Original Research

Hybrid pig versus Göttingen minipig-derived cartilage and chondrocytes show pig line-dependent differences

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Abstract

Minipigs are widely used as a large animal model for cartilage repair. However, many *in vitro* studies are based on porcine chondrocytes derived from abundantly available premature hybrid pigs. It remains unclear whether pig line-dependent differences exist which could limit the comparability between *in vitro* and *in vivo* results using either hybrid or miniature pig articular chondrocytes. Porcine knee joint femoral cartilage was isolated from 3- to 5-month-old hybrid pigs and Göttingen minipigs. Cartilage from both pig lines was analysed for thickness, zonality, cell content, size and proteoglycan deposition. Cultured articular chondrocytes from both pig lines were investigated for gene and/or protein expression of cartilage-specific proteins such as type II collagen, aggrecan, the chondrogenic transcription factor Sox9, non-specific type I collagen and the cell-matrix receptor β1-integrin. Cartilage was significantly thinner in the miniature pig compared to the hybrid pig, but the differences between the medial and lateral femur condyles did not reach a significant level. Knee joint cartilage zone formation started only in the minipig, whereas cellularity and cell diameters were comparable in both pig lines. Blood vessels could be detected in the hybrid pig but not the minipig cartilage. Sulphated proteoglycan deposition was more pronounced in cartilage zones II–IV of both pig lines. Minipig chondrocytes expressed type II and I collagen, Sox9 and β1-integrin at a higher level than hybrid pig chondrocytes. These distinct line-dependent differences should be considered when using hybrid pig-derived chondrocytes for tissue engineering and Göttingen minipigs as a large animal model.

Keywords: Chondrocytes, cartilage, articular, minipig, hybrid pig

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Introduction

Immature hybrid pig cartilage is often used for tissue engineering since it is easily accessible, e.g. from the local abattoir. The collagen architecture of porcine and human cartilage revealed more similarities when compared to that of other species.[1] Cultivated hybrid pig-derived chondrocytes share several properties with human chondrocytes, such as a lower proliferation rate and less rapid loss of type II collagen expression as a feature of dedifferentiation compared with that of other large animals such as sheep and horse cartilage.[2] Healthy human cartilage for chondrocyte isolation and in vitro experiments is limited; therefore, porcine chondrocytes serve very often as a useful in vitro model. Since pig knee joint cartilage is thicker than that of other domestic larger animal species such as sheep and goats,[3,4] the pig could also be a valuable large animal model for orthopaedic research on articular cartilage repair. In view of the very rapid and vast body growth of hybrid pigs, resulting in the logistic problems of handling and housing adults, they are usually not practicable as a large animal model. Immature hybrid pigs show intrinsic cartilage healing,[5] and in cartilage repair studies, the pigs usually stay for six or 12 months post surgery to fully assess the outcome of cartilage repair.[6,7] For these reasons, minipigs are used more often than hybrid pigs as a large animal model. The minipig model allows studying chondral defect repair since the cartilage in the lateral femur condyles is thick enough for defect filling and scaffold fixation. A cartilage thickness of 1–2 mm has been reported for the porcine femur condyle cartilage.[4,8] The 'Göttingen minipig' was developed in the 1960s at the University of Göttingen (Germany) by cross-breeding three pig lines: the Vietnamese pot-bellied pig, the Minnesota minipig and the German Landrace.[9] In contrast to hybrid pigs, which are cross-breeds of several European races, e.g. German Landrace, Large White and Piétrain pig,[10] minipigs are smaller, friendlier and easier to handle (Table 1). The reason behind breeding the Göttingen minipig was the increasing need for a model which has the anatomical and physiological characteristics of the wild boar (Sus scrofa) but

Table 1 Difference between hybrid pig and Göttingen minipig

	Hybrid pig	Göttingen minipig
Character	Stress-sensitive, aggressive [33]	Friendly, calm [34]
Adult weight*	200–300 kg [13, 35]	30–45 kg [13, 36]
Adult age	18–36 months [13, 37]	10, 5–13 [4], 18–22 months ([8], www.minipigs.dk)
Husbandry conditions: food and place	For hybrid pigs more expensive than for Göttingen minipigs	

*The adult age refers to the development of the body weight.

