Histochem Cell Biol (2009) 131:219–229
DOI 10.1007/s00418-008-0516-6

ORIGINAL PAPER

Differing in vitro biology of equine, ovine, porcine and human articular chondrocytes derived from the knee joint: an immunomorphological study

G. Schulze-Tanzil · R. D. Müller · B. Kohl · N. Schneider · W. Ertel · K. Ipaktchi · H. Hünigen · O. Gemeinhardt · R. Stark · T. John

Accepted: 15 September 2008 / Published online: 7 October 2008
© Springer-Verlag 2008

Abstract For lack of sufficient human cartilage donors, chondrocytes isolated from various animal species are used for cartilage tissue engineering. The present study was undertaken to compare key features of cultured large animal and human articular chondrocytes of the knee joint. Primary chondrocytes were isolated from human, porcine, ovine and equine full thickness knee joint cartilage and investigated flow cytometrically for their proliferation rate. Synthesis of extracellular matrix proteins collagen type II, cartilage proteoglycans, collagen type I, fibronectin and cytoskeletal organization were studied in freshly isolated or passaged chondrocytes using immunohistochemistry and western blotting. Chondrocytes morphology, proliferation, extracellular matrix synthesis and cytoskeleton assembly differed substantially between these species. Proliferation was higher in animal derived compared with human chondrocytes. All chondrocytes expressed a cartilage-specific extracellular matrix. However, after monolayer expansion, cartilage proteoglycan expression was barely detectable in equine chondrocytes whereby fibronectin and collagen type I deposition increased compared with porcine and human chondrocytes. Animal-derived chondrocytes developed more F-actin fibers during culturing than human chondrocytes. With respect to proliferation and extracellular matrix synthesis, human chondrocytes shared more similarity with porcine than with ovine or equine chondrocytes. These interspecies differences in chondrocytes in vitro biology should be considered when using animal models.

Keywords Chondrocytes · Collagen type II · Proteoglycans · Knee joint · Cytoskeleton

G. Schulze-Tanzil (✉) · R. D. Müller · B. Kohl · W. Ertel · T. John
Department of Trauma and Reconstructive Surgery,
Charité-Campus Benjamin Franklin, FEM,
Krahmerstrasse 6-10, 12207 Berlin, Germany
e-mail: gundula.schulze-tanzil@charite.de

G. Schulze-Tanzil
Department of Cell and Neurobiology, Centre of Anatomy,
Charité-University of Medicine, Schumannstrasse 20-21,
10098 Berlin, Germany

N. Schneider
Equine Clinic, Department of Clinical Sciences,
Faculty of Veterinary Medicine, University of Liège,
Bd de Colonster, 20, 4000 Sart Tilman, Belgium

H. Hünigen · O. Gemeinhardt · R. Stark
Institute of Veterinary Anatomy, Freie Universität Berlin,
Koserstrasse 20, 14195 Berlin, Germany

K. Ipaktchi
Department of Orthopedic Surgery,
Denver Health Medical Center, Denver, CO, USA

Introduction

Articular chondrocytes from various animal species are often used for in vitro and in vivo tissue engineering studies or to elucidate the pathogenesis of cartilage disorders. However, there appear to exist substantial interspecies differences which have to be accounted for, when using animal chondrocytes for in vitro and in vivo models. A literature review reveals that only limited data exists regarding the interspecies comparison of animal chondrocytes in vitro (Akens and Hurtig 2005; Giannoni et al. 2005). In the presented study, we focused our attention on the knee joint because of high susceptibility to cartilage injury in humans. What makes the knee joint especially interesting is the fact that there are more anatomical similarities between large animal species and humans as compared to other lower

limb joints, (e.g. the human ankle joint) and it was therefore chosen as cell source for this study (Dye 1987). The chondrocyte differentiated phenotype is characterized by a specific synthetic pattern including production of the cartilage marker collagen type II, deposition of cartilage-specific proteoglycans and typical morphologic correlates such as a mostly rounded cell morphology (Benya and Shaffer 1982). This phenotype is the precondition for cartilage tissue engineering applications. Loss of differentiated chondrocyte functions is a typical feature of prolonged chondrocyte expansion in monolayer culture and accompanies chondrocyte proliferation (Benya and Shaffer 1982; Schulze-Tanzil et al. 2002). Dedifferentiated chondrocytes produce less cartilage-specific collagen type II, but show an extracellular matrix containing increasing amounts of ubiquitous matrix proteins such as collagen type I and fibronectin, and cells exhibiting fibroblast-like appearance (Benya and Shaffer 1982). Extracellular matrix interaction of chondrocytes is an important survival precondition (Yang et al. 1997; Hirsh et al. 1997; Cao et al. 1999). Studies found integrins to play an essential role in this cross-talk mediating cell–matrix signal transduction (Knudson and Loeser 2002). Transmembranous integrin receptors are connected to cytoskeleton and interact with proteins such as vinculin and talin to form focal cell matrix adhesions (Archer and Francis-West 2003). These integrin interacting cytoskeletal signaling proteins are responsible for chondrocyte cytoskeleton structure and determine chondrocytes shape. However, cytoskeleton organization changes during chondrocyte dedifferentiation, leading to thick F-actin filaments, so called "stress fibers" (Martin et al. 1999; Zwicky and Baici 2000).

This study was designed to analyze some immunomorphological features characterizing chondrocyte differentiated phenotype and to assess proliferation capacity in knee joint derived articular chondrocytes of large animal species and human. Additionally, expression of specific and dedifferentiation associated extracellular matrix components as well as cytoskeletal organization was assessed.

Materials and methods

Cartilage biopsy origin and chondrocytes culture

Full thickness cartilage from femur condyles and tibia plateaus of adult human ($n = 5$, age range 50–76 years), porcine ($n = 5$, age range 3–10 months), ovine ($n = 4$, adult) and equine ($n = 2$, bilateral knees, adult) knee joints was harvested within 6 h after death. Identical enzymatic isolation protocol was used for chondrocyte isolation of all cartilage species (Schulze-Tanzil et al. 2002, 2004). Articular cartilage was minced into 1–3 mm fragments and enzymatically digested with 1% pronase (Roche Diagnostics GmbH, Mannheim, Germany, 7 U/mg) for 2 h at 37°C and subsequently with 0.2% (v/v) collagenase (Sigma-Aldrich, Munich, Germany, 266 U/mg) for 4 h at 37°C. Isolated chondrocytes were resuspended in growth medium [Ham's F-12/Dulbecco's modified Eagle's medium (50/50, Biochrom-Seromed, Munich, Germany) containing 10% fetal calf serum (FCS, Biochrom-Seromed), 25 µg/mL ascorbic acid (Sigma-Aldrich), 50 IU/mL streptomycin, 50 IU/mL penicillin, 2.5 µg/mL amphotericin B, essential amino acids, L-glutamine (all: Biochrom-Seromed)] and seeded at 21,000 cells/cm^2 in 6-well plates for all experiments.

