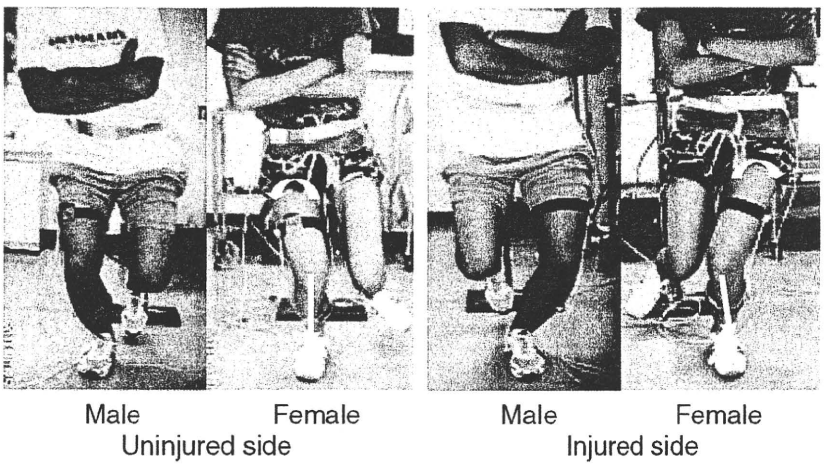

Fig. 4 Comparing gender differences from the coronal view, female ACL-injured subjects demonstrated significantly more external hip rotation and knee valgus in both the injured and uninjured legs than male subjects

participating sports. However, the results were compatible with the study by Zeller et al. [27], which showed that female healthy subjects demonstrated more hip flexion than male healthy subjects despite greater rectus femoris muscle activation during single leg squatting. They discussed how quadriceps muscle could not be seen as the culprit in the lack of knee control and concluded that the activation patterns of the hip musculature were necessary. Thus, the effect of selection bias of the female controls was not clearly demonstrated in the current study.

The uninjured leg of male ACL-injured subjects did not exhibit any significant characteristics except for less external knee rotation compared to that of the male controls. The kinematic differences in the ACL-injured subjects shown in the study indicate the features of the ACL-injured leg.

In comparison with the ACL-injured and -uninjured side, the kinematics of the ACL-injured leg exhibited more knee varus than the uninjured leg in both male and female ACL-injured subjects. These results suggest that an ACL-injured knee could be maintained in balance more easily by maintaining a more varus position. The link between knee valgus loading and resultant increases in ACL strain has been demonstrated previously in vivo as well as in cadaveric studies [32, 33]. Therefore, for ACL-injured subjects, maintaining the knee position with more varus in ACL may have the potential for stabilizing an injured knee by reducing ACL dependency while maintaining the knee's position. This fact suggests that additional varus load stresses the ACL-injured knee.

In comparison with gender differences, the kinematics of female subjects exhibited more external hip rotation and knee valgus than male subjects in both the injured and uninjured legs. During landing, females often display valgus knee positioning. This position is defined as "dynamic valgus" and incorporates the movement of internal hip rotation and adduction [14]. By studying the mechanism of non-contact ACL injuries, it was reported that there is a "position of no return", described as a loss of control at the hip and pelvis [7]. Hip and trunk-pelvis-hip control is assumed to be lost before ACL rupture. ACL rupture reportedly results from internal hip rotation and adduction, knee valgus, and external tibial rotation on a pronated, externally rotated foot. These gender differences in joint kinematics suggest that increased dynamic knee valgus contributes to ACL non-contact injury risk in women and that the hip plays an important role in controlling knee valgus.

With regard to hip adduction, no statistically significant difference was found between female and male subjects, even though females exhibited more knee valgus in comparison with males. This result raises the possibility that the female pelvis is inclined to the squatting leg side in order to adjust for the dysfunction of hip abductors. Therefore, the relative angle of hip adduction corresponding to the pelvis may have exhibited no statistical differences for male subjects.

Single leg squatting is considered to be a simple and safe clinical examination in comparison with single leg landing; therefore, it could be possible to perform it on many subjects at an out-patient clinic. In future studies, collecting kinematics data and comparing data of ACL-injured subjects from various kinds of sports and sports levels will be needed to establish the differences of knee kinematics during single leg half squatting and to develop an ACL-injury prevention method based on the results. The measurement setup can be also indicated to reveal the kinematic change in ACL-reconstructed knees. Such a study would reveal different kinematic characteristics of the ACL-reconstructed knees. Developing an adequate training regiment for each patient based on kinematics data will be important as rehabilitation immediately after ACL

Table 4 Comparison between uninjured and injured knees at angle of unified knee flexion in ACL-injured subjects during single leg squatting

Motion	Mean joint angle (°)					
	Male			Female		
	Uninjured	Injured	*P* values	Uninjured	Injured	*P* values
Knee						
Flexion[a]	63.9 ± 18.3	65.1 ± 17.9		66.5 ± 13.3	66.1 ± 12.2	
Varus	13.6 ± 10.5	20.7 ± 11.1	0.0052[b]	5.0 ± 12.2	7.2 ± 12.5	0.0486[b]
External rotation	12.1 ± 32.0	0.7 ± 36.8	0.0057[b]	21.4 ± 28.9	3.4 ± 34.8	0.1142[n.s.]
Hip						
Flexion	29.9 ± 14.0	35.0 ± 17.1	0.6185[n.s.]	28.1 ± 15.6	32.4 ± 16.8	0.2973[n.s.]
Varus	9.7 ± 7.7	10.4 ± 8.6	0.8681[n.s.]	10.3 ± 7.9	12.6 ± 7.5	0.0984[n.s.]
External rotation	2.7 ± 8.4	1.0 ± 9.3	0.0419[b]	8.5 ± 7.6	7.5 ± 7.6	0.4347[n.s.]

[a] An approximation of the ACL-injured leg's knee flexion angle was extracted from the data of the uninjured leg

[b] Level of significance ($\alpha < 0.05$)

reconstruction. Additional studies are needed to evaluate the relationship between hip morphology, general joint laxity, and other factors in female ACL-injured subjects.

Regarding study limitations, the first limitation of this study is that the knee flexion angle was not able to be controlled in either injured subjects or control subjects. An approximation of the injured leg's knee flexion angle was needed for extracting the knee flexion angle of the uninjured leg for unifying the knee flexion angle. We compared all the joint angles except the knee flexion angle in both the injured and uninjured legs. Comparing the injured and uninjured knees, the exact same results as shown in Table 4 were measured from the data at maximum knee flexion. Therefore, we considered the results to be valid for this study that evaluated hip and knee joint angles at maximum knee flexion while performing the single leg squat. The second limitation is that the physical activity of the female control group is higher than that of the male control group, and the sport item is very limited to the case of playing volleyball. This difference of physical activity and difference of sport item may have affected the results of kinematics of single leg squatting in the female control group.

Conclusion

Comparing the ACL-injured subjects with the healthy controls, female ACL-injured subjects demonstrated significantly more external hip rotation and knee flexion, and less hip flexion than the female controls during single leg half squatting, while male ACL-injured subjects did not exhibit any significant characteristics except for knee rotation.

