Osteoarthritis and Cartilage 21 (2013) 481–490

Osteoarthritis and Cartilage

Repair of osteochondral defects with recombinant human type II collagen gel and autologous chondrocytes in rabbit

H.J. Pulkkinen †*, V. Tiitu †, P. Valonen ‡, J.S. Jurvelin §, L. Rieppo §, J. Töyräs §‖, T.S. Silvast §, M.J. Lammi †¶, I. Kiviranta #††

† *Institute of Biomedicine, University of Eastern Finland, Kuopio, Finland*
‡ *A. I. Virtanen Institute, University of Eastern Finland, Kuopio, Finland*
§ *Department of Applied Physics, University of Eastern Finland, Kuopio, Finland*
‖ *Department of Clinical Neurophysiology, Kuopio University Hospital, Kuopio, Finland*
¶ *Biocenter Kuopio, University of Eastern Finland, Kuopio, Finland*
# *Department of Orthopaedics and Traumatology, University of Helsinki, and Helsinki University Central Hospital, Helsinki, Finland*
†† *Department of Orthopaedics and Traumatology, Jyväskylä Central Hospital, Jyväskylä, Finland*

ARTICLE INFO

Article history:
Received 29 August 2012
Accepted 7 December 2012

Keywords:
Animal model
Autologous cell
Cartilage tissue engineering
Collagen
Hydrogel
Recombinant protein

SUMMARY

Objective: Recombinant human type II collagen (rhCII) gels combined with autologous chondrocytes were tested as a scaffold for cartilage repair in rabbits *in vivo*.
Method: Autologous chondrocytes were harvested, expanded and combined with rhCII-gel and further pre-cultivated for 2 weeks prior to transplantation into a 4 mm diameter lesion created into the rabbit's femoral trochlea ($n = 8$). Rabbits with similar untreated lesions ($n = 7$) served as a control group.
Results: Six months after the transplantation the repair tissue in both groups filled the lesion site, but in the rhCII-repair the filling was more complete. Both repair groups also had high proteoglycan and type II collagen contents, except in the fibrous superficial layer. However, the integration to the adjacent cartilage was incomplete. The O'Driscoll grading showed no significant differences between the rhCII-repair and spontaneous repair, both representing lower quality than intact cartilage. In the repair tissues the collagen fibers were abnormally organized and oriented. No dramatic changes were detected in the subchondral bone structure. The repair cartilage was mechanically softer than the intact tissue. Spontaneously repaired tissue showed lower values of equilibrium and dynamic modulus than the rhCII-repair. However, the differences in the mechanical properties between all three groups were insignificant.
Conclusion: When rhCII was used to repair cartilage defects, the repair quality was histologically incomplete, but still the rhCII-repairs showed moderate mechanical characteristics and a slight improvement over those in spontaneous repair. Therefore, further studies using rhCII for cartilage repair with emphasis on improving integration and surface protection are required.

Introduction

The advances in regenerative medicine and biomaterial fabrication have made it possible to use tissue engineered transplants and/or biocompatible materials in the cartilage repair procedures. Different collagens in a variety of formulas (*e.g.*, sponges, membranes or gels) have been used as biomaterial scaffold for chondrocytes in the articular cartilage repair and tissue engineering[1–6]. Collagen, especially type II collagen, forms a natural environment for the chondrocytes since it is the predominant matrix molecule in cartilage[7]. The chondrocytes interact with the surrounding matrix, *e.g.*, *via* integrin receptors[8], which attach to type II collagen. A positive feedback from the surrounding matrix is essential for the cells to maintain their chondrocytic phenotype and matrix production[9] and to prevent apoptosis[10].

Type II collagen has been used as a scaffold for chondrocytes[6,11,12], although the use of type I or III collagen is more common[4,13–16]. The collagen materials that have been tested in patients include porcine type I/III collagen membrane[15,16] and type I collagen gel from rat tail[13]. Although immunological reactions

* Address correspondence and reprint requests to: H.J. Pulkkinen, Institute of Biomedicine, Anatomy, University of Eastern Finland, PO Box 1627, 70211 Kuopio, Finland. Tel: 358-45-1278218; Fax: 358-17-163032.
E-mail address: hertta.pulkkinen@uef.fi (H.J. Pulkkinen).

1063-4584/$ – see front matter
http://dx.doi.org/10.1016/j.joca.2012.12.004

have not been detected, the use of animal derived components in humans always raises safety issues. Previous feasibility studies of collagen as a scaffold for cartilage repair have used materials isolated from various animal sources, such as pigs[4,12] or cattle[3,6]. However, we have had access to recombinant human type II collagen (rhCII) produced in yeast cultivations[17,18]. This recombinant technique produces hydroxylated triple helical collagen, which has thermal stability similar to native collagen when coexpressed with recombinant prolyl hydroxylase[18]. The feasibility of this material for chondrocyte cultivation was demonstrated in our previous study, in which a mixture of rhCII-gel and chondrocytes was injected subcutaneously into the backs of nude mice[19]. Six weeks after the implantation, a piece of cartilaginous tissue with type II collagen and proteoglycans (PGs) was formed[19].

Collagen as a scaffold for chondrocytes is presumably biodegradable[20]. Therefore, collagen scaffolds are supposed to offer only initial support for the cells, and to be degraded in the course of time. Preferably the degradation of the initial scaffold collagen is accompanied with simultaneous collagen production that replaces the implanted collagen in a remodeling process. This is the main hypothesis behind all tissue engineering protocols using biodegradable materials, but it is an open question whether the process of remodeling can take place and to be restricted only to lesion site. The biodegradation of recombinant collagens has not been studied yet, but it is possible that the more random structure in the manufactured collagen can cause them to be more vulnerable for enzymatic degradation. The aim of this work was to test rhCII in a cartilage defect model *in vivo*. In the present study, the rhCII was used for the first time in cartilage tissue repair in the form of hydrogel-like scaffold for chondrocytes.

Method

Collagen gel transplantation

A total of 15 mature female New Zealand white rabbits (age 9 months) were used for this experiment. The Institutional Animal Care and Use Committee of the University of Kuopio and the Provincial Government approved the study plan (experiment number 04-82). Rabbits were randomly divided into two groups and defects to the articular cartilage were created as follows: (1) defects repaired with pre-cultivated rhCII-gel seeded with previously expanded autologous chondrocytes (rhCII-repair, $n = 8$); and (2) defects left empty (spontaneous repair, $n = 7$). The contralateral joints of spontaneous repair group were used as the control group for intact articular cartilage.

The rabbits in the rhCII-group underwent two surgical operations; in the first operation a biopsy of cartilage for chondrocyte isolation was harvested from the margin of the femoral trochlea of the left knee joint. The operations were performed under anesthesia (Domitor 0.35 ml/kg, Orion Pharma, Espoo, Finland and Ketaminol 0.6 ml/kg, Intervet International B.V., Boxmeer, the Netherlands). Subsequently, the cartilage biopsies (10–20 mg) were washed with sterile phosphate-buffered saline (PBS). Cartilage was digested with 120 units/ml of collagenase (Sigma, St. Louis, MO, USA) in Dulbecco's modified medium (DMEM, Euroclone, Pero, Italy) at 37°C and 7% CO_2 overnight. The next day, the isolated cells were washed with PBS and suspended in DMEM. After enzymatic digestion the cells were expanded in cell cultures for approximately 2–3 weeks. The expanded cells were trypsinized and the gels were prepared as follows: 300 μl of DMEM containing 3 million chondrocytes was combined with 300 μl of rhCII-solution as previously described[19].