Chondrocyte proliferation assay

Monolayer chondrocytes (passage 1) were detached using 0.05% trypsin/1.0 mM EDTA solution, followed by rinsing of cell suspension in PBS before 15 min incubation in the dark with 5 µM/mL 5-carboxyfluorescein diacetate succinimidylester (CFDA-SE) solution (Molecular Probes, Invitrogen, CA, USA) at 37°C. After removal of excessive staining by washing in PBS, the cells were seeded in 6-well plates at 21,000 cells/cm^2 and further cultivated for 48 h. Cells were detached, resuspended in FACS staining buffer [1% bovine serum albumin (BSA)/PBS/0.01% NaN_3]. The CFDA-SE staining intensity was assessed by flow cytometry using a FACSCalibur™ flow cytometer and CellQuest™ software (Becton Dickinson, Heidelberg, Germany). Further flow cytometric data analysis was performed using FlowJo™ software for PC-based computer (Tree Star Inc., Ashland, OR, USA).

Indirect immunofluorescence microscopy and phalloidin-FITC staining

Chondrocytes (passage 0, 48 h in culture) or expanded chondrocytes (1 week in culture, passage 1) were cultured on cover slips for 48 h before fixing in 4% paraformaldehyde for 15 min. Cells were washed with Tris-buffered saline (TBS 0.05 M Tris, 0.015 M NaCl, pH 7.6), overlaid with protease-free donkey serum (5% diluted in TBS) for 10 min at room temperature (RT), permeabilized by 0.1% Triton X-100 for 6 min, rinsed and incubated with primary antibodies (polyclonal rabbit-anti-collagen type II, I (1:50, AcrisAntibodies, Hiddenhausen, Germany), monoclonal mouse-anti-fibronectin and adult cartilage proteoglycans (1:40, Dianova, Hamburg, Germany; 1:20, Chemicon, International, Temecula, CA, USA), monoclonal mouse-anti-(human)-vinculin (1:40, Chemicon) or phalloidin-FITC (Sigma-Aldrich) in a humid chamber 1 h at RT. Controls included replacement of primary antibodies with respective isotype mouse IgG1 (Caltag, Hamburg, Germany) diluted at the same concentration as used for primary antibodies. Cells were subsequently washed with TBS

before incubation with donkey-anti-rabbit-Alexa-Fluor® 488 or -Alexa-Fluor® 555 (Invitrogen, CA, USA) or donkey-anti-mouse-Cy-3 (Invitrogen) coupled secondary antibodies (diluted 1:200 in TBS), respectively, for 1 h at RT. Labeled cells were rinsed several times with TBS before being mounted with fluoromount mounting medium (Southern Biotech, Biozol Diagnostica, Eching, Germany) and examined using fluorescence microscopy (Axioskop 40, Carl Zeiss, Jena, Germany).

Western blot analysis for β1-integrins and vinculin

Western blotting was used to determine protein synthesis in 48-h old chondrocytes monolayer cultures (passage 1). Chondrocyte monolayers were washed with PBS solution (Biochrom-Seromed), whole cell proteins were extracted by incubation with lysis buffer [25 mM HEPES, pH 7.5, 0.1% Triton X-100, 5 mM $CaCl_2$, 2 mM DTT, 1 mM EGTA (Carl-Roth, Karlsruhe, Germany)], proteinase inhibitors (proteinase complete mini, Roche Diagnostics, Grenzach-Wyhlen, Germany) on ice for 30 min and cell debris was removed by centrifugation. Supernatants were stored at −70°C until use. Total protein concentration of whole cell extracts was measured using Bradford protein assay (Roti-Nanoquant, Carl-Roth) and BSA as standard. Samples were separated by Tris–glycine SDS-PAGE (12% acrylamide) under reducing conditions before being transferred to a nitrocellulose membrane (Carl-Roth), using a transblot apparatus (Biorad Laboratories, Munich, Germany). Equal protein loading was controlled by the use of Ponceau S staining (Sigma-Aldrich) and β-actin house-keeping protein expression. Membranes were blocked using blocking buffer (3%BSA/PBS/0.05% Tween20) 1 h at RT and incubated overnight at 4°C with the primary antibodies monoclonal anti-β1-integrin (BD Transduction, Laboratories, Hamburg, Germany) and vinculin (Sigma-Aldrich) or monoclonal β-actin (Sigma-Aldrich) diluted 1:1,000 in blocking buffer. Membranes were washed with PBS/0.05% Tween20 and incubated with horseradish peroxidase conjugated secondary goat-anti-mouse IgG antibodies (1:5,000) (DakoCytomation, Hamburg, Germany) for 2 h at RT. Specific binding was detected by nonradioactive chemiluminescence using RotiLumin™ (Carl-Roth) and a LAS 3000 Image Reader (Fujifilm, Düsseldorf, Germany). Protein bands were semiquantified by densitometric scanning (AlphaDigiDoc, Alpha Innotech, San Leandro, CA, USA).

Statistical Analysis

All values are expressed as mean SEM. Data were analyzed using Student's *t* test and one way ANOVA, followed by Bonferroni post hoc testing (GraphPad). Statistical significance was set at a p value of 0.05.

Results

Knee articular chondrocytes morphology varies between animal species

Knee joint articular chondrocytes cultured in monolayer (passage 0, 48 h post isolation) exhibited a rounded or elongated shape with small cytoplasmatic processes and focal cell–cell contacts. Immediately after isolation, human chondrocytes had a more polymorphic and polydendritic shape, porcine cells had a sharp-edged appearance, ovine cells presented a cuboid shape and equine were round shaped (Fig. 1A1–D1).

