytes

Next, we investigated the effect of LiCl, inhibitor of GSK-3β, on 2DG-induced dedifferentiation of chondrocytes. As shown in Figure 6A, Western blot analysis revealed that LiCl treatment leads to accumulation of β-catenin and a reduction of type II collagen expression. Phosphorylated c-jun was also markedly increased by LiCl treatment, which is similar to what Ryu *et al.* reported. In addition, MG132, a blocker of β-catenin degradation by the

inhibition of 26S proteasome, also resulted in an increase of β-catenin and c-jun, and a decrease of type II collagen expression (Figure 6A). Treatment of LiCl or MG132 also caused enhanced expression of β-catenin and reduced expression of type II collagen, as determined by luciferase assay (Figure 6B). These results indicate that 2DG causes increased expression of β-catenin. Next we examined whether the accumulation of β-catenin regulates 2DG-induced dedifferentiation by using S37A β-catenin (a stable non-ubiquitinatable form
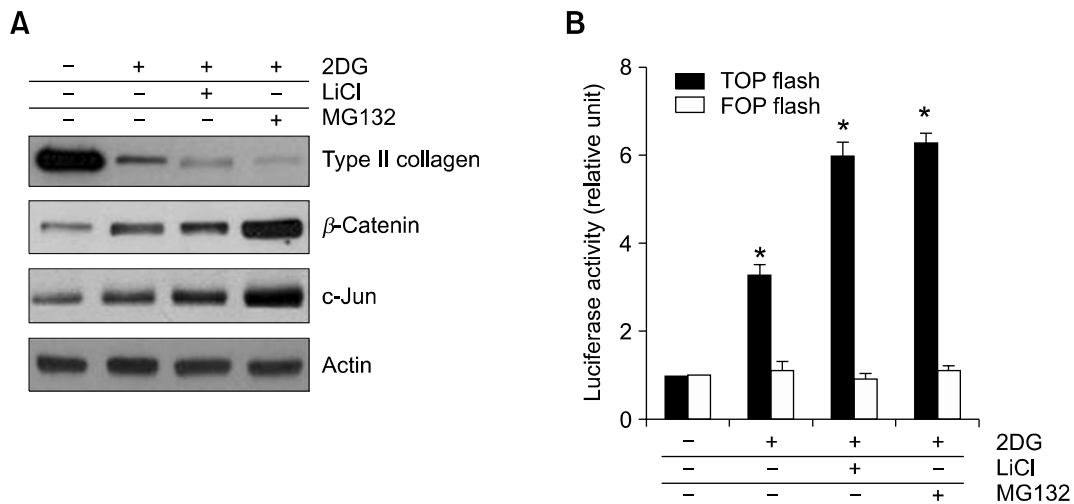
A

B

Figure 6. Accumulation of β-catenin induces dedifferentiation in rabbit articular chondrocytes. (A) Chondrocytes were untreated or treated with 5 mM 2DG for 24 h in the presence of 10 mM lithium chloride (LiCl) or 40 μM proteasomal inhibitor Z-Leu-Leu-Leu-CHO (MG132). Expressions of type II collagen, β-catenin, c-jun and actin were detected by Western blot analysis. Actin was used as a loading control. (B) Chondrocytes were transfected with active (TOPFlash) or inactive (FOPFlash) TCF/LEF reporter gene and then treated with the vehicle alone as a control (con), 5 mM 2DG or 5 mM 2DG with 10 mM LiCl or 40 μM MG132, and TCF/LEF reporter activity was monitored by luminometer. Data are presented as results of a typical experiment (A) and as mean values with standard deviation (B) (n = 4). *, $P < 0.01$ compared with untreated cells.

