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AG-041R, a cholecystokinin-B/gastrin receptor antagonist, stimulates the repair of osteochondral defect in rabbit model

Toru Nakanishi [a], Kenzo Kawasaki [a], Yuji Uchio [a], Hiroko Kataoka [a], Masaharu Terashima [b], Mitsuo Ochi [a,*]

[a] *Department of Orthopaedics, Shimane Medical University, 89-1 Enya-cho, Izumo, Shimane 693-8501, Japan*
[b] *Department of Biochemistry, Shimane Medical University, 89-1 Enya-cho, Izumo, Shimane 693-8501, Japan*

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Abstract

A newly synthesized compound (AG-041R), 3R-1-(2,2Diethoxyethyl)-((4methylphenyl) amino-carbonyl methyl)-3-((4methylphenyl) ureido-indoline-2-one), is a cholecystokinin-B/gastrin receptor antagonist which has stimulatory effects on the matrix synthesis of chondrocytes in vitro. In this study, we examined the effect of AG-041R on the repair of osteochondral defects (cylindrical, 4 mm diameter) in the patellar groove of the rabbit knee joint. At the time of operation, 100 μl of 1 μM of AG-041R was administered, followed by 200 μl with an osmotic pump for 14 days. Histological and biochemical evaluations were performed at 12 and 24 weeks after surgery. The histological score of the AG-041R-treated group, the quantity of glycosaminoglycan and the ratio of chondroitin sulfate in the AG-041R-treated tissue were significantly higher than in the untreated group. Moreover, the degeneration of cartilage around the defect was suppressed in the AG-041R-treated group. These findings suggest that AG-041R is effective for the repair of osteochondral defects.

Keywords: Cholecystokinin-B/gastrin receptor antagonist; Chondrocyte; Articular cartilage; Repair; (Rabbit); Osteochondral defect

1. Introduction

Since articular cartilage has a limited capability of spontaneous healing, several surgical procedures for repair of osteochondral defects such as osteochondral graft (Hangody et al., 1998; Outerbridge et al., 1995), autologous chondrocyte implantation (Brittberg et al., 1994; Peterson et al., 2000), or transplantation of tissue-engineered cartilage-like tissues (Ochi et al., 1998, 2001a, in press) have recently been introduced. Although good results have been obtained, surgical intervention is a prerequisite to this method.

An ideal and less invasive procedure to repair articular cartilage defects is to inject chondrogenic small molecules into the joint. Growth factors influence a variety of cell activities including proliferation, migration, matrix synthesis and differentiation. Many of these factors, including basic fibroblast growth factor (bFGF), insulin-like growth factors (IGF), bone morphogenic proteins (BMP) and transforming growth factor-β (TGF-β), have been shown to affect chondrocyte metabolism and chondrogenesis (Matsumura et al., 2000; Matsusaki et al., 1998; Shida et al., 1996). Although these agents may affect the formation of cartilage repair tissue of an osteochondral defect, they have been reported to yield undesirable complications such as synovitis or osteophyte formation (Van Beuningen et al., 1994). Therefore, agents which are free of undesirable complications are ideal for the repair of osteochondral defects.

AG-041R, 3R-1-(2, 2 Diethoxyethyl)-((4 methylphenyl) amino-carbonyl methyl)-3-((4 methylphenyl) ureido-indoline-2-one), is a cholecystokinin-B/gastrin receptor antagonist, which was originally developed to treat gastric ulcers (Chiba et al., 1998; Fukui et al., 1998). In a preclinical toxicological study on rats, oral administration of a high dose of AG-041R was found to stimulate systemic cartilage hyperplasia, including the trachea, the intervertebral disk and the articular cartilage. Following this unexpected discovery, daily intra-articular injections of a high dose of AG-041R for 3 weeks into the normal knee joints of rats have induced of cartilage hyperplasia in marginal regions of the femoral condyle without other tissues being affected (Kitamura et al., 2001). In addition, AG-041R was shown to have a

* Corresponding author. Tel.: +81-853-20-2240; fax: +81-853-20-2236.
E-mail address: mitsuo@shimane-med.ac.jp (M. Ochi).

