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RESEARCH ARTICLE

Jellyfish collagen matrices conserve the chondrogenic phenotype in two- and three-dimensional collagen matrices

Judith Sewing[1,2]*, Matthias Klinger[3] and Holger Notbohm[2]
[1]*CRM Coastal Research & Management, Tiessenkai, Kiel, Germany*
[2]*Institute of Virology and Cell Biology, University of Lübeck, Lübeck, Germany*
[3]*Institute for Anatomy, University of Lübeck, Lübeck, Germany*

Abstract

Cartilage is a tissue with a very low capability of self-repair and the search for suitable materials supporting the chondrogenic phenotype and thus avoiding fibrotic dedifferentiation for matrix-associated chondrocyte transplantation (MACI) is ongoing. Jellyfish collagen was thought to be a suitable material mainly because of its good availability and easy handling. Collagen was extracted from jellyfish *Rhopilema esculentum* and the spreading of porcine chondrocytes on two (2D) and three dimensional (3D) collagen matrices examined in comparison with vertebrate collagens, placenta collagen and a commercially available matrix from porcine collagen type I (Optimaix®). In 2D, most chondrocytes kept their round shape on jellyfish collagen and vertebrate collagen type II compared with vertebrate collagen type I. This was also confirmed in 3D experiments, where chondrocytes preserved their phenotype on jellyfish collagen, as indicated by high collagen II/(II + I) ratios (≥54 % and ~92 % collagen type II in mRNA and protein, respectively) and no proliferation during 28 days of cultivation. These observations were discussed with a view to potential structural differences of jellyfish collagen, which might influence the integrin-mediated adhesion mechanisms of vertebrate cells on jellyfish collagen. This probably results from a lack of integrin-binding sites and the existence of an alternative binding mechanism such that cells kept their round shape on jellyfish collagen, preventing chondrocytes from dedifferentiation. Thus, collagen from *R. esculentum* is a very suitable and promising material for cartilage tissue engineering. Copyright © 2015 John Wiley & Sons, Ltd.

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1. Introduction

Cartilage is, because of its low metabolic rate, a tissue with a very limited capacity of self-repair. Hence several approaches have been made to stimulate the regeneration (e.g. by microfracturing or by transplanting autologous chondrocytes). A very promising method is the matrix-associated chondrocyte transplantation matrix-associated chondrocyte transplantation (MACI) (Behrens *et al*., 1999). Here, matrices are loaded *in vitro* with autologous cells and subsequently implanted into the lesion. The matrices are thought to ensure a homologous spreading of the cells *in situ* and to support the chondrogenic phenotype, thereby refilling the lesion with newly synthesized material. Nevertheless, no complete repair of the defect with hyaline, articular cartilage has yet been observed (Anders *et al*., 2008).

Collagen is a very suitable material as it is bioresorbable and allows a natural cell–matrix interaction. It is a very smart material as it can be utilized as a gel or sponge, or in molecular or fibrillar form and it can be conjugated with other substances such as glycosaminoglycans (GAG) (Galois *et al*., 2006; Murphy *et al*., 2010). Collagen type I is most commonly used for the construction of matrices,

*Correspondence to: Judith Sewing, CRM Coastal Research & Management, Tiessenkai 12, D–24159 Kiel, Germany. E-mail: judith.sewing@crm-online.de

sometimes in combination with type III collagen (Nehrer *et al*., 1997; Pieper *et al*., 2002; Freyria *et al*., 2004; Schlegel *et al*., 2008). However, collagen type II, being the major collagen in cartilage is more suitable for the purpose (Nehrer *et al*., 1997, 1998; Freyria *et al*., 2009), but it is known to induce arthritis (DeSimone *et al*., 1983). Mainly vertebrate collagens (VC) are utilized today, but the use of porcine or bovine collagens holds the risk of transferring diseases such as bovine spongiform encephalopathy (BSE) (Standard Guide F 2212 ASTM International), which is not the case with invertebrate collagens (Prusiner, 1998; Imran and Mahmood, 2011). A suitable alternative to VC II might therefore be jellyfish collagen (JFC), not least because of good availability and easy handling.

The main interest in JFC rose with its utilization as food in Asia (Kimura *et al*., 1983). Since that time, its application in the treatment of rheumatoid arthritis (Hsieh, 2005), its antioxidative activity (Zhuang *et al*., 2009) and its potential use in biomedical applications (Calejo *et al*., 2009; Addad *et al*., 2011), and as three dimensional (3D) matrix (Song *et al*., 2006) were examined, and it proved to possess qualities comparable to vertebrate collagen (VC). Meanwhile the suitability of JFC for tissue engineering of cartilage has been proved (i.e. reconstruction of nasal septum and knee respectively), indicating that this collagen supports the chondrogenic phenotype and even promotes redifferentiation of cells previously cultured in a monolayer, without showing any cytotoxicity (Bermüller *et al*., 2013; Hoyer *et al*. 2014). However, a comparison with other materials was still pending, as was the elucidation of the direct impact of JFC on porcine chondrocytes. Our aim was therefore to test the suitability of JFC matrices for chondrocytes concerning survival, conservation of phenotype and matrix reproduction in comparison with vertebrate collagen type I and II. Spreading assays were conducted to obtain an overview of the adhesion behaviour of porcine chondrocytes (pChs) to JFC in comparison with VCs. In a second step, 3D JFC matrices were compared with self-manufactured matrices from human placenta collagen (HPC) and to Optimaix® (Matricel, Germany). Optimaix is a 3D matrix derived from porcine collagen intended for cell culture applications, representing porcine matrices already used in MACI. HPC served as control to investigate the effect of the manufacturing process as well as the biochemical properties of the collagens on porcine chondrocytes. The indicator for a suitable matrix was the conservation of the chondrogenic phenotype of freshly isolated pChs, which was considered to be evident in high collagen II expression on mRNA and protein level and low proliferation rates. Histological analyses supported the results visually.

2. Materials and methods

All chemicals were of p.a. grade. Unless stated otherwise, all steps were carried out at 4 °C.

2.1. Collagen extraction and scaffold construction

Collagen from cured jellyfish *Rhopilema esculentum* (Liroy B.V., Rotterdam, Netherlands) was extracted by pepsin digestion as published in Stötzel *et al*. (2012). It was stored at –20 °C and freeze-dried shortly before use.