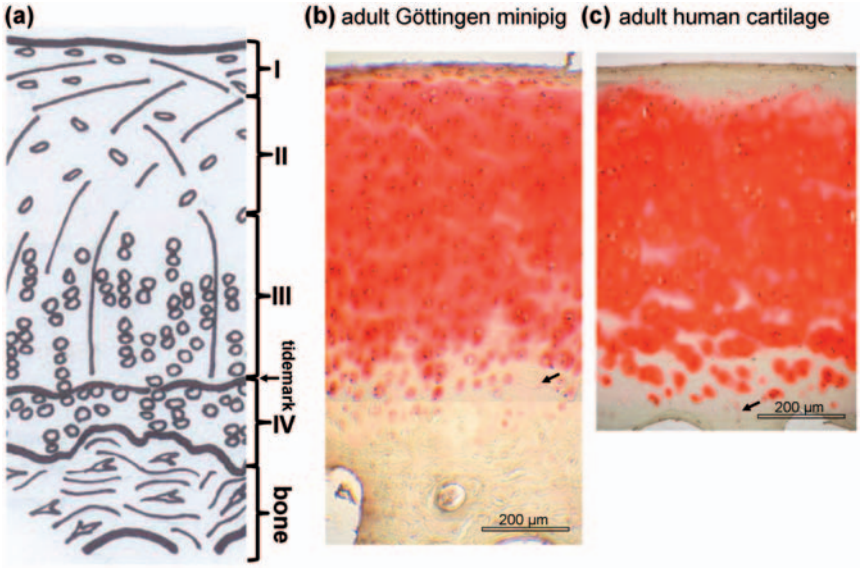

Figure 1 Zonation of the articular cartilage. (a) A scheme of the zonation in adult joint cartilage is depictured: cell distribution and collagen fiber arrangement are foreshadowed. (b, c) Medial femur condyle cartilage of an adult minipig (3.5 years old) and cartilage of a human femoral head was stained with Safranin-O. I: superficial layer; II: intermediate layer; III: deep layer; IV: calcified layer. Arrow: tidemark. Scale bar: 200 µm. (A color version of this figure is available in the online journal)

remained smaller, economical and manageable.[11,12] There exists a white line and a coloured line of the Göttingen minipig.[9,14] Inside the European Union, the Göttingen minipig could only be obtained from the raiser Ellegaard in Denmark (www.minipigs.dk) and is still rather expensive compared to hybrid pigs. Worldwide, there are two other breeding facilities: Marshall BioResources in United States (www.marshallbio.com) and Oriental Yeast Co Ltd. in Japan (www.oycbio.jp). These miniature pigs have an inbreeding coefficient below 10%.[15] Köhn et al.[16] could show differences in the growth development of both pig lines; the hybrid pig shows the classical sigmoid growth pattern, whereas the Göttingen minipig revealed an almost linear growth curve.[13]

So, the question arises whether these both pig lines might show differences in joint macroanatomy and cartilage histology, or whether divergent articular chondrocyte biology is detectable which might influence the outcome of orthopaedic cartilage repair studies. Therefore, the aim of this study was to address these questions using in situ and in vitro analyses of minipig and hybrid pig-derived cartilage and isolated chondrocytes.

Material and methods

Pig-derived cartilage and chondrocytes

Porcine articular cartilage was isolated from healthy knee joints of 3- to 5-month-old hybrid pigs (n = 3) and Göttingen minipigs (n = 5) (Ellegaard Göttingen Minipigs A/S, Soroe Landevej 302, DK-4261 Dalmose, Denmark). Additionally, joint cartilage of the medial femoral condyle of one adult minipig (3.5 years) and one human femoral head (86 years old) were used for histology, as shown in Figure 1. Chondrocytes from another five-month-old hybrid pig were isolated and are shown in Figure 5(b1-b2) but excluded from the other experiments due to ossification in culture. Cartilage for chondrocyte isolation was derived from the trochlea groove, patella and the medial and lateral femur condyles. The hybrid pigs and minipigs from which the joint cartilage was harvested were finalized at the Charité animal experimental research unit at the end of other surgical studies which were approved by the National Animal Care and Use Committee.