The rhCII-cell constructs were cultivated for 2 weeks prior to the next surgical operation to minimize the influence of early stage contraction in the gels. Some extra gels were made for performing live/dead analyzes using fluorescent dyes and confocal microscope[21] in order to monitor viability of the rabbit chondrocytes. In the second surgical operation an osteochondral lesion (4 mm in diameter, 3 mm in depth) was drilled into the right femoral trochlea (patellar groove) ($n = 8$) and filled with the pre-cultivated rhCII-cell construct. Before implantation, the gel grafts were punched with a 4 mm biopsy punch (Kai Industries, Oyana, Gifu, Japan). The precise size of the gel grafts allowed tight fitting of the implant into the defect. For the spontaneous repair group, similar lesions were created ($n = 7$) and left empty. At the end of the experiment, the contralateral knees ($n = 7$) from this spontaneous repair group were used for harvesting intact tissue from patellar groove as a control reference. After 6 months, the animals were sacrificed and the distal ends of femurs were stored at −20°C in PBS with protease inhibitors[22]. Prior freezing the samples were photographed and the filling of the samples was analyzed from the photographs using ImageJ software (version 1.36b, National Institute of Health, USA).

Micro-computed tomography (μCT) analysis

To analyze the subchondral bone structure, the knee joint samples were scanned with a μCT scanner (SkyScan 1172, SkyScan, Kontich, Belgium). Tube voltage of 100 kV was applied with voxel size of $15 \times 15 \times 15$ μm^3. The μCT images were analyzed with the CT Analyzer software version 1.9.1.0 (SkyScan).

The three dimensional (3D)-analysis was performed for two separate manually selected volumes of interest (VOI) for each sample [VOI selection in the Fig. 1(C)]. The VOI1 (a cylinder with 4 mm diameter and 2 mm in height) was placed within the deep part of the healed osteochondral lesion, while VOI2 (a cylinder with 4 mm diameter and 1 mm height) was placed below the lesion.

Histology

After formalin fixation for 24 h the osteochondral samples were decalcified using 10% ethylenediaminetetraacetic acid (EDTA) (Merck KGaA, Darmstadt, Germany) and subsequently, embedded and cut into 3- or 5-μm-thick sections perpendicular to the joint surface. PGs were stained with toluidine blue and safranin O. Bone and cartilage tissues were defined using the Masson's trichrome staining. Histopathological examination to analyze possible immunological reaction was performed from hematoxylin and eosin (HE) stained sections by experienced pathologist. The modified O'Driscoll's score (maximum score 30) for cartilage repair was used to evaluate the repair quality from the safranin O stained sections[23]. Three observers scored the blinded samples independently, and the scores were averaged and rounded to the nearest integer.

The tissue thicknesses used in the mechanical testing analysis were determined using the Toluidine blue stained sections. Thicknesses were determined using ImageJ software at the center of the repaired cartilage and at the corresponding site for the intact cartilage, leaving out the outermost, loose fibrillated layer.

Type II collagen was detected using the mouse monoclonal antibody E8[24]. Anti-type I collagen (Abcam, Cambridge, UK) was used with overnight incubation (1:100) at 4°C, after 30 min protease and 1 h hyaluronidase treatment at 37°C. For the immunostainings, negative controls without primary antibody (1% Bovine Serum Albumin (BSA) in PBS) were included. The Envision + System-horseradish peroxidase (HRP) (Dako, Glostrup, Denmark) was used for staining.

Enhanced polarized light microscopy

Sample sections (5-μm-thick) were cut, dewaxed in xylene, and rehydrated as previously described[25]. Tissue glycosaminoglycans (GAGs) were digested with bovine testicular hyaluronidase, type IV (Sigma, St. Louis, MO, USA) using 1000 units/ml in PBS for 18 h at

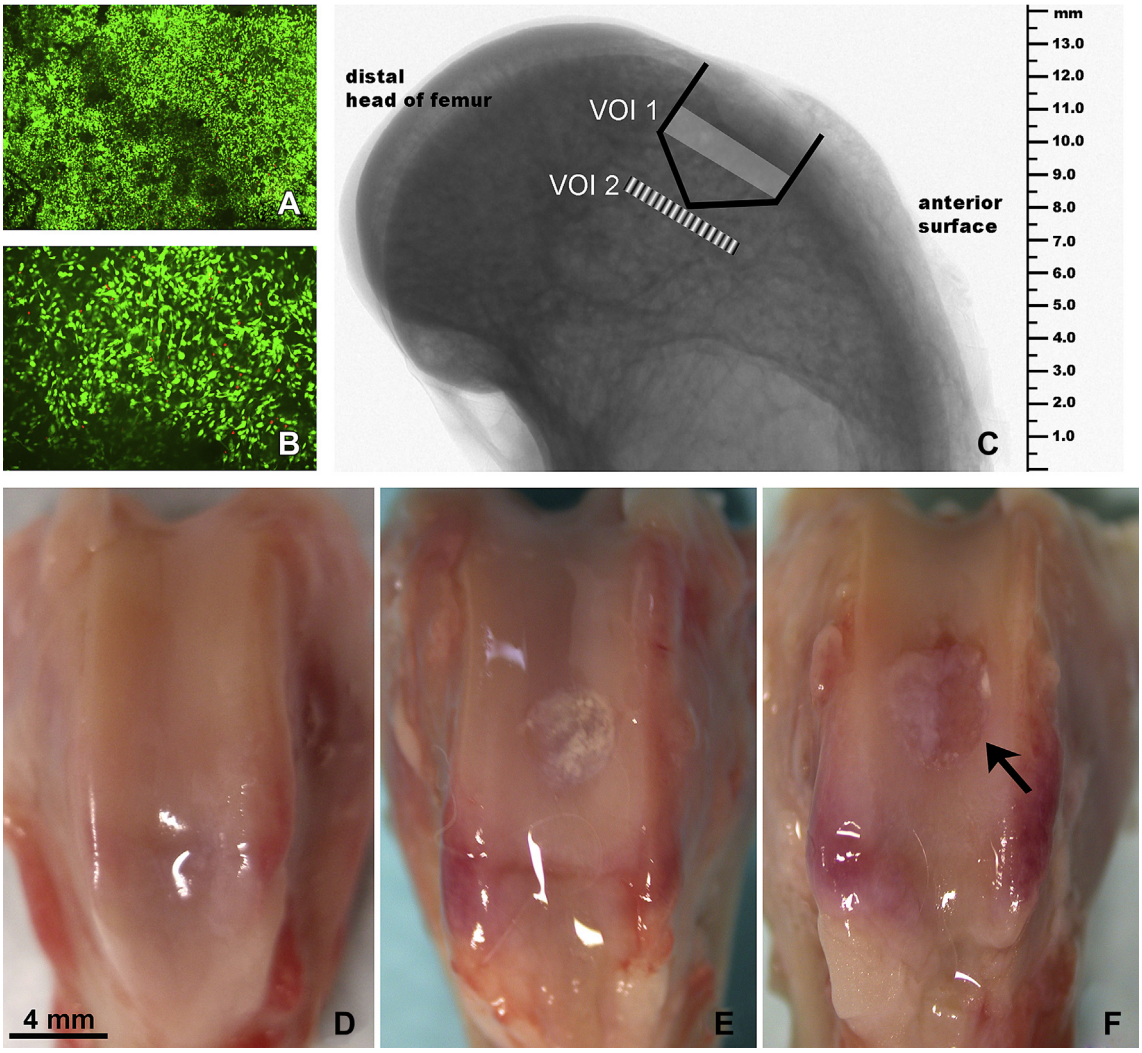

Fig. 1. Viability of lapine chondrocytes was analyzed after 4 weeks of *in vitro* cultivation from rhCII-gels made for this purpose. The image shows dead cells in red and living cells in green color in different magnifications (A, B). The selection of VOI illustrated in the μCT-projection image as follows (C); VOI1: the volume analyzed in the osteochondral lesion site (gray). VOI2: the volume analyzed under the drilled lesion (black and white stripes). The black lines indicate the drilled osteochondral lesion (diam. = 4 mm, depth = 3 mm). Photographs of (D) intact, (E) rhCII-gel repair and (F) spontaneous repair in the rabbit femoral trochlea after 6 months of operations. The repair site is clearly visible in the rhCII and spontaneous repair. In the rhCII-repairs, the defects were filled with repair tissue. In the spontaneous repair, the defects were not fully filled with repair tissue, as shown with the black arrow.