For the preparation of human placenta collagen I/III based upon Miller (1984), a placenta was obtained from a patient of the department of gynaecology and obstetrics of the university hospital Lübeck after giving written informed consent. The placenta was rinsed several times in tap water, dissected into small pieces and washed for 12 h in 1 M NaCl. After swelling the tissue in 0.1 M NaCl and 0.1 M NaH_2PO_4 at pH 1.5 overnight the material was homogenized with an ultraturrax (IKA, Staufen, Germany) and subsequently digested over night with 2500 U/mg pepsin derived from porcine gastric mucosa (Roche, Germany) at a calculated concentration of 10 mg enzyme per gram of dried tissue. Non-digested material was separated by ultracentrifugation at 140 000 *g* for 1 h. The total collagen contained in the supernatant was precipitated with 4.5 M NaCl and pelleted by ultracentrifugation. These pellets were resuspended and dialysed against 0.05% acetic acid for 2 days. The total collagen was purified in a second precipitation step with 1.2 M NaCl followed by ultracentrifugation. Upon resuspension and dialysis of the resulting pellets against 0.05% acetic acid, collagen type V was extracted by 0.7 M NaCl precipitation and pelleted by ultracentrifugation. The remaining supernatant contained collagen type I and III, which was again dialysed and finally lyophilized.

For JFC and HPC scaffold construction, lyophilized collagen was suspended over night at 20 mg/ml in 0.05% acetic acid and injected into polyoxymethylene (POM) casting moulds, enclosing the scaffold from all sides and leaving two little holes for injection. The scaffolds were frozen at a rate of 0.1–0.2 °C/min down to –20 °C and afterwards freeze-dried in a UVS400A Lyophilizator (Thermo Scientific, Waltham, MA, USA) at room temperature. They measured 3 mm in height and 6 mm in diameter. Crosslinking was then induced with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; Thermo Scientific, Germany), which has been previously proved to be a non-toxic crosslinker (e.g. Bermüller *et al*., 2013). Scaffolds were incubated in a 0.5% w/v EDC solution and finally washed as described elsewhere (Bermüller *et al*., 2013). The EDC concentration was adjusted beforehand to achieve rigidities comparable to the stiffness of Optimaix to rule out undesired effects owing to variations in stiffness (Engler *et al*., 2006; Schuh *et al*., 2010).

Porcine collagen I (GP2; Matrix BioScience GmbH, Germany) and bovine collagen II (University of Lübeck) served as vertebrate collagen controls.

2.2. Analysis of scaffolds

Stiffness was measured as described elsewhere (Bermüller *et al*., 2013).

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In order to measure thermal resistance by differential scanning calorimetry (DSC), the washed and thoroughly dried scaffolds were sealed in aluminium melting pans (BO 14–3003 and 14–3017; Perkin Elmer) and measured in a Pyris 6 DSC (Perkin Elmer) at a heating rate of 2 °C/min. The melting temperature was calculated as a function of the peak temperature using acquisition software (Perkin Elmer).

For scanning electron microscopy (SEM), the samples were placed on aluminium slides and rotary sputtered with platinum followed by examination in a SEM 505 (Philips, Eindhoven, Netherlands). The pore size was measured on SEM-images with AxioVision software (Zeiss, Germany).

2.3. Cell isolation and culture in 3D

Porcine chondrocytes were isolated as described previously (Schuh *et al*., 2010). Hyaluronidase and collagenase II were purchased from Worthington (Lakewood, USA) and collagenase P from Roche. The scaffolds were placed in a 96-well plate and seeded with 60 µl of cell suspension in RPMI medium (Biochrom, Germany) to a final cell concentration of 1×10^6 cells per scaffold. Squeezed JFC and HPC scaffolds were soaked in cell suspension and Optimaix scaffolds (Matricel) were seeded following the manufacturer's instructions by dripping cell suspension on the dry sponge. Each scaffold was immediately overlaid with 200 µl RPMI medium and cells were allowed to settle for 3–4 h at 37 °C and 5% CO_2. The scaffolds were then transferred to 24-well plates coated with 2% w/v alginate and cultured for 4 weeks with RPMI medium supplemented with 35 µM 2-phospho-L-ascorbic acid trisodium salt (Sigma, Germany). Medium was changed every 2–3 days, samples from different time points were washed briefly in phosphate-buffered saline (PBS) and frozen immediately in liquid nitrogen. Scaffolds intended for histology were embedded in CryoMolds with Tissue Tek (Sakura, Torrance, CA, USA) and stored at –20 °C. A portion of the isolated porcine chondrocytes intended for the spreading assay was frozen as described in Schuh *et al*. (2010).

2.4. Spreading assay

A 96-well plate (TPP Techno Plastic Products AG, Switzerland) was coated with 30 µg/ml collagen (JFC, HPC, VC I and II) in 0.05 % acetic acid and allowed to dry for 2 days under sterile conditions to generate a coating of 4.5 µg/cm^2. To avoid denaturation of JFC, all types of coating were crosslinked with 0.5% EDC as described above and finally potentially non-occupied areas were blocked with 1 % BSA (fraction V; Roth, Germany). Frozen pChs were thawed, cultivated for one night at high density of 13×10^6 cells/T75 flask, harvested and resuspended in an appropriate volume of RPMI medium. Approximately 20,000 cells were then seeded per 96-well and incubated for 24 h. The different phenotypes were microscopically assessed with two images taken from three replicates, using an Axiovert 40C microscope (Zeiss). Cells were counted manually supported by using Image J 1.45 s software (National Institutes of Health, Bethesda, MD, USA) whereby distinction was made between flattened cells and all others, the latter including round cells and edge cases (Schlegel *et al*., 2008).

2.5. Analysis of cell culture on scaffolds

For all analyses except histology, scaffolds were incubated in buffers chosen with a view to downstream processing and homogenized in Precellys tubes with 2.8 mm spheres in a homogenizer (both Peqlab, Germany) for 3×15 s at 5000 rpm.

mRNA for reverse-transcription polymerase chain reaction (RT-PCR) was purified using the NucleoSpin® RNA II kit (Macherey & Nagel, Germany). For this purpose, the scaffolds were homogenized in 353.5 µl of RA 1 buffer containing β-mercaptoethanol (Sigma). The RNA concentration was assessed with NanoDrop 2000c (Thermo Scientific, Germany) and, if necessary, RNA was concentrated with 5 M ammonium acetate (Ambion®; Life Technologies Corp, USA) and redissolved in water. Then 500 ng RNA were then transcribed to cDNA (Revert Aid TM H Minus First Strand cDNA Synthesis Kit; Fermentas, Germany) and RT-PCR was carried out in an iCycler (BioRad, Germany) using the Maxima® Probe/ROX qPCR Master Mix (Fermentas). Primer and probe sequences are provided in Table 1.