The ACL-injured leg demonstrated more knee varus than the uninjured leg in both male and female ACL-injured subjects. With regard to gender differences in ACL-injured subjects, female subjects demonstrated significantly more external hip rotation and knee valgus in both the injured and uninjured legs than male subjects.

The single leg half squat was a reproducible way to simplify the single leg landing position, and it was performed repeatedly and safely. Therefore, a large amount of data collection could be performed on many subjects.

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Mesenchymal stem cell-based therapy for cartilage repair: a review

Hideyuki Koga · Lars Engebretsen · Jan E. Brinchmann · Takeshi Muneta · Ichiro Sekiya

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Abstract Articular cartilage injury remains one of the major concerns in orthopaedic surgery. Mesenchymal stem cell (MSC) transplantation has been introduced to avoid some of the side effects and complications of current techniques. The purpose of this paper is to review the literature on MSC-based cell therapy for articular cartilage repair to determine if it can be an alternative treatment for cartilage injury. MSCs retain both high proliferative potential and multipotentiality, including chondrogenic differentiation potential, and a number of successful results in transplantation of MSCs into cartilage defects have been reported in animal studies. However, the use of MSCs for cartilage repair is still at the stage of preclinical and phase I studies, and no comparative clinical studies have been reported. Therefore, it is difficult to make conclusions in human studies. This requires randomized clinical trials to evaluate the effectiveness of MSC-based cell therapy for cartilage repair.

Keywords Mesenchymal stem cells · Cartilage repair · Cell transplantation · Chondrocytes

H. Koga (✉) · L. Engebretsen
Oslo Sports Trauma Research Center,
Norwegian School of Sports Sciences,
PO Box 4014, Ullevaal Stadion, 0806 Oslo, Norway
e-mail: koga-z@rg7.so-net.ne.jp

L. Engebretsen
Orthopaedic Center, Ullevaal University Hospital,
0407 Oslo, Norway

L. Engebretsen
Faculty of Medicine, University of Oslo, 0407 Oslo, Norway

J. E. Brinchmann
Laboratory for Cell Therapy, Institute of Immunology,
Rikshospitalet University Hospital, 0027 Oslo, Norway

T. Muneta
Section of Orthopaedic Surgery, Graduate School,
Tokyo Medical and Dental University, 1-5-45 Yushima,
Bunkyo-ku, Tokyo 113-8519, Japan

I. Sekiya
Section of Cartilage Regeneration, Graduate School,
Tokyo Medical and Dental University, 1-5-45 Yushima,
Bunkyo-ku, Tokyo 113-8519, Japan

Introduction

Cartilage defects have very limited intrinsic healing capacity. Partial thickness defects that do not penetrate the subchondral bone do not usually repair spontaneously [25], while repair of full thickness cartilage defects that penetrate the subchondral bone depends on the circumstances, such as age, defect size and location [17]. Small defects can repair spontaneously with production of hyaline cartilage, whereas larger defects will only repair with production of fibrous tissue or fibrocartilage which are biochemically and biomechanically different from normal hyaline cartilage. As a result, degeneration subsequently occurs which may progress to osteoarthritic change in some cases [91].

Various surgical methods have been proposed to regenerate articular cartilage. However, they all have inherent problems, leaving many patients with inadequately treated cartilage lesions. Recently, mesenchymal stem cells (MSCs) have been suggested as a source of cells for cell-based treatment of cartilage lesions. MSCs are known to play important roles in development, post-natal growth, repair and regeneration of mesenchymal tissues. They are easily isolated, and retain high expansion potential and multipotentiality that includes chondrogenic

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differentiation potential. Based on these properties, MSCs are potentially an attractive cell source for cartilage regenerative medicine. With regard to in vivo studies, transplantation of MSCs into full thickness articular cartilage defects has been attempted under various conditions. Although many successful results have been reported, a number of questions, such as from which tissue MSCs are suitable or what conditions are appropriate for cartilage repair, still exist, limiting clinical applications for cartilage injury. Currently, very few clinical studies of MSC transplantation for cartilage repair have been reported.

This paper briefly describes some of the problems associated with currently used methods for repair of cartilage lesions, followed by a review of the existing literature on MSC-based cell therapy for articular cartilage repair. We focus on three major parts in the process; transplanted cells, scaffolds and growth factors, and their differential performance in vitro and in MSC-based cell therapy in animal studies. Finally we review clinical studies on MSC therapy for cartilage repair, and discuss MSC therapy compared with autologous chondrocyte implantation (ACI), which is an established procedure worldwide [7, 10, 59, 62, 73]. The intention of this paper is to review whether MSC-based cell therapy can be an alternative for treatment of articular cartilage injury.

Current surgical treatment for articular cartilage injury

Bone marrow stimulation

Bone marrow stimulation is a technique in which subchondral bone is penetrated. One of the expectations for the penetration is to induce bone marrow-derived chondroprogenitors into the cartilage lesion. The procedure may also enhance the expression of cytokines to promote cartilage repair [57]. This technique includes drilling [75], abrasion [41] and microfracture [61, 93]. At present, these techniques are preferred by the majority of orthopaedic surgeons as they are easy to perform, need no special surgical instruments and are more cost effective than others [103]. However, cartilage defects only repair with fibrous tissue or fibrocartilage by these methods, probably because the number of chondroprogenitors induced from bone marrow is too small to promote repair with hyaline cartilage, and the results are often followed by degeneration in the repaired tissue [93].

Mosaicplasty

Mosaicplasty is a procedure whereby autologous osteochondral plugs are transplanted into the cartilage defect. By this method, the repaired tissue is predominantly composed of hyaline cartilage [33, 60]. However, donor site morbidity [78] and the limited availability of autologous osteochondral plugs limit the usefulness of this method, particularly for repair of large lesions.

Autologous chondrocyte implantation

The clinical use of ACI was first reported by Brittberg et al. in 1994 [10], following animal studies which had shown its effectiveness [29]. In this method, chondrocytes are obtained from a biopsy taken from a non-weight bearing part of the cartilage of the patients, and are expanded in vitro, followed by the injection of a suspension of expanded chondrocytes into cartilage defects, covered with autologous periosteal flap. Although clinical results of the original ACI were reported as promising [62, 73], this procedure has some potential disadvantages, such as leakage of transplanted cells, invasive surgical method, hypertrophy of periosteum [32, 50] and loss of chondrogenic phenotype of expanded chondrocytes in monolayer culture [8]. Second-generation ACI was introduced to improve these problems, and biomaterials such as collagen type I gel [69], hyaluronan-based scaffold [59] and collagen type I/III membrane [7] were applied to secure cells in the defect area, to restore chondrogenic phenotype by way of three-dimensional culture [28] and to replace the periosteum as defect coverage. At present, only two prospective studies comparing the original and second-generation ACI are available [7, 59] and both studies showed no significant differences in the short term clinical outcomes. As for the first generation ACI, the newly regenerated cartilage often consists of fibrous tissues [35, 96] possibly due to limited number of chondrocytes and their low proliferation potential. Bony overgrowth which causes thinning of the regenerated cartilage and violation of the tidemark is also of concern [1]. Moreover, this method still sacrifices healthy cartilage. These aspects limit ACI in the treatment of large defects and may increase the long-term risk of developing osteoarthritis.