37°C. The sections were left unstained, embedded in DPX mounting medium (Difco, East Molesey, UK), and examined with a computerized Leitz-Ortholux POL microscope using 5.7 × 5.7 μm^2 pixel size. The degree of parallelism of the collagen network, *i.e.*, the parallelism index, and the orientation angle of the collagen fibrils were determined[26]. A higher PI (maximum value is 1) indicates that the majority of the collagen fibrils are running in the same direction at that given site.

Fourier-transform infrared imaging spectroscopy (FT-IRIS)

The 5-μm-thick sections were prepared similarly to those used for polarized light microscopy (but without the digestion of GAGs), and transferred onto 2-mm-thick infrared-transparent ZnSe-windows. FT-IRIS was utilized to determine the amide I (1584–1720 cm^{-1}) absorbance of the sections as a measure of collagen content, whereas the carbohydrate region (984–1140 cm^{-1}) was used to estimate the PG content[27]. Measurements were done using a Perkin Elmer Spectrum Spotlight 300 FTIR imaging system (Perkin Elmer, Cambridge, UK) at 4 cm^{-1} spectral resolution and with a pixel size of 25 × 25 μm^2.

Mechanical testing

A stepwise creep testing protocol (indenter diameter = 544 μm, 3 × 84.4 kPa, ramp rate = 0.015 mm/s corresponding to strain rate of 0.017–0.002, creep time 1200 s) was used to analyze mechanical properties[28]. The samples were thawed and balanced in a PBS bath containing protease inhibitors[22]. The indentation site was at the center of the lesions ($n = 8$ for rhCII-repair and $n = 7$ for spontaneous repair) or in the corresponding site in the intact tissue ($n = 7$). The equilibrium and dynamic moduli were calculated from the second indentation step[29] by assuming Poisson ratios of 0.1 and 0.5, respectively. Tissue thicknesses to calculate mechanical properties were obtained from histological sections.

Statistical analyses

Statistical tests were performed using the SPSS Statistic 17.0 software (SPSS Inc., Chigaco, IL, USA). Mann–Whitney test was used for the independent samples, and Wilcoxon's signed-ranks test for analysis of the dependent samples. Statistical significance

was set at P less than 0.05. The results are presented as means (95% confidence intervals (CIs)).

Results

Chondrocyte viability in rhCII-gels

The viability assay of the transplanted rhCII-gels confirmed that after 4 weeks of *in vitro* cultivation the lapine chondrocytes were alive inside the rhCII-gel [Fig. 1(A and B)]. This finding is comparable to our earlier results, where bovine chondrocytes maintained their viability *in vitro*[21] and subcutaneously *in vivo*[19].

Macroscopical appearance

Six months after the operation, repair tissue mostly filled the created lesion sites. The repairs had macroscopically smooth surface, but the color of the repair differed from the surrounding cartilage [Fig. 1(D–F)]. In three spontaneous repair sample the lesions showed partial filling and macroscopically 79 ± 35% (mean ± standard deviation (SD); $n = 6$; range 10–100%) of the spontaneous repair areas were filled up to the level of adjacent cartilage. One rhCII-repaired lesion was partially filled and an average of 99 ± 3% ($n = 6$; range 93–100%) of the rhCII-repaired areas were completely filled. This difference was insignificant ($P = 0.310$). Some peripheral cartilage areas of the femoral trochleae were swollen and hyperemic [Fig. 1(E and F)]. This reaction was apparent in both repair groups. The intact control cartilage had a smooth trochlear area [Fig. 1(D)].

Histology

The lesion sites were clearly visible histologically in both repair groups, and detachment of the repair tissue from the adjacent cartilage could be noticed (Fig. 2). The detachment was partly due to the tissue processing, as the ruptures showed different appearances in the different sections taken from the same sample [Fig. 2(B) vs Fig. 2(H)]. In general, the integration of the rhCII-implant into the bone was good [Fig. 2(B) and (E)].

The average value for cartilage thickness was 372 ± 71 μm for the intact tissue, 411 ± 71 μm for the rhCII-repair and 273 ± 100 μm for the spontaneous repair. These differences between intact and rhCII-repaired cartilage ($P = 0.463$), intact and spontaneously repaired cartilage ($P = 0.219$) and between rhCII and spontaneously repaired samples ($P = 0.094$) were insignificant. The superficial layer of the rhCII and spontaneous repair was fibrous and hypocellular [Fig. 2(E and F)], and had only a weak staining for PGs. PGs were abundant in the deeper zones, and tissue appearance was similar to cartilage. However, the tidemark was partially incomplete. In the adjacent tissue next to the repaired area, the cellularity was nearly normal with mild clustering and slight fibrillation. No signs of inflammation (macrophages, necrosis, lymphocytes or neutrophils) were observed in the HE-stained sections (pictures not shown) in neither of the repaired cartilage groups.