To measure proliferation, scaffolds were homogenized in 500 µl of 1% Triton X100 in PBS, centrifuged and DNA content in the supernatant was analysed using Quant-iT™ PicoGreen® dsDNA Kit (Invitrogen) on a Mithras 940 plate reader (Berthold Technologies) in black 96-well plates (Nunc; Thermo Scientific, Germany). The DNA standard included in the kit was used to plot a

Table 1. Sequences of primers (PM) and probes (PO)

Name	Producer	Type	
COL1A1 S	TIB Molbio	PM	5′-GTGATCTGCGACGAAATCAAGAA
COL1A1 A	TIB Molbio	PM	5′-GGGTGACACCTCGCCTTC
COL1A1 TM	TIB Molbio	PO	5′-6AFM-TGTCCCAGCGCCAGAGTCCCT – BBQ
51_COL_FW1	Microsynth	PM	5′-GGGCAACGACGGTCAACCAG
51_COL_REV1	Microsynth	PM	5′-CTTGGCACCGGGAGCACCAG
51_COL_PROBE1	Microsynth	PO	5′FAM-CCAGGACCGCCAGCAGGACCCACG-BHQ1

standard curve. The mean of two or three measurements of each scaffold represent one replicate.

Protein was analysed immunologically on slot blot. Scaffolds were homogenized in 250 µl of 0.1% pepsin in acetic acid at pH 2.8 and incubated overnight at 4 °C on a shaker to digest all non-collagenous proteins. Digestion was stopped by adding one drop of 1 M Tris-Base pH 7. After centrifugation at 16 000 **g** for 20 min, supernatants were lyophilized and subsequently resuspended in an appropriate volume of water. The following steps were carried out at room temperature. Samples and positive controls of bovine collagen type II and porcine collagen type I (University of Lübeck) were loaded onto a nitrocellulose membrane (BioRad) using a 72-well slot-blot array system (Minifold™ II; Whatman, Germany) where sample volume of the replicates was adjusted to obtain a sufficient fluorescence signal. Membranes were dried, blocked for 2 h in blocking solution, composed of 5% skimmed milk powder (Fluka; Sigma Aldrich, Germany) in TBS-T (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.05% Tween 20) and incubated for 2 h with 0.13 ml/cm^2 of the respective primary antibodies, each diluted in blocking solution. The antibodies were 1:1250 diluted mouse anti type I collagen (ab90395, mouse monoclonal; Abcam, Germany) and 1:1000 diluted mouse anti II collagen (II-II-6B3 Developmental Studies Hybridoma Bank, University of Iowa, USA). Membranes were washed in TBS-T, equilibrated for 10 min in alkaline phosphatase (AP) buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) and stained in 0.2 ml/cm^2 with BCIP/NBT (Roth) in AP buffer. Finally, membranes were rinsed in water and dried. images were taken in a scanner and analysed densitometrically with Image J 1.45 s.

For histological analyses, scaffolds embedded in Tissue Tek were vertically sectioned using a Leica CM3050 cryotome to 10 µm slices from the centre of the scaffolds. Sections were mounted onto microscope slides and left to air-dry overnight at room temperature. All staining was carried out at room temperature.

For Alcian blue staining, samples were fixed in 4% formalin for 1 h, washed briefly in PBS and water and then incubated for 2 min in 3% acetic acid. Sulphated GAGs were stained overnight with 0.5 % Alcian blue in 0.5% acetic acid. After washing the slides in water, cell nuclei were counterstained for 3–5 min in nuclear fast red ammonium salt. Following a final wash, the slides were air dried and covered with Entellan (Merck, Germany). The dyes were purchased from Roth.

Immunostaining for collagen type II was performed following Schuh *et al*. (2010). Staining for collagen I was carried out with a dilution of the primary antibody of 1:50. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma). All samples were analysed with an Axioplan 2 microscope (Zeiss) using the acquisition software.

2.6. Statistical analyses

Statistical analyses were performed with Graph Pad Prism software 5.03 (GraphPad Software, Inc., USA). Statistical significance was calculated with one-way ANOVA followed by Dunn's multiple comparison test with a significance level of $p < 0.05$. All results are displayed as scatter plots or as mean ± standard deviation.

3. Results

3.1. Properties of collagen scaffolds

Stiffness was variable according to different EDC concentrations, whereas a saturation of crosslinks was achieved at a concentration of about 1 % EDC (Figure 1). Scaffold types JFC, HPC and Optimaix differed in some features, mainly in pore size, owing to the method of preparation (Table 2). Both JFC and HPC scaffolds had a comparable pore size in a honeycombed structure, whereas pores in Optimaix scaffolds were of a tunnel-like structure with very long pores (multiples of diameter; see also Figure 2). Nevertheless, they all showed a comparable stiffness from 10.4 ± 0.6 to 13.4 ± 2.2 kPa and appropriate crosslinking, which became evident in an increased melting temperature ≥ 50 °C.

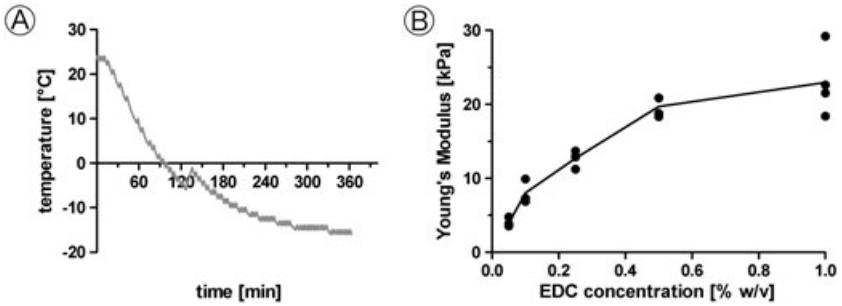

Figure 1. (A) Typical thermogram during scaffold freezing. The freezing rate was calculated from the linear slope between +5 and –5 °C (here –0.2 °C/min). The peak after 120 min is caused by heat of crystallization of the solvent. (B) Correlation of crosslinker concentration (1-ethyl-3-(3-dimethylaminopropyl)carbodiimid hydrochloride, EDC) and Young's Modulus of the scaffolds. With increasing EDC concentration an increase of stiffness occurred, whereas owing to saturation no higher stiffness was expected at concentrations > 1.0% (n = 3–4)

Table 2. Summary of physical and general properties of scaffolds

Name	Height × diameter (mm)	Collagen	Concentration of original material (mg/ml)	Stiffness (kpa)	Melting point (°C)	Pore size (length × width) (μm)
HPC	3 × 6	Type I/III with residual V, human	20	10.4 ± 0.6	68.5 ± 0.7	200 × 70
JFC	3 × 6	Type II like, jellyfish	20	12.3 ± 3.7	55.5 ± 0.4	190 × 90
Optimaix	3 × 5	Type I with residual elastin, porcine*	15*	13.4 ± 2.2	51.6 ± 0.8	90 (width)

HPC, human placenta collagen; JFC, jellyfish collagen. Optimaix is a commercially available matrix from porcine collagen type I (Matricel).
*Data from Saxena *et al*. (2009).