A

B
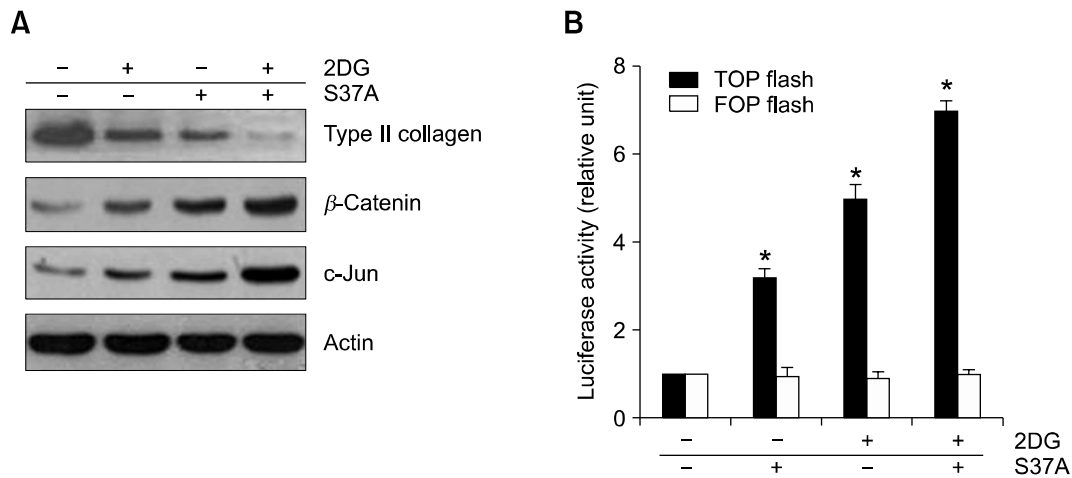
Figure 7. Ectopic expression of S37A β-catenin causes phenotype loss of chondrocytes. (A) Chondrocytes were transfected with empty vector or S37A β-catenin for 6 h, and treated with the vehicle alone as a control (con) or 5 mM 2DG for 24 h. After 24 h incubation, expressions of type II collagen, β-catenin, c-Jun and β-actin were detected by Western blot analysis. Actin was used as loading control. (B) Chondrocytes were transfected with active (TOPFlash) or transfected with inactive (FOPFlash) TCF/LEF reporter gene and S37A β-catenin, and treated with the vehicle alone as a control or with 5 mM 2DG, and TCF/LEF reporter activity was monitored by luminometer. Data are presented as results of a typical experiment (A) and as mean values with standard deviation (B) (n = 4). *, $P < 0.01$ compared with untreated cells.

of β-catenin). Consistent with the results shown in Figure 6, we found that ectopic expression of S37A β-catenin accelerated a reduction of type II collagen of chondrocytes (Figure 7A). Consistent with the expression levels of β-catenin, c-jun, known as a β-catenin target gene, increased as demonstrated by Western blot analysis (Figure 7A). Transfection of S37A β-catenin dramatically increased TCF/LEF activity (Figure 7B). These results revealed that 2DG induced dedifferentiation by translocation of β-catenin into the nucleus.

Dedifferentiation by 2DG occurs via β-catenin accumulation, not by ER-stress

Because our current and previous findings indicated that 2DG caused endoplasmic reticulum stress (ER-stress) response in articular chondrocytes, we next examined the relationship between 2DG-induced dedifferentiation and ER stress. The level of GRP94 (an ER stress inducible gene) was expressed during the up-regulation of β-catenin and down-regulation of type II collagen expression by 2DG. The addition of salubrinal (an inhibitor of ER stress) to the 2DG treated chondrocytes inhibited GRP94 expression. However, expression of β-catenin and type II collagen did not appear to change (Figure 8). These results indicate that 2DG-induced dedifferentiation is regulated by the β-catenin pathway but not by an ER stress response.