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stimulatory effect on the proliferation and matrix synthesis of chondrocytes in collagen gel culture system in vitro (Ochi et al., 2001b). Therefore, we hypothesized that AG-041R would be a potent therapeutic reagent, providing a minimally invasive method for treating articular cartilage defects. The purpose of this study was to investigate the effects of AG-041R on the repair of osteochondral defects in rabbit articular cartilage.

2. Materials and methods

2.1. Osteochondral defect models

Male adolescent Japanese white rabbits ($n=24$), weighing between 2.5 and 3.0 kg were anesthetized with a mixture of ketamine (40 mg/kg body weight) and xylazine (1.5 mg/kg body weight) intramuscularly. Knees were bilaterally drilled through the medial parapatellar approach. Along the midline of the patellar groove, a hole of 4 mm diameter was punctured using an electric drill with a 4-mm drill-bit. Bleeding was observed from the cancellous bone, indicating that this defect was not a partial-thickness defect but a cylindrical, full-thickness defect (5 mm in depth) extending into the cancellous bone.

2.2. Administration of AG-041R

AG-041R (a gift from Chugai Pharmaceutical, Tokyo, Japan), was dissolved in dimethylsulfoxide (DMSO) to prepare a 100 mM stock solution, which was serially diluted with sterile saline to 1 μM AG-041R for stimulating proliferation of chondrocytes cultured in collagen gel composites (Ochi et al., 2001b). One knee joint space in each animal was injected with 300 μl of 1 μM AG-041R solution (experimental group). Initially, 100 μl of 1 μM AG-041R solution was injected intra-articularly on completion of the drilling after the operation. AG-041R (200 μl) was continuously administered for 2 weeks after the operation using an osmotic pump (Model 2002; Alzet, Palo Alto, CA, USA) connected to silastic medical tubing (0.76 mm ID/1.22 mm OD) (Alzet), where a 20-mm length of the tubing was introduced into the joint cavity through the capsule. The osmotic pump was implanted subcutaneously in the hind region of the legs. The pump had a nominal pumping rate of 0.5 μl/h over a 2-week period. The other knee joint space in each animal was administered with an equivalent volume of the vehicle (without AG-041R) on operation and consistently supplied with an osmotic pump in a manner similar to the experimental group to serve as a control (control group). All animals were allowed to walk freely without any splinting.

2.3. Macroscopic observation

Twelve knees for each group were analyzed at 12 and 24 weeks after operation. The regenerated tissue in the defects was examined macroscopically when the animals were sacrificed by administering an overdose of sodium pentobarbital. The knees were assessed for contractures and adhesions, and the surfaces of the regenerated area were inspected for color, integrity, contour and smoothness. The degree of synovitis and spur formation was also evaluated according to the assessment protocol reported by Grande et al. (1989).

2.4. Histological evaluation

A histological evaluation was performed on six specimens from each group, at 12 and 24 weeks. After macroscopic observation, the distal portion of each femur was removed. The removed femurs were then fixed in 4% paraformaldehyde for 3 days. Each specimen was decalcified in 10% formic acid buffered with citric acid and embedded in paraffin after cutting the defect horizontally in half through its center. Sections (5 μm thickness) were cut in the horizontal plane and stained with safranin O-fast green. The sections were examined microscopically and scored according to the histological grading scale (0–14 points, normal cartilage achieves a total score of 0 points) reported by Wakitani et al. (1994) for regenerated tissue of osteochondral defects and the histological–histochemical grading (0–14 points, normal cartilage achieves a total score of 0 points) reported by Mankin et al. (1971) for scoring degenerative change of the articular cartilage around osteochondral defects.

2.5. Matrix formation

After macroscopic observation, the regenerated tissue in the osteochondral defects of both sides in 12 animals (six animals at 12 weeks and six animals at 24 weeks) was resected. After the dry weight of the regenerative tissue was measured, the quantity of glycosaminoglycan synthesized in regenerative tissue was monitored as described by Shinmei et al. (1992) to estimate matrix formation in the regenerated tissue. Glycosaminoglycan components, chondroitin sulfate and hyaluronic acid were digested by unsaturated disaccharides with chondroitinase ABC and hyaluronidase SD (Seikagaku, Tokyo, Japan), and separated using high performance liquid chromatography (HPLC). The sum of the amounts of chondroitin sulfate and hyaluronic acid per unit dry weight of regenerative tissue was considered as total glycosaminoglycan synthesis, and the ratios of two isomers of chondroitin sulfate, chondroitin-4-sulfate and chondroitin 6-sulfate, were measured to assay the quality of the matrix, as previously reported (Kawasaki et al., 1999; Matsusaki et al., 1998; Uchio et al., 2000).