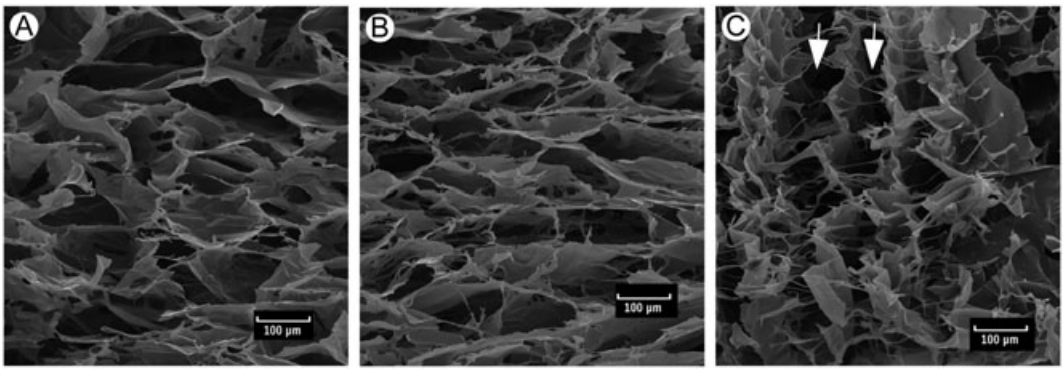

Figure 2. Scanning electron photomicrographs of scaffolds. (A) Jellyfish collagen (JFC) scaffold, (B) human placenta collagen (HPC) scaffold, (C) Optimaix; arrowheads indicate channel directions. Bars: 100 μm

3.2. Spreading assay

No significant difference in the number of attached cells per visual field was detectable 24 h after seeding (Figure 3A), whereas a significant difference was observed between the fractions of non-flattened cells: 80.1 ± 7 and 93.2 ± 1.6 % of cells on JFC and VC type II, respectively, retained a round shape, whereas 38.7 ± 12.1 and 53.8 ± 17.5 % on HPC and collagen I, respectively, retained a round shape (Figure 3B). The data indicate that pChs recognized a difference between these collagens through the adhesion of their receptors.

3.3. 3D cell culture

During 28 days of cell culture, no shrinking of any type of matrix was observed. mRNA analyses were expressed as the portion of type II from the sum of both collagens I

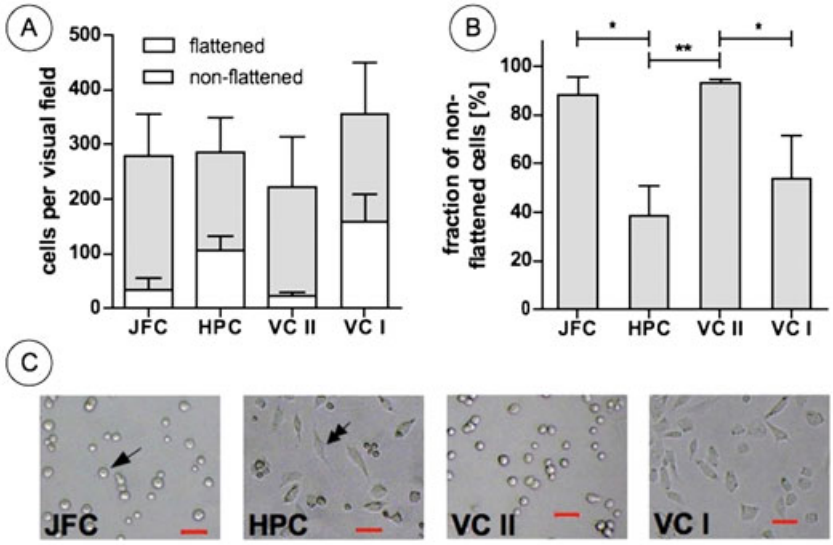

Figure 3. Results from spreading assay of porcine chondrocytes (pChs) on different substrates after 24 h. (A) flattened and non-flattened cells per visual field. Sums of cells were not significantly different ($n = 6$). (B) Fractions of non-flattened cells per visual field. *$p < 0.05$; **$p < 0.01$. (C) Details of pictures from microscope with examples of flattened (double arrows) and round cells (normal arrows). JFC, jellyfish collagen; HPC, human placenta collagen; VC, vertebrate collagen. Bars: 64 μm. [Colour figure can be viewed at wileyonlinelibrary.com]

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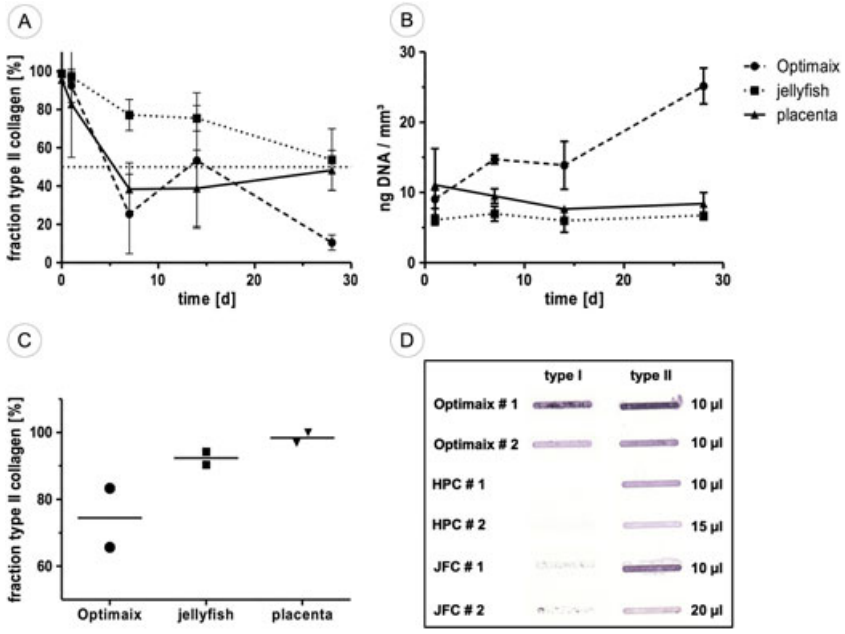