After expansion (passage 1, 1-week culture), human, porcine and equine chondrocytes developed a progressively more flattened polymorphic morphologies whereby ovine chondrocytes rapidly exhibited an elongated fibroblastic shape. Porcine cells maintained their characteristic sharp edged appearance (Fig. 1A2–D2). Toluidin blue staining revealed at higher magnification round cell nuclei containing several nucleoli in all chondrocytes. Additionally,

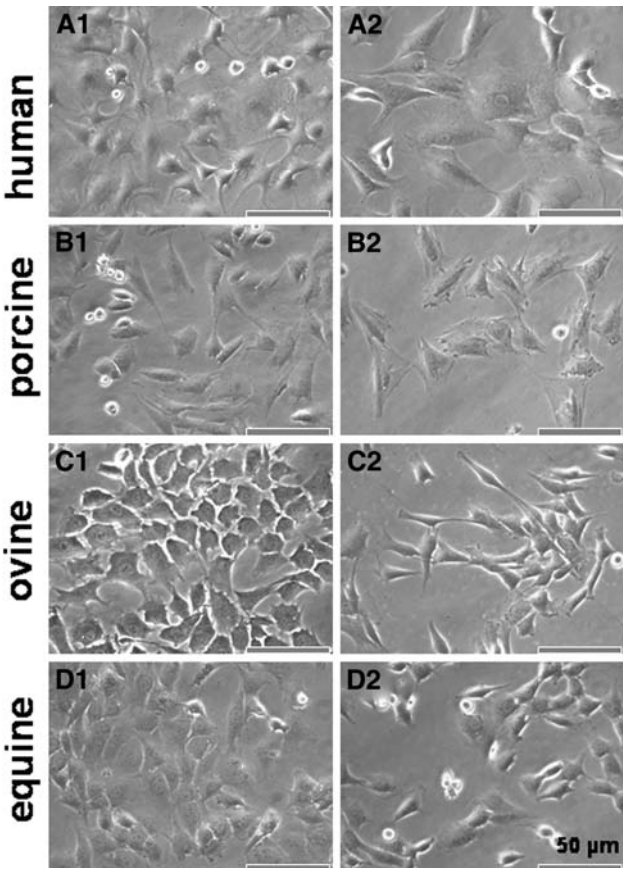

Fig. 1 Light microscopic morphology of human, porcine, ovine and equine primary articular chondrocytes. Articular chondrocyte cultures (*passage 0* A1–D1, *passage 1* A2–D2) deriving from the human (**a**), porcine (**b**), ovine (**c**) and equine (**d**) knee joint are shown. *Scale bar* 50 µm

chondrocytes contained numerous cytoplasmatic vacuoles and a well-structured cytoplasm. Equine-derived chondrocytes revealed more cytoplasmatic vacuoles in passage 0 than other species located predominately at the cell periphery (data not shown).

Higher proliferation rate in animal versus human chondrocytes

CFDA-SE accumulates in cells after the staining process and then distributes equally onto daughter cells during proliferation. Therefore, a low proliferation rate correlates with high CFDA-SE fluorescence intensities. At passage 1, the proliferation assay revealed a higher mean fluorescence intensity for CFDA-SE after 48 h in human chondrocytes compared to large animal species indicating lower human chondrocyte proliferation rate (Fig. 2). Equine chondrocytes exhibited the highest proliferation rate followed by ovine and porcine chondrocytes whereas human chondrocytes had the lowest proliferation rate of all. This proliferation trend was also discernible via light microscopy, using immunolabeling for the nuclear proliferation marker ki67 which occurs during cell division. A higher number of cells showed nuclear staining for ki67 in ovine and equine compared to human chondrocytes (data not shown). Flow cytometric analysis revealed that detached human chondrocytes had a higher cell size and granularity corresponding to their lower proliferation rate with regards to animal-derived chondrocytes (data not shown).

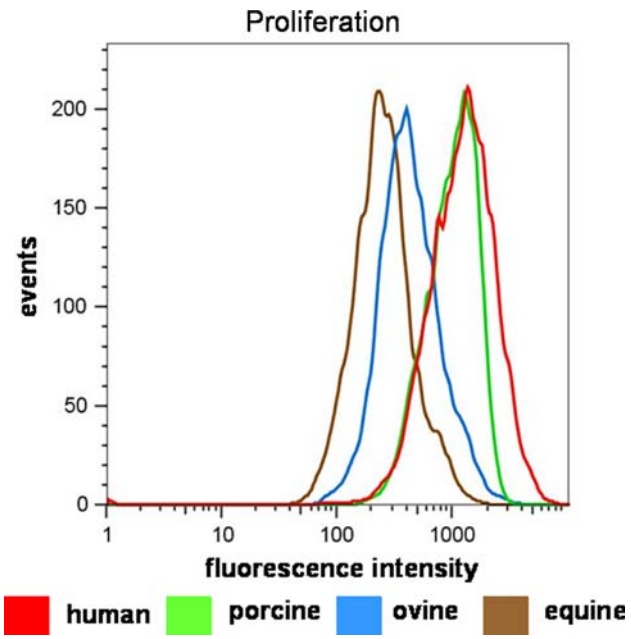

Fig. 2 Proliferation capacity of human, porcine, ovine and equine primary articular chondrocytes. Chondrocytes of passage 1 were stained with CFDA-SE and then cultured for 48 h in monolayer until detached and measured for fluorescence intensity using flow cytometry. A representative experiment of three independent experiments using chondrocytes from different donors is shown

Equine chondrocytes downregulate cartilage proteoglycan expression and increase fibronectin and collagen type I synthesis during culture

Chondrocytes of all investigated species were double-immunolabeled for collagen type II (Fig. 3A1–D1, A2–D2) or fibronectin (Fig. 4A1–D1, A2–D2) combined with phalloidin-FITC to analyze cytoplasma organization simultaneously as well as collagen type I and adult cartilage proteoglycans (Fig. 5A1–D2, A2–D2). Human and animal derived chondrocytes expressed extracellular collagen type II (Fig. 3A1–D1, A2–D2). However, some chondrocytes were strongly collagen type II positive whereas others exhibited only a moderate labeling. The deposition of collagen type II in equine chondrocytes of passage 0 was unique in that there were very long filamentous fibrils of collagen type II interconnected to neighboring cells, thereby forming dense networks of collagen type II in culture (Fig. 3D1). Also ovine chondrocytes of passage 0 were found to produce high amounts of collagen type II with thin fibrils intimately surrounding the chondrocytes (Fig. 3C1).

Investigation of fetal ovine chondrocytes revealed rounded cages of collagen type II around some cells with a very high ability for collagen type II production, whereas other cells were collagen type II negative, indicating a premature differentiation state (data not shown). The collagen type II expression decreased in all species during expansion and was barely detectable in equine chondrocytes at passage 1 (Fig. 3A2–D2).

The immunolabeling for adult cartilage proteoglycans showed a heterogeneous expression pattern in all species with some chondrocytes producing high amounts of proteoglycans and others having only weak proteoglycan production. Proteoglycans were released from cells in small vesicles (Fig. 5D1, white arrow) and deposited directly around the cell, where they largely remained cell-associated. Only in freshly isolated ovine chondrocyte cultures, cartilage proteoglycans were localized further away from their chondrocytes origin (Fig. 5C1). However, in equine and ovine chondrocytes, cartilage proteoglycan expression was barely detectable during further cell expansion (Fig. 5C2, D2).

The deposition of fibronectin in long extracellular filaments, particularly at cell–cell contact areas was very similar in chondrocyte cultures of all studied species and was detectable during the whole investigation period (Fig. 4A1–D2). After passaging equine chondrocytes revealed a higher fibronectin (Fig. 4D2) synthesis rate compared to human, porcine and ovine chondrocytes. Collagen type I was produced by all chondrocyte species during the culture time.