2.6. Statistical analysis

Results were expressed as the mean values plus or minus one standard deviation (S.D.). The unpaired Student's t test,

where $p < 0.05$ was considered to be significant, was employed to statistically evaluate the Wakitani score, Mankin score, chondroitin sulfate contents, and chondroitin-6-sulfate to chondroitin-4-sulfate ratios with respect to different concentrations of AG-041R. All calculations were done using the StatView Ver. 4.5 statistical software package (Abacus Concepts, Berkeley, CA, USA).

3. Results

3.1. Macroscopic observations

In the experimental group, the defects at 12 and 24 weeks which were covered with white, glossy regenerative tissue, appeared slightly irregular and could be distinguished from the surrounding normal cartilage (Fig. 1A). The surrounding cartilage of the repaired tissue in the defect showed no evidence of degenerative change at 12 and 24 weeks. No reactions occurred on the opposite patellar cartilage, and no sign of synovitis or osteophyte formation was present during the investigation period.

In the control group, the defects were partially covered with white regenerative tissue at 12 weeks. At 24 weeks after operation, the defect portrayed an irregular surface and a fissure was observed (Fig. 1B). Although the defect was easily distinguished, brightness manifesting cartilage around the defect faded at 12 and 24 weeks. Moreover, some degenerative changes of articular cartilage around the defects were observed at 24 weeks.

3.2. Histological evaluation

In the experimental group, the defect was filled with cartilage-like tissue stainable with safranin O-fast green. At 12 weeks, the articular surface of the repair cartilage was smooth, and appeared to be restored up to the original bone–articular junction (Fig. 2A). The cells in the repaired tissue remained round or polygonal in shape, morphologically similar to the articular chondrocytes. At 24 weeks after operation, despite the regenerated tissue showing some slight degenerative changes, such as roughness of the repaired cartilage surface and reduction of safranin O staining (Fig. 2B), there were limited degenerative changes in the articular cartilage around the defect. No evidence of any cartilage structures with osteophyte, chondrophyte formations or synovitis were noted in the joint space.

On the other hand, the regenerative tissues of the defect in the control group were partially covered with remaining partial defects observed at 12 weeks; the extracellular matrix showed reduced staining with safranin O-fast green (Fig. 2C). The cell morphology of repaired tissues was fibroblastic and spindle-shaped, thus lacking the characteristics pertinent to cartilage. At 24 weeks, the defects were covered with fibrous tissue with a rough repaired surface. The articular cartilage around the defect showed degenerative changes such as cluster formation of chondrocytes, less staining with safranin O-fast green, and thin articular cartilage at 12 weeks after operation. These degenerative changes were more pronounced at 24 weeks (Fig. 2D).