Figure 4. Response of cells on different matrices in terms of mRNA-expression, proliferation and protein synthesis in a 28-day culture period. (A) Reverse-transcription polymerase chain reaction analysis of collagen I and II expression, shown as fraction of type II collagen of the sum ($n = 4$). (B) DNA content over 28 days of cell culture ($n = 3$–4). (C,D) Slotblot protein analysis of protein expression after 28 days of culture. Dots represent measurements of two scaffolds: (C) densitometric analysis of blot; (D) slotblot; applied volumes for each replicate are indicated. JFC, jellyfish collagen; HPC, human placenta collagen. Optimaix is a commercially available matrix from porcine collagen type I. [Colour figure can be viewed at wileyonlinelibrary.com]

and II in per cent. Freshly isolated cells at moment of seeding expressed almost 100 % collagen II (Figure 4). During culture, the cells on JFC scaffolds expressed a greater portion of collagen II (at least 75.4 ± 6.6 %) than the cells on HPC scaffolds (38.4 ± 13.8 %) and Optimaix (25.4 ± 20.1 %). On day 28, cells on JFC expressed 53.8 ± 16.1 % collagen type II and cells on HPC slightly less (48.2 ± 10.4 %). The cells on Optimaix expressed the lowest percentage of collagen II mRNA (10.5 ± 4.0 %).

The collagen II protein expression on day 28 was also calculated as per cent of the total collagen. None of the scaffolds themselves reacted with the antibodies (data not shown). The cells on JFC (~94 %) and HPC (~98 %) showed clearly higher expressions of collagen II than cells on Optimaix (~75 %). These results reflect the data from RT-PCR for JFC and Optimaix, but not for HPC, which showed a lower collagen II mRNA expression. This discrepancy might result from a post-transcriptional regulation of mRNA or different mRNA and protein metabolic rates (Fu *et al.*, 2007).

The cell seeding on Optimaix and JFC scaffolds proved to be similarly effective. In contrast, the HPC scaffolds took up about twice as many cells as JFC (Figure 4). In JFC and HPC scaffolds, no distinct alteration in the DNA content was detectable, whereas in Optimaix the DNA content increased continuously from 9.1 ± 1.3 to 25.2 ± 2.5 ng/cm^3, indicating proliferation.

The histological staining of glycosaminoglycans and cell nuclei after 28 days of culture showed a homologous spreading of the cells in the scaffolds with more patches of newly synthesized GAGs in JFC and HPC scaffolds (Figure 5). In all scaffold types the largest deposits were found on the edges. After immunostaining, HPC and Optimaix matrices themselves showed initially a slight collagen I staining, which was clearly distinguishable from newly synthesized collagen by structure (Figure 6).

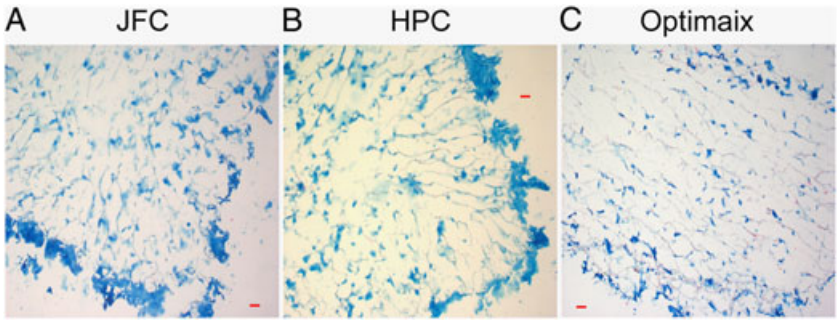

Figure 5. Alcian blue staining of scaffolds after 28 days in culture, 10 µm. (A) Jellyfish collagen (JFC) matrix; (B) human placenta collagen (HPC) matrix; (C) Optimaix (a commercially available matrix from porcine collagen type I). Bars: 100 µm. [Colour figure can be viewed at wileyonlinelibrary.com]