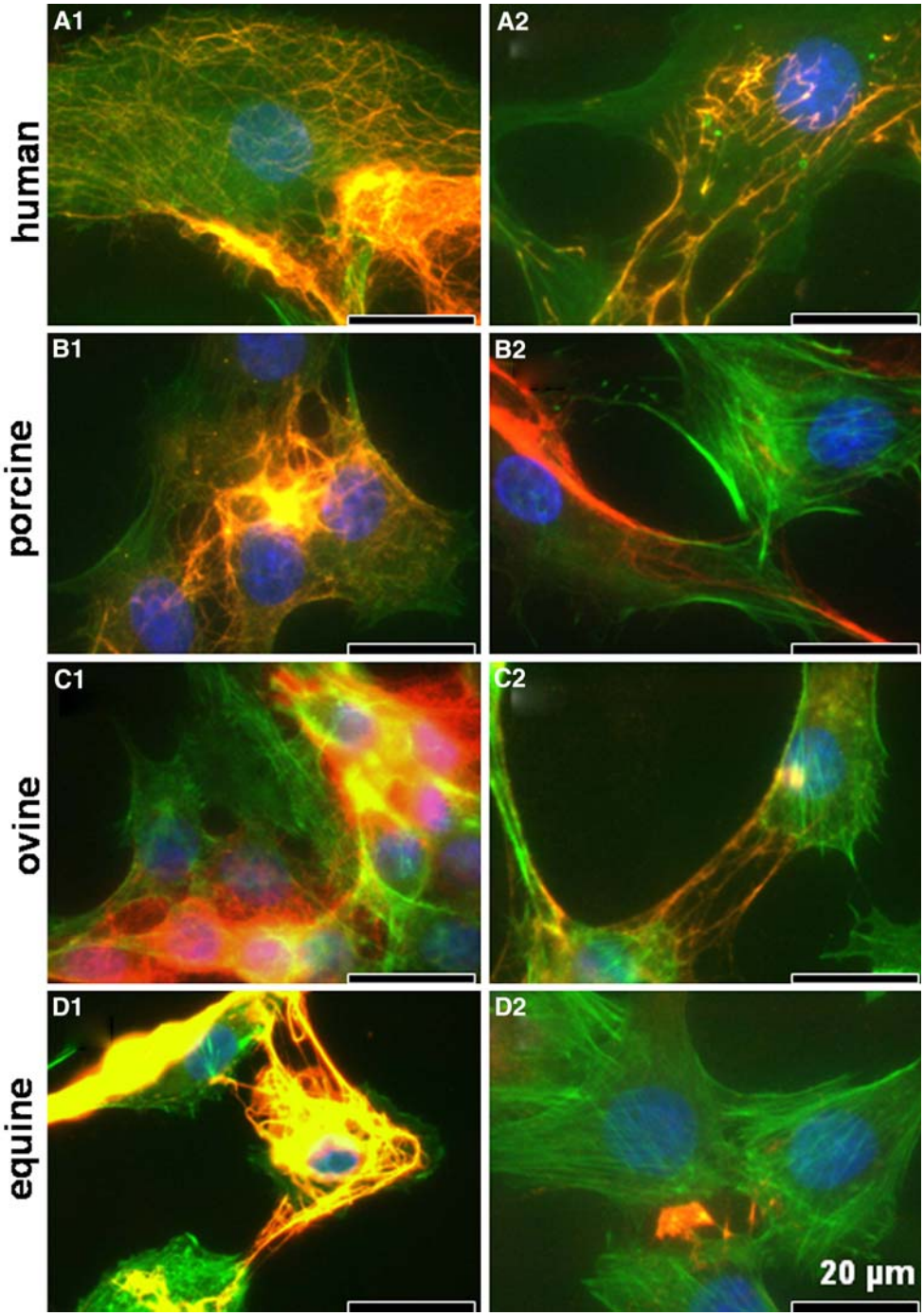

Fig. 3 Extracellular deposition of collagen type II in human- and animal-derived chondrocytes. Chondrocytes 48 h after enzymatic isolation (*passage 0* A1–D1 or *passage 1* A2–D2) of human (**a**), porcine (**b**), ovine (**c**) and equine (**d**) origin were extracellularly double-immunolabeled with specific anti-collagen type II antibodies and Alexa-Fluor® 555 (*red*) coupled secondary antibodies and subsequently intracellularly stained with phalloidin-FITC (*green*). Chondrocytes cell nuclei were counterstained using DAPI (*blue*). *Scale bar* 20 µm

Collagen type I production was found to be increased in equine chondrocyte cultures during expansion compared to freshly isolated cells (Fig. 5A1–D2).

Chondrocytes cytoskeletal assembly varies between animal species and changes during in vitro expansion

Chondrocytes of passage 0 (Fig. 6A1–D1) and 1 (Fig. 6A2–D2) were double-immunolabeled with phalloidin-FITC staining and vinculin antibodies to visualize their cytoskeletal assembly and integrin-associated focal adhesion contacts. Cytoskeletal vinculin was found to be expressed in large focal and fibrillar adhesions at the ends of F-actin cables by chondrocytes of all species and arranged at the cell periphery in a coronar manner (Fig. 6A1–D2, white arrows focal adhesion sites). Thick F-actin stress fiber bundles and focal adhesion sites became more and more prominent during cell expansion in all