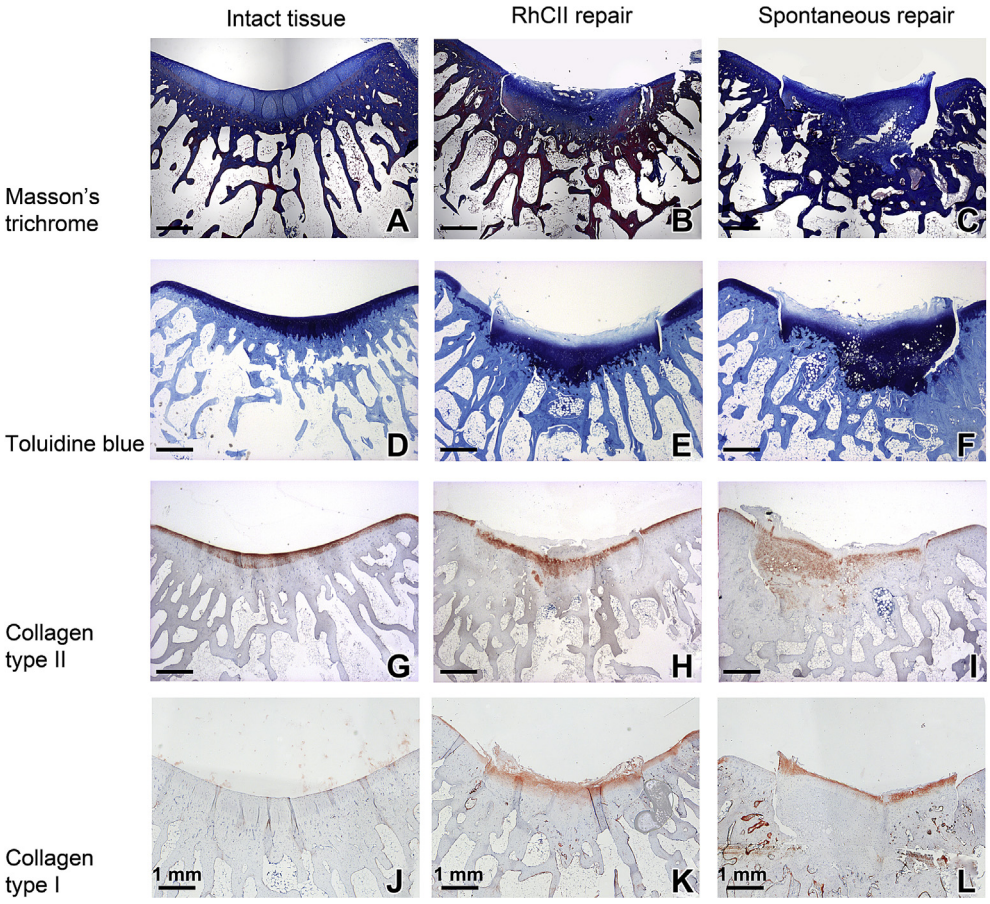

Fig. 2. Histological stainings in the intact and repaired joint areas as indicated above. Masson's trichrome staining (A–C) showed presence of cartilage and bone in the repaired tissue (B and C). Both rhCII-repair and spontaneous repair tissues show peripheral disintegration, which can be either real or an artifact, as seen in the images C vs I. In the repair tissues the superficial layer is fibrous and the lack of cells and PGs is shown, *e.g.*, in images E and F of the toluidine blue stainings (D–F). Immunohistological stainings of type II (G–I) and I (J–L) collagens in the intact and repaired joint areas as indicated in the photograph panel show type II collagen in the deeper regions of repair tissue and type I collagen in the superficial layer.

Immunostaining for type II collagen was present in all zones of the normal intact articular cartilage [Fig. 2(G)]. In the repair tissues, type II collagen was present in the deeper parts of the repair tissue, but not in the hypocellular superficial layer of both repair groups [Fig. 2(H and I)]. In the most superficial layer, the hypocellular part of the repair tissue contained type I collagen [Fig. 2(K and L)] in both repair groups. Often a thin layer of type I collagen specific staining was detected also in the healthy adjacent cartilage surrounding the repair tissues in both repair groups [Fig. 2(K and L)]. The lack of staining for type I collagen in the intact cartilage [Fig. 2(J)] shows also the specificity of the immunostaining and lack of type I collagen in the intact cartilage.

The histological scoring showed that in both of the repair groups the quality of the tissue was significantly lower than in the intact cartilage (Table I). The overall score for intact tissue ($n = 7$) was 29.1 (0.7), whereas in the rhCII-repaired cartilage ($n = 6$) the score was 12.5 (1.4), and in the spontaneous repair ($n = 7$) the score was 14.1 (2.7), presented as mean (95% CIs). There were no significant differences between the repair groups in any category that was estimated. However, based on the histological scoring the rhCII-repaired tissue showed closer resemblance to the intact in the category of tidemark and cellular characteristics ($P = 0.366$, and 0.051 respectively), whereas the spontaneous repair gained a better result in the category of nature of the predominant tissue ($P = 0.141$).

μCT

The structure of regenerated bone was studied with μCT in the vicinity of the injury (VOI1) and deeper in the bone (VOI2). A number of parameters given by the analysis software were analyzed, but there were no major differences in most of them when compared with the intact bone tissue (Table II). In the VOI1 of the rhCII-repair group, the trabeculae thickness (TbTh) was significantly smaller, and their number (TbN) higher than in the intact tissue. In the rhCII-group the VOI2 area showed thicker, but fewer trabeculae, than the corresponding area in intact control group. In the spontaneous repair group, only the TbTh in VOI1 differed significantly from the intact control tissue (Table II).

Enhanced polarized light microscopy and FT-IRIS

Polarized light microscopic analysis was utilized to evaluate the status of the collagen network [the degree of parallelism Figs. 3(A), 4(A), fibril orientation Figs. 3B, 4B and 5(A–C), and anisotropy Fig. 5(D–F)] in intact vs the rhCII and the spontaneous repair tissues. In addition, the collagen and PG contents were calculated from FT-IRIS data [Figs. 3(C–D), 4(C–D), and 5(G–I)]. The organization of the collagen network differed in both repair groups in comparison to intact cartilage. The overall fibril orientation in the rhCII-repair tissue was significantly less perpendicular-to-surface than in the intact cartilage [Fig. 4(B)], while the parallelism index in the spontaneous repair tissue was lower than in the intact one [Fig. 4(A)]. However, the total collagen content in either of the repair samples, evaluated by FT-IRIS, did not differ significantly from the control group [Fig. 4(C)]. PG content was lower in the superficial part of the repair tissue in both repair groups Fig. 3(D)], and in the rhCII-repair group also in the deeper repair tissue [Fig. 3(D)].

The depth-wise analysis of the histological samples indicated that in both repair groups the fibril orientation differed significantly from the intact tissue [Fig. 3(B)] practically through the whole depth of the repair tissue. In the rhCII-repair tissue, the parallelism index was close to that in the intact cartilage [Fig. 3(A)], while the spontaneous repair tissue had a more inferior quality in this respect, showing significantly lower values in the middle and deep zones of the repair tissue [Fig. 3(A)]. The intact cartilage had highest values for both the collagen [Figs. 3(C) and 4(C)] and PG contents [Figs. 3(D) and 4(D)].

Biomechanical testing

There were no significant differences in the values of equilibrium modulus in different groups [Fig. 6(A)], although the spontaneous repair tissue was somewhat softer than the intact (66%) and rhCII-repair tissues (34%). Similar findings were observed in the dynamic modulus, where the spontaneous repair tissue showed the lowest values, which were 65% lower than intact and 34% lower than rhCII-repaired cartilage (insignificant differences) [Fig. 6(B)]. Exact *P*-values are given in the Fig. 6.

Discussion

There were no major differences in the macroscopical appearances of the repaired tissue between the rhCII and spontaneous repair, besides that the filling of the repair area was more complete in the rhCII-repaired lesions. The surfaces of the repair tissues were visibly detectable, but smooth, and no large fissures were seen.

Table I
The detailed results of histological grading (modified O'Driscoll) in the intact, rhCII and spontaneously repaired cartilage. Exact *P*-values are presented for each statistical analysis

	Scoring values in each repair group			*P*-values		
	Intact	RhCII	Spontaneous	Int. vs RHCII	RhCII vs Spont.	Int. vs Spont.
Matrix staining	2.3	0.5	1.0	0.001*	0.138	0.047*
Tidemark	1.0	0.7	0.5	0.366	0.445	0.031*
Nature of the predominant tissue	4.0	1.7	2.0	0.001*	0.628	0.141
Surface regularity	3.0	0.5	1.0	0.001*	0.181	0.016*
Structural integrity	2.0	0.2	0.4	0.001*	0.138	0.016*
Thickness	2.0	1.0	1.1	0.001*	0.445	0.016*
Bonding to adjacent tissue	2.0	0.4	0.4	0.001*	0.945	0.016*
Hypocellularity	3.0	1.2	1.6	0.001*	0.181	0.016*
Chondrocyte clustering	1.9	1.1	1.0	0.001*	0.366	0.016*
Cellular characteristics	3.0	2.3	2.3	0.051	1.000	0.016*
Fibrillation	3.0	2.4	2.4	0.008*	0.836	0.031*
Presence of fibrous tissue over graft	1.0	0.3	0.1	0.001*	0.234	0.016*
Presence of subchondral bone healing	1.0	0.2	0.1	0.001*	0.445	0.016*
Total	29.1	12.5	14.1	0.002*	0.880	0.000*
95%CI	0.7	1.4	2.7			

Asterisks (*) indicate statistically significant differences between groups (*$P < 0.05$).