Comparison of treatments currently in use for cartilage repair

Jakobsen et al. [40] evaluated quality and outcome of 69 clinical cartilage repair studies using microfracture, autologous osteochondral transplantation (mosaicplasty), autologous periosteal transplantation or ACI. Data from 3,987 surgical procedures in these studies were assessed. More than half of the studies were retrospective, and only four studies were prospective, randomized and controlled trials [35, 44]. No significant differences in outcome were found between the four techniques, and large variations in the reported outcomes were seen within each treatment

modality. The low-methodological quality found in these studies indicates that caution is required when interpreting results after surgical cartilage repair. It was concluded that firm recommendations on which procedure to choose cannot be provided at this time.

Mesenchymal stem cells

Friedenstein [23] first established the existence of MSCs and showed that bone marrow contains cells that can differentiate into bone and cartilage. His initial work has been extended by a number of investigators and it has been reported that MSCs isolated from bone marrow have self-renewal potential and multilineage differentiation potential including chondrogenesis [42, 74, 76, 81]. MSCs can be isolated from a variety of adult mesenchymal tissues, have extensive proliferation potential and are easily expanded without loss of their multilineage differentiation potential within several passages. Therefore, MSCs are perceived as an attractive cell source for regenerative medicine for cartilage injury.

Chondrogenesis of MSCs

Chondrogenesis of MSCs was first reported by Ashton et al. [5] and a defined medium for in vitro chondrogenesis of MSCs was first described by Johnstone et al. [42], who used micromass culture with transforming growth factor-beta (TGF-β) and dexamethasone. Sekiya et al. [85, 88] reported that addition of bone morphogenetic proteins (BMPs) enhanced chondrogenesis under the conditions employed by Johnstone et al. [42]. Currently, the micromass culture is widely used to evaluate chondrogenic potential of MSCs in vitro.

However, this in vitro chondrogenesis does not mimic cartilage formation during development. During micromass culture, MSCs increase expressions of both collagen type II (chondrocyte marker) and type X (hypertrophic chondrocyte marker) [6, 39]. Furthermore, MSCs continue to express collagen type I [94]. Other cytokines such as insulin-like growth factor (IGF) [71] and parathyroid hormone-related peptide (PTHrP) [43] have been tried for better differentiation cocktails, but it is still difficult to obtain in vitro MSC-based cartilage formation comparative with native cartilage tissue.

When bone marrow-derived mesenchymal cells were subcutaneously transplanted in special diffusion chambers, some cells differentiated into cartilage ectopically [5]. Also, MSCs implanted into osteochondral defects differentiated into chondrocytes [99]. On the other hand, after cartilage pellets differentiated from MSCs in vitro were transplanted subcutaneously, these pellets disappeared [19] or calcified with vascular invasion [72]. This indicates the importance of signals from the microenvironment to induce cartilage formation of MSCs and to maintain its phenotypes [15, 47, 99].

MSCs from various mesenchymal tissues

Although bone marrow is considered to be a well-accepted source of MSCs, stem cells are present in a variety of mesenchymal tissues other than bone marrow and can be isolated from them, such as synovium [18], periosteum [24], skeletal muscle [11], adipose tissue [110], trabecular bone [82] and umbilical cord blood [55]. These MSCs are similar irrespective of their origin in that they have colony-forming ability and in vitro chondrogenic, osteogenenic and adipogenic potentials [20, 30]. Recently, there are increasing number of studies describing the specific properties of MSCs, including chondrogenic potential, dependent on their origin [16, 37, 48, 56, 70, 77, 79, 83, 86, 105, 109].

Some comparative studies showed that MSCs from bone marrow had more in vitro chondrogenic potential than those from adipose tissue [16, 37, 56, 77, 79, 86, 105]. Sakaguchi et al. [83] harvested human bone marrow, synovium, periosteum, muscle and adipose tissue, and isolated and expanded MSCs in a similar condition. They demonstrated that MSCs derived from synovium had higher chondrogenic potential than those from other mesenchymal tissues. Yoshimura et al. [109] also demonstrated higher chondrogenic differentiation potential of MSCs from synovium in rats in a similar way.

One drawback of these studies is that the evaluation of in vitro chondrogenesis may not represent the chondrogenic potential of MSCs transplanted into cartilage defects. Park et al. [70] showed that MSCs from bone marrow and periosteum were superior to cells isolated from fat with respect to forming hyaline cartilaginous tissue when transplanted into cartilage defect in rats. Koga et al. [48] compared in vivo chondrogenic potential among various MSCs in rabbits and demonstrated that MSCs from synovium and bone marrow had a higher potential to repair cartilage defect than those from skeletal muscle and adipose tissue.

Which is a better MSC source for cartilage regeneration, bone marrow or synovium? Nimura et al. [67] reported that MSCs from synovium expanded much faster than those from bone marrow when cultured with autologous human serum. This is an advantage of MSCs from synovium. However, bone marrow is easier to harvest than synovium, which is a reason why bone marrow is more widely accepted as an MSC source.

Suitable conditions for cartilage repair with MSCs

Cell density

In ACI, chondrocyte density used for clinical treatment was 10^6 cells/ml or less when they chondrocytes embedded in a gel [7, 59, 69]. In MSC transplantation, higher density appears to be required. Koga et al. reported that 5×10^7 MSCs/ml embedded in collagen type I gel repaired cartilage defect successfully whereas 10^6 MSCs/ml resulted in failure in rabbit [48]. MSCs divided sparsely during in vitro [38, 87] and in vivo [47] chondrogenesis, whereas viable MSCs were decreased by apoptosis [38].

Growth and differentiation factors

Growth and differentiation factors including members of the TGF-β superfamily, IGF-1 or FGF have been shown to stimulate the chondrogenic differentiation of MSCs. Although some studies have reported that transplantation of undifferentiated MSCs into cartilage defects provided good results [1, 47, 99], these factors such as TGF-β [31, 54], BMPs [66], IGF-1[26] and, particularly, a combination of TGF-β and BMPs [88] have been shown to enhance cartilage repair in combination with MSCs in vitro and in animal studies. Such stimulations could be achieved by direct administration of recombinant growth and differentiation factors or by transfer of the respective genes, and each study showed that cartilage defects transplanted with enhanced MSCs led to better repair than those with untreated MSCs. These results suggest that the use of appropriate differentiation factors could improve cartilage repair by MSC transplantation. Presently, the differentiation factors cannot be ranked according to efficiency. There are some disadvantages with the use of growth factors. Direct administration into the injured site requires high dosages or repeated injections due to relatively short half-lives of these proteins in vivo. This technique is very expensive and may lead to unanticipated adverse effects [3, 98]. Gene therapy has been suggested as an alternative method by which these proteins can be provided for a prolonged period of time directly at the site of cell transplantation [27]. However, a large amount of work still has to be carried out to prove that gene therapy is sufficiently safe to allow clinical use [22]. Even if the growth factors are used only during in vitro differentiation, there are problems regarding availability of good manufacturing practice format, price and the fact that no combination of differentiation factors has been able to turn off collagen type I and collagen type X genes [6, 39, 94]. A better strategy might be to construct scaffolds which are able to deliver differentiation factors directly to the cells embedded in the scaffold. Currently some bioscaffolds exist that can elicit a controlled action and reaction to the surrounding tissue environment (bioactive), and others that exhibit a controlled chemical breakdown and resorption with ultimate replacement by regenerating tissue (resorbable). New generation biomaterials are also being designed to stimulate regeneration of living tissues using tissue engineering and in situ tissue regeneration methods. These materials will lead to limitless possibilities for cartilage regeneration [34, 108]. However, they require further investigations for clinical use.