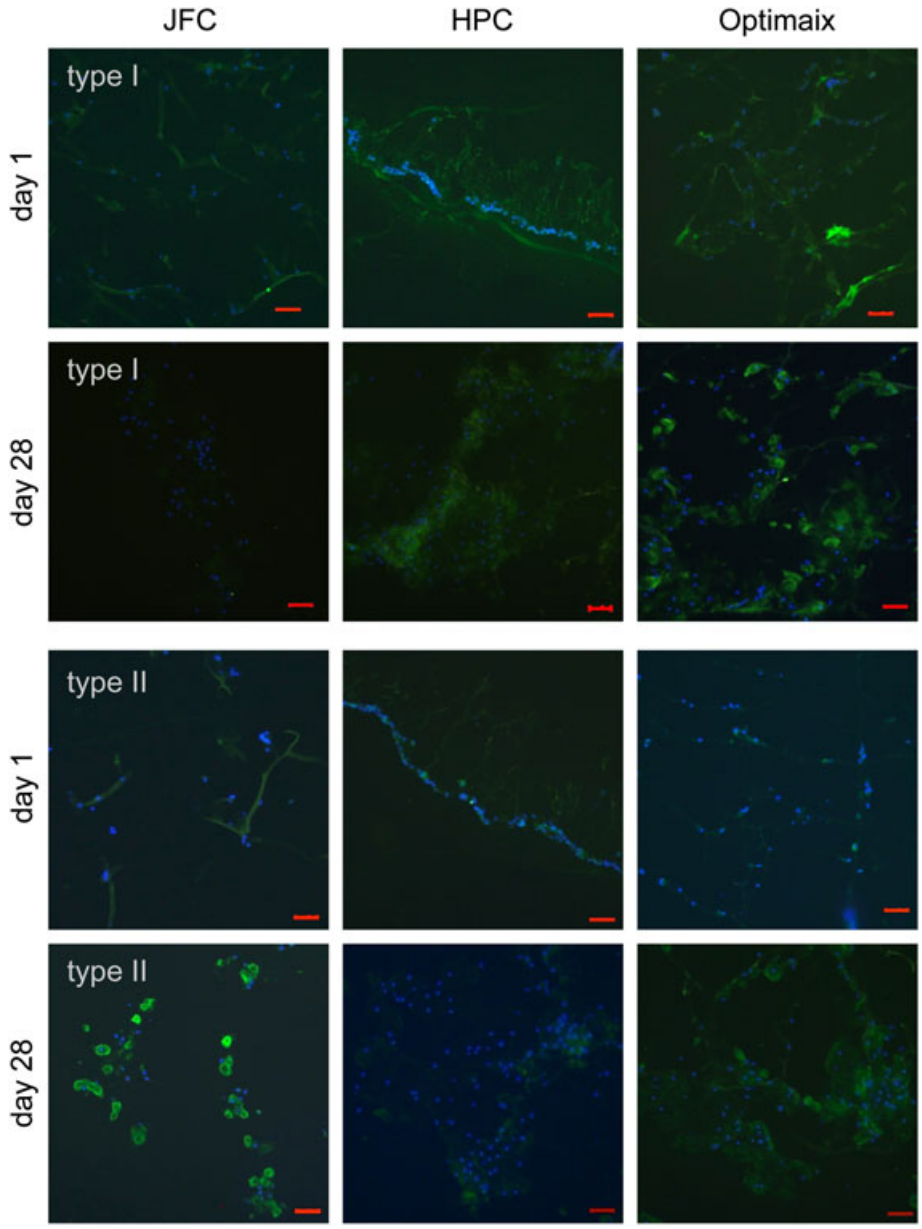

Figure 6. Immunological staining of samples for collagen type I and type II, respectively, after 1 day and 28 days in cell culture; 10 µm slices. Bars: 50 µm. JFC, jellyfish collagen; HPC, human placenta collagen. Optimaix is a commercially available matrix from porcine collagen type I. [Colour figure can be viewed at wileyonlinelibrary.com]

None of the matrices exhibited collagen type II staining. After 28 days of cell culture, JFC and HPC matrices contained more collagen type II than type I, which was clearly reversed in the Optimaix matrix. These findings underlined the results of the protein analysis.

4. Discussion

We examined the spreading of pChs on JFC compared with VC and later analysed the ability of JFC, HPC and Optimaix 3D matrices to conserve the chondrogenic phenotype and thus to produce constructs close to hyaline-like cartilage *in vitro*.

Collagens from jellyfish are comparable to VC in appearing as homotrimers or heterotrimers and have a typical glycine content of about 30%. Nevertheless, collagens from Cnidaria are known to be rather highly glycosylated (Kimura *et al.*, 1983; Miura and Kimura, 1985) with a low hydroxyproline content, associated with a low melting temperature (Miura and Kimura 1985; Mizuno *et al.*, 2003). It was possible to prove these facts and the homotrimeric composition of collagen from *R. esculentum* (Zhuang *et al.*, 2009) in this laboratory (data not shown) which prompted the hypothesis that JFC closely resembles vertebrate collagen type II and might thus be very suitable for the cultivation of chondrocytes.

In a spreading assay pChs developed different phenotypes on JFC, HPC and VC collagen type I and II,

respectively, with the chondrogenic phenotype being best conserved on JFC and VC type II. We presume that this effect results from the mechanism with which pChs attach themselves to collagen. Differentiated chondrocytes appear to be of spherical to ovoid shape (Nehrer *et al*., 1997; Benya and Shaffer 1982; Schlegel *et al*., 2008) combined with high expression of collagen type II, low expression of collagen type I (Schuh *et al*., 2010) and a low proliferation rate (Nehrer *et al*., 1997; Freyria *et al*., 2009). In contrast, the flattening of chondrocytes is associated with an increase in actin organization (Glowacki *et al*., 1983; Benya *et al*., 1988), resulting in a dedifferentiation of cells, as cell morphology and actin cytoskeleton organization promote the entry into the cell cycle (Iwig *et al*., 1995). These cellular responses are driven by the type of receptor with which cells bind to the extracellular matrix (ECM).

Chondrocytes bind to vertebrate fibrillar collagens through a multitude of receptors, mainly integrins, DDR2 and syndecans (reviewed in Leitinger and Hohenester, 2007; Couchman 2010). Integrins α1β1, α2β1, α10β1 and α11β1 recognize the GXX GER-motif; this could be GFPGER, GLPGER or GASGER (Xu *et al*., 2000). Representatives of integrins have been found in invertebrates and in jellyfish (Reber-Müller *et al*., 2001), although a direct binding to collagen has not yet been described (Ağbaş and Sarras, 1994; Heino *et al*., 2009), binding via intermediate proteins such as fibronectin has been discussed (Heino *et al*., 2009). In sequences from *Hydra vulgaris* (NCBI accession ABG80452.1), *Hydra magnapapillata* (XP_002164888.1) and jellyfish *Podocoryna carnea* (CAA08789.1), although the GXX GER-motif is not present, slightly diverging sequences exist, which probably allow attachment of mammalian cells to JFC via integrins with a lower affinity (Xu *et al*., 2000; Addad *et al*., 2011).

In addition, DDR-like proteins have been found in invertebrates, but it is not yet known whether they can bind to collagen (Heino *et al*., 2009). Owing to a missing binding motif (Konitsiotis *et al*., 2008; Xu *et al*., 2011) in the sequences analysed here, binding of mammalian cells to JFC via DDR might be excluded.

It has been shown that JFC evokes flattening of osteoblasts to a lesser degree than VC I. Instead of integrins, mammalian cells mainly attach to JFC via heparan sulphate (up to 80 %; Addad *et al*., 2011), i.e. transmembrane homodimeric syndecans (Couchman 2010). Syndecan-4 induces building of focal adhesions together with α5β1-integrin, but it also regulates the contraction of cytoskeleton (Xian *et al*., 2010) by the activation of RhoA by a PKCα or directly by α-actinin (Xian *et al*., 2010; Okina *et al*., 2012). In contrast, transmembrane integrins regulate the cytoskeleton by the activation of RhoA by tyrosine kinases FAK and Src (Xian *et al*., 2010). Although syndecans also directly influence the cytoskeleton, it is conceivable, because of the different signal cascades, that cell reaction in gene expression might be different.

The clear difference in the phenotypes on collagen I and collagen II might not be based on the integrin binding, as analyses of the sequences of human collagen I (α1: P02452.5; α2: P08123.7) and II (CAA34683.1) showed a similar number of integrin binding sites. In contrast, a hydrate shell of the hydrophilic sugars of the highly glycosylated collagen type II might lead to steric hindrance of the cells during binding (Yang *et al*., 1993), thus impairing cell attachment and spreading (Liao *et al*., 2009).

Aside from the quality of the binding, the quantity also plays a crucial role. Following a receptor saturation model (Gaudet *et al*., 2003) a cell is rounded at very low or very high substrate density, whereas at moderate substrate density the cell has to flatten to bind all receptors. This model might explain the conservation of spherically shaped chondrocytes in 3D matrices with no binding sites (Benya and Shaffer, 1982) as well as in those with abundant binding sites (Galois *et al*., 2006), and the influence of substrate density in 2D surfaces (Engler *et al*., 2004; Erickson *et al*., 2009). Hence, the rounded phenotype on JFC might result from the scarcity of integrin binding sites. In contrast, VC I offers many integrin binding sites but not in high density, resulting in a flattening of cells. Finally, VC II with a similar number of integrin binding sites leads to rounding of the cells, probably because of its high glycosylation. Adhesion to collagen is a very complex event that depends on many factors, including the receptors themselves, which influence each other (Morgan *et al*., 2007; Sweeney *et al*., 2008). Presumably, several binding processes overlap and interact, which prevents us from achieving a comprehensive interpretation of our results. Hence, individual binding processes of mammalian cells to JFC will have to be elucidated in further projects.