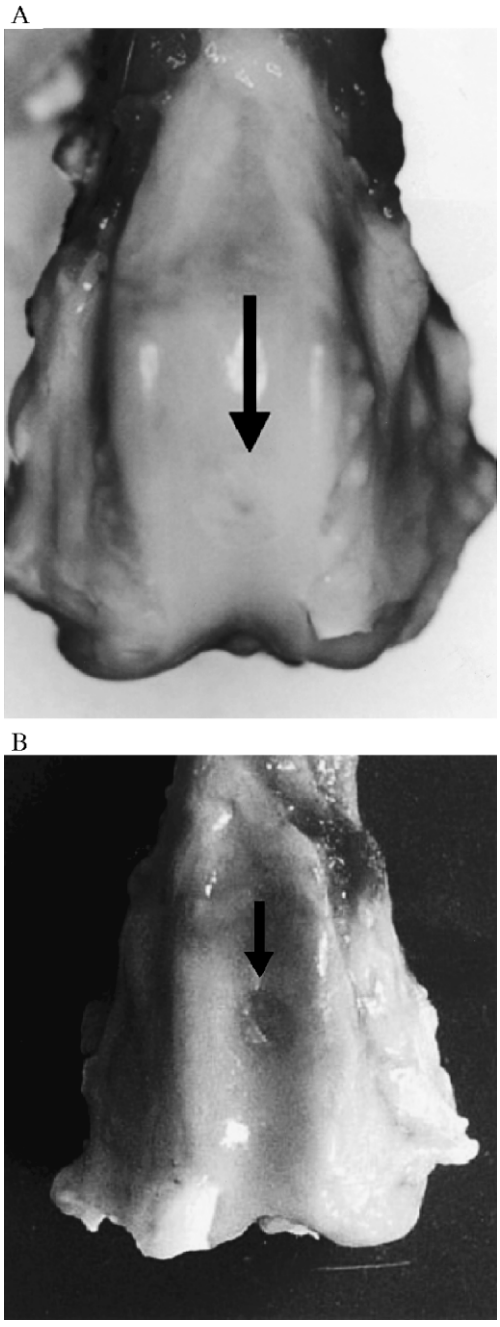

Fig. 1. Gross appearance of the 4 mm diameter osteochondral defects treated with (A) or without (B) AG-041R at 24 weeks after operation. (A) In the AG-041R-treated group, the defect is covered with white, glossy regenerative tissue but is slightly irregular. No evidence of degenerative change and no sign of synovitis or osteophyte formation are observed at 24 weeks. (B) In the control group, the defects are partially covered with repaired tissue. The brightness representing articular cartilage around the defect faded and some irregularity is observed at 24 weeks.