Culture serum

MSCs have been expanded with foetal bovine serum (FBS) for research as well as for clinical use [36, 99]. However, supplementation with FBS has several risks, such as disease transmission and immune reaction [58, 89, 104]. Increasing the safety of MSC transplantation requires the use of safe and effective substitute for FBS. Autologous human serum has been investigated as a substitute for FBS. Several studies have compared the proliferative effects of autologous human serum and FBS on MSCs from bone marrow with variable results. Some reported that FBS was superior to human serum [52]. Others reported that their proliferative effects were similar [92, 106], and still others demonstrated that MSCs proliferated more in human serum than in FBS [45, 64, 90, 95]. The discrepancies among them may be from serum dose difference, from variations among donor sera, from differences in sample numbers and also from differences in harvested sites [2]. For other MSCs, Nimura et al. [67] reported that MSCs from synovium expanded more in human serum than in FBS through platelet-derived growth factor signalling, while opposite results were obtained with MSCs from bone marrow. As for chondrogenic potential, MSCs from bone marrow [90] and synovium [67] precultured in autologous human serum showed lower in vitro chondrogenic potential than in FBS, whereas in vivo chondrogenic potential of rabbit MSCs from synovium was similar [67].

Human platelet lysate (hPL) has also been reported as a substitute. Some reports showed that MSCs from bone marrow proliferated more in hPL than in FBS and retained their differentiation potentials including chondrogenesis, while there is a disadvantage that the amount of hPL obtained from a patient is less than half that of human serum [12, 13, 21, 53, 84]. Although there is no comparative study between human serum and hPL, they might be an effective and more beneficial substitute for FBS.

Clinical studies on MSC transplantation for cartilage repair

Presently only one prospective clinical study of MSC transplantation for cartilage repair has been published, in

Table 1 Clinical studies on MSC transplantation for cartilage repair

Authors	Year	No. of patients	Results and comments
Wakitani et al.	2002	24	Prospective, randomized study
			Bone marrow MSC versus cell-free scaffold
			No significance in clinical results
			Better arthroscopic and histological score in MSC group
Wakitani et al.	2004	2	Case reports transplanted in patellar defects
			Bone marrow MSC
			All are clinically improved
Wakitani et al.	2007	3	Three case reports involving nine defects in five knees
			Bone marrow MSC
			All are clinically improved
Kuroda et al.	2007	1	A case report
			Bone marrow MSC
			Clinically improved

which bone marrow-derived MSCs were resuspended in a collagen type I gel and transplanted with autologous periosteal flap [100]. In this study, patients with knee osteoarthritis who underwent a high tibial osteotomy were treated with a cell-containing scaffold with a periosteal flap transplanted into a cartilage defect in the medial femoral condyle and compared with patients who were transplanted with cell-free scaffold with a periosteal flap transplanted into a similar lesion. Although the cell-treated group showed no significant improvement clinically compared with the control group, the arthroscopic and histological score was better in MSC-transplanted group. There were also three case reports from the same group which reported that patients' clinical symptoms had improved [51, 101, 102] (Table 1). However, there is still no comparative clinical study with other surgical methods.

Comparison of chondrocyte and MSC transplantation for cartilage repair

As mentioned before, MSC transplantation has some advantages in cartilage repair over ACI. MSCs can be isolated from various tissues without harvesting healthy articular cartilage and are easily expanded without loss of their chondrogenic potential at early passages. Therefore, MSCs are an attractive alternative cell source not only for patients with focal cartilage lesion but also for those with osteoarthritis [65, 68]. However, there are also some disadvantages and risks to use MSCs for cartilage repair. Some reports showed that MSCs-derived chondrocytes expressed hypertrophy-related genes leading to cell death or calcification followed by vascularization when implanted subcutaneously or intramuscularly, whereas articular chondrocytes resisted calcification and vascular invasion [19]. When MSCs were transplanted into cartilage defects, while they could differentiate into chondrocytes according to local microenvironment in articular joints, the thickness of the regenerated cartilage became thinner than the original thickness and the tidemark was violated [1, 47, 99]. Transformation of MSCs is also one of the concerns. It has been reported that MSCs can undergo spontaneous transformation after long-time culture [80, 97], although they can be managed safely during the standard ex vivo expansion period and such transformation is considered to be very rare [9]. Further investigation to solve these problems is required.

A few animal studies have been published comparing ACI and MSC transplantation for cartilage repair. In these studies, no significant differences were observed in histological score between the group transplanted with chondrocytes and with MSCs [1, 107], although repaired tissue with MSCs appeared better in cell arrangement, subchondral bone remodelling, and integration with surrounding cartilage [107].

Less invasive technique for MSC transplantation

Treatment with MSCs (and chondrocytes) requires the transplantation of a cell and scaffold composite. If periosteal coverage is needed, the method is quite invasive as it requires harvesting the periosteum and fixation with suturing to the neighbouring cartilage. Moreover, hypertrophy and ossification are of concern [32, 50, 69]. Without periosteum, a scaffold is needed to keep the cells at the injured site; the scaffolds are derived from animals, thereby increasing the risk of disease transmission and immune reaction [14]. The easiest and the least invasive method might be intra-articular injection; however, with this technique, most of the injected cells adhered to synovial tissues [63], which might increase the risk of adverse effects such as synovial proliferation, and only a small portion of the cells adhered to the cartilage defects [49]. Recently, some

papers reported less invasive methods without scaffolds to adhere transplanted cells effectively [4, 46, 49]. If clinically successful, such methods may extend the indications for MSC-based cell therapy for cartilage repair.

Conclusions

As articular cartilage defects have very limited intrinsic healing capacity, development of new methods for treatment for cartilage defects is of major importance for orthopaedic surgeons. Although various surgical methods have been attempted, including bone marrow stimulation technique, mosaicplasty and ACI, each of them has some disadvantages. MSC-based cell therapy is expected to be an alternative for cartilage repair because MSCs are easily isolated from a variety of mesenchymal tissues, have high proliferative potential and have chondrogenic potential. In animal studies, transplantation of MSCs into cartilage defects has been attempted under different circumstances, and a number of publications exist on cell source, cell density, growth and differentiation factors, culture serum and scaffold. Recently, less invasive techniques for MSC transplantation into cartilage defects have also been developed, and the clinical use of MSCs may well have a bright future. However, the technique is still in the state of preclinical and phase I studies, and there is no comparative clinical study with other surgical methods. Moreover, some concerns still exist about the chondrogenesis and genetic stability of MSCs. Randomized clinical trials are needed to evaluate the effectiveness of MSC-based cell therapy for cartilage repair.