In 3D matrices, our analyses showed that pChs kept their chondrogenic phenotype on JFC matrices comparable to matrices from placenta collagen, as indicated by a high collagen II ratio in protein and mRNA levels and a low proliferation rate. Usually, histological analyses hardly allow a quantitative evaluation, and therefore merely serve to visually confirm data. A deposition of GAG was detected by Alcian blue staining. It was not appropriate to quantify the amount of GAG, as a major characteristic of hyaline cartilage is the collagen composition (i.e. a high type II content) (Galois *et al*., 2006). All scaffolds showed a uniform distribution of cells with no remarkable shrinking even after 28 days in culture, although this was not always the case in previous works (Nehrer *et al*., 1997; Pieper *et al*., 2002; Freyria *et al*., 2009). Comparison of the present data with literature was difficult because of different experimental set-ups and data description. In particular, the use of a ratio of collagen I and collagen II (Galois *et al*., 2006) in previous works would have made results comparable. With a portion of ~54 % (mRNA) and ~94 % (protein) newly synthesized collagen II the JFC matrix is comparable to other materials (Nehrer *et al*., 1998; Pieper *et al*., 2002; Freyria *et al*., 2004). The differences between the results from JFC and Optimaix might be ascribed to the different pore structures (Nehrer *et al*., 1997; Murphy *et al*., 2010),

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as the HPC matrix with a similar pore structure to JFC matrices showed comparable results. But the spreading assay demonstrated that the differences could also be ascribed in some part to the different collagens, although it should be heeded that adhesion in 2D and 3D is clearly different (reviewed in Cukierman *et al*., 2001). The remarkable difference between the conserving potential of HPC in 3D compared with the large number of flattened cells in 2D is probably attributable to the differences in stiffness, which is a multiple on collagen-coated plastic compared with the 3D matrix. However, as the 3D matrices in the present experiment showed a very similar stiffness of about 10–13 kPa, an effect of stiffness could be excluded here.

A low stiffness of the matrix is known to support the chondrogenic phenotype (Galois *et al*., 2006; Freyria *et al*., 2009; Schuh *et al*., 2010). In 2D, a preferable stiffness is ~4 kPa (Schuh *et al*., 2010), which was not achievable in scaffolds without shrinking. Whether this value would also be preferable in 3D has yet to be determined, but owing to the limited binding of cells to JFC via integrins it is questionable whether stiffness plays a role anyway. However, it should be kept in mind that a product for medical application should be inherently stable.

This study has shown the potential of JFC to conserve the chondrogenic phenotype of porcine chondrocytes in 2D and 3D matrices, which might originate from a different binding mechanism of mammalian cells to JFC because of poor integrin binding sites. Together with its good availability and handling, JFC is thus a most promising material for the application in biomedical devices, especially for the regeneration of human cartilage.

Conflict of interest

The authors declare no conflict of interest.

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References

Addad S, Exposito J-Y, Faye C *et al*. 2011; Isolation, characterization and biological evaluation of jellyfish collagen for use in biomedical applications. *Mar Drugs* **9**: 967–983.

Ağbaş A, Sarras MP. 1994; Evidence for cell surface extracellular matrix binding proteins in *Hydra vulgaris*. *Cell Adhes Commun* **2**: 59–73.

Anders S, Schaumburger J, Schubert T *et al*. 2008; Matrixassoziierte autogene Chondrozytentransplantation (MACT): Minimalinvasive Technik am Kniegelenk. *Oper Ortho Traumatol* **20**: 208–219.

Behrens P, Ehlers EM, Köchermann KU *et al*. 1999; New therapy procedure for localized cartilage defects. Encouraging results with autologous chondrocyte implantation. *MMW Fortschr Med* **141**: 49–51.

Benya PD, Shaffer JD. 1982; Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell* **30**: 215–224.

Benya PD, Brown PD, Padilla SR. 1988; Microfilament modification by dihydrocy tochalasin B causes retinoic acid-modulated chondrocytes to reexpress the differentiated collagen phenotype without a change in shape. *J Cell Biol* **106**: 161–170.

Bermüller C, Schwarz S, Elsaesser AF *et al*. 2013; Marine collagen scaffolds for nasal cartilage repair: prevention of nasal septal perforations in a new orthotopic rat model using tissue engineering techniques. *Tissue Eng Part A* **19**: 2201–2214.

Calejo MT, Morais ZB, Fernandes AI. 2009; Isolation and biochemical characterisation of a novel collagen from *Catostylus tagi*. *J Biomat Scie Polym Ed* **20**: 2073–2087.

Couchman JR. 2010; Transmembrane signaling proteoglycans. *Annu Rev Cell Dev Biol* **26**: 89–114.

Cukierman E, Pankov R, Stevens DR *et al*. 2001; Taking cell-matrix adhesions to the third dimension. *Science* **294**: 1708–1712.

DeSimone DP, Parsons DB, Johnson KE *et al*. 1983; Type II collagen-induced arthritis. A morphologic and biochemical study of articular cartilage. *Arthritis Rheum* **26**: 1245–1258.

Engler A, Bacakova L, Newman C *et al*. 2004; Substrate compliance versus ligand density in cell on gel responses. *Biophys J* **86**: 617–628.

Engler AJ, Sen S, Sweeney H *et al*. 2006; Matrix elasticity directs stem cell lineage specification. *Cell* **126**, 677–689.

Erickson IE, Huang AH, Sengupta S *et al*. 2009; Macromer density influences mesenchymal stem cell chondrogenesis and maturation in photocrosslinked hyaluronic acid hydrogels. *Osteoarthr Cartil* **17**: 1639–1648.

Freyria A-M, Cortial D, Ronzière M-C *et al*. 2004; Influence of medium composition, static and stirred conditions on the proliferation of and matrix protein expression of bovine articular chondrocytes cultured in a 3-D collagen scaffold. *Biomaterials* **25**: 687–697.

Freyria A-M, Ronzière M-C, Cortial D *et al*. 2009; Comparative phenotypic analysis of articular chondrocytes cultured within type I or type II collagen scaffolds. *Tissue Eng Part A* **15**: 1233–1245.

Fu N, Drinnenberg I, Kelso J *et al*. 2007; Comparison of protein and mRNA expression evolution in humans and chimpanzees. *PLoS One* **2**: e216.