Discussion

2DG serves as a competitive inhibitor of glucose. In cancer cells, 2DG regulates cell responses such as proliferation and apoptosis by inhibiting glycolysis and glycosylation (Wick *et al.*, 1957; Jain *et al.*, 1985; Kaplan *et al.*, 1990). Cartilage is a unique tissue, since it functions under normal conditions and uses glucose as the main energy substrate and main precursor for glycosaminoglycan synthesis (Kim and Conrad, 1976; Sweeney *et al.*, 1993; Cho *et al.*, 2003). Despite the importance of an appropriate glucose supply in cartilage homeostasis, the effects and the mechanisms underlying 2DG on various responses, such as differentiation of articular chondrocytes, are still poorly understood. Cartilage and chondrocytes were destroyed under effects of mechanical stress and non-mechanical stress (Kaarniranta *et al.*, 1998; Ragan *et al.*, 2000; Salter *et al.*, 2001; Fermor *et al.*, 2002; Sironen *et al.*, 2002; Oliver et al., 2005). ER stress is involved in various diseases including autoimmune disease such as rheumatoid arthritis and neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Purcell *et al.*, 2003; Forman *et al.*, 2003; Gow and Sharma, 2003; Zhao *et al.*, 2005; Paschen and Mengesdorf, 2005). Oliver *et al* (2005) showed that ER stress by tunicamycin decreased aggrecan but not type II collagen expression in the C-28/I2 chondrocytes cell line. Downregulation of β-catenin expression is a critical
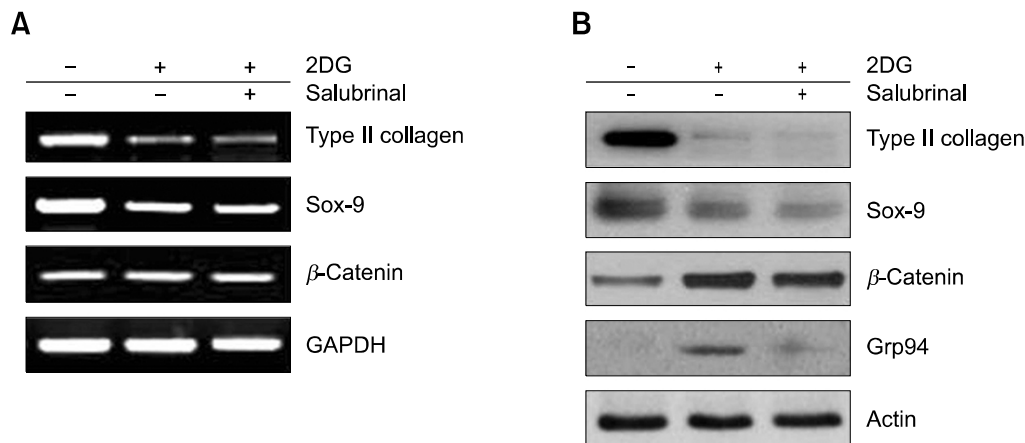
A

−	+	+	2DG
−	−	+	Salubrinal

Type II collagen

Sox-9

β-Catenin

GAPDH

B

−	+	+	2DG
−	−	+	Salubrinal

Type II collagen

Sox-9

β-Catenin

Grp94

Actin

Figure 8. Dedifferentiation by 2DG occurs through β-catenin accumulation in rabbit articular chondrocytes. (A) Chondrocytes were untreated or treated with 5 mM 2DG for 24 h in the presence of 20 μM ER- stress inhibitor salubrinal. Expressions of type II collagen, SOX-9, β-catenin and GAPDH were determined by RT-PCR. GAPDH was used as a loading control. (B) Primary chondrocytes were untreated or treated with 5 mM 2DG for 24 h in the presence of 20 μM salubrinal, ER-stress inhibitor. Expressions of type II collagen, SOX-9, β-catenin, Grp94 and β-actin were determined by Western blot analysis. Data are presented as results of a typical experiment.