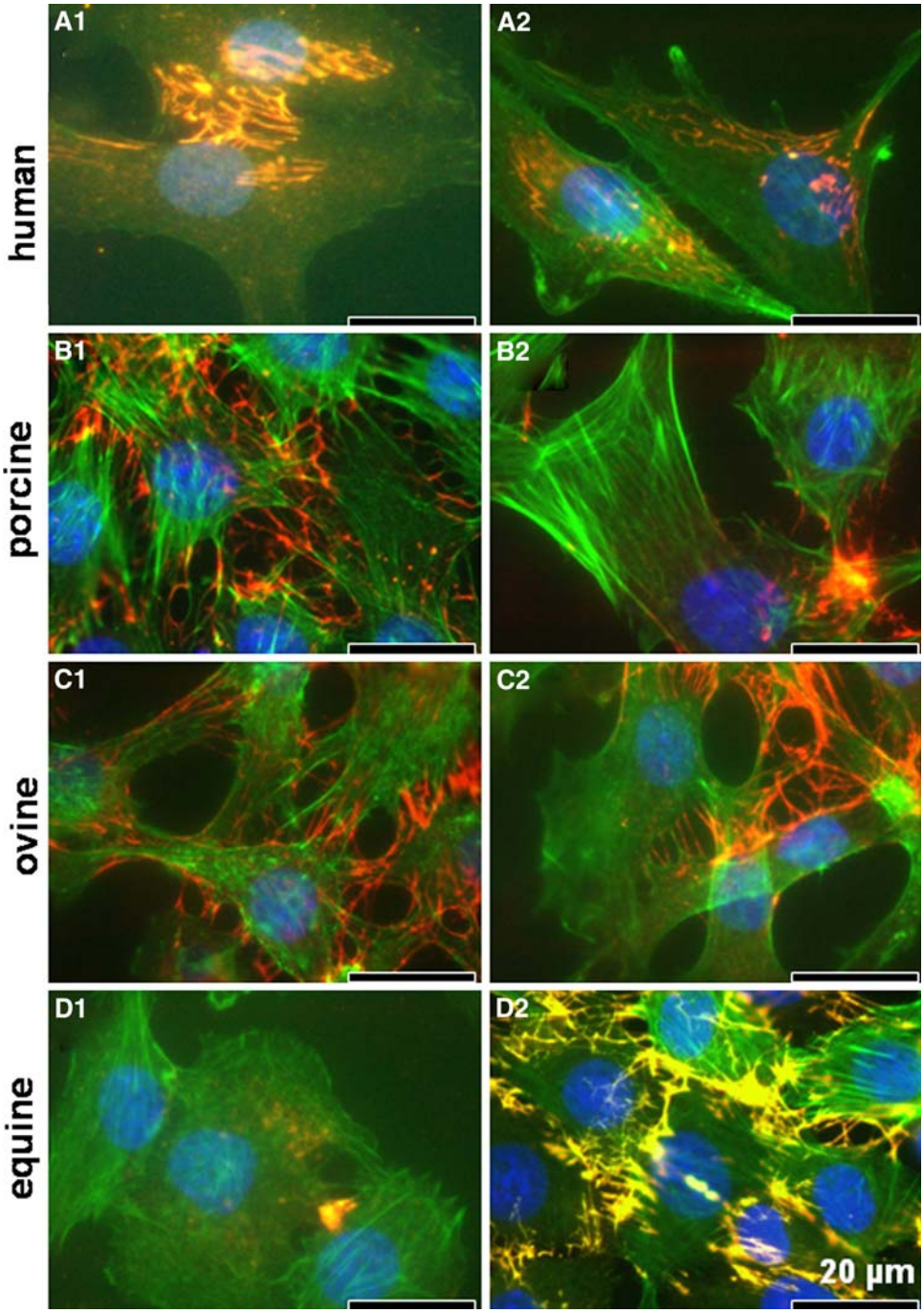

Fig. 4 Extracellular deposition of fibronectin in human- and animal-derived chondrocytes. Chondrocytes 48 h after enzymatic isolation (*passage 0* A1–D1 or *passage 1* A2–D2) of human (**a**), porcine (**b**), ovine (**c**) and equine (**d**) origin were extracellularly double-immunolabeled with specific anti-fibronectin antibodies and Cy3 (*red*) coupled secondary antibodies and subsequently intracellularly stained with phalloidin-FITC (*green*). Chondrocytes cell nuclei were counterstained using DAPI (*blue*). *Scale bar* 20 µm

species (Fig. 6A2–D2). Stress fibers were most pronounced in equine and porcine chondrocytes (Fig. 6D2 and B2). In equine chondrocytes, vinculin fibers appeared thicker, (Fig. 6D1, D2) whereas in porcine chondrocytes they were found to be longer (Fig. 6B2) than in other species. Vinculin fibers were more irregularly distributed in ovine chondrocytes compared to other species (Fig. 6C1 and C2). Human chondrocytes revealed a weaker F-actin staining with regards to other species during cell expansion (Fig. 6A2).

β1-Integrin and vinculin expression differs between animal and human chondrocytes

Whole cell protein extracts of expanded chondrocytes were investigated using western blot analysis. β1-integrins (Fig. 7a) and vinculin (Fig. 7b) were discernible in all chondrocyte species. However, β1-integrin expression was found to be most pronounced in equine chondrocytes. There was a significant difference comparing β1-integrin expression in equine versus the other chondrocyte species ($p < 0.01$). β1-integrin