Galois L, Hutasse S, Cortial D *et al*. 2006; Bovine chondrocyte behaviour in three-dimensional type I collagen gel in terms of gel contraction, proliferation and gene expression. *Biomaterials* **27**: 79–90.

Gaudet C, Marganski WA, Kim S *et al*. 2003; Influence of type I collagen surface density on fibroblast spreading, motility, and contractility. *Biophys J* **85**: 3329–3335.

Glowacki J, Trepman E, Folkman J. 1983; Cell shape and phenotypic expression in chondrocytes. *Proc Soc Exp Biol Med* **172**: 93–98.

Heino J, Huhtala M, Käpylä J *et al*. 2009; Evolution of collagen-based adhesion systems. *Int J Biochem Cell Biol* **41**: 341–348

Hoyer B, Bernhardt A, Lode A *et al*. 2014; Jellyfish collagen scaffolds for cartilage tissue engineering. *Acta Biomater* **10**: 883–892.

Hsieh PY-H. 2005; Auburn University, US Patent 6894029.

Imran M, Mahmood S. 2011; An overview of animal prion diseases. *Virol J* **8**: 493.

Iwig M, Czeslick E, Müller A *et al*. 1995; Growth regulation by cell shape alteration and organization of the cytoskeleton. *Eur J Cell Biol* **67**: 145–157.

Kimura S, Miura S, Park Y-H. 1983; Collagen as the major edible component of jellyfish (*Stomolophus nomural*). *J Food Sci* **48**: 1758–1760.

Konitsiotis AD, Raynal N, Bihan D *et al*. 2008; Characterization of high affinity binding motifs for the discoidin domain receptor DDR2 in collagen. *J Biol Chem* **283**: 6861–6868.

Leitinger B, Hohenester E. 2007; Mammalian collagen receptors. *Matrix Biol* **26**: 146–155.

Liao H, Zakhaleva J, Chen W. 2009; Cells and tissue interactions with glycated collagen and their relevance to delayed diabetic wound healing. *Biomaterials* **30**: 1689–1696.

Miller EJ. 1984; Chemistry of the collagens and their distribution. In Extracellular Matrix Biochemistry, Piez KA, Reddi AH (eds). Elsevier, New York, NY; 41–81.

Miura S, Kimura S. 1985; Jellyfish mesogloea collagen. Characterization of molecules as

alpha 1 alpha 2 alpha 3 heterotrimers. *J Biol Chem* **260**: 15352–15356.
Mizuno K, Hayashi T, Bächinger HP. 2003; Hydroxylation-induced stabilization of the collagen triple helix. *J Biol Chem* **278**: 32373–32379.
Morgan MR, Humphries MJ, Bass MD. 2007; Synergistic control of cell adhesion by integrins and syndecans. *Nat Rev Mol Cell Biol* **8**: 957–969.
Murphy CM, Haugh MG, O'Brien FJ. 2010; The effect of mean pore size on cell attachment, proliferation and migration in collagen-glycosaminoglycan scaffolds for bone engineering. *Biomaterials* **31**: 461–466.
Nehrer S, Breinan HA, Ramappa A *et al*. 1997; Matrix collagen type and pore size influence behaviour of seeded canine chondrocytes. *Biomaterials* **18**: 769–776.
Nehrer S, Breinan HH, Ashkar S *et al*. 1998; Characteristics of articular chondrocytes seeded in collagen matrices in vitro. *Tissue Eng* **4**: 175.
Okina E, Grossi A, Gopal S, Multhaupt HAB, Couchman JR. 2012; Alpha–actinin interactions with syndecan-4 are integral to fibroblast-matrix adhesion and regulate cytoskeletal architecture. *Int J Biochem Cell Biol* **44**: 2161–2174.
Pieper JS, van der Kraan PM, Hafmans T *et al*. 2002; Crosslinked type II collagen matrices: preparation, characterization and potential for cartilage engineering. *Biomaterials* **23**: 3183.
Prusiner SB. 1998; Prions. *Proc Natl Acad Sci U S A* **95**: 13363–13383.
Reber-Müller S, Studer R, Müller P *et al*. 2001; Integrin and talin in the jellyfish *Podocoryne carnea*. *Cell Biol Int* **25**: 753–769.
Saxena AK, Ainoedhofer H, Höllwarth ME. 2009; Esophagus tissue engineering: *in vitro* generation of esophageal epithelial cell sheets and viability on scaffold. *J Pediatr Surg* **44**: 896–901.
Schlegel W, Nürnberger S, Hombauer M *et al*. 2008; Scaffold-dependent differentiation of human articular chondrocytes. *Int J Mol Med* **22**: 691–699.
Schuh E, Kramer J, Rohwedel J *et al*. 2010; Effect of matrix elasticity on the maintenance of the chondrogenic phenotype. *Tissue Eng Part A* **16**: 1281–1290.
Song E, Yeon Kim S, Chun T *et al*. 2006; Collagen scaffolds derived from a marine source and their biocompatibility. *Biomaterials* **27**: 2951–2961.
Stötzel S, Schurink M, Wienk H *et al*. 2012; Molecular organization of various collagen fragments as revealed by atomic force microscopy and diffusion-ordered NMR spectroscopy. *Chemphyschem* **13**: 3117–3125.
Sweeney SM, Orgel JP, Fertala A *et al*. 2008; Candidate cell and matrix interaction domains on the collagen fibril, the predominant protein of vertebrates. *J Biol Chem* **283**: 21187–21197.
Xian X, Gopal S, Couchman JR. 2010; Syndecans as receptors and organizers of the extracellular matrix. *Cell Tissue Res* **339**: 31–46.
Xu H, Raynal N, Stathopoulos S *et al*. 2011; Collagen binding specificity of the discoidin domain receptors: binding sites on collagens II and III and molecular determinants for collagen IV recognition by DDR1. *Matrix Biol* **30**: 16–26.
Xu Y, Gurusiddappa S, Rich RL *et al*. 2000; Multiple binding sites in collagen type I for the integrins alpha1beta1 and alpha2beta1. *J Biol Chem* **275**: 38981–38989.
Yang CL, Rui H, Mosler S *et al*. 1993; Collagen II from articular cartilage and annulus fibrosus. Structural and functional implication of tissue specific posttranslational modifications of collagen molecules. *Eur J Biochem* **213**: 1297–1302.
Zhuang Y, Hou H, Zhao X *et al*. 2009; Effects of collagen and collagen hydrolysate from jellyfish (*Rhopilema esculentum*) on mice skin photoaging induced by UV irradiation. *J Food Sci* **74**: H183–H188.