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In Vivo Pharmacokinetics of Ketoprofen after Patch Application in the Mexican Hairless Pig

Masafumi Horie[a], Ichiro Sekiya[b,*], Tomomasa Nakamura[a], Hozumi Tanaka[d], Kotaro Maekawa[e], Masaru Nakanishi[e], Takeshi Muneta[a,c], and Eiji Kobayashi[f]

[a]*Section of Orthopedic Surgery, Tokyo Medical and Dental University, Tokyo, Japan*
[b]*Section of Cartilage Regeneration, Tokyo Medical and Dental University, Tokyo, Japan*
[c]*Global Center of Excellence Program, International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo Medical and Dental University, Tokyo, Japan*
[d]*Center for Experimental Medicine, Jichi Medical University, Tochigi, Japan*
[e]*Fundamental Research Laboratories, Hisamitsu Pharmaceutical Co., Inc., Ibaraki, Japan*
[f]*Division of Organ Replacement Research, Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan*

ABSTRACT: To evaluate the pharmacokinetics of topical drugs, *in vitro* permeation studies are performed using sacrificed pig skin or human tissues resected at surgery; however, these methods have their limitations in *in vivo* pharmacokinetics. This study examined the usefulness of Mexican hairless pigs for *in vivo* pharmacokinetic study, especially the drug concentration in the tissues. A ketoprofen patch was applied on the back of Mexican hairless pigs for 24 h, followed by sequential collection of blood specimens from 0 to 36 h ($n = 3$). Also, the skin, subcutaneous fat, fascia and muscle from the center of the site of application were excised at 12 h after the application ($n = 4$). Ketoprofen was first detected in the plasma at 8 h, the concentration increasing up to 24 h; the plasma concentration began to decrease after the removal of the ketoprofen patch. Ketoprofen concentrations in the tissues decreased with increasing depth of the tissues, but the values in the deep muscles, being the lowest among the tissues examined, were still higher than those in the plasma. While the data of drug concentration in human tissue are difficult to test, the Mexican hairless pig model appears to be attractive for *in vivo* pharmacokinetic studies of topically applied ketoprofen. Copyright © 2009 John Wiley & Sons, Ltd.

Key words: ketoprofen; Mexican hairless pig; patch; pharmacokinetics; topical application

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used widely for pain relief in musculoskeletal disorders [1]. Although oral formulations of NSAIDs are currently popular, they are associated with a high incidence of adverse effects, including stomach irritation, hepatotoxicity and kidney failure [2,3]. In order to minimize the incidence of systemic events related to oral formulations of NSAIDs, topical forms of the drugs have been developed and the ketoprofen patch is one such product [4].

To evaluate the pharmacokinetics of topically applied drugs, *in vitro* permeation studies [5,6] and *in vivo* pharmacokinetic studies have been performed using small animals [7]; however, the results obtained from such studies may not be directly applicable to humans. There have been several reports describing the NSAID concentrations in human tissues after transdermal

*Correspondence to: Section of Cartilage Regeneration, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo, 113-8519 Japan.
E-mail: sekiya.orj@tmd.ac.jp

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application [8–11], but the information from these studies remains limited. Larger animals will be preferable for *in vivo* pharmacokinetic studies for obtaining results applicable to humans.

Miniature pigs are used widely for medical research because of their easy handling and raising [12]. Among miniature pigs, it was considered that hairless pigs might be highly suitable for the evaluation of topical drugs, because the structure of the hairless pig skin is similar to that of human [13]. This study evaluated the *in vivo* pharmacokinetics of ketoprofen applied topically on the back of Mexican hairless pigs. The results demonstrated the usefulness of the Mexican hairless pig for the study of NSAID patches.

Materials and Methods

Animals

To collect sequential blood specimens, three Mexican hairless pigs [14] (National Livestock Breeding Center, Ibaraki, Japan) aged 12 months old and weighing 23.1–38.0 kg were used. To obtain en bloc tissue specimens from the back, four Mexican hairless pigs aged 5–22 months old and weighing 9.8–30.8 kg were used. All Mexican hairless pigs used were bred under specific pathogen-free (SPF) conditions. All the pigs had free access during the study period to food and water in a postoperative care cage which was 40 cm in width, 121 cm in depth, and 109 cm in height. The schemes of the animal experiments had been investigated and permitted by The Judging Committee of Experimental Animal Ethics of Jichi Medical University.

Topical application of ketoprofen

The sparse hairs on the back of the animals were shaved with an electrical clipper and the application area was swept with dry cotton. The ketoprofen patch (10 cm × 7 cm; Hisamitsu Pharmaceutical Co., Inc., Tokyo, Japan) containing 20 mg of ketoprofen was applied on the back of the Mexican hairless pigs. The medial margin of the patch was located at 3 cm to the left of the spinous processes of the thoracic vertebrae.

Plasma preparation

One day before the experiment, a central venous catheter was placed in the right medial cervical vein in all the animals under general anesthesia for blood sampling. Five milliliter blood samples were collected in heparinized syringes at 0, 1, 2, 4, 6, 8, 12 and 24 h, after which the ketoprofen patch was removed; thereafter, blood samples were collected again at 28, 32 and 36 h. The collected blood samples were immediately centrifuged at 3000 rpm for 15 min. Plasma was separated and the plasma samples were cryopreserved at −20°C until analysis.

Tissue sampling

The ketoprofen patches were applied on the backs of the animals for 12 h. Immediately after removal of the patches and wiping off of the drug remaining on the skin surface with wet cotton, the skin, subcutaneous fat, fascia and muscle at the center of the patch application site were excised en bloc (2 cm × 2 cm × 3 cm) under anesthesia induced by intramuscular injection of 10 mg/kg of ketamine, 2 mg/kg of xylazine and 0.02 mg/kg of atropine. The specimens were then divided into five sections; skin, subcutaneous fat, fascia, superficial muscle up to 5 mm thickness, and the remaining deeper muscle. All specimens were cryopreserved at −20°C until the analysis.

Ketoprofen concentration analysis

Fifty milligrams of tissues were homogenized in methanol. Ketoprofen in the homogenates and plasma (0.25 ml) was acidified and extracted by liquid–liquid extraction with diethyl ether. After evaporation of the organic phase, the residue was dissolved in a methanol/water mixture and transferred into vials. Ketoprofen extracted from the plasma and tissues was assayed by high-performance liquid chromatography with positive ion spray ionization tandem mass spectrometry detection (LC-MS-MS; 2695 separation module (Waters) and API-4000 (Applied Biosystems/MDS SCIEX).

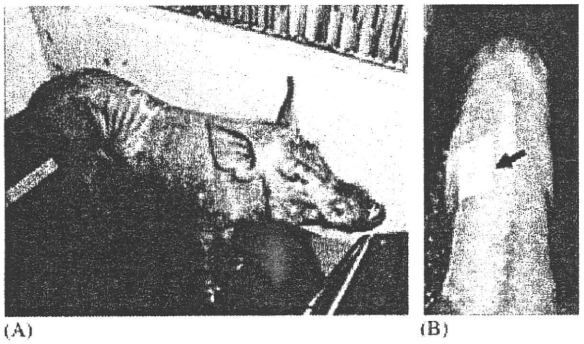
Results and Discussion

Mexican hairless pigs are descendants of Iberian pigs. The name 'hairless' derives from its main

characteristic, namely, the absence of hair (or sparse hair) on the skin (Figure 1A).