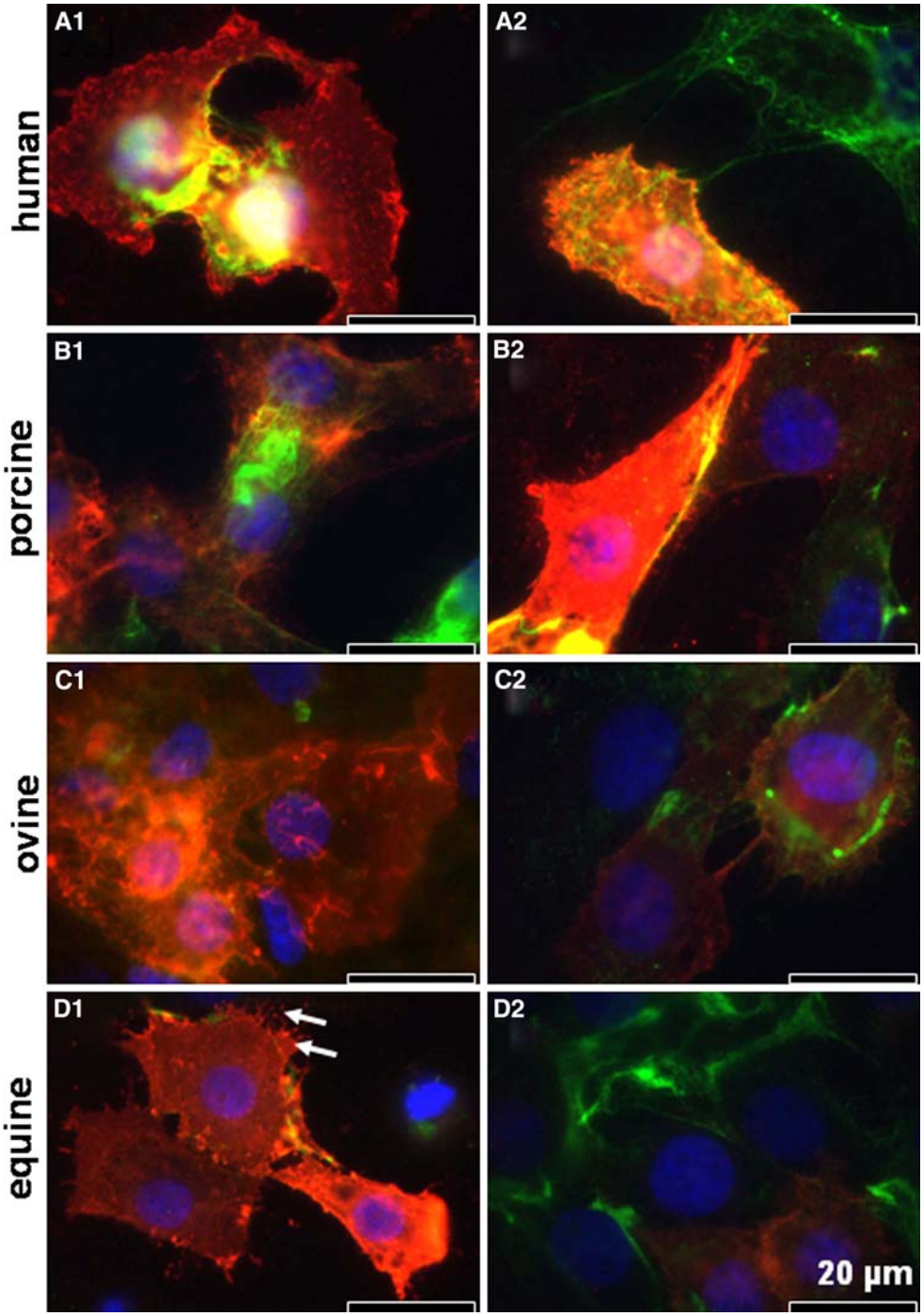

Fig. 5 Extracellular deposition of cartilage proteoglycans and collagen type I in human- and animal-derived chondrocytes. Chondrocytes 48 h after enzymatic isolation (*passage 0* A1–D1 or *passage 1* A2–D2) of human (**a**), porcine (**b**), ovine (**c**) and equine (**d**) origin were extracellularly double-immunolabeled with specific anti-cartilage proteoglycan (*red*) and collagen type I (*green*) antibodies and Cy3 (*red*) or Alexa-Fluor® 488 (*green*) coupled secondary antibodies. Chondrocytes cell nuclei were counterstained using DAPI (*blue*). *White arrows* indicate small vesicles containing proteoglycans are released from chondrocytes. *Scale bar* 20 µm

expression between the remaining other species showed no statistic difference. Vinculin production was significantly higher in equine compared to ovine and porcine chondrocytes when compared to ovine chondrocytes ($p < 0.05$) at passage 1.

Discussion

Until now, only few studies have analyzed interspecies differences of articular chondrocytes deriving from multiple animal sources (Akens and Hurtig 2005; Giannoni et al. 2005). However, chondrocytes isolated from large animals such as sheep, swine and horse are regularly used since decades for in vitro models of cartilage reconstruction, tissue engineering and in order to elucidate the pathogenesis of inflammatory and degenerative cartilage diseases (Vittur et al. 1994; Hamilton et al. 2005; Peretti et al. 2006; Thomas et al. 2007). As such, large animal models play an important role in development and evaluation of cartilage repair strategies it is thus especially interesting to investigate

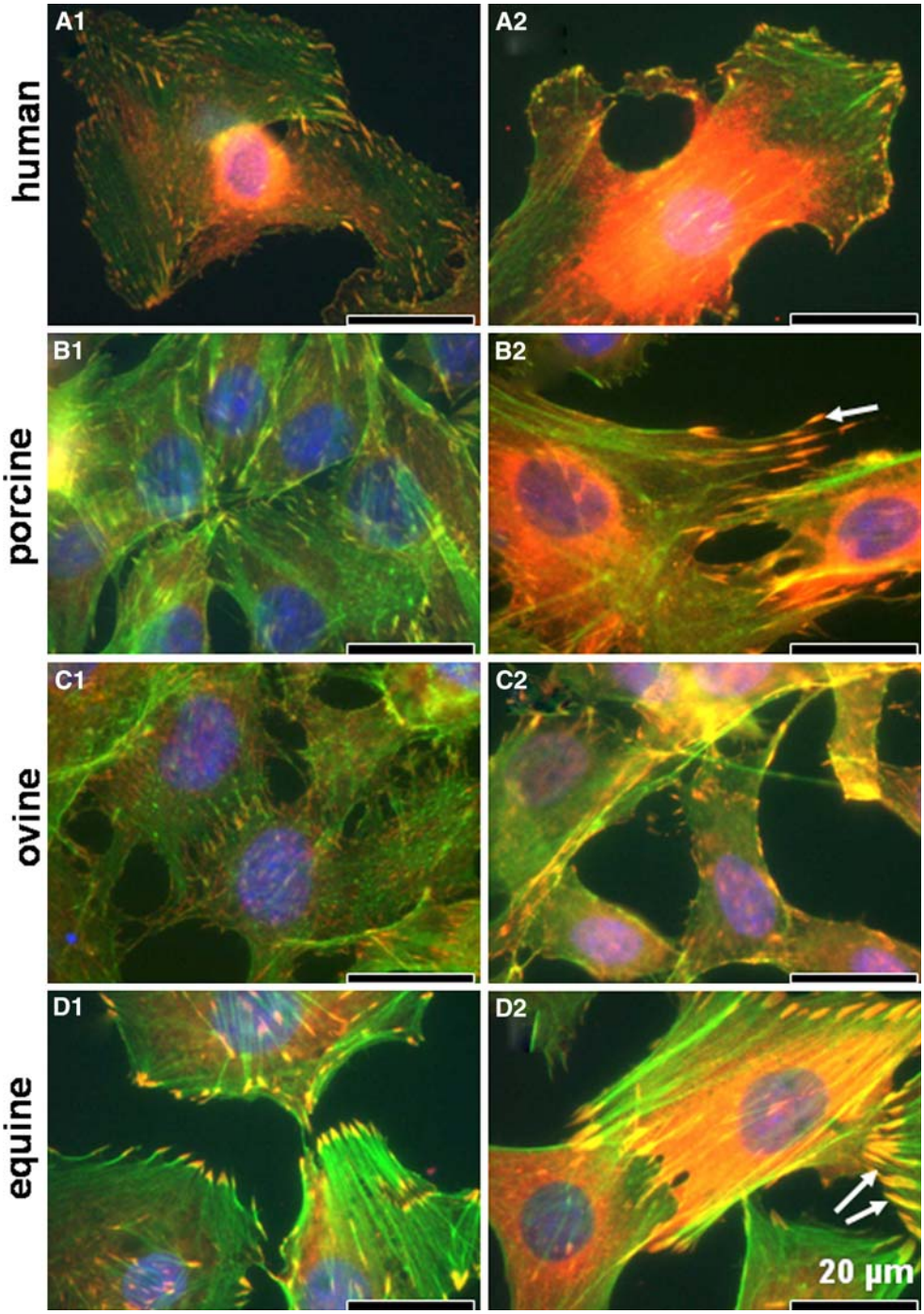

Fig. 6 Cytoskeletal changes in passaged human or animal-derived chondrocytes. Chondrocytes from *passage 0* (A1–D1) and *1* (A2–D2) from the different species (**a** human, **b** porcine, **c** ovine, **d** equine) were cultured on cover slides, stained with phalloidin-FITC (*green*) and immunolabeled intracellularly with anti-vinculin antibodies (*red*) to show their cytoskeletal assembly and focal adhesion site formation. Chondrocytes cell nuclei were counterstained using DAPI. *White arrows* indicate focal adhesion sites of the chondrocytes. *Scale bar* 20 µm