Table 2 Primer sequences used for RTD-PCR analysis

Primer	Primer sequences	Assay-ID	Ref Seq (mRNA)	Amplicon size (bp)	Supplier
Human COL2A1	Not published by manufacturer	Hs00264051_m1	NM_033150.2	124	ABI
Human SOX9	Not published by manufacturer	Hs00165814_m1	NM_000346.2	102	ABI
S. scrofa ACAN	Not published by manufacturer	Ss03374823_m1	NM_00116465	60	ABI
Human ITGB1	Not published by manufacturer	Hs00559595_m1	NM_033666.1	75	ABI
S. scrofa ACTB	Not published by manufacturer	Ss03376081_u1	AK237086.1	77	ABI
Mouse ACTB	TGGGACGACATGGAGAA (F) GAAGGTCTCAAACATGATCTGG (R) GCCCCCCTGAACCCTAA (P)	241014	NM_007393	147	Qiagen
Human COL1A1	GGCAACGATGGTGCTAA (F) GACCAGCATCACCTCTGTC (R) AATGCCTGGTGAACGTG (P)	241462	NM_000088	138	Qiagen

F: forward primer; R: reverse primer; P: probe; ABI: Applied Biosystems by Life Technologies Ltd (Darmstadt, Germany); Qiagen: Qiagen GmbH (Hilden, Germany).

Histological staining procedures

Osteochondral tissue cubes from the lateral and medial femoral condyles were fixed in 4% paraformaldehyde (48 h), decalcified in 100 mmol/L EDTA solution (pH 8.0, Merck, Darmstadt, Germany) and embedded in paraffin. Paraffin sections (thickness: 6 µm) were used for histological staining. For haematoxylin eosin (HE) staining, sections were stained for 4 min in Harris haematoxylin solution (Sigma-Aldrich, Munich, Germany), rinsed in tap water and counterstained for 4 min in eosin (Carl Roth GmbH, Karlsruhe, Germany).

For Safranin-O staining, sections were stained for 10 min in Weigert's iron haematoxylin solution (Carl Roth), rinsed for 10 min in running tap water and stained for 5 min in 0.001% Fast Green (Sigma) solution. Subsequently, they were rinsed in 1% acetic acid and stained with 0.1% Safranin-O (Merck) for 5 min. Cartilage thickness was measured light microscopically using ImageJ software.

Finally, all sections were rinsed with aqua dest and subsequently dehydrated in an ascending alcohol series. Then, the sections were embedded with Entellan (Merck). All slices were analysed by light microscopy (Axioskop 40 microscope: Carl Zeiss Jena, Germany). Photos of the sections were taken using an Olympus camera XC30 (Olympus Soft Imaging Solutions GmbH, Muenster, Germany).

Cell counting in articular cartilage

Cartilage was divided into four zones (Figure 1). Cells were counted in corresponding areas of the medial and lateral condyles of the hybrid pig and Göttingen minipig cartilage. Cell diameters and cartilage thickness were measured.

Isolation and culturing of porcine chondrocytes

Cartilage samples were subsequently minced into 1 mm fragments and enzymatically digested with 20 mg/mL pronase (Roche, Basel, Switzerland) diluted in Ham's F-12/Dulbecco's modified Eagle's medium (DMEM) 1:1 (Biochrom AG, Berlin, Germany) for 1 h at 37°C and subsequently digested with 1 mg/mL collagenase (SERVA Electrophoresis GmbH, Heidelberg, Germany) diluted in growth medium (Ham's F-12/DMEM 1:1 containing 1% fetal calf serum (FCS), 50 IU/mL streptomycin, 50 IU/mL penicillin, 0.5 µg/mL partricin, essential amino acids, 2 mM L-glutamine [all: Biochrom AG] and 25 µg/mL ascorbic acid [Sigma-Aldrich]) for 16 h at 37°C. Isolated chondrocytes were resuspended at 28,000 cells/cm^2 in T175 flasks in growth medium with 10% FCS.

Gene expression analysis using real-time detection polymerase chain reaction

Gene expression was determined using real-time detection polymerase chain reaction (RTD-PCR). Porcine articular chondrocytes (passage 0) were cultured for six days until 70% confluence. Chondrocyte total RNA was isolated using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA quantity and quality were evaluated with the RNA NanoDrop (Thermo Scientific, Bremen, Germany). Equal amounts of total RNA (500 ng in a volume of 20 µL) were reverse transcribed using the Qiagen QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. One microlitre aliquots (16.7 ng) of the cDNA were amplified by RTD-PCR in a 20 µL reaction mixture using specific primer pairs (Table 2) for type II collagen, aggrecan, Sox9 and the house-keeping gene β-actin (all: Applied Biosystems, Foster City, USA), type I collagen and the house-keeping gene β-actin (all: Qiagen). Assays were performed using the TaqMan Gene Expression Assay (Applied Biosystems) or the Quantitect Gene Expression Assay (Qiagen) in an Opticon 1 – Real-Time-Cycler (Opticon^{TM} RTD-PCR, Bio-