A topical patch containing 20 mg of ketoprofen was applied to the back of Mexican hairless pigs for 24 h (Figure 1B). Blood was collected at 0, 1, 2, 4, 6, 8, 12, 24 (prior to patch removal) 28, 32 and 36 h after the patch application. Ketoprofen was first detected in the plasma at 8 h, and the concentration increased steadily up to 24 h (Figure 2). After removal of the ketoprofen patch, the plasma concentration decreased, but the drug could still be detected until the 36 h time-point.

Next, the ketoprofen concentrations in the tissue specimens were measured. Ketoprofen patches were applied on the back of Mexican hairless pigs for 12 h. Immediately after removal of the patch, the skin, subcutaneous fat, fascia and muscle at the center of the patch application site (Figure 3A) were excised en bloc (2 cm × 2 cm × 3 cm). The muscle was divided into superficial muscle up to 5 mm thickness and the remaining deep muscle. The highest ketoprofen concentration was obtained in the skin, followed by that in the subcutaneous fat, fascia, superficial muscle and deep muscle, in that order (Figure 3B). Interestingly, ketoprofen concentrations in the tissues decreased with increasing depth of the tissues, even though the composition of the tissues, tissue permeability to ketoprofen and the vascularity in the layers are totally different. The ketoprofen concentration in the deep muscle was the lowest among the tissues examined, but it was still higher than that in the plasma. There was no marked difference in ketoprofen concentrations in the



(A) (B)

Figure 1. Mexican hairless pig. (A) Typical appearance of a 12-month-old Mexican hairless pig. (B) Ketoprofen patch (arrow) applied on the back of the Mexican hairless pig

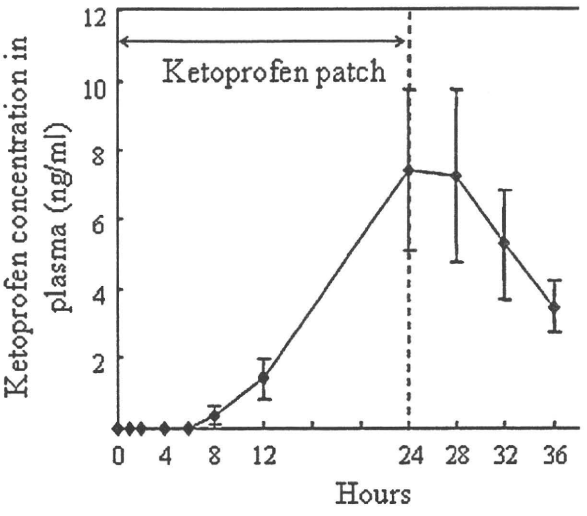


Figure 2. Ketoprofen concentrations in the plasma. Average values with SEM are shown ($n = 3$)

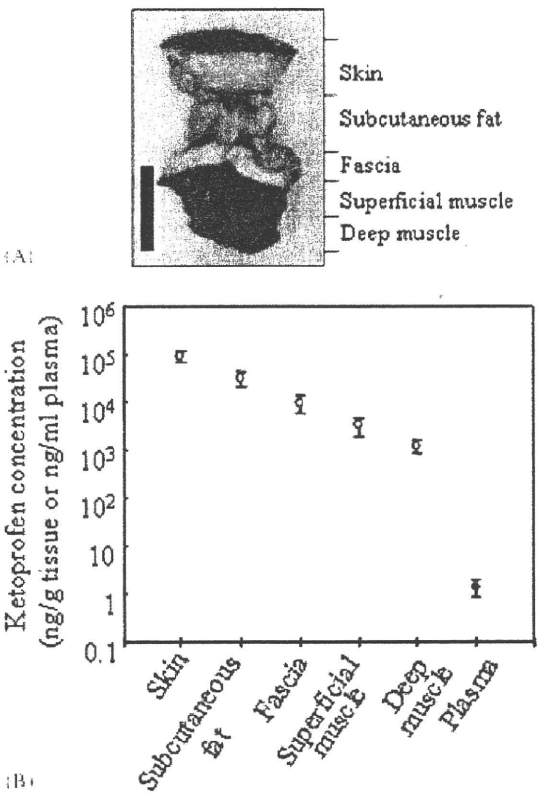


Figure 3. Ketoprofen concentration in the tissues. (A) Tissues from the center of the patch application site were excised en bloc and examined. Scale bar: 10 mm. (B) Ketoprofen concentration in each tissue at 12 h after the ketoprofen patch application. Average values with SEM are shown ($n = 4$)

tissues and plasma between small and big animals in this study.

In our previous research conducted in humans, the ketoprofen concentrations in the plasma reached their maximum at 13 h and decreased thereafter when a 20 mg ketoprofen patch was applied on the back of humans for 24 h (data not shown). The pharmacokinetics of ketoprofen in the plasma was thus different in between humans and Mexican hairless pigs. One of the possible explanations is that the difference in the thickness of the stratum corneum, which represents a rate-limiting factor for percutaneous absorption. Since the stratum corneum in Mexican hairless pigs is thicker than that in humans, the percutaneous absorption rate in the Mexican hairless pig could be expected to be slower.

At 12 h after the application, even though the ketoprofen concentrations in the plasma did not reach their peak levels, the ketoprofen concentrations in the tissues under the patch application site decreased with increasing depth of the tissues. Furthermore, the concentrations in the deep muscles were still higher than the plasma concentrations at this time-point. These findings indicate that ketoprofen may be directly delivered to the deep tissues without passing through the blood stream.

At present, in order to evaluate the pharmacokinetics of topically applied drugs, *in vitro* permeation studies are often performed using the skin from the sacrificed mouse, rat [7] or pig [5,15]. Our study has the advantage of being able to obtain the living animal data that include the effect of blood flow.

The percutaneous absorption of ketoprofen from topical application is known to be influenced by differences in skin structure at various regions of the body [16]. In a clinical situation, ketoprofen patches are applied not only to the back, but also to varied positions of the entire body in humans. In this study, the ketoprofen patches were applied only to the back of the pigs. When investigating the abdominal site or leg of large four-footed animals previously, some failures were experienced. For example, the patches peeled off or slipped from the abdominal site from kicks of the hind legs, and the abdominal sites became dirty from the feces or urine. In order to avoid these technical failures, only the back skin of large four-footed animals was investigated.

It is difficult to obtain data of drug concentrations in tissues from non-human primates. The FDA recommends having a pre-clinical model in pigs (personal communications). Based on the results of our study, the Mexican hairless pig may serve as a suitable model for *in vivo* pharmacokinetic analysis of topically applied drugs, especially ketoprofen, in solid tissues, which is difficult to obtain in humans.