possible interspecies differences regarding large animal cartilage biology and to test the transferability of animal experiment data to human cartilage studies. Chondrocytes proliferative activity and their synthetic profile for cartilage-specific extracellular matrix proteins represent key parameters. Both are essential to fill in cartilage defects and produce a sufficient—hyaline-like—stable articular cartilage matrix. In the presented study, isolated chondrocytes of all investigated species revealed distinct morphological differences and diverging proliferative activities in culture which became even more evident during higher passages. Overall, higher proliferation rates were found in animals as compared to human chondrocytes, suggestive of a generally higher regenerative potential in animal cartilage defect coverage via resident or transplanted chondrocytes. However, the proliferation rate of chondrocytes is also age-dependent as shown for various species (Allen et al. 2004; Kim et al. 2005; Tran-Khanh et al. 2005). Most studies using animal chondrocytes were performed with cartilage from young adults or immature individuum as analyzed in the present

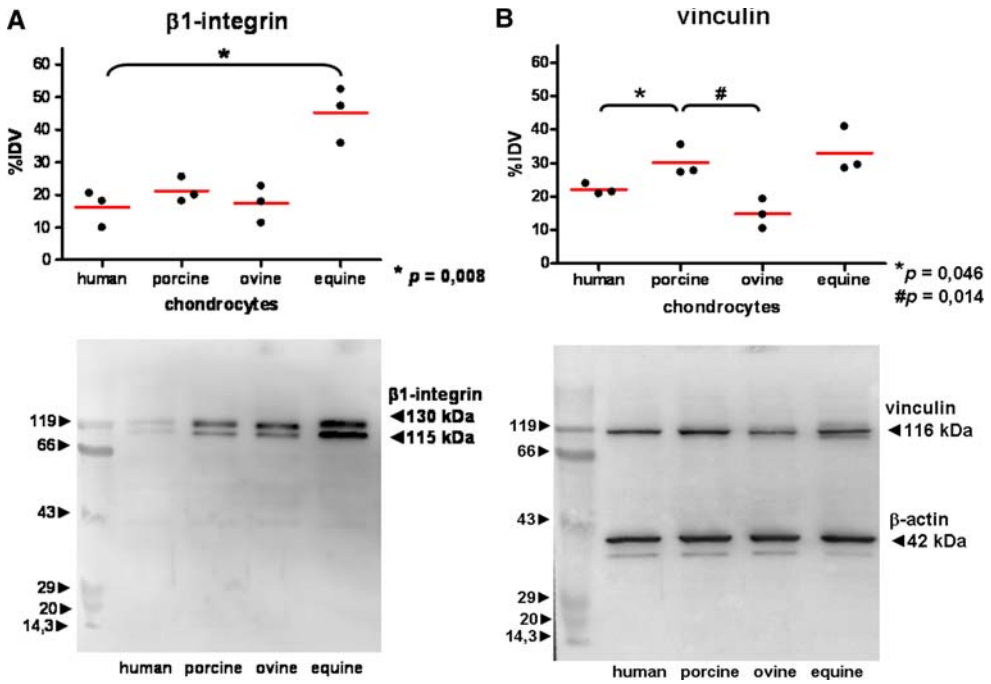

Fig. 7 Expression of the cell matrix receptor β1-integrin and the cytoskeletal focal adhesion protein vinculin in human, porcine, ovine and equine primary articular chondrocytes. Whole cell proteins of chondrocytes (*passage 1*) were extracted at day 2 of culture, separated by SDS-PAGE and immunoblotted using specific antibodies directed against the cell–matrix receptor β1-integrin (**a**) and vinculin (**b**). Blots were reprobed with an antibody directed against the house-keeping protein β-actin as an internal reference (**b**). The results of densitometric evaluations of three independent experiments are shown

study and not aging cartilage. The own results concerning the proliferation rate confirm previous data (Giannoni et al. 2005) comparing sheep- and dog-derived chondrocytes to human articular chondrocytes. Of note is the fact that these authors investigated hip joint derived chondrocytes. It is known that chondrocytes derived from cartilage of different joints or joint locations generally also show biological differences (Little and Ghosh 1997).

One might hypothesize that the observed morphological differences in chondrocytes between species correlating with variations in the composition and architecture of the cartilage extracellular matrix, cell–matrix receptor expression patterns and particularly the cytoskeletal organization of chondrocytes might be a means to compensate for different biomechanical conditions between studied species. It was previously shown, that even within the same species, the composition of the extracellular articular cartilage matrix differed based on harvested joint location amount of mechanical loading (Little and Ghosh 1997; Murray et al. 1999).

Our findings reveal distinct immunomorphological differences in the extracellular matrix synthesis of articular large animal chondrocytes compared to human articular chondrocytes. A notable finding was the high production of very long collagen type II fibers at onset of culturing, forming pericellular cages in equine chondrocytes (Fig. 3D1). These collagen type II fibers were found to be strongly attached to neighboring chondrocytes cell membranes. Though also ovine chondrocytes produced higher amounts of collagen type II compared to human and porcine chondrocytes, their fibers were characteristically shorter and thinner. Equine chondrocytes produced much more fibronectin in the first passage compared to freshly isolated equine chondrocytes and passaged chondrocytes of other study species. Another interesting finding was the fact that expression of cell matrix β1-integrin receptors, mediating cell-matrix interaction, was higher in equine cells compared to other species chondrocytes. This strong integrin expression might be related to the dense cell-associated extracellular matrix networks found around the equine chondrocytes (Fig. 3D1, D2). This might also explain an observed retention of parts of their collagen type II matrix after enzymatic isolation procedures (unpublished results).

Additionally, we studied the expression of the β1-integrin subunit, which is usually associated with various α-chain subtypes located in cartilage integrin receptors which recognize different extracellular matrix ligands and is a part of the $\alpha 5\beta$1-fibronectin receptor. We found a rapid decrease in cartilage proteoglycan and simultaneous increase in unspecific fibronectin as well as collagen type I synthesis in equine chondrocytes as compared to humans in the first passage after 7 days of culture. These data suggest an early onset of chondrocyte dedifferentiation in equine chondrocytes correlating well with their

high proliferative activity and the observed very early fundamental cytoskeletal changes. Cytoskeletal components were well discernible in all investigated chondrocytes species. The occurence of thin F-actin bundles was already visible at the beginning of monolayer culture. After short-term culture, F-actin polymerization was less pronounced in human compared to animal chondrocytes but increased in all species during cell expansion already as early as during the first passage. These findings support other reports (Martin et al. 1999; Zwicky and Baici 2000; Hamilton et al. 2005). Especially equine and porcine cells showed rapid formation of stress fiber bundles spanning throughout the chondrocyte. Vinculin represents a link between other cytoskeletal components and integrin-attached focal extracellular matrix contacts (Zaidel-Bar et al. 2004). Coronal vinculin distribution was particularly visible in equine and porcine chondrocytes as shown by immunohistochemistry and underlined by the semi-quantification of the western blot analysis.