Table II
Bone healing in osteochondral repairs in rabbits: calculated parameters from μCT-analysis from VOI 1 (injured area or equivalent site in the intact) and VOI 2 (below the injury) for intact cartilage and rhCII and spontaneously repaired cartilage. Data presented as mean (95% CI). Exact *P*-values are given for differences between rhCII-intact and spontaneous-intact. There were no significant differences of the measured bone parameters between rhCII and spontaneous repair groups

Abr.[†]	Unit	Intact		RhCII		*P*-value (rhCII vs intact)	Spontaneous		*P*-value (spont. vs intact)
		Average	CI 95%	Average	CI 95%		Average	CI 95%	
VOI 1									
BS/BV	mm^{-1}	4.03	(0.33)	4.29	(0.30)	0.635	5.63	(1.87)	0.225
BV/TV	%	74.3	(6.25)	69.5	(4.68)	0.295	59.7	(13.7)	0.156
TBPf	mm^{-1}	−4.74	(2.52)	−9.74	(3.02)	0.073	−6.81	(3.98)	0.313
SMI		−1.16	(1.45)	−2.56	(0.85)	0.138	−1.3	(1.47)	1.000
TbTh	mm	**0.54**	(0.08)	**0.36***	(0.05)	0.008	**0.36***	(0.09)	0.031
TbN	mm^{-1}	**1.41**	(0.20)	**1.97***	(0.22)	0.005	1.69	(0.34)	0.156
TbSp	mm	0.28	(0.04)	0.33	(0.05)	0.234	0.38	(0.09)	0.156
VOI 2									
BS/BV	mm^{-1}	5.26	(0.18)	5.21	(0.51)	0.805	5.25	(1.18)	0.596
BV/TV	%	38.9	(1.33)	39.76	(3.64)	0.318	40.3	(9.54)	0.844
TBPf	mm^{-1}	−0.73	(0.68)	−0.68	(0.55)	0.902	−0.43	(4.77)	0.297
SMI		0.39	(0.21)	0.48	(0.19)	0.620	0.92	(0.57)	0.063
TbTh	mm	**0.21**	(0.01)	**0.25***	(0.02)	0.017	0.21	(0.03)	0.813
TbN	mm^{-1}	**1.86**	(0.05)	**1.63***	(0.14)	0.011	1.8	(0.23)	0.688
TbSp	mm	0.41	(0.03)	0.48	(0.06)	0.073	0.41	(0.04)	0.844

Asterisks (*) indicate statistically significant differences in the given parameter (bolded) in comparison to intact (*$P < 0.05$).

[†] Abbreviations (and short descriptions): BS/BV = Bone surface-to-volume ratio (ratio of solid surface-to-volume), BV/TV = Bone volume fraction (ratio of bone volume to total volume), TBPf = Trabecular bone pattern factor (index of relative convexity or concavity of the total bone surface, higher TBPf means a more disconnected trabecular structure), SMI = Structural model index (indicates the relative prevalence of plate (SMI value O), cylinder (3) or sphere (4) like structures in the material, negative values indicate enclosed cavities), TbTh = Trabecular thickness, TbN = Trabecular number, TbSp = Trabecular separation.

Microscopically, the superficial layer contained mild fibrillation and small fissures. It also contained type I collagen, was hypocellular, and had low PG content. Instead, in the deeper areas of the repair tissue the amount of PGs was normal, or close to it, and type II collagen was present. The origin of the type II collagen in the rhCII-repaired cartilage was not authenticated, because the antibody used for the immunostaining cannot distinguish between the human and lapine type II collagen. Presumably part of the type II collagen detected in the rhCII-repaired cartilage was produced by the transplanted chondrocytes, and part of it was originating from the rhCII. Collagenous matrixes are presumed to be biodegradable[20], but the rate of the degradation is not known. Therefore, without labeling of the implanted collagen scaffold and knowledge of the degradation rate, it is not possible to estimate the origin of the type II collagen. The remodeling of the collagen structure is poorly understood, and there are no studies describing remodeling or re-organization of the collagenous structure inside cartilage repair.

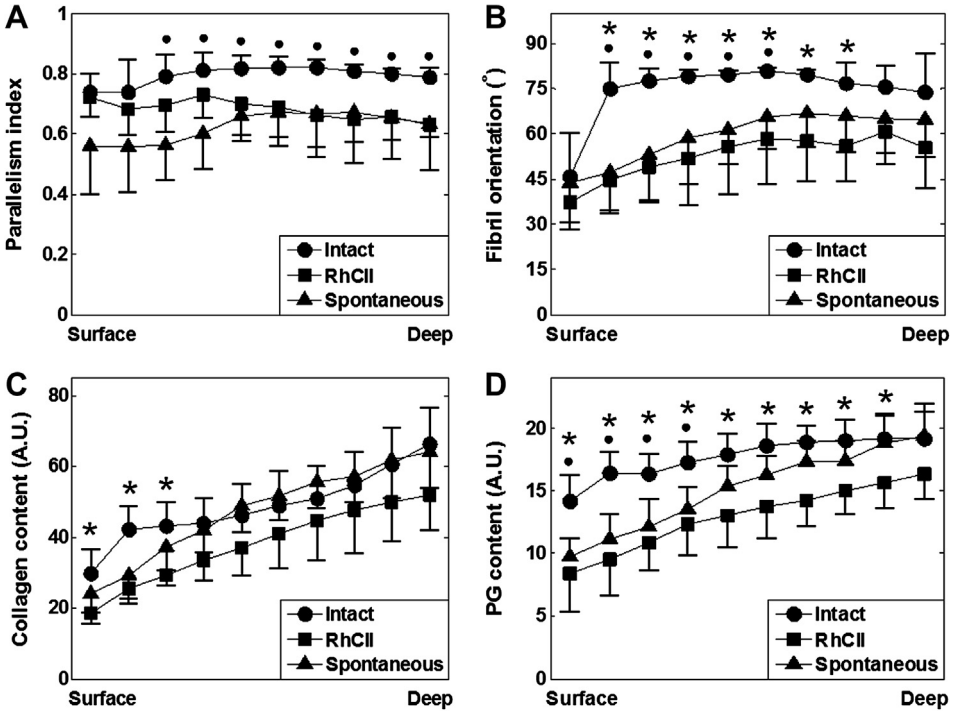

Fig. 3. The parallelism index (A), collagen orientation (B), collagen content (C) and PG content (D) in relation to the depth of the tissue in the intact ($n = 7$), rhCII-gel ($n = 7$) and spontaneous ($n = 6$) repair groups. A.U. = absorbance unit (amide I absorption). Data is presented as mean (error bars show 95% CIs). Statistically significant differences ($P < 0.05$) are indicated with symbols as follows: ● = between intact and spontaneous, and * = between intact and rhCII-gel repair.