Implications

This study evaluated the *in vivo* pharmacokinetics of ketoprofen applied topically in Mexican hairless pigs. Peak plasma levels were observed 24 h after the ketoprofen patch application on the back and the ketoprofen concentrations in the tissues decreased with increasing depth of the tissues. Mexican hairless pigs are attractive models for the pharmacokinetic study of topically applied ketoprofen.

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Methylation Status of CpG Islands in the Promoter Regions of Signature Genes During Chondrogenesis of Human Synovium–Derived Mesenchymal Stem Cells

Yoichi Ezura, Ichiro Sekiya, Hideyuki Koga, Takeshi Muneta, and Masaki Noda

Objective. **Human synovium–derived mesenchymal stem cells (MSCs) can efficiently differentiate into mature chondrocytes. It has been suggested that DNA methylation is one mechanism that regulates human chondrogenesis; however, the methylation status of genes related to chondrogenic differentiation is not known. The purpose of this study was to investigate the CpG methylation status in human synovium–derived MSCs during experimental chondrogenesis, with a view toward potential therapeutic use in osteoarthritis.**

Methods. **Human synovium–derived MSCs were subjected to chondrogenic pellet culture for 3 weeks. The methylation status of 12 regions in the promoters of 10 candidate genes (*SOX9*, *RUNX2*, *CHM1*, *FGFR3*, *CHAD*, *MATN4*, *SOX4*, *GREM1*, *GPR39*, and *SDF1*) was analyzed by bisulfite sequencing before and after differentiation. The expression levels of these genes were analyzed by real-time reverse transcription–polymerase chain reaction. Methylation status was also examined in human articular cartilage.**

Results. **Bisulfite sequencing analysis indicated that 10 of the 11 CpG-rich regions analyzed were hypomethylated in human progenitor cells before and after 3 weeks of pellet culture, regardless of the expression levels of the genes. The methylation status was consistently low in *SOX9*, *RUNX2*, *CHM1*, *CHAD*, and *FGFR3* following an increase in expression upon differentiation and was low in *GREM1* and *GPR39* following a decrease in expression upon chondrogenesis. One exceptional instance of a differentially methylated CpG-rich region was in a 1-kb upstream sequence of *SDF1*, the expression of which decreased upon differentiation. Paradoxically, the hypermethylation status of this region was reduced after 3 weeks of pellet culture.**

Conclusion. **The DNA methylation levels of CpG-rich promoters of genes related to chondrocyte phenotypes are largely kept low during chondrogenesis in human synovium–derived MSCs.**

The mechanisms underlying human articular chondrogenesis are largely unknown. Chondrogenesis per se is a complex multistep process. In humans, this mainly occurs in the developing skeleton and during the healing of fractures. It begins with recruitment, proliferation, and condensation of mesenchymal progenitor cells at predetermined embryonic sites, which leads to the formation of a precartilaginous primordium (1). Commitment of primordial progenitors to partially differentiated chondrocytes proceeds to serial proliferation/differentiation of the early chondrocytes, resulting in organization of typical columnar structures with layers of differentiating chondrocytes (1). Importantly, these processes are conducted entirely by multiple cellular interactions and are believed to be programmed in genomic sequences at various rates. However, epigenetic regulation may also be involved, since epigenetic control of gene expression appears to be an important aspect of general embryonic development as well as the differentiation processes of somatic cells (2–5).

Genomic DNA methylation, modification of nucleosome histone tails, and chromosome remodeling are essential contributors to the mechanisms of epigenetic control (2,6). Among these mechanisms, genomic DNA methylation is the most fundamental process. It occurs

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Yoichi Ezura, MD, PhD, Ichiro Sekiya, MD, PhD, Hideyuki Koga, MD, PhD, Takeshi Muneta, MD, PhD, Masaki Noda, MD, PhD: Tokyo Medical and Dental University, Tokyo, Japan.

Address correspondence and reprint requests to Yoichi Ezura, MD, PhD, Department of Molecular Pharmacology, Tokyo Medical and Dental University, 3-10 Kanda-Surugadai 2-Chome, Chiyoda-ku, Tokyo 101-0062, Japan. E-mail: ezura.mph@mri.tmd.ac.jp.

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as a heritable modification during cellular replication and lineage differentiation, and it is essential for early embryonic development. DNA methylation consists of the addition of a methyl group to the 5′ cytosine in a CpG dinucleotide, which favors genomic integrity and ensures proper regulation of gene expression, largely contributing to gene silencing (3). The important roles of DNA methylation in X chromosome inactivation, genomic imprinting, as well as early embryonic development have been clearly delineated (4,5). Indeed, a recent report of a comprehensive analysis of embryonic stem cells subjected to neurogenesis indicated that CpG methylation at a specific locus may have an important function in regulating the lineage commitment of progenitor cells (7,8). Its potential roles during late-stage development, however, have been examined in only a limited number of investigations concerning some specialized types of cells (3,7–11).

Articular chondrocyte maturation, or aging, has been one topic of interest with regard to regulation through DNA methylation. However, only a few in vitro studies have indicated some possible involvement of DNA methylation in the maturation of cultured primary chondrocytes (12) and growth plates (13) or in the progression of degenerative joint diseases (14,15). Thus, it has not been determined whether epigenetic control is indeed involved in the in vivo process of chondrogenesis during normal development, and in particular, no data from human studies have been reported.

Chondrogenic processes have been investigated in vivo by means of 2 well-known methods of culturing mesenchymal cells: so-called "chondrogenic pellet culture" (16,17) and "micromass culture" (18,19). Both methods efficiently produce cartilaginous structures within 2–3 weeks from undifferentiated mesenchymal cells isolated from embryonic limb buds or wing buds (16–18). The established murine cell line C3H10T1/2 has also been used in these analyses (19). More recently, the chondrogenic pellet culture method has frequently been used in analyses of pluripotent mesenchymal progenitor cells from adult human tissues, which are also referred to as mesenchymal stem cells (MSCs) (20,21). Our previous studies of the therapeutic use of MSCs in degenerative articular joint diseases showed that among the various MSC types we examined, human synovium–derived MSCs appeared to be most potent for chondrogenesis (22). Since these differences were detectable even in analyses of cells harboring the same genetic makeup, the results indicate that differences other than genomic sequences contribute to the divergent differentiating potentials for cellular lineage specifications.

In the present study, we examined whether epigenetic controls would be diverse among different sets of MSCs. To examine the question of whether promoter sequences of genes that are critical in lineage specification differ in their epigenetic status, we investigated the DNA methylation status of 10 candidate genes in chondrogenic pellet cultures of human synovium–derived MSCs. Candidate genes included both chondrogenic and nonchondrogenic genes. This study is the first to perform bisulfite sequencing analyses of DNA methylation (23) in freshly isolated human MSCs and in differentiated populations of MSCs cultured by in vitro chondrogenic assay.

MATERIALS AND METHODS

Tissue sampling and isolation of human mesenchymal cells. Knee joint synovial tissues were obtained from 3 volunteer donors undergoing therapeutic orthopedic surgery at the University Hospital of Tokyo Medical and Dental University. Informed consent was obtained from each patient, and the study was approved by the Institutional Review Board. Knee joint articular cartilage samples were obtained from an additional patient with osteoarthritis (OA), who gave informed consent. All of the patients were undergoing knee surgery for OA or trauma, and DNA methylation status was examined in all 4 specimens.