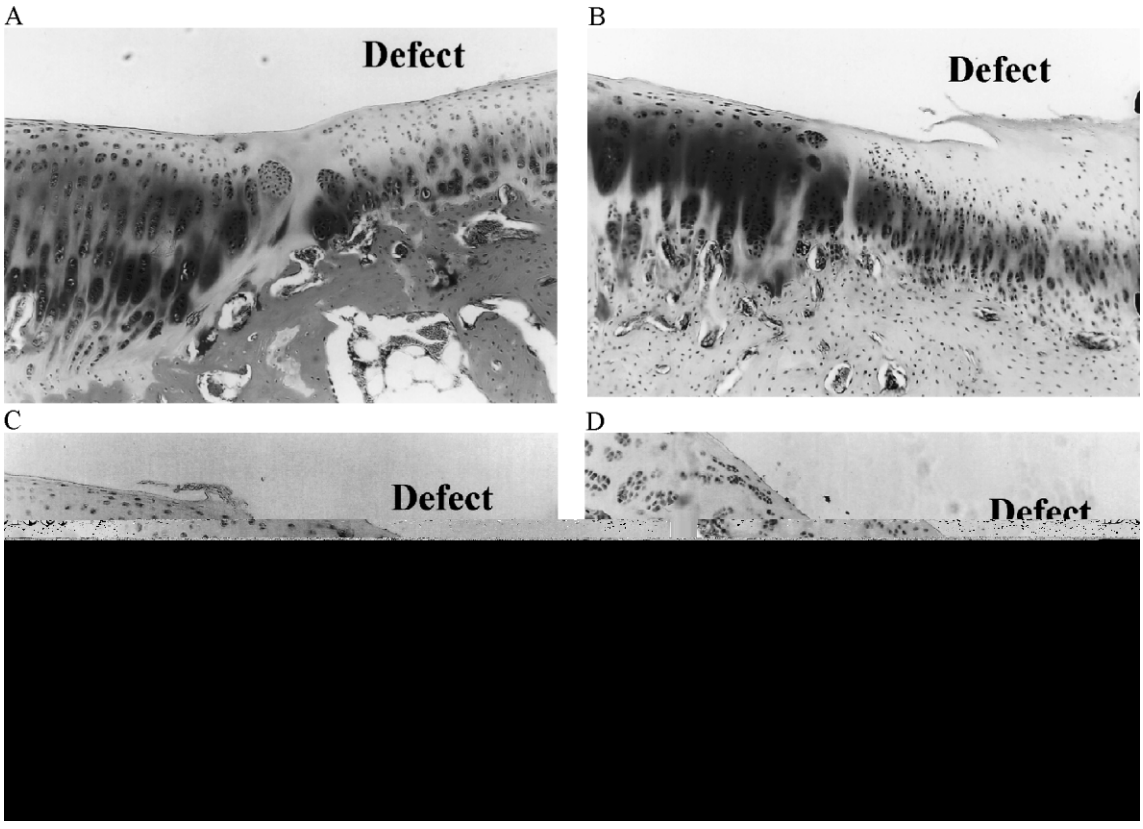

Fig. 2. The histological appearances of the AG-041R-treated (A, B) and untreated (C, D) groups. (A) The defect treated with AG-041R is filled with cartilage-like tissue stained with safranin O-fast green. The articular surface of the repair cartilage is almost smooth at 12 weeks after operation (safranin O-fast green, × 20). (B) The regenerated tissues at 24 weeks after operation show signs of degenerative changes, such as roughness of the repaired cartilage surface with reducted safranin O staining. However, the cartilage around the defect is shown to be intact (safranin O-fast green, × 20). (C) The defect without AG-041R is partially covered with fibrous-like tissue, in which the extracellular matrix is weakly or not all stained with safranin O-fast green at 12 weeks and the surface of the repaired tissue is irregular (safranin O-fast green, × 20). (D) The defects are covered with fibrous tissue with a rough repaired surface, and extracellular matrices are not stained with safranin O-fast green. The articular cartilage around the defect shows degenerative change such as cluster formation of chondrocytes, with reduced staining with safranin O-fast green at 24 weeks (safranin O-fast green, × 20).

3.3. Histological scoring

Table 1 shows the Wakitani score of regenerative tissue and Mankin score of the cartilage around the defect. Compared to the control groups, the mean Wakitani scores of repaired tissue at 12 and 24 weeks were significantly ($P < 0.05$) lower. In addition, compared to the control groups, the mean Mankin scores of the cartilage surrounding the defects in the experimental group at 12 and 24 weeks were significantly ($P < 0.05$) ranked.

Table 1
Histological scoring (six knees per each group at each time)

Weeks	Group	Wakitani score	Mankin score
12	experimental group	4.2 ± 1.5[a]	1.2 ± 0.9[a]
	control group	9.3 ± 2.1	5.2 ± 1.8
24	experimental group	5.3 ± 1.7[a]	2.1 ± 1.8[a]
	control group	10.2 ± 2.4	6.9 ± 3.1

Values are given as the average and plus or minus standard deviation.
[a] $P < 0.05$ vs. control group.

3.4. Matrix formation

The quantity of glycosaminoglycan and compositions of chondroitin sulfate in the regenerative tissue (Table 2) served as an indicator of the extracellular matrix formation. The total amount of glycosaminoglycan in the experimental group was significantly higher than in the control group at

Table 2
Quantity of glycosaminoglycan and composition ratio of chondroitin sulfate isomers in the regenerative tissue (six knees per each group at each time)

Weeks	Group	Total glycosaminoglycan synthesis (nmol/mg dry weight)	Ratios of chondroitin 6-sulfate to chondroitin 4-sulfate
12	experimental	53.3 ± 5.5[a]	1.23 ± 0.15[a]
	control	43.3 ± 6.1	0.93 ± 0.21
24	experimental	48.3 ± 6.2[a]	1.02 ± 0.13[a]
	control	34.3 ± 7.5	0.83 ± 0.14

Values are given as the average and plus or minus standard deviation.
[a] $P < 0.05$ vs. control group.

12 and 24 weeks. To analyze the quality of synthesized chondroitin sulfate, the ratio of the two isomers, chondroitin 6-sulfate and chondroitin 4-sulfate accumulated at 12 and 24 weeks, was calculated. The ratios of chondroitin 6-sulfate so chondroitin 4-sulfate in the experimental group were significantly higher than those of the control group at 12 and 24 weeks.

4. Discussion

In the present report, we clearly showed the stimulatory effects of AG-041R on chondrogenesis in the rabbit knee osteochondral defects histologically and biochemically in vivo. Moreover, administration of AG-041R into joints with osteochondral defects suppressed the degenerative change of the cartilage around the defect without forming chondrophytes, osteophytes or synovitis. There were no reports on other such cholecystokinin-B/gastrin receptor antagonists.