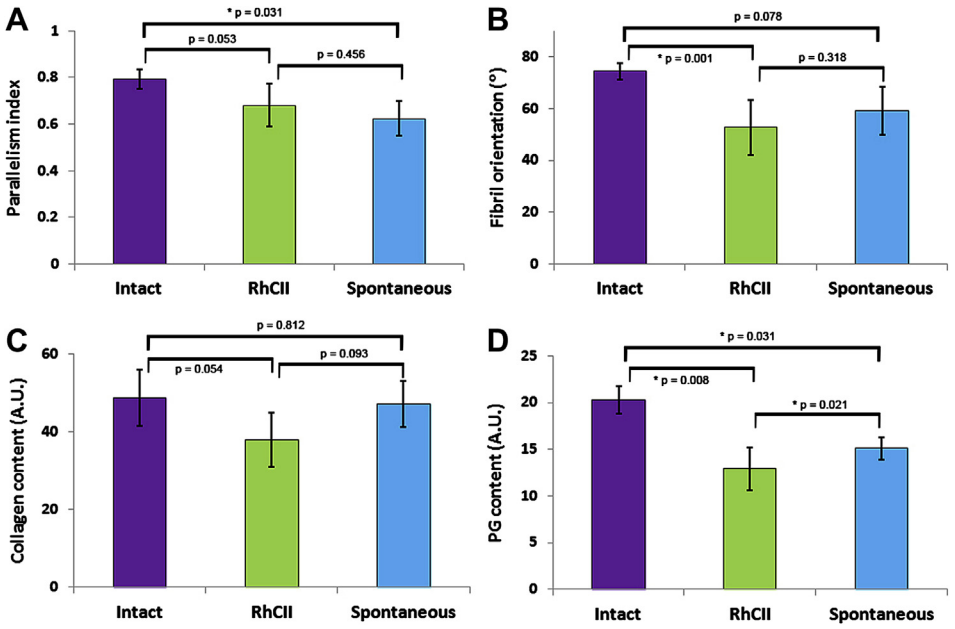

Fig. 4. The averages of parallelism index (A), the collagen fibril orientation (B), collagen content (C) and PG content (D) in the intact ($n = 7$), rhCII-gel ($n = 7$) and spontaneous ($n = 6$) repair groups. A.U. = absorbance unit (amide I absorption). Data is presented as mean (error bars show 95% CIs).

In the intact articular cartilage type I collagen could not be detected, but in both of the repaired groups, a thin layer of type I collagen staining was detected in the surface of the adjacent non-injured cartilage. This finding might be an indication of the phenomenon where the focal articular cartilage injury systemically affects the whole joint, not just the defected area or repair tissue, possible *via* some soluble mediators.

The organization of the collagen fibrils in the repairs differed from the intact cartilage. The anisotropy level might be influenced by the diameter of the collagen fibrils, which has been recorded to thinner than intact in repaired cartilage[30,31]. In mature intact cartilage the collagens are organized in a depth-dependent manner, but during the development the architecture varies a lot[28,32,33], and in the rabbits femur the collagens are horizontally oriented until 6 weeks of age[34]. In the present study, the collagen fibrils were more horizontally oriented in the repairs than in intact cartilage. In pigs, the collagen network of spontaneous repair tissue was more randomly arranged and showed reduced parallel fibril orientation still at 1 year after the operation, although the collagen fibril number and diameters were similar to normal tissue[30]. These findings confirm that the maturation of the collagen fibrils in the injured cartilage is a time-consuming process, and it is still not evident whether it is even possible to achieve such a localized maturation of collagen fibrils in the injured adult cartilage.

In this study, the mechanical properties of the repaired cartilage were similar to intact tissue, although loss of PGs and fibrous tissue was detected in the most superficial part of the repair tissues. During growth the mechanical properties of cartilage show

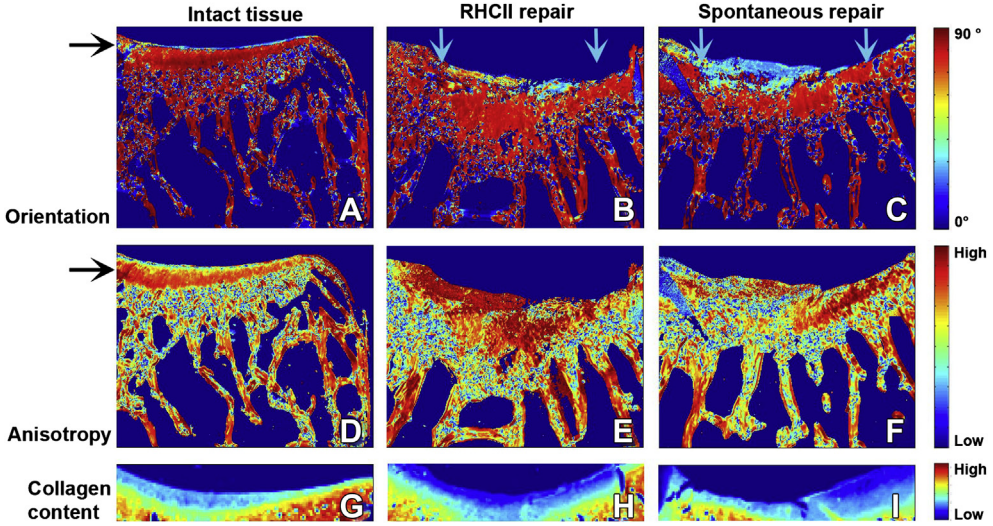

Fig. 5. Polarized light microscopy images demonstrating the collagen fibril orientation and parallelism in the intact and repair tissues as indicated in the photograph panels. The red color represents the perpendicular-to-surface and blue color the parallel-to-surface orientation in Figs. (A–C). In Figs. (D–F) the red color is indicating high and blue color low anisotropy. Collagen content distribution in cartilage is shown in Figs. (G–I) in the FTIR images with red color representing high and blue color low collagen content.

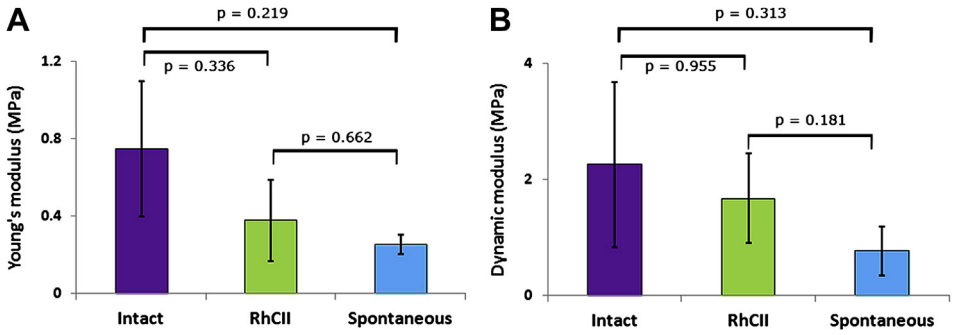

Fig. 6. The equilibrium modulus (Young's modulus or elastic modulus E) (A) and dynamic modulus (B) of the intact ($n = 7$), rhCII ($n = 8$) and spontaneously ($n = 6$) repaired tissue. Data is presented as mean (error bars show 95% CIs).

significant changes in conjunction with biochemical and structural alterations[28]. In developing rabbits the highest dynamic modulus was measured in the maturing femoral cartilage at 6 weeks of age[28]. In the present study the relatively high dynamic modulus in the rhCII-repaired cartilage implies that the deeper part of the repair tissue had restored its functional composition. The parallelism of the collagen fibrils in the rhCII-repair tissue was close to that in the intact cartilage, which may have resulted in better biomechanical properties, particularly in the dynamic modulus, over the spontaneous repair tissue. Furthermore, the horizontally organized collagen fibrils potentially improved the dynamic modulus in the rhCII-repairs. However, there was high variation in the mechanical measurements, because the heterogeneous structure of the repaired samples made the mechanical measurements challenging. The potential variations in the stiffness of underlying bone can also complicate the determination of cartilage mechanical properties using indentation tests, despite the fact that the subchondral bone is assumed to be infinitely stiff. No major changes in the bone μCT parameters were observed, which could explain the changes in the dynamic moduli of the rhCII-repaired cartilage.