Human synovium–derived MSCs were obtained as described previously (22). Briefly, synovial tissues were minced, digested with collagenase, separated with a nylon filter, and the cells were plated at a clonal density in complete α-minimum essential medium containing 10% fetal bovine serum and antibiotics. After 14 days of expansion, cells were replated at a density of 50 cells/cm^2 (passage 1). Half of the cells were harvested for DNA isolation at this point, and the remainder were trypsinized for use in the chondrogenesis assay. We used 3 lines of human synovium–derived MSCs from 3 individual patients in the present study.

In vitro chondrogenesis assay. Chondrogenic pellet culture was performed according to the protocol described elsewhere (22). Briefly, a suspension of 8×10^5 human synovium–derived MSCs was placed into a 15-ml conical-bottom BD-Falcon polypropylene test tube (BD Biosciences, San Jose, CA) and centrifuged at 1,500 revolutions per minute for 10 minutes. The culture medium was replaced with "chondrogenesis medium," which consisted of 500 ng/ml of recombinant human bone morphogenetic protein 2 (Asteras Pharmaceutical, Tokyo, Japan), 10 ng/ml of transforming growth factor β3 (R&D Systems, Minneapolis, MN), $10^{-7}M$ dexamethasone (Sigma-Aldrich Japan, Tokyo, Japan), and 50 mg/ml of ITS+ (insulin–transferrin–selenium) Premix (BD Biosciences), and the pellets were cultured at 37°C in a CO_2 incubator. Pellets prepared in the same way were placed in 6–12 test tubes on standing Styrofoam, and the medium was changed every 3–4 days until day 21, and genomic DNA was then isolated from each pellet. Chondrocytic differentiation was ascertained *both* by histologic analysis and by real-time reverse transcription–polymerase chain reaction (RT-PCR)

Table 1. Down-regulated genes examined for CpG sites in the promoters

Gene	Size of TSS-containing CpG island, bp	Upstream inclusion	No. of satellite CpG islands*	CpG site density†		
				TSS islands	Upstream regions	Satellite islands
BCLX	375	−100 bp	3	M	M to L	H to M
CAP2	678	−30 bp	0	H to M	M	–
CNN1	134	Exon 1 only	0	M	L	–
DDAH1	1,229	Includes upstream	0	H	H	–
DIPA	1,256	−110 bp	3	H	M	H to M
FGF5	480	−700 bp	3	H	H	M
FZD2	1,227	−150 bp	3	H	H to M	M
GPR39	103	−150 bp to TSS	2	L	L to M	H
GREM1	1,766	−700 bp	0	H	H	–
ITGA3	1,137	−800 bp	2	H to M	H to M	M
KIAA1199	753	−500 bp	0	H	H	–
KTRS	749	−200 bp	0	H to M	M	–
PODXL	1,280	−500 bp	2	H	H	M
SDF1A	1,346	−800 bp	3	H	H	H
SOX4	132	Exon 1 only	0	M	L	–
TIMP2	1,282	−500 bp	1	H	H	M

* Numbers of satellite CpG islands estimated within the 2-kb upstream region.
† Classified according to the percentage of CpG ratios in the island, as high (H), medium (M), or low (L). TSS = transcription start site.

analysis of pellets cultured in parallel, as described previously (22,24).

Candidate gene selection and primer design. As possible targets for epigenetic regulation through DNA methylation, 10 candidate genes were selected from 3 categories: genes encoding transcription factors important for chondrocyte lineage commitment, genes up-regulated in chondrogenic pellet cultures, and genes down-regulated in chondrogenic pellet cultures.

For the genes encoding transcription factors important for chondrocyte lineage commitment, we first examined the promoter methylation status of 6 genes encoding key transcription factors for chondrogenesis: runt-related transcription factor 2 (*RUNX2*), zinc-finger protein osterix (*OSTERIX*), natural killer 3 homeobox 2 (*NKX3-2*), sex-determining region Y–type high mobility group box 5 (*SOX5*), *SOX6*, and *SOX9*. Since the expression of these factors is tightly regulated in most cells, these genes would be expected to be important targets of epigenetic control. Examination of the number and size of CpG islands and the density of CpG sites in upstream and downstream flanking sequences of the transcription start sites of *OSTERIX*, *NKX3-2*, *SOX5*, and *SOX6* was eliminated from the study targets, since they had no CpG islands within the regions. We therefore selected *SOX9* and *RUNX2*, which had CpG islands containing transcription start sites.

For genes up-regulated in chondrogenic pellet cultures and genes down-regulated in chondrogenic pellet cultures, we reexamined the gene expression profile data previously reported by one of us (IS) (25). After considering 40 representative signature genes whose expression changed significantly during 3 weeks of pellet culture (Tables 1 and 2) and after evaluating CpG site densities in the promoter regions of these genes, we selected 7 candidate genes that were possibly important for epigenetic regulation through DNA methylation. These were chondromodulin 1 (*CHM1*), fibroblast growth factor receptor 3 (*FGFR3*), and chondroadherin (*CHAD*) for genes up-regulated in chondrogenic pellet cultures, and for genes down-regulated in chondrogenic pellet cultures, these were *SOX4*, Gremlin 1 (*GREM1*), G protein–coupled receptor 39 (*GPR39*), and stromal cell–derived factor 1 (*SDF1*). We also added matrilin 4 (*MATN4*) for genes up-regulated in chondrogenic pellet cultures, although its promoter CpG islands were away from transcription start sites. This promoter sequence was used as a reference for a region with low CpG density. Target sites for bisulfite sequencing analysis (23) were determined according to the prediction calculated with MethPrimer software (26) (available at www.urogene.org/methprimer/index1.html). Primer sets were designed to be basically within 1 kb upstream of the transcription start sites.

The gene symbols shown in Tables 1 and 2 are consistent with the National Center for Biotechnology Information Entrez Gene style. Sizes of transcription start site–containing CpG islands and adjacent CpG islands are given in basepairs. CpG site densities within the regions were estimated from the diagram provided by MethPrimer software output.

DNA isolation and bisulfite sequence analysis. Genomic DNA was isolated from human synovium–derived MSCs before (day 0; control) and after 21 days of pellet culture (day 21). At least 3 pellets per patient were analyzed separately. No significant differences between the pellets were detected. For DNA isolation, ~5 million undifferentiated human synovium–derived MSCs or pellets were directly digested with 1 mg/ml of proteinase K (Sigma-Aldrich Japan) at 50°C for 16 hours, and the extracted DNA was purified using a QIAamp Micro DNA kit (Qiagen, Tokyo, Japan). Sample concentrations were calculated from the absorbance at 260 nm, as measured with an ND-1000 instrument (NanoDrop Technologies, Wilmington, DE). As a reference sample, genomic DNA was isolated from the articular cartilage of a patient with OA. Cartilage from the posterior condyle of the distal femur was placed on the viewing platform of a stereomicroscope and was dissected with a scalpel into 4 layers: the tangential