

## Three-dimensional scaffold-free fusion culture: the way to enhanced chondrogenesis of *in vitro* propagated human articular chondrocytes

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### Abstract

Cartilage regeneration based on isolated and culture-expanded chondrocytes has been studied in various *in vitro* models, but the quality varies with respect to the morphology and the physiology of the synthesized tissues. The aim of our study was to promote *in vitro* chondrogenesis of human articular chondrocytes using a novel three-dimensional (3-D) cultivation system in combination with the chondrogenic differentiation factors transforming growth factor beta 2 (TGF- $\beta$ 2) and L-ascorbic acid. Articular chondrocytes isolated from six elderly patients were expanded in monolayer culture. A single-cell suspension of the dedifferentiated chondrocytes was then added to agar-coated dishes without using any scaffold material, in the presence, or absence of TGF- $\beta$ 2 and/or L-ascorbic acid. Three-dimensional cartilage-like constructs, called single spheroids, and microtissues consisting of several spheroids fused together, named as fusions, were formed. Generated tissues were mainly characterized using histological and immunohistochemical techniques. The morphology of the *in vitro* tissues shared some similarities to native hyaline cartilage in regard to differentiated S100-positive chondrocytes within a cartilaginous matrix, with strong collagen type II expression and increased synthesis of proteoglycans. Finally, our innovative scaffold-free fusion culture technique supported enhanced chondrogenesis of human articular chondrocytes *in vitro*. These 3-D hyaline cartilage-like microtissues will be useful for *in vitro* studies of cartilage differentiation and regeneration, enabling optimization of functional tissue engineering and possibly contributing to the development of new approaches to treat traumatic cartilage defects or osteoarthritis.

### Introduction

Although articular cartilage shows remarkable durability, this tissue has no, or very low ability to self-repair and untreated lesions may result in osteoarthritis.<sup>1</sup> The low potential for spontaneous regeneration spurred the development of cell therapies, such as the autologous chondrocyte transplantation (ACT), which aims to functionally and painlessly repair articular cartilage defects.<sup>2</sup> However, such techniques cannot guarantee cartilage regeneration and there have not been enough studies to prove long-term efficacy.<sup>3,4</sup> Consequently, there is a high demand for improved cartilage regeneration techniques, especially for young active patients with traumatic lesions or symptoms of cartilage degeneration. Numerous studies have been performed using chondrocytes isolated from bovine, rabbit, or sheep cartilage.<sup>5-7</sup>

However, the obtained data and such animal-based concepts are not necessarily transferable to the human joint situation.<sup>8,9</sup> Detailed biochemical and molecular studies with human chondrocytes have been hampered by a number of factors such as the scarce availability of human tissue, the very low number of cells available from biopsy samples, and the limited proliferative capacity and high phenotypic instability of cultured chondrocytes.<sup>10</sup> Our aim was to establish a valid human chondrocyte model for *in vitro* chondrogenesis with relevance for both fundamental research and clinical approaches. To this end, we wanted to generate 3-D *in vitro* cartilage-like microtissues using a special culture system mimicking the first step of chondrogenesis in vertebrates, the condensation of mesenchymal cells.<sup>11,12</sup> Optionally, differentiation promoting bioactive molecules could be added but without the aid of any scaffold material. After expansion of isolated chondrocytes in monolayer culture, accompanied by dedifferentiation of the cells, 3-D tissues were induced and the chondrogenic stimuli TGF- $\beta$ 2 and L-ascorbic acid were applied. Previous studies using different subtypes of the TGF- $\beta$  superfamily as differentiation stimulators in our 3-D *in vitro* microtissues revealed best effects on chondrocyte redifferentiation when TGF- $\beta$ 2 was added to the culture medium.<sup>13</sup> To evaluate the quality of the tissue constructs we used hyaline cartilage typical differentiation markers like collagen type II and proteoglycans as essentials of functional cartilage extracellular matrix and the small intracellular protein S100. As a kind of counterstain we also looked for the expression of collagen type I, typically marking fibrocartilage and fibrous tissues and therefore also dedifferentiated chondrocytes.

During this project, chondrocytes were cul-

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Key words: *in vitro* cartilage, 3-D cell culture, fusion culture technique, tissue engineering, cell differentiation, extracellular matrix, immunohistochemistry.

Contributions: ML, planning, execution, analysis of the study, results discussing, manuscript writing; FM, planning, execution and analysis of the study; KM, study execution; KK, technical assistance; US, manuscript discussing and proofreading; UA, study planning and analysis, results discussion, manuscript design and discussion.

Conflicts of interest: the authors declare that no competing financial or personal interests exist.

Acknowledgments: The work presented in this paper was made possible by funding from the Ministerium für Wissenschaft, Forschung und Kultur (MWFK, Ministry of Science, Research, and Culture), Brandenburg, Germany. This work was also supported by funding from the Bundesministerium für Bildung und Forschung (BMBF, Federal Ministry of Education and Research, PtJ-Bio, 0315883), Germany. The authors would like to thank colleagues at the Translational Centre for Regenerative Medicine (TRM) at the University of Leipzig for discussion and proofreading of the manuscript.

Received for publication: 8 August 2013.

Accepted for publication: 18 September 2013.

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European Journal of Histochemistry 2013; 57:e31  
doi:10.4081/ejh.2013.e31

tured for about 40 days in a 3-D environment partly enriched with differentiation factors. The engineered cartilaginous microtissues were analysed histologically and immunohistochemically to determine the amount and distribution of tissue-specific matrix components. Since the presence of cartilage matrix molecules and protein markers are needed to assess the quality of the generated microtissues, histological and immunohistochemical detection techniques *in situ* were the methods of choice.<sup>14</sup> The objectives of this study were to demonstrate *in vitro* chondrogenesis by human articular chondrocytes from elderly patients in a new 3-D two-step tissue generat-

ing system and the formation of cartilage-like microtissues without using any scaffold or supporting gel matrix. Moreover, our cartilage engineering method could be a useful model system for studying both the metabolism of chondrocytes and their activity in a 3-D configuration. In addition, in case of an autologous application, the generated cartilage microtissues may be even suitable as transplants, especially for traumatic defects.

## Materials and Methods

### Cell source and monolayer culture of human articular chondrocytes

Articular cartilage was obtained from human femoral condyles of patients undergoing knee surgery due to osteoarthritis. Independent experiments were performed with cartilage from six different patients. An informed, written consent was obtained from all patients. Cartilage tissue was peeled from the condyles with a sterile scalpel and chondrocytes were isolated from the surrounding matrix by mechanical mincing of the tissue with a scalpel followed by enzymatic treatment. The diced tissue was placed in commercial available Alpha medium (Biochrom, Berlin, Germany) and HAM's F12 (1:1) (Biochrom) with collagenase type II (350 U/mL; Biochrom). The closed tube was placed on a shaker (Eppendorf Thermomixer comfort; Eppendorf, Hamburg, Germany) at 300 rpm interval mixing and was incubated at 37°C for 20 h. The extracted chondrocytes were centrifuged at 300xg for 5 min. The supernatant was removed and the pellet was resuspended with 10 ml of Alpha medium plus HAM's F12 enriched with 1% L-glutamine (Biochrom), and 10% human serum (serum pool from voluntary donors); this was the basal medium. The cells were counted using a hemocytometer (Neubauer chamber; LO - Laboroptik, Lancing, UK) and the cell viability was determined using trypan blue (Biochrom). The chondrocytes were plated at a cell density of  $2 \times 10^4$  cells/cm<sup>2</sup>. The cells were expanded in monolayer culture at 37°C and 5% CO<sub>2</sub> for two passages and subcultured by enzymatic dissociation with a solution of 0.05% trypsin and 0.02% EDTA (Biochrom) at a ratio of 1:3.

### Generation of cartilaginous microtissues

To induce microtissue formation chondrocytes were seeded in agarose-coated (Sigma-Aldrich, Steinheim, Germany) wells of 96-well plates at a concentration of  $3 \times 10^5$  cells/well in 200  $\mu$ L basal medium per well. Stable chondrocyte aggregates, which formed after only two days, were then cultivated under different conditions to promote redifferentiation.

### Generation of fusion tissues and chondrogenic redifferentiation conditions

Induction of fused aggregates was achieved by combining five single spheroids in one well of a 96-well plate after the initial two-day formation of the individual spheroids. Single spheroids and fusion tissues were cultivated under four different conditions containing different bioactive molecules to induce redifferentiation. The *in vitro* aggregates were cultured either in basal medium (bm), or in basal medium supplemented with 50  $\mu$ g/mL L-ascorbic acid (Sigma-Aldrich), 5 ng/mL TGF- $\beta$ 2 (R&D Systems, Wiesbaden-Nordenstadt, Germany), or 50  $\mu$ g/mL L-ascorbic acid and 5 ng/mL TGF- $\beta$ 2. The microtissues were grown under these conditions for six weeks. The medium was changed three times a week.

### *In vitro* cartilage analysis

After 6 weeks, *in vitro* tissue constructs were harvested and prepared for further analysis. The construct diameter was calculated from the flat area by image analysis. This was assessed with an inverted phase contrast microscope CKX 41 (Olympus, Hamburg, Germany), a DP 71 digital camera (Olympus) and imaging software Cell<sup>F</sup> (Soft Imaging Systems, Münster, Germany). The tissue constructs were rinsed in PBS, embedded in Neg-50 frozen section medium (Richard-Allan Scientific, Kalmazoo, USA), and sectioned using a cryomicrotome (Microm GmbH, Walldorf, Germany). The 7  $\mu$ m cryosections on glass slides (Superfrost Plus Menzel Gläser; Menzel, Braunschweig, Germany) were air-dried and directly analysed, or stored at -20°C.

### Histology and immunohistochemistry

Prior to analysis, monolayer chondrocytes from passage 2 and tissue cryosections on glass slides from undigested parts of the hyaline cartilage of the condyles were fixed in a two-step process. They were first fixed in 4% formaldehyde (AppliChem, Darmstadt, Germany) at 4°C for 10 min, then the slides were incubated in a 1:1 mixture of methanol/acetone (Roth, Karlsruhe, Germany) at -20°C for 10 min. After this fixation procedure the slides were rinsed in PBS for 3 to 5 min. Histological staining was performed with hematoxylin and eosin (H&E) (AppliChem) for morphological analysis of cells, and Safranin O-Fast Green (AppliChem) to detect glycosaminoglycans (GAGs). Fixed chondrocytes and cryosections were stained immunohistochemically for human collagen type I, type II, and S100. First slides were rinsed with PBS and incubated for 20 min at room temperature (RT) with normal goat serum (Dianova, Hamburg, Germany) diluted 1:50 in

PBS/0.1% BSA (Roth, Karlsruhe, Germany) to block unspecific binding. Primary antibodies were diluted in PBS/0.1% BSA as follows: anti-collagen type I (1:1000; MP Biomedicals, Ohio, USA), anti-collagen type II (1:1000; MP Biomedicals), and anti-S100 (1:400; DakoCytomation, Glostrup, Denmark). The cells and cryosections were then incubated with the primary antibodies in a humidified chamber overnight at 4°C. Slides were washed three times with PBS and incubated in a humidified chamber for 1 h in the dark at RT with Cy3-conjugated goat anti-mouse (collagen type I and II) and goat anti-rabbit (S100) antibody (Dianova) diluted 1:600 in PBS/0.1% BSA including DAPI (1  $\mu$ g/mL; Fluka, Seelze, Germany) to stain cell nuclei. Slides were washed three times with PBS and subsequently cells and tissue sections were mounted in fluorescent mounting medium (DakoCytomation) and covered with a cover slip to prevent fluorescence bleaching. Finally, slides were stored in the dark at 4°C until analysis by fluorescence microscopy. Cryosections of native human articular cartilage were used as a positive control for collagen type II and S100 and as a negative control for collagen type I. In addition, all experiments included the replacement of primary antibodies by PBS as a negative control for unspecific binding of the secondary antibody.

### Phase contrast microscopy of cell culture

Photos of single spheroids and fusions were captured in black and white by using the light microscope CKX 41 equipped with the camera DP 71 (Olympus), and documented using Cell<sup>F</sup>-Imaging Software for Life Science Microscopy (Soft Imaging Systems).

### Color microscopy of histological specimens

Results of histological analyses were documented using the microscope BX 41 equipped with the camera Color View I (Olympus) and cell<sup>P</sup>-Imaging software (Soft Imaging Systems).

### Fluorescence microscopy for immunohistochemical analysis

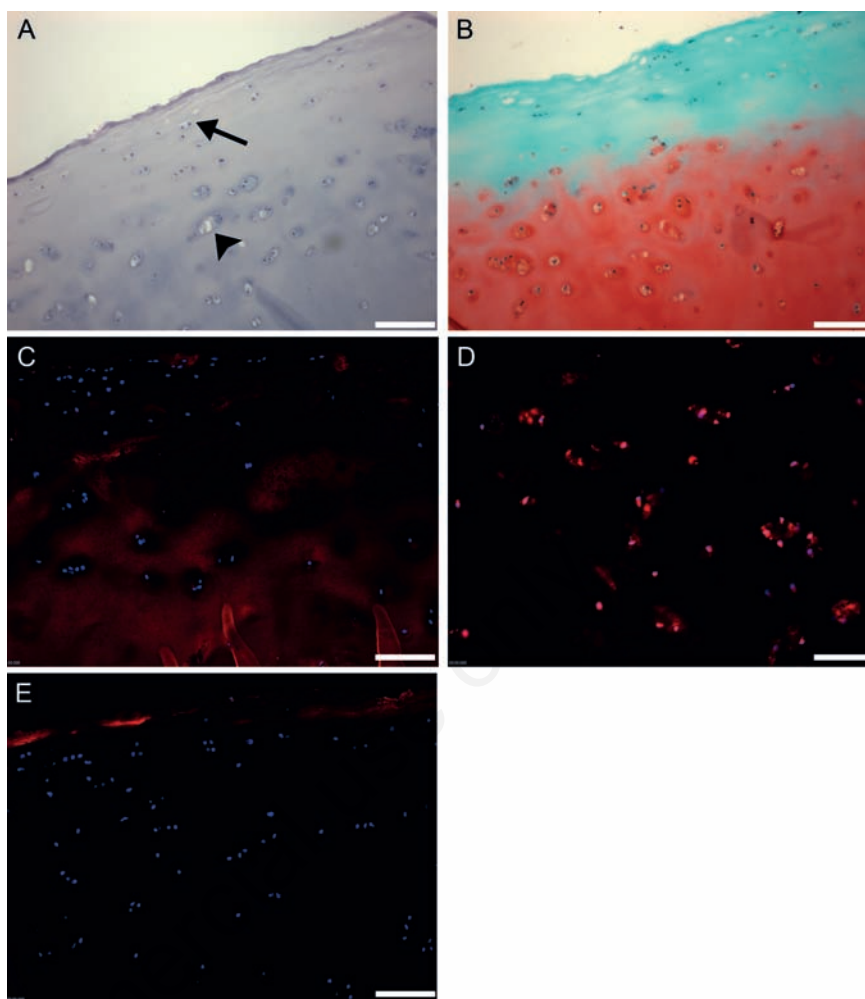
Fluorescence of Cy3-conjugated antibodies and DAPI staining of immunohistochemically stained cells and cryosections was visualised using the computer-assisted fluorescence microscope system IX81 with xenon burner MT20 (Olympus). Image documentation and evaluation was performed using the digital camera F-View II (Olympus) and Cell<sup>F</sup>-Imaging Software for Life Science Microscopy (Soft Imaging Systems).

## Results

### Analysis of human articular cartilage tissue

Human hyaline cartilage samples were isolated from the femoral condyles of six different donors. The resulting cell yields from each donor differed due to varying amounts of available cartilage tissue with healthy macroscopic appearance (Table 1). The cell viability after the isolation process was above 92% in all cases (Table 1).

The histological and immunohistochemical analyses of a representative sample are shown in Figure 1. The human hyaline cartilage showed the typical structure of elongated flattened cells in the superficial zone and rounded cells frequently arranged in small isogenous groups (lacunae) in the middle zone of the tissue. As obvious in Figure 1A, the chondrocytes are separated from each other by the extracellular matrix (ECM). At least 70% of the tissue showed proteoglycan deposition. As expected for cartilage tissues the surface regions of the tissue sections used in this study were stained by Fast Green, implying that proteoglycans had been degraded (Figure 1B). The tissue showed strong expression of collagen type II (Figure 1C red), which was reduced in the surface regions, confirming a change in matrix composition in this zone (Figure 1C). S100 proteins were expressed by most of the cells and were restricted to the cytoplasm (Figure 1D). As also expected, collagen type I was not expressed in native hyaline cartilage except a typical thin layer at the surface (Figure 1E). Comparable histological and immunohistochemical results were obtained with the cartilage samples from the five other donors (*data not shown*).



**Figure 1.** Histological and immunohistochemical analyses of native human articular cartilage cryosections. A) HE-staining with cell nuclei in dark blue embedded in light blue ECM; B) SO-staining indicating proteoglycans (red); C) indirect immunofluorescence for collagen type II (red); D) indirect immunofluorescence for S100 (red dots intracellular); E) indirect immunofluorescence for collagen type I (red); C-E) cell nuclei stained in blue with DAPI. Arrow indicates flattened chondrocytes in the superficial zone of the tissue; arrowhead shows lacunae in the middle zone of the tissue. Scale bars: 100  $\mu$ m.

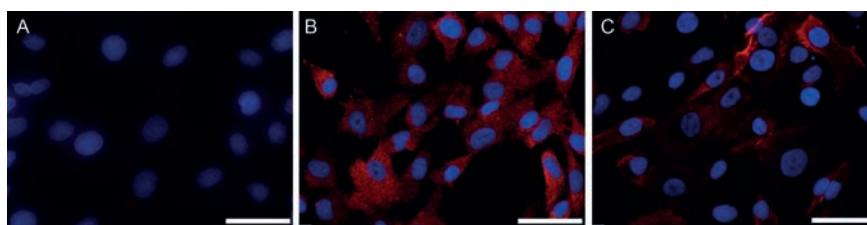
**Table 1.** Characterization of donors, tissues and freshly isolated chondrocytes used for engineering *in vitro* tissues.

Donor	Gender	Age	Macroscopic appearance of articular cartilage	Weight of cartilage tissue used for cell isolation	Cell yield*	Cell viability
1	Female	56	Smooth surface	3.2 g	$9.5 \times 10^6$	95.2%
2	Female	68	Mainly smooth surface with some discontinuities	2.8 g	$8.2 \times 10^6$	96.8%
3	Male	63	Smooth surface	4.0 g	$11.0 \times 10^6$	97.1%
4	Female	76	Mainly smooth surface with slight superficial fibrillation	2.5 g	$7.2 \times 10^6$	92.4%
5	Male	73	Mainly smooth surface with some discontinuities	2.3 g	$7.6 \times 10^6$	95.7%
6	Male	81	Partly uneven surface with slight superficial fibrillation	1.5 g	$5.3 \times 10^6$	93.8%

\*Total number of cells isolated from healthy (unaffected) parts of cartilage tissue covering the condyles.

## Dedifferentiation of human chondrocytes cultured as monolayer

During cell expansion in monolayer culture, chondrocytes of each donor dedifferentiated and acquired a fibroblastic cell shape (*data not shown*). The chondrocytes in passage 2 of monolayer culture, *i.e.* after about 4 population doublings, expressed very low levels of collagen type II or none at all (Figure 2A). By contrast, S100 was still expressed in all cells at passage 2 in a typical punctate pattern (Figure 2B). Furthermore, atypical collagen type I was already expressed by most cells even after such a short time in culture (Figure 2C).



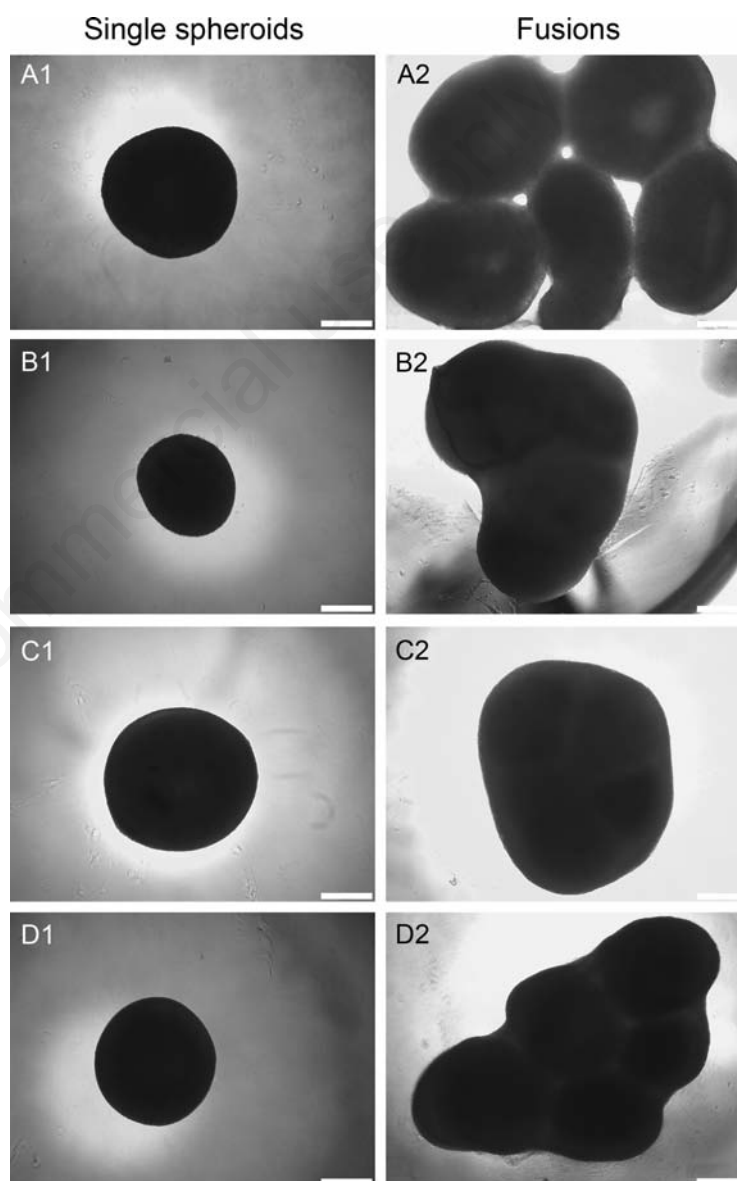
**Figure 2.** Immunohistochemical analysis of human chondrocytes in monolayer culture at passage 2 (after ~4 population doublings). A) indirect immunofluorescence for collagen type II; B) indirect immunofluorescence for S100; C) indirect immunofluorescence for collagen type I (antigens stained in red by Cy3 labelling; cell nuclei stained in blue with DAPI). Collagen type II was downregulated, whereas collagen type I was upregulated due to dedifferentiation of chondrocytes in monolayer culture. Chondrocyte-specific S100 was still expressed in all cells. Scale bars: 50  $\mu\text{m}$ .

## Generation of *in vitro* cartilage microtissues by special 3-D culture systems

In 3-D culture microtissues formed as single spheroids or fusion cultures (Figure 3). In general, stable spheroids were formed within two days without addition of L-ascorbic acid or TGF- $\beta$ 2. The spheroids became more compact in the following 2-3 weeks of culture, but then remained a constant size until harvesting at 6 weeks. The diameter of single spheroids was usually between 800 and 1400  $\mu\text{m}$  (Figure 3). Apart from differences in size, single spheroids showed no observable morphological changes under different medium supplement conditions (Figure 3 column single spheroids). By contrast, the presence of TGF- $\beta$ 2 and/or L-ascorbic acid seemed to influence the fusion grade of the formation of microtissues. In each medium type, except the basal medium (Figure 3 A2), single spheroids coalesced into a fairly compact microtissue representing a coherent aggregate (Figure 3 B2, C2, and D2). Although spheroids in the basal medium merged at some surface regions, they formed a rather fragile aggregate in which each single spheroid remained well-defined and distinct from each other (Figure 3 A2).

## Cell-matrix morphology and proteoglycan synthesis in spheroids and fused microtissues

Figure 4 shows HE-staining of representative cryosections of single spheroids and fused microtissues. In the basal medium, the cell- and matrix distribution in single spheroids and fusions was in part similar. In fusions, regions with increased ECM production were visible, represented by a greater distance between chondrocytes (Figure 4 A2 and A3 stars). The addition of L-ascorbic acid alone resulted in unfavourable faint tissue constructs reflected by fissured cryosections (Figure 4 B1). However, once more, fused constructs were more compact with increased ECM synthesis (Figure 4 B2 and B3 stars).



**Figure 3.** Single spheroids and fused microtissues formed by human articular chondrocytes in medium with different supplements added. A1-A2) basal medium (bm); B1-B2) bm + L-ascorbic acid; C1-C2) bm + TGF- $\beta$ 2; D1-D2) bm + TGF- $\beta$ 2 + L-ascorbic acid. Morphological appearance of all microtissues was comparable. Single spheroids cultured in bm + L-ascorbic acid were smaller than others. Fusion grade was promoted by supplementation with TGF- $\beta$ 2 and/or L-ascorbic acid. Scale bars: 500  $\mu\text{m}$ .

TGF- $\beta$ 2 alone or in combination with L-ascorbic acid resulted in increased matrix synthesis (Figure 4C and D stars) but fusions in particular developed a native cartilage-like morphology. The outer rim of spheroids and fusions had a high matrix content and contained quite flat elongated cells when grown in basal medium enriched with TGF- $\beta$ 2 and L-ascorbic acid (Figure 4 D2, arrow). The presence of the cytokine TGF- $\beta$ 2 in the culture medium induced increased synthesis of proteoglycans, regardless of the fusion culture technique (Figure 5C). Addition of TGF- $\beta$ 2 and L-ascorbic acid led to the most positive staining for GAGs (Figure 5D). By contrast, L-ascorbic acid alone did not stimulate proteoglycan synthesis in the single spheroid *in vitro* tissues; the GAGs were barely detectable (Figure 5 B1). Of note, the culture of several spheroids as fused microtissues enabled the induction of proteoglycan synthesis independent of differentiation factors (Figure 5, columns 2 and 3).

### Expression of cartilage-specific proteins in spheroids and fused microtissues

The fusion culture itself induced the synthesis of collagen type II compared with single spheroid tissue constructs even in the basal medium (Figure 6 A1,A2). Without adding specific differentiation factors or adding L-ascorbic acid alone collagen type II was not detectable via immunohistochemistry in single spheroids (Figure 6 A1,B1). TGF- $\beta$ 2 alone, or in combination with L-ascorbic acid, promoted the redifferentiation of chondrocytes already in single spheroids, leading to collagen type II expression (Figure 6 C1,D1). Supplementing the fusion culture with TGF- $\beta$ 2 alone, or in combination with L-ascorbic acid showed even higher levels of collagen type II expression (Figure 6 C2,D2).

The S100 expression was also increased when single spheroids were cultured as fused microtissues (Figure 7, column 2). Again, the differentiating effects of TGF- $\beta$ 2 alone or in combination with L-ascorbic acid contributed to the upregulation of the intracellular S100 protein (Figure 7 C1,D1). It is remarkable that the S100 expression correlated with the localisation of collagen type II within the sections of single spheroids and fusions, especially in the presence of TGF- $\beta$ 2 alone or plus L-ascorbic acid (Figures 6 and 7). While collagen type II was expressed strongly in the inner part of the microtissues its expression in the outer zones was very weak (Figure 6 C,D). Similar expression patterns were observed for S100, especially in those microtissues grown in medium supplemented with TGF- $\beta$ 2 and L-ascorbic acid (Figure 7 D1,D2). Considering collagen type I as a marker of dedifferentiated cartilage,

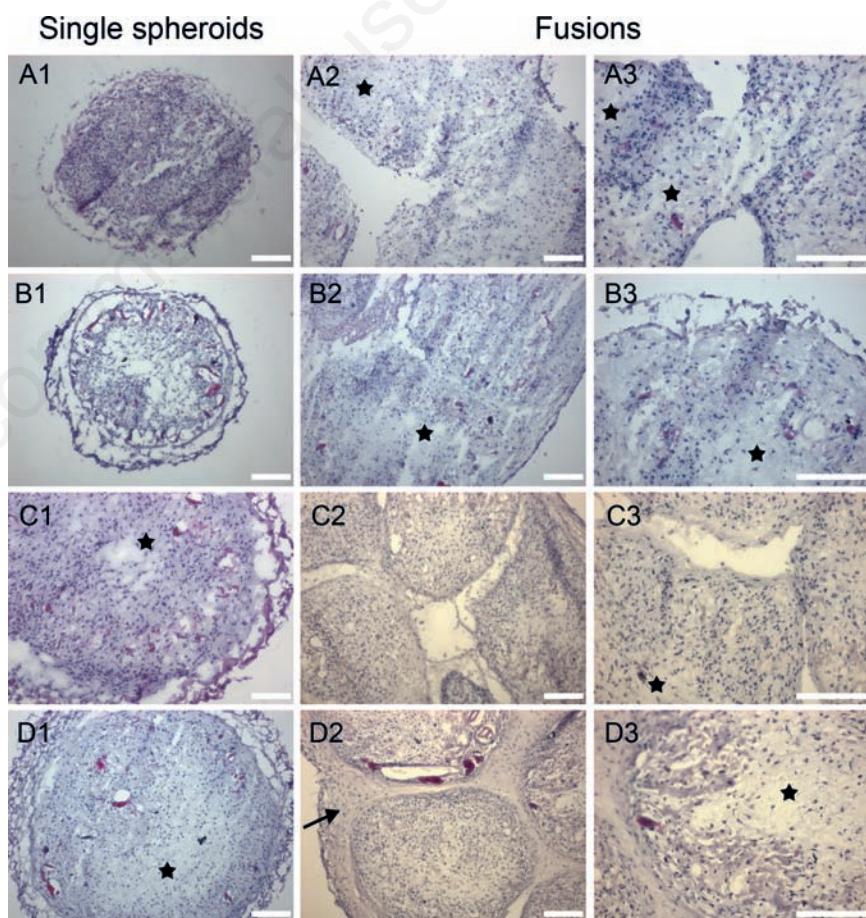
Figure 8 shows reduced expression of this protein in the presence of TGF- $\beta$ 2 and TGF- $\beta$ 2 plus L-ascorbic acid (Figure 8 C,D). In both the single spheroids and fused microtissues, collagen type I expression was restricted to the outer zone of the tissues (Figure 8 C,D). In addition, it is noteworthy that collagen type II was almost absent in regions where collagen type I was upregulated and *vice-versa* (comparison of Figure 6 C,D with Figure 8 C,D).

### Discussion

The tissue engineering system described here opens up possibilities to generate functional human cartilage microtissues for use in *in vitro* studies of chondrogenesis and therapeutic cartilage regeneration *in vivo*. Using autologous cells for engineering cartilage has the advantage of reducing the risk of immuno-

logical rejection and transmitting diseases.

Cell cycle arrested chondrocytes isolated from cartilage tissue restarted to proliferate again in monolayer culture. When serially cultured they stopped synthesizing cartilage-specific macromolecules and instead synthesized molecules normally expressed by mesenchymal cells characteristic of other connective tissues (Figure 2).<sup>15</sup> Many attempts to preserve a chondrocyte-specific phenotype in different culture systems have been reported.<sup>16-18</sup> Chondrogenic redifferentiation was achieved by growing human or animal chondrocytes on biodegradable scaffolds.<sup>19-21</sup> However, many synthetic polymer matrices degrade, creating an acidic environment that is harmful to the implanted cells and the surrounding tissues.<sup>22,23</sup> We also felt that the self-organisation of a tissue without forcing the cells into a scaffold structure may be more physiological and may allow a better fit into a given articular cartilage defect site due to the ability of the

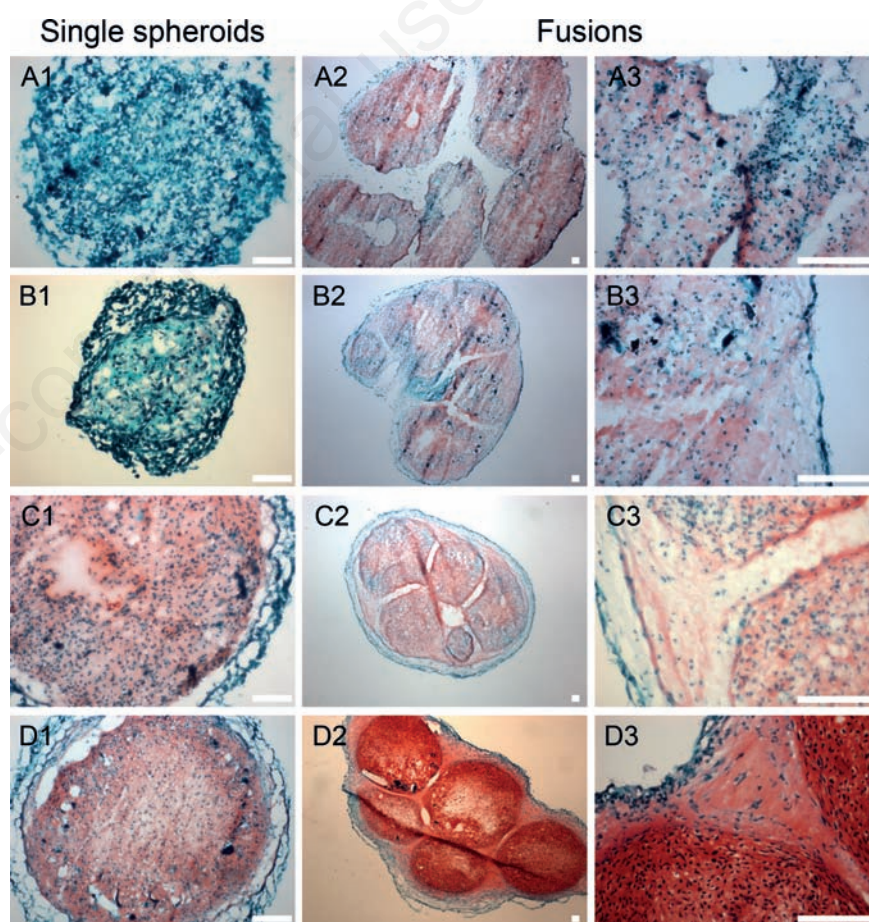


**Figure 4.** Hematoxylin-Eosin-staining of cryosections of *in vitro* cartilage-like tissues (light blue or violet ECM between cell nuclei in dark blue). Comparison of different culture conditions. A1-A3) basal medium (bm); B1-B3) bm + L-ascorbic acid; C1-C3) bm + TGF- $\beta$ 2; D1-D3) bm + TGF- $\beta$ 2 + L-ascorbic acid. Stars indicate regions of increased ECM synthesis, arrow shows fibrous tissue-like outer layer. Fusion formation and supplementation with TGF- $\beta$ 2 resulted in increased matrix production. Scale bars: 100  $\mu$ m.

embedded cells to adapt to the topology of that defect.<sup>24</sup> In contrast to the approaches mentioned above, our system is based on scaffold-free induction of cartilage-like *in vitro* tissues which imitates the initial step of *in vivo* chondrogenesis. Hayes and colleagues reported also chondrogenic characteristics by engineering neocartilaginous grafts scaffold-free, though they used articular chondrocytes isolated from 7-day-old calves.<sup>25</sup> Many of the reports that we cite here generally demonstrate promising redifferentiation of animal chondrocytes in 3-D culture systems. However, the generation of 3-D cartilage tissue structures from adult human chondrocytes particularly derived from elderly donors is far more challenging. Chondrocytes isolated in huge numbers from healthy joints of young animals can be directly used for 3-D culture,<sup>25</sup> whereas *in vitro* studies with human chondrocytes usually need monolayer expansion steps. Furthermore, chondrocytes from various animal knee joints differ in their *in vitro* biology from cells isolated from the corresponding human articular surface.<sup>26</sup> These remarkable differences between animal and human chondrocytes led to the decision to use human chondrocytes in this study. We wanted to develop an *in vitro* cartilage engineering system with potential application in the clinic. The resulting single spheroids and fusions grown in 3-D culture illustrate a gradual redifferentiation of cells in these microtissues. This redifferentiation process was influenced biochemically by supplementing the medium with TGF- $\beta$ 2. A number of studies have emphasised the role of TGF- $\beta$  and insulin-like growth factor-I (IGF-I) as important mediators in promoting tissue repair through increased production of major articular cartilage matrix components.<sup>27-30</sup> However, the effect of TGF- $\beta$  on matrix metabolism in chondrocytes is controversial. Conflicting reports have demonstrated that TGF- $\beta$  leads to both increases<sup>31,32</sup> and decreases<sup>33</sup> in proteoglycan synthesis. In our study, TGF- $\beta$ 2 was an effective promoter for chondrogenic differentiation in the 3-D culture systems. Combining TGF- $\beta$ 2 with L-ascorbic acid in the culture medium also led to chondrogenic redifferentiation in microtissues. The supplementation with L-ascorbic acid is frequently reported to mediate pro-differentiating effects towards the chondrogenic lineage,<sup>34</sup> but the effect of L-ascorbic acid on proteoglycan synthesis by chondrocytes is debatable.<sup>35,36</sup> Our results show that L-ascorbic acid alone could not stimulate proteoglycan synthesis (Figure 5 B1). GAG expression was detected in fusions cultivated with and without L-ascorbic acid, indicating that the fusion culture itself stimulated GAG expression rather than L-ascorbic acid (compare Figure 5 A2,A3 with B2,B3). It is generally believed that L-ascorbic acid modulates col-

lagen production through its effect on prolyl hydroxylation.<sup>37</sup> Interestingly, adding L-ascorbic acid alone did not increase collagen type II expression in single spheroids or in fusions (Figure 6 A1,A2,B1,B2). The combination of L-ascorbic acid with TGF- $\beta$ 2 led to improved chondrogenic differentiation in microtissues, represented by an increase in collagen type II and S100 expression paralleled by a downregulation of collagen type I, which was restricted to the periphery of the microtissues. Remarkably, collagen type I was strongly detectable at the surface but hardly in the depth of the *in vitro* tissues cultured with TGF- $\beta$ 2 (Figure 8 C,D) as occurs within the superficial zone of native articular cartilage (Figure 1E). An even higher provoking effect on the differentiation degree of chondrocytes in the *in vitro* tissues was induced by the newly established two-step fusion culture technique. Here, collagen type II (Figure 6 A2-D2) as well

as hyaline typical proteoglycans (Figure 5 A2-D2) were identifiable throughout the matrix of fused microtissues, confirming the hyaline nature of these tissues. This two-step aggregation system, implemented by fusing preformed aggregates, presumably using cell-cell and/or cell-matrix interactions, is a novel way to generate cartilage-like microtissues. The fusion culture technique improved cartilage tissue formation compared with single spheroids in each medium condition. We hypothesize that several mechanisms may cause the differentiation promoting effect in fusions. In the development of skeletal tissues, cell condensation is the pivotal stage that facilitates the selective regulation of chondrogenesis specific genes. Furthermore, cell-to-cell interactions and gap junction-dependent communication are crucially involved in chondrogenic differentiation.<sup>38,39</sup> During the fusion process the surfaces of preformed *in vitro* tissues were



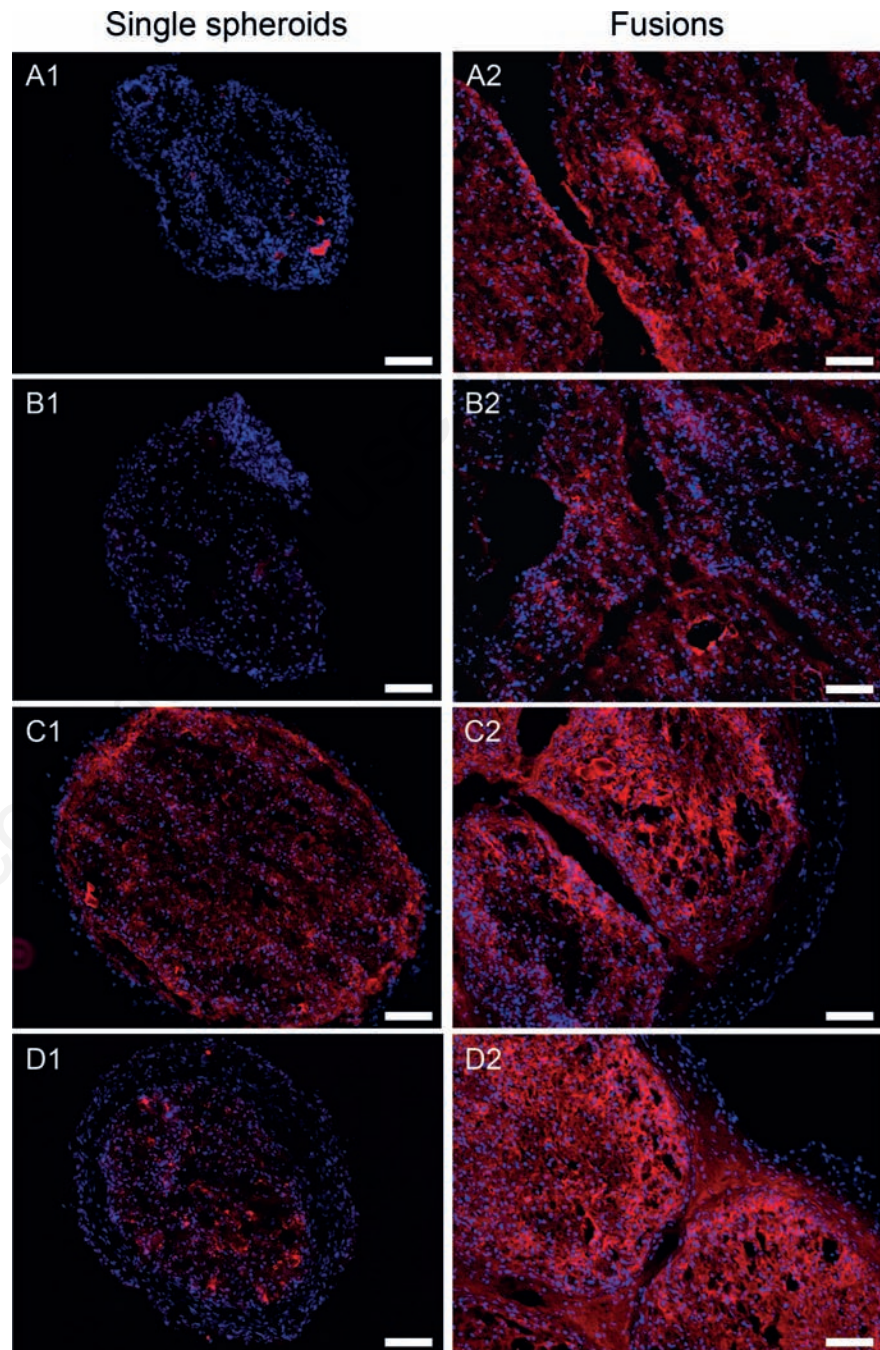
**Figure 5.** Safranin O-staining (red = proteoglycans) of cryosections of *in vitro* cartilage-like tissues. Comparison of different culture conditions. A1-A3) basal medium (bm); B1-B3) bm + L-ascorbic acid; C1-C3) bm + TGF- $\beta$ 2; D1-D3) bm + TGF- $\beta$ 2 + L-ascorbic acid. Independently from TGF- $\beta$ 2 and L-ascorbic acid, fusion cultivation strongly stimulated the synthesis of proteoglycans. Fusion culture with TGF- $\beta$ 2 + L-ascorbic acid synergistically promoted the production of proteoglycans. Scale bars: 100  $\mu$ m.

arranged in close contact (Figure 3 D2 and Figure 5 D2) enabling cell-to-cell interactions and gap junction-mediated communication, resulting in the promotion of cartilaginous differentiation.<sup>40</sup> The cell-cell adhesion proteins N-cadherin and neural cell adhesion molecule (NCAM) are important molecules in mediating cell-cell interaction during chondrogenic differentiation.<sup>41</sup> Following the aggregation of cells, the levels of cell-cell adhesion molecules drop and the differentiation process proceeds. The pre-cartilage ECM formed after this cohesion process enables the cells also to migrate and fibronectin is one of the well known components which might support this migration process.<sup>42</sup> Additionally, gap junctional intercellular communication may play a major role in cartilaginous differentiation thereby influencing linked processes such as altered  $\text{Ca}^{2+}$  or second messenger exchange.<sup>43</sup> Adult articular cartilage chondrocytes exist as individual cells embedded in the ECM, and direct intercellular communication via gap junctions occurs mainly among the flattened chondrocytes facing the outer cartilage layer.<sup>40,44</sup> However, chondrocytes extracted from adult articular cartilage and grown in primary culture express connexin 43 (Cx43) and form functional gap junctions capable of sustaining the propagation of intercellular calcium waves.<sup>40,45</sup> Our single spheroids imitate the process of mesenchymal condensation as one of the earliest steps during cartilage development *in vivo*. However, the aggregation of several single spheroids into fused microtissues seems to enhance this differentiation impulse in cartilage formation. Remarkably, this effect was not achieved just seeding  $1.5 \times 10^6$  cells in one agarose-coated well to generate a bigger tissue aggregate in a one-step procedure containing as many cells as five spheroids fused together (*data not shown*).

Moreover, we confirm here our previous finding that self-aggregation of dedifferentiated proliferating chondrocytes stopped the cell division process and resulted in a subsequent expression of the S100 protein, regardless of the medium composition or culture conditions.<sup>14</sup> The expression of S100 was even higher in the presence of TGF- $\beta$ 2 and in all microtissues generated *via* the fusion technique (Figure 7). S100 is able to demonstrate cartilage and chondrocyte differentiation because it is known that reduced S100 expression in human articular chondrocytes is correlated with cumulative population doublings.<sup>46</sup> S100, an intracellular calcium binding protein, was proposed as marker of chondrogenic cells already in 1992 and was found to be expressed during early chondrogenic differentiation processes (reviewed in Giovannini *et al.*<sup>46</sup>). A recent publication even used the S100 protein as the exclusive marker to identify chondro-

cytes which really enhance the status of this cartilage differentiation marker.<sup>47</sup> The different S100 proteins, *e.g.* S100A1 and S100B, are involved in a wide range of intracellular processes such as cell-to-cell communication, cell shape, cell structure and growth. They are also essential in intracellular calcium depend-

ent signal transduction, in which they act in a similar manner to cytokines.<sup>48</sup> In addition, there exist a large pool of further subtypes (24 known) of the S100 protein family with various cellular functions.<sup>49</sup> Recent studies reported on special functions of S100 subtypes in chondrocytes. S100A10 seems to play a role in inflam-



**Figure 6.** Immunohistochemistry for collagen type II. Detection of collagen type II protein expression (red staining) in the generated microtissues. Cell nuclei stained in blue with DAPI. Comparison of different cultivation conditions. A1-A2) basal medium (bm); B1-B2) bm + L-ascorbic acid; C1-C2) bm + TGF- $\beta$ 2; D1-D2) bm + TGF- $\beta$ 2 + L-ascorbic acid. Collagen type II expression was enhanced in all fused microtissues and further improved in the presence of TGF- $\beta$ 2. Scale bars: 100  $\mu\text{m}$ .

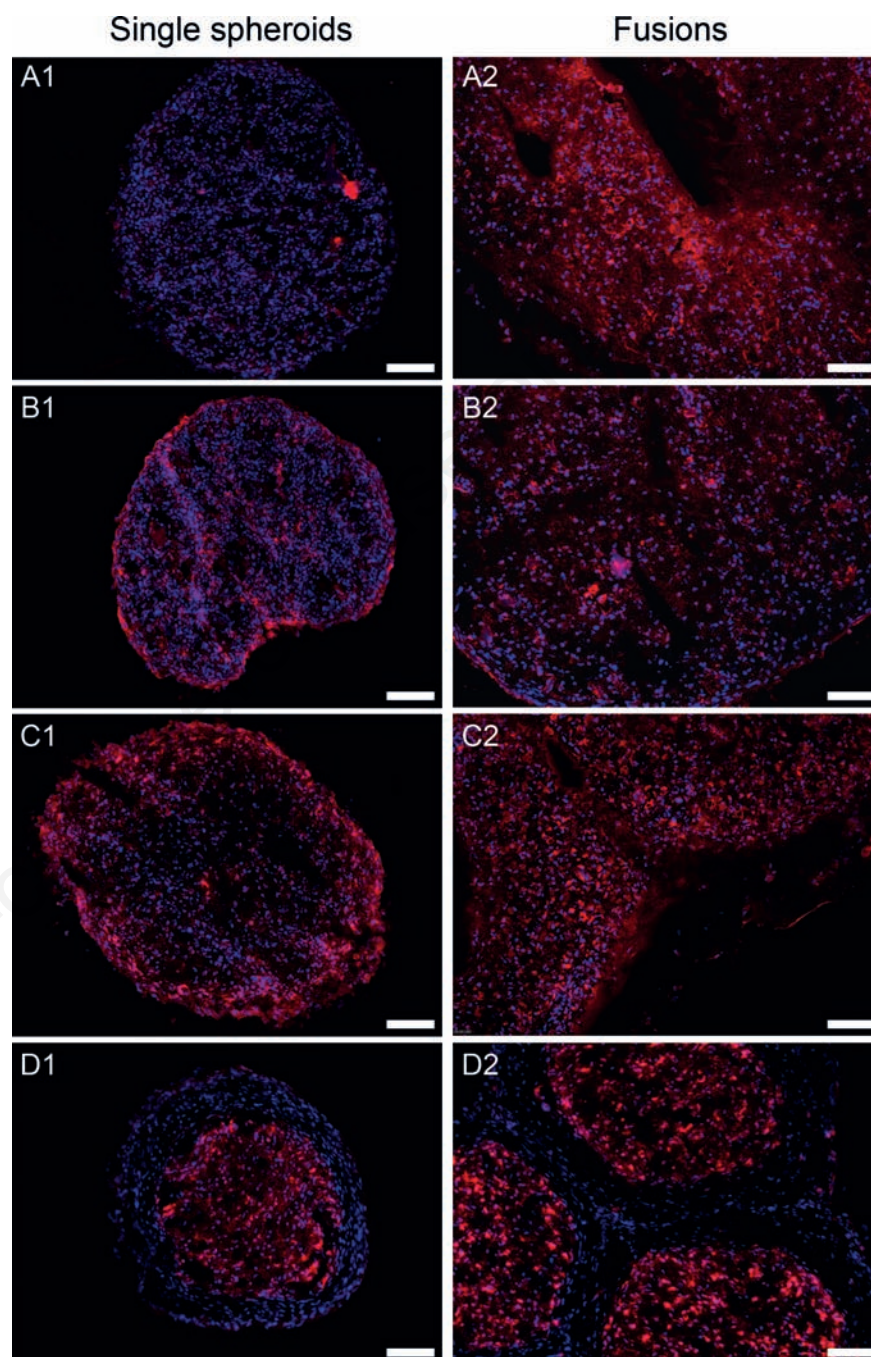
matory responses in human chondrocytes,<sup>50</sup> whereas S100A12 seems to be involved in the pathogenesis of osteoarthritis by upregulating matrix metalloproteinase 13 (MMP13) and vascular endothelial growth factor (VEGF) both associated with the progression of osteoarthritis.<sup>51</sup> These observations are in line with the statement of Donato *et al.* that particular S100 proteins could be induced in pathological circumstances in a cell type that does not express it in normal physiological conditions.<sup>49</sup> To confirm chondrogenic differentiation in 3-D tissues we focused on the detection of S100A1 and S100B, both recognized by the used S100 antibody.

To express collagen type II in microtissues, an advanced stimulation of the differentiation process was required, *i.e.* by fusion formation and/or biochemically. The sequential appearance of the chondrocyte marker proteins S100 and collagen type II in differentiating microtissues fits with the role of S100 proteins as targets of the SOX trio (SOX9 and its coactivators SOX5 and SOX6) transcription factors<sup>52</sup> and that the transcription factor SOX9 has essential roles in successive steps of the chondrocyte differentiation pathway.<sup>53,54</sup> Our data and the cited literature show that S100 is commonly used as an early chondrospecific cellular marker, and that it is valuable for the quality control of cells in culture and even in *in vitro* tissues. In addition, S100 is an ideal marker to discriminate chondrocytes from other mesenchymal cells in connective tissues, such as osteoblasts or fibroblasts, allowing exclusion of contaminating cells in engineered cartilage-like tissue constructs. The co-expression of collagen type II and S100 at the protein level in particular regions of microtissues became clearly visible in single spheroids and fusions cultured in TGF- $\beta$ 2 containing medium. This important observation confirms immunofluorescence as the method of choice to visualize cartilage-specific protein expression. Furthermore, computer-assisted fluorescence colour evaluation was applied for initial semi-quantitative analyses of the immunohistochemically stained tissue sections (*data not shown*). The obtained preliminary results showed for instance that the percentage of the Cy3-indicated collagen type II expression in fusions in basal medium was a multiple compared to that in single spheroids (80-95% in fusions compared to 10-15% in single spheroids). However, setting the baseline for the detection of the fluorescence signals is partly operator dependant. Therefore, this method provided only semi-quantitative data. Nevertheless, the visualisation of the immunofluorescence results already convincingly showed the differences between fusions and single spheroids.

In conclusion, this study provides a new

two-step-based engineering process to create scaffold-free neocartilaginous tissues even using adult human chondrocytes. The culture of expanded and dedifferentiated human articular chondrocytes in a 3-D environment result-

ed in the formation of differentiated cartilaginous microtissues. Furthermore, we observed no phenomena associated with chondrocyte hypertrophy or cartilage calcification, such as collagen type X expression (*data not shown*).



**Figure 7. Immunohistochemistry for S100.** Detection of S100 protein expression (red staining) in the generated microtissues. Cell nuclei stained in blue with DAPI. Comparison of different culture conditions. A1-A2) basal medium (bm); B1-B2) bm + L-ascorbic acid; C1-C2) bm + TGF- $\beta$ 2; D1-D2) bm + TGF- $\beta$ 2 + L-ascorbic acid. S100 expression was increased when fusions were formed. S100 was predominantly expressed in the centre of the tissues when cultured in the presence of TGF- $\beta$ 2 + L-ascorbic acid. Scale bars: 100  $\mu$ m.

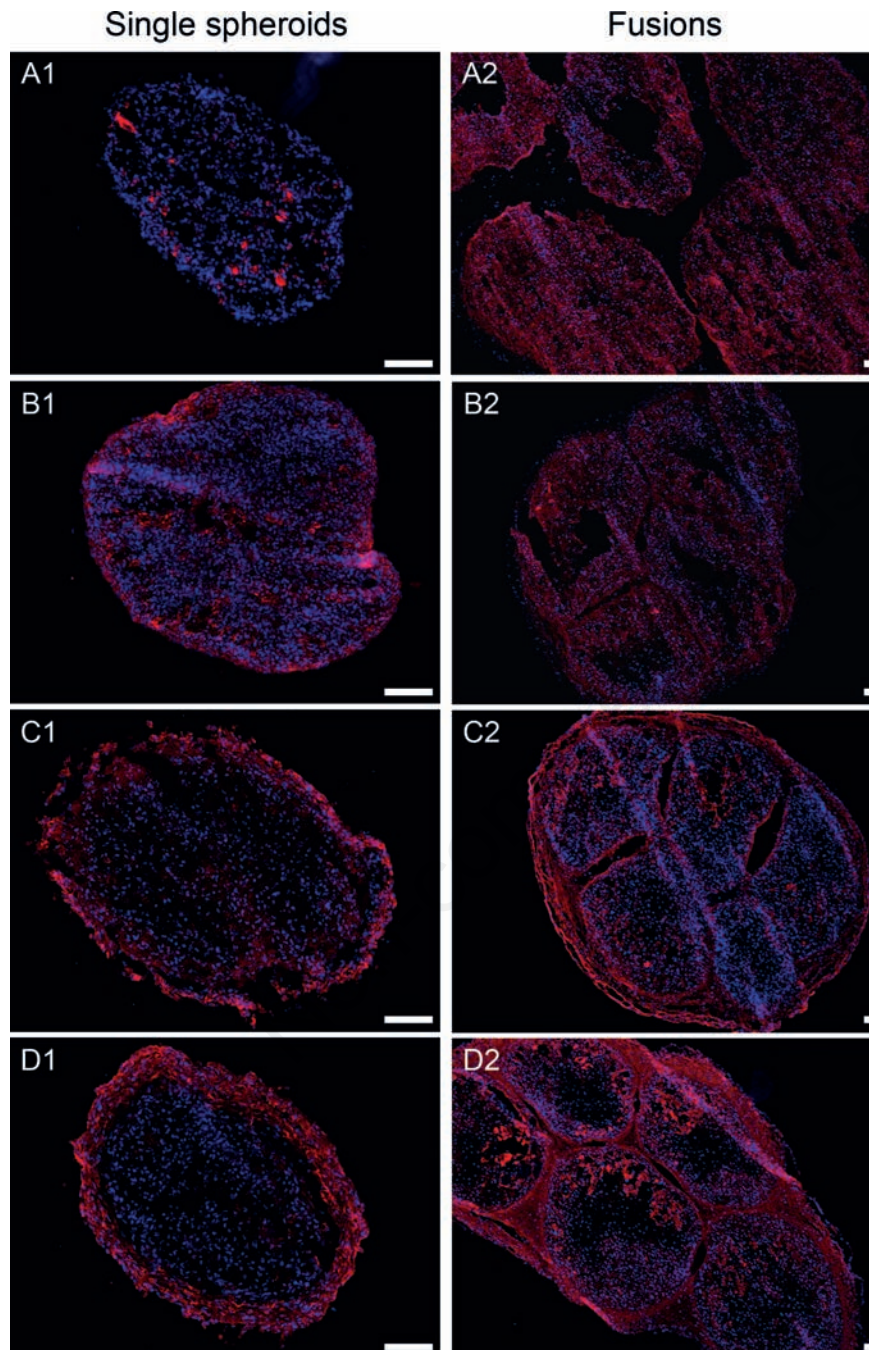


We provide evidence that our newly established fusion culture technique promotes chondrogenic differentiation *in vitro*, triggering the setup of a self-made ECM mainly composed of collagen type II and proteoglycans. In single spheroids there was little expression of

these cartilage markers in basal medium and in the presence of L-ascorbic acid, but this limited matrix production was overcome by the formation of fused microtissues. The fusion culture together with adequate stimulatory growth factors synergistically induced re-

expression of the cartilage phenotype and provides a platform technology to generate *in vitro* scaffold-free human transplants applicable for use in the clinic to regenerate traumatic cartilage defects or even treat osteoarthritis.

However, growth factors are sometimes considered to be critical when used in the clinic and they augment the costs for manufacturing transplants. Therefore, it is of particular importance that our new fusion technology can generate well differentiated transplantable microtissues even without further support by growth factors.



**Figure 8.** Immunohistochemistry for collagen type I. Detection of collagen type I protein expression (red staining) in the generated microtissues. Cell nuclei stained in blue with DAPI. Comparison of different culture conditions. A1-A2) basal medium (bm); B1-B2) bm + L-ascorbic acid; C1-C2) bm + TGF- $\beta$ 2; D1-D2) bm + TGF- $\beta$ 2 + L-ascorbic acid. The expression of the dedifferentiation marker collagen type I was reduced and restricted to the periphery of the microtissues when cultured in basal medium enriched with TGF- $\beta$ 2. Scale bars: 100  $\mu$ m.

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