Earlier data have demonstrated that the integration of the repair tissue with the adjacent healthy cartilage is problematic[35,36] and several factors present in the damaged synovial joint can inhibit or weaken the integration[37,38]. In the present study no integration enhancers were used, and the lateral integration of the repair tissue was incomplete in both rhCII and spontaneous repair groups. Although some part of this disintegration was an artifact due to the tissue processing, the bonding of the repair tissue was not tight enough to remain in contact with the adjacent tissue through the sample processing. Conversely, the attachment to the subchondral bone occurred well in both repair groups. In inspection of the repair tissue the tidemark area seemed to better restored in the rhCII-treated lesions, and it is possible that the use of rhCII have provided some protection to the remodeling of the tidemark during the repair growth.

Although collagen has been used in various formulas previously, the differences in the experimental set-ups make the comparisons rather difficult. Occasionally, the type I/III collagen membrane has improved the repair[39–42], although benefits have not always been noticed, especially for cell-free scaffolds[14,41,43]. In general the combination of collagen with seeded cells tends to give better repair result[2,12,44]. In the cartilage repair surgery, it is difficult to show direct evidence that the implanted scaffold has remained in the repair area, and for biodegradable materials it is impossible to show residency of the construct after degradation has taken place. The pre-cultivation of the constructs was previously used successfully to aid fitting of the collagen gels in to the defect[4] and to help initial attachment of the repair constructs. The pre-cultivation of the rhCII appeared to provide sufficient attachment of the implants also in our study, and no loose constructs were detected. Since we used osteochondral drilling to create a lesion, the repair site was also filled with blood from the bone marrow, bringing mesenchymal stem cells to the repair site. Previous study using fluorescently labeled chondrocytes for implantation shows that implanted cells can persist in the defect site up to 10 weeks[45]. In this study, the implanted cells were not labeled and, therefore, their presence in the repair tissue cannot be confirmed. However, migration of reparative cells from the bone marrow cannot be excluded, and it is possible that the implanted and migratory cells together participated to the formation of the extracellular matrix in the repair tissue.

In conclusion, the use of rhCII resulted in a slight improvement of the filling and the biomechanical properties of the repair tissue, although histologically the tissue quality did not reach level of the native cartilage. Since no adverse effects of using rhCII emerged during this study the rhCII was shown to be biocompatible for cartilage repair. Therefore, there are no known obstacles for the rhCII to be considered as a scaffold material for human patients, in which the full benefit of using human specific collagen would be achieved.

Author contributions

Hertta J. Pulkkinen: Conception and design, Collection and assembly of data, Analysis and interpretation of the data, Drafting and revising the article and final approval.

Virpi Tiitu: Conception and design, Collection and assembly of data, Drafting the article and final approval.

Piia Valonen: Conception and design, Collection and assembly of data, Drafting the article and final approval.

Jukka S. Jurvelin: Analysis and interpretation of the data, Technical support, Critical revision of the article and final approval.

Lassi Rieppo: Analysis and interpretation of the data, Technical support, Critical revision of the article and final approval.

Juha Töyräs: Analysis and interpretation of the data, Technical support, Critical revision of the article and final approval.

Tuomo S. Silvast: Analysis and interpretation of the data, Technical support, Critical revision of the article and final approval.

Mikko J. Lammi: Conception and design, Obtaining of funding, Drafting and revising the article and final approval.

Ilkka Kiviranta: Conception and design, Obtaining of funding, Drafting the article and final approval.

Role of the funding source

This study was funded by these grant sponsors: Academy of Finland (grant no 206113 and 128177), Sigrid Juselius Foundation, the ComBio project of the Finnish Funding Agency for Technology and Innovation, and TBDP National Doctoral Programme of

Musculoskeletal Disorders and Biomaterials. The rhCII-material was provided without cost by Fibrogen Europe.

Conflict of interest

The study sponsors detailed above did not involve in the study design, data collection or analysis, or in the writing of the manuscript, neither did they affect the decision to submit the manuscript for publication. The authors do not have any relationships that could lead to a financial conflict of interest.

Acknowledgments

Authors would like to thank Juho Marjanen, Sanna Miettinen, Eija Rahunen, Kari Kotikumpu and Eija Sedergren-Varis for technical assistance,Yevgeniya Kobrina for FTI-IRIS measurements, and Reijo Sironen, MD, PhD, for histopathological evaluation.