key for chondrogenic differentiation from mesenchymal cells and a decrease of β-catenin is necessary for phenotypes maintenance of differentiated chondrocytes (Ryu *et al*., 2002). Yoon *et al*. (2002) suggested that phorbol 12-myristate 13-acetate (PMA) inhibited chondrogenesis via a decrease of β-catenin in chondrifying mesenchimal cells. Also, Blom *et al*. (2009). reported that β-catenin was upregulation in osteiarthritic mice. These previous findings suggested that β-catenin might be regulated in dediferentiated chondrocytes by 2DG. The results of this study show that 2DG causes dedifferentiation through β-catenin in rabbit articular chondrocytes. Ryu *et al*. (2002) reported that stimulators such as IL-1β and RA caused dedifferentiation via β-catenin in rabbit articular chondrocytes. Consistent with the above report, in the present study, β-catenin is up regulated at protein level during dedifferentiation by 2DG but not at the transcriptional level. These results suggest that increased β-catenin may be related to dedifferentiation by 2DG in chondrocytes. Therefore, in this study, we focused on the β-catenin pathway and its regulation mechanism by 2DG. Our findings showed that 2DG-induced dedifferentiation in a time- and dose-dependent manner, as demonstrated by the reduction of sulfated proteoglycan accumulation and type II collagen expression. Previous studies have demonstrated that stimulators such as IL-1β-(Goldring *et al*., 1994; Demoor-Fossard *et al*., 2001) and RA-induced (Cash *et al*., 1997; Weston *et al*., 2000) β-catenin expression in chondrocytes is mediated by decreased activities of GSK3-β. Therefore, we searched whether the phospho-GSK3-β level

increased in 2DG treated cells. Our results showed that β-catenin expression and phospho-GSK3-β expression was upregulated by 2DG synchronously with the downregulation of type II collagen. β-catenin expression by post-translational modification was induced by inhibition of GSK3-β with LiCl or 26S proteasome with MG132. Increased β-catenin significantly downregulated type II collagen and sulfated proteoglycan accumulation. Next, we examined the effect of S37A β-catenin, a stable non-ubiquitinatable form of β-catenin, in chondrocytes. Transfection of S37A β-catenin led to the same results. These results were confirmed by transfecting the TOPflash and FOPflash plasmid, the promoter region which contains binding sites for the β-catenin transcription factor. Then we investigated whether the increased activities of the TCF/LEF promoter resulted from the positive effect of 2DG on β-catenin expression. 2DG also caused endoplasmic reticulum (ER) stress in cells (Harding *et al*., 2002; Zhang and Kaufman, 2004; Schroder and Kaufman, 2005), which suggests that 2DG may induce dedifferentiation as part of the ER stress response of the chondrocytes. Next we examined whether dedifferentiation was regulated by 2DG-induced ER stress. As shown in Figure 8, the expression of grp94, an ER stress marker protein, was increased by 2DG treatment. We also observed that 2DG-induced grp94 expression was blocked with salubrinal, an inhibitor of ER stress, in chondrocytes. However, the expression of type II collagen and SOX-9 did not change by salubrinal treatment. This suggests that dedifferentiation by 2DG is regulated by the β-catenin pathway but not by the ER stress pathway. In summary, our result

collectively indicates that 2DG regulates dedifferentiation via the β-catenin pathway in rabbit articular chondrocytes.

Methods

Culture of primary chondrocytes and experimental conditions

Rabbit articular chondrocytes were isolated from joint cartilage of 2-week-old New Zealand White rabbits by enzymatic digestion as described previously. Briefly, cartilage slices were dissociated enzymatically for 6 h in 0.2% collagenase type II (381 units/ml of solid, Sigma, St.Louis, MO) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen). After collecting individual cells by brief centrifugation, the cells were suspended in DMEM supplemented with 10% (v/v) bovine calf serum, 50 μg/ml streptomycin, and 50 units/ml penicillin and were then plated on culture dishes at a density of 5×10^4 cells/cm^2. The medium was changed every 2 days after seeding. After 3 days in culture, the medium was changed with glucose-free medium and cells were treated with the indicated pharmacological reagents.