This chondrogenic activity was first discovered in an oral chronic toxicological study in rats during the development of AG-041R as a gastric agent (Kitamura et al., 2001). The previous study has shown that intra-articular injections of a high dose of AG-041R into normal rat knee joints for 3 weeks induces chondrophyte formation in the marginal region of the femoral condyle, where chondorogenic progenitor cells exist (Kitamura et al., 2001). These findings raise the possibility that the target cells of AG-041R in the osteochondral defect model are chondroblasts and chondrocytes within the defective region. The histological study of the origin and differentiation of cells in the repair of osteochondral defects of articular cartilage showed that by 12 days, an increasing number of the mesenchymal cells had differentiated into chondroblasts and many of these were chondrocytes located in lacunae surrounded by a safranin O-stained cartilage matrix (Shapiro et al., 1993). Thus, continuous administration of AG-041R using an osmotic pump for 2 weeks is regarded as long enough for AG-041R to work on these target cells and can promote the repair of osteochondral defects.

In this study, the amount of glycosaminoglycan and the ratios of chondroitin 6-sulfate to chondroitin 4-sulfate in restored tissue in the experimental group were higher than those in the control group. As shown in our previous report (Ochi et al., 2001a,b), AG-041R stimulates the matrix synthesis. In normal articular cartilage, the ratio of chondroitin 6-sulfate to chondroitin 4-sulfate of glycosaminoglycan is high, indicating cartilage maturation (Bayliss et al., 1999). Therefore, AG-041R treatment not only causes an increase in glycosaminoglycan but also improves the quality of repaired tissue.

It is generally recognized that large osteochondral defects stimulate degenerative changes of normal articular cartilage around the defect (Browne and Branch, 2000). When repairing osteochondral defects, preventing the progress of degenerative change of the articular cartilage as well as completely repairing the osteochondral lesion are considered important. Our histological observations at 12 and 24 weeks also suggested that the degenerative changes of articular cartilage around the osteochondral defect were not as obvious as in the control group. Our previous study of AG-041R demonstrated the stimulatory effects of AG-041R on metabolism of chondrocytes in vitro (Ochi et al., 2001b). On this stimulatory effect for chondrocytes, administration of AG-041R into joints with osteochondral defects suppressed degenerative change of the cartilage around the defects. Therefore, AG-041R may affect not only the repair of osteochondral defects but also prevention of degenerative change of normal cartilage around the osteochondral defect in clinical applications.

Since the proliferation of chondrocytes was stimulated by 0.1–1 μM AG-041R and inhibited by 10 μM in vitro in our previous study (Ochi et al., 2001b), we administered 1 μM of AG-041R into the rabbit knee joints. AG-041R of 1 μM functioned only in osteochondral defects to stimulate chondrogenesis without inducing synovitis or osteophyte formation. AG-041R has the physicochemical properties of metabolic stability and lipophilicity. These properties of AG-041R demonstrate its greater suitability for clinical application than cartilage-repairing growth factors, which have a short half-life in vivo besides promoting osteophyte formation (Van Beuningen et al., 1994). As a less invasive approach, intra-articular administration of AG-041R has potential for the treatment of articular cartilage defects.

The mechanism of AG-041R for these chondrogenic effects is still unclear. As AG-041R is a cholecystokinin-B/gastrin receptor antagonist, AG-041R inhibited proliferation and gastrin-induced release of histamine in the epithelial cell by blocking the cholecystokinin-B/gastrin receptor (Ding et al., 1997; Chiba et al., 1998). In contrast, AG-041R stimulated cartilage formation with enhanced proliferation of chondrocytes and matrix synthesis in vitro (Kitamura et al., 2001; Ochi et al., 2001b) and in vivo (present study). Since we obtained the preliminary data that AG-041R significantly increased the TGF-β1 mRNA levels in mouse embryonal carcinoma-derived cell line ATDC5 cells using Northern blot analysis (unpublished data), the mechanism of AG-041R for chondrocytes may be quite different from that for epithelial cells, and the effect of this compound may not be related to its biopotency as a gastrin receptor antagonist. However, the question of whether chondrocytes or chondrocyte progenitor cells possess the cholecystokinin-B/gastrin receptor, or whether other cholecystokinin-B/gastrin receptor antagonists also stimulate the cartilage formation or not, remains unresolved. Further studies are warranted to clarify the mechanism for cartilage formation by AG-041R, and are now being undertaken in my laboratories.

In conclusion, AG-041R stimulated chondrogenesis of osteochondral defects without undesirable complications such as osteophyte formation or synovitis when injected into rabbit knee joints. Therefore, this novel synthetic compound may be useful for promoting the repair of osteochondral defects.

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