References

1. Harris JD, Brophy RH, Siston RA, Flanigan DC. Treatment of chondral defects in the athlete's knee. Arthroscopy 2010;26: 841–52.
2. Wakitani S, Kimura T, Hirooka A, Ochi T, Yoneda M, Yasui N, *et al*. Repair of rabbit articular surfaces with allograft chondrocytes embedded in collagen gel. J Bone Joint Surg Br 1989;71:74–80.
3. Frenkel SR, Toolan B, Menche D, Pitman MI, Pachence JM. Chondrocyte transplantation using a collagen bilayer matrix for cartilage repair. J Bone Joint Surg Br 1997;79:831–6.
4. Kawamura S, Wakitani S, Kimura T, Maeda A, Caplan AI, Shino K, *et al*. Articular cartilage repair. Rabbit experiments with a collagen gel-biomatrix and chondrocytes cultured in it. Acta Orthop Scand 1998;69:56–62.
5. Chajra H, Rousseau CF, Cortial D, Ronziere MC, Herbage D, Mallein-Gerin F, *et al*. Collagen-based biomaterials and cartilage engineering. Application to osteochondral defects. Biomed Mater Eng 2008;18:S33–45.
6. Funayama A, Niki Y, Matsumoto H, Maeno S, Yatabe T, Morioka H, *et al*. Repair of full-thickness articular cartilage defects using injectable type II collagen gel embedded with cultured chondrocytes in a rabbit model. J Orthop Sci 2008;13: 225–32.
7. Poole AR, Kojima T, Yasuda T, Mwale F, Kobayashi M, Laverty S. Composition and structure of articular cartilage: a template for tissue repair. Clin Orthop Relat Res 2001;391:S26–33.
8. Salter DM, Hughes DE, Simpson R, Gardner DL. Integrin expression by human articular chondrocytes. Br J Rheumatol 1992;31:231–4.
9. Hirsch MS, Lunsford LE, Trinkaus-Randall V, Svoboda KK. Chondrocyte survival and differentiation in situ are integrin mediated. Dev Dyn 1997;210:249–63.
10. Cao L, Lee V, Adams ME, Kiani C, Zhang Y, Hu W, *et al*. Beta-integrin–collagen interaction reduces chondrocyte apoptosis. Matrix Biol 1999;18:343–55.
11. Nehrer S, Breinan HA, Ramappa A, Hsu HP, Minas T, Shortkroff S, *et al*. Chondrocyte-seeded collagen matrices implanted in a chondral defect in a canine model. Biomaterials 1998;19:2313–28.
12. Lee CR, Grodzinsky AJ, Hsu HP, Spector M. Effects of a cultured autologous chondrocyte-seeded type II collagen scaffold on the healing of a chondral defect in a canine model. J Orthop Res 2003;21:272–81.
13. Efe T, Theisen C, Fuchs-Winkelmann S, Stein T, Getgood A, Rominger MB, *et al*. Cell-free collagen type I matrix for repair of cartilage defects-clinical and magnetic resonance imaging results. Knee Surg Sports Traumatol Arthrosc 2012;20: 1915–22.
14. Christensen BB, Foldager CB, Hansen OM, Kristiansen AA, Le DQ, Nielsen AD, *et al*. A novel nano-structured porous polycaprolactone scaffold improves hyaline cartilage repair in a rabbit model compared to a collagen type I/III scaffold: in vitro and in vivo studies. Knee Surg Sports Traumatol Arthrosc 2012;20:1192–204.
15. Bartlett W, Skinner JA, Gooding CR, Carrington RW, Flanagan AM, Briggs TW, *et al*. Autologous chondrocyte implantation versus matrix-induced autologous chondrocyte implantation for osteochondral defects of the knee: a prospective, randomised study. J Bone Joint Surg Br 2005;87: 640–5.
16. Zheng MH, Willers C, Kirilak L, Yates P, Xu J, Wood D, *et al*. Matrix-induced autologous chondrocyte implantation (MACI): biological and histological assessment. Tissue Eng 2007;13: 737–46.
17. Myllyharju J, Nokelainen M, Vuorela A, Kivirikko KI. Expression of recombinant human type I–III collagens in the yeast *Pichia pastoris*. Biochem Soc Trans 2000;28:353–7.
18. Baez J, Olsen D, Polarek JW. Recombinant microbial systems for the production of human collagen and gelatin. Appl Microbiol Biotechnol 2005;69:245–52.
19. Pulkkinen HJ, Tiitu V, Valonen P, Jurvelin JS, Lammi MJ, Kiviranta I. Engineering of cartilage in recombinant human type II collagen gel in nude mouse model in vivo. Osteoarthritis Cartilage 2010;18:1077–87.
20. Lee CH, Singla A, Lee Y. Biomedical applications of collagen. Int J Pharm 2001;221:1–22.
21. Pulkkinen HJ, Tiitu V, Valonen P, Hämäläinen ER, Lammi MJ, Kiviranta I. Recombinant human type II collagen as a material for cartilage tissue engineering. Int J Artif Organs 2008;31: 960–9.
22. Rieppo J, Töyräs J, Nieminen MT, Kovanen V, Hyttinen MM, Korhonen RK, *et al*. Structure–function relationships in enzymatically modified articular cartilage. Cells Tissues Organs 2003;175:121–32.
23. O'Driscoll SW, Keeley FW, Salter RB. Durability of regenerated articular cartilage produced by free autogenous periosteal grafts in major full-thickness defects in joint surfaces under the influence of continuous passive motion. A follow-up report at one year. J Bone Joint Surg Am 1988;70:595–606.
24. Holmdahl R, Rubin K, Klareskog L, Larsson E, Wigzell H. Characterization of the antibody response in mice with type II collagen-induced arthritis, using monoclonal anti-type II collagen antibodies. Arthritis Rheum 1986;29: 400–10.
25. Hyttinen MM, Arokoski JP, Parkkinen JJ, Lammi MJ, Lapveteläinen T, Mauranen K, *et al*. Age matters: collagen birefringence of superficial articular cartilage is increased in young guinea-pigs but decreased in older animals after identical physiological type of joint loading. Osteoarthritis Cartilage 2001;9:694–701.
26. Rieppo J, Hallikainen J, Jurvelin JS, Kiviranta I, Helminen HJ, Hyttinen MM. Practical considerations in the use of polarized light microscopy in the analysis of the collagen network in articular cartilage. Microsc Res Tech 2008;71:279–87.
27. Camacho NP, West P, Torzilli PA, Mendelsohn R. FTIR microscopic imaging of collagen and proteoglycan in bovine cartilage. Biopolymers 2001;62:1–8.
28. Julkunen P, Harjula T, Iivarinen J, Marjanen J, Seppänen K, Närhi T, *et al*. Biomechanical, biochemical and structural correlations in immature and mature rabbit articular cartilage. Osteoarthritis Cartilage 2009;17:1628–38.

29. Hayes WC, Keer LM, Herrmann G, Mockros LF. A mathematical analysis for indentation tests of articular cartilage. J Biomech 1972;5:541–51.
30. Långsjö TK, Vasara AI, Hyttinen MM, Lammi MJ, Kaukinen A, Helminen HJ, *et al*. Quantitative analysis of collagen network structure and fibril dimensions in cartilage repair with autologous chondrocyte transplantation. Cells Tissues Organs 2010;192:351–60.
31. Changoor A, Nelea M, Methot S, Tran-Khanh N, Chevrier A, Restrepo A, *et al*. Structural characteristics of the collagen network in human normal, degraded and repair articular cartilages observed in polarized light and scanning electron microscopies. Osteoarthritis Cartilage 2011;19:1458–68.
32. Williamson AK, Chen AC, Sah RL. Compressive properties and function–composition relationships of developing bovine articular cartilage. J Orthop Res 2001;19:1113–21.
33. Rieppo J, Hyttinen MM, Halmesmäki E, Ruotsalainen H, Vasara A, Kiviranta I, *et al*. Changes in spatial collagen content and collagen network architecture in porcine articular cartilage during growth and maturation. Osteoarthritis Cartilage 2009;17:448–55.
34. Julkunen P, Iivarinen J, Brama PA, Arokoski J, Jurvelin JS, Helminen HJ. Maturation of collagen fibril network structure in tibial and femoral cartilage of rabbits. Osteoarthritis Cartilage 2010;18:406–15.
35. Shapiro F, Koide S, Glimcher MJ. Cell origin and differentiation in the repair of full-thickness defects of articular cartilage. J Bone Joint Surg Am 1993;75:532–53.
36. Khan IM, Gilbert SJ, Singhrao SK, Duance VC, Archer CW. Cartilage integration: evaluation of the reasons for failure of integration during cartilage repair. A review. Eur Cell Mater 2008;16:26–39.
37. Schaefer DB, Wendt D, Moretti M, Jakob M, Jay GD, Heberer M, *et al*. Lubricin reduces cartilage–cartilage integration. Biorheology 2004;41:503–8.
38. Englert C, McGowan KB, Klein TJ, Giurea A, Schumacher BL, Sah RL. Inhibition of integrative cartilage repair by proteoglycan 4 in synovial fluid. Arthritis Rheum 2005;52:1091–9.
39. Haddo O, Mahroof S, Higgs D, David L, Pringle J, Bayliss M, *et al*. The use of chondrogide membrane in autologous chondrocyte implantation. Knee 2004;11:51–5.
40. Russlies M, Behrens P, Wunsch L, Gille J, Ehlers EM. A cell-seeded biocomposite for cartilage repair. Ann Anat 2002;184:317–23.
41. Willers C, Chen J, Wood D, Xu J, Zheng MH. Autologous chondrocyte implantation with collagen bioscaffold for the treatment of osteochondral defects in rabbits. Tissue Eng 2005;11:1065–76.
42. Giordano M, Aulisa AG, Mastantuoni G, Gigante A, Guzzanti V. Pridie's marrow stimulation technique combined with collagen matrix for cartilage repair. A study in a still growing sheep model. Int J Immunopathol Pharmacol 2011;24:101–6.
43. Dorotka R, Windberger U, Macfelda K, Bindreiter U, Toma C, Nehrer S. Repair of articular cartilage defects treated by microfracture and a three-dimensional collagen matrix. Biomaterials 2005;26:3617–29.
44. De Franceschi L, Grigolo B, Roseti L, Facchini A, Fini M, Giavaresi G, *et al*. Transplantation of chondrocytes seeded on collagen-based scaffold in cartilage defects in rabbits. J Biomed Mater Res A 2005;75:612–22.
45. Dell'Accio F, Vanlauwe J, Bellemans J, Neys J, De Bari C, Luyten FP. Expanded phenotypically stable chondrocytes persist in the repair tissue and contribute to cartilage matrix formation and structural integration in a goat model of autologous chondrocyte implantation. J Orthop Res 2003;21:123–31.