Western blot analysis

For Western blot analysis, whole cell lysates were prepared by extracting protein using a buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS supplemented with protease inhibitors and phosphatase inhibitors. Proteins were size-fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. The nitrocellulose sheet was blocked with 5% non-fat dry milk in Tris-buffered saline. The following antibodies were used: anti-type II collagen (santa cruz, CA), anti-SOX-9 (santacruz, CA), anti-β-catenin (santacruz, CA), anti-pGSK3-β (Cell Signaling, MA), anti-c-jun (santacruz, CA), anti-GRP94 (santacruz, CA), and anti- β-actin (santacruz, CA). Blots were developed using a peroxidase-conjugated secondary antibody and a chemiluminescence system.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIZOL (Life Technology, MD) according to the manufacturer's protocol. The following primers and conditions were used for PCR in rabbit articular chondrocytes : for type II collagen (370-bp product), 5'-GACCCCATGCAGTACATGCG-3' (sense) and 5'-AGCCGCCATTGATGGTCTCC-3' (antisnese) with an annealing temperature of 52°C; for SOX-9 (386-bp product), 5'-GCGCGTGCAGCACAAGAAGGACCACCCGGATTAC-AAGTAC-3' (sense) and 5'-CGAAGGTCTCGATGTTGGA-GATGACGTCGCTGCTCAGCTC-3' (antisense) with an annealing temperature of 60°C; for β-catenin (480-bp product), 5'-GAAAATCCAGAGTGGACAATGGCTACT-3' (sense) and 5'-ACCATAACTGCAGCCTTAACC-3' (antisense) with an annealing temperature of 52°C; for glyceraldehydes 3-phosphate dehydrogenase (GAPDH; 299-bp product), 5'-TCACCATCTTCCAGGAGCGA-3' (sense) and 5'-CACA-ATGCCGAAGTGGTCGT-3' (antisense) with an annealing temperature of 50°C.

Alcian blue staining

The cells were fixed with 95% methanol at -20°C for 2 min and stained with 0.1% Alcian blue in 0.1 M HCl overnight. The chondrocytes were washed three times with PBS buffer and 6 M guanidine HCl was added for 6 h. Production of sulfated proteoglycan was measured at 620 nm by Enzyme-Linked Immunosorbent Assay (ELISA).

Immunofluorescence staining

Rabbit chondrocytes were fixed in 3.5% paraformaldehyde in PBS for 15 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS. The fixed cells were washed three times with PBS and incubated with for 2 h with antibodies against type II collagen, β-catenin or β-actin. The cells were washed and incubated with secondary antibodies for 1 h, washed with PBS, and observed under a fluorescence microscope.

Immunohistochemical staining

The cartilage explants (125 mm^3) were fixed in 4% paraformaldehyde in PBS for 24 h at 4°C, dehydrated with graded ethanol, embedded in paraffin, and sectioned into 4 μm slices as described previously. The sections were stained by standard procedures using Alcian blue or antibody against type II collagen, and visualized by developing with a kit purchased from DAKO (Carpinteria, CA).

Transfection and reporter gene assays

Retroviral vector (5 μg) containing cDNA for S37A β-catenin was transfected to chondrocytes using CarriGene reagent (Kinovate Life Sciences, CA), following the procedure recommended by the manufacturer. The transfected cells, which were cultured in complete medium for 24 h, were used in further assay as indicated in each experiment. To investigate β-catenin-TCF/LEF signaling, cells were transiently transfected with 1 μg TCF/LEF reporters, TOPFlash (optimal LEF-binding site) or FOPFlash (mutated LEF-binding site) (Upstate Biotech., Lake Placid, NY), and 1 μg pCMV-β-galactosidase. Following incubation with the indicated pharmacological reagents for 48 h, luciferase activity was measured and normalized for transfection efficiency using β-galactosidase activity.

Determination of cell viability

Chondrocytes were seeded on 96 well plates at 2×10^4 cells/well. After incubation with the specific concentrations of 2DG (Sigma, Steinheim, Germany), cell viablity (ATCC; LCG Promochem, Zurich, Switzerland) was performed according to the manufacturer's instructions.

Data analysis and statistics

The results are represented as the mean SD and compared using one-factor ANOVA for comparing individual treatments with their respective control values.

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