The presented data suggests that early dedifferentiation-associated changes were evident in chondrocytes of all studied species during the first week of culture. These changes were most pronounced in equine chondrocytes, but also clearly evident in ovine chondrocytes.

In summary, the data presented in this study reveals a distinct interspecies difference in chondrocytes isolated from large animal as compared to human derived cartilage tissue. The variability in the in vitro stability of the differentiated phenotype between humans and large animals has to be especially considered when using expanded chondrocytes from large animals for cartilage defect repair models. With regards to cartilage-specific matrix expression and proliferation rate, porcine chondrocytes seem to share more similarities with human derived chondrocytes than the other investigated chondrocyte species. This study presents significant differences between articular chondrocytes from large animal species and humans which calls for special caution when comparing experimental results from animal chondrocytes versus human articular chondrocytes.

Acknowledgments We thank Navid Dedashthi for her assistance. The authors would like to thank Dr. Ruth Hirschberg from the Institute of Veterinary Anatomy of the Free University of Berlin for her support. This study was supported by the Rahel Hirsh program of the Charité Medical School, Berlin, Germany, the Hypatia Program of the Technical University of Applied Sciences Berlin, the Deutsche Arthrosehilfe e.V., and of the Sonnenfeld Foundation, Berlin, Germany.

References

Akens MK, Hurtig MB (2005) Influence of species and anatomical location on chondrocyte expansion. BMC Musculoskeletal Disord 6:23

Allen RT, Robertson CM, Harwood FL, Sasho T, Williams SK, Pomerleau AC, Amiel D (2004) Characterization of mature vs aged rabbit articular cartilage: analysis of cell density, apoptosis-related gene expression and mechanisms controlling chondrocyte apoptosis. Osteoarthritis Cartil 12:917–923

Archer CW, Francis-West P (2003) Cells in focus: the chondrocyte. Int J Biochem Cell Biol 35:401–404

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

Cao L, Lee V, Adams ME, Kiani C, Zhang Y, Hu W, Yang BB (1999) β1-Integrin-collagen interaction reduces chondrocyte apoptosis. Matrix Biol 18:343–355

Dye SF (1987) An evolutionary perspective of the knee. J Bone Joint Surg Am 69(7):976–983

Giannoni P, Crovace A, Malpeli M, Maggi E, Arbico R, Cancedda R, Dozin B (2005) Species variability in the differentiation potential of in vitro expanded articular chondrocytes restricts predictive studies on cartilage repair using animal models. Tissue Eng 11:237–248

Hamilton DW, Riehle MO, Monaghan W, Curtis AS (2005) Articular chondrocyte passage number: influence on adhesion, migration, cytoskeletal organisation and phenotype in response to nano- and micro-metric topography. Cell Biol Int 29:408–421

Hirsh MS, Lunsford LE, Trinkhaus-Randall V, Svoboda KK (1997) Chondrocyte survival and differentiation in situ are integrin mediated. Dev Dyn 210:249–263

Knudson W, Loeser RF (2002) CD44 and integrin matrix receptors participate in cartilage homeostasis. Cell Mol Life Sci 59:36–44

Kim HJ, Park SR, Park HJ, Choi BH, Min BH (2005) Potential predictive markers for proliferative capacity of cultured human articular chondrocytes: PCNA and p21. Artif Organs 29:393–398

Little CB, Ghosh P (1997) Variation in proteoglycan metabolism by articular chondrocytes in different joint regions is determined by post-natal mechanical loading. Osteoarthritis Cartilage 5:49–62

Martin I, Vunjak-Novakovic G, Yang J, Langer R, Freed LE (1999) Mammalian chondrocytes expanded in the presence of fibroblast growth factor-2 maintain the ability to differentiate and regenerate three-dimensional cartilaginous tissue. Exp Cell Res 253:681–688

Murray RC, Zhu CF, Goodship AE, Lakhani KH, Agrawal CM, Athanasiou KA (1999) Exercise affects the mechanical properties and histological appearance of equine articular cartilage. J Orthop Res 17:725–731

Peretti GM, Xu J-W, Bonassar LJ, Kirchhoff H (2006) Review of injectable cartilage engineering using fibrin gel in mice and swine model. Tissue Engineering 12:1151–1168

Schulze-Tanzil G, de Souza P, Villegas Castrejon H, John T, Merker H-J, Scheid A, Shakibaei M (2002) Redifferentiation of dedifferentiated human chondrocytes in high-density cultures. Cell Tissue Res 308:371–379

Schulze-Tanzil G, Mobasheri A, de Souza P, John T, Shakibaei M (2004) Loss of chondrogenic potential in dedifferentiated chondrocytes correlates with deficient Shc-Erk interaction and apoptosis. Osteoarthritis Cartilage 12:448–458

Thomas CM, Fuller CJ, Whittles CE, Sharif M (2007) Chondrocyte death by apoptosis is associated with cartilage matrix degradation. Osteoarthritis Cartilage 15:27–34

Tran-Khanh N, Hoemann CD, McKee MD, Henderson JE, Buschmann MD (2005) Aged bovine chondrocytes display a diminished capacity to produce a collagen-rich, mechanically functional cartilage extracellular matrix. J Orthop Res 23:1354–1362

Vittur F, Grandolfo M, Fragonas E, Godeas C, Paoletti S, Pollesello P, Kvam BJ, Ruzzier F, Starc T, Mozrzymas JW, Martina M,

DeBernard B (1994) Energy metabolism, replicative ability, intracellular calcium concentration, and ionic channels of horse articular chondrocytes. Exp Cell Res 210:130–136

Yang C, Li SW, Helminen HJ, Khillan JS, Bao Y, Prockop DJ (1997) Apoptosis of chondrocytes in transgenic mice lacking collagen II. Exp Cell Res 235:370–373

Zaidel-Bar R, Cohen M, Addadi L, Geiger B (2004) Hierarchical assembly of cell matrix adhesion complexes. Biochem Soc Trans 34(Part 3):416–420

Zwicky R, Baici A (2000) Cytoskeletal architecture and cathepsin B trafficking in human articular chondrocytes. Histochem Cell Biol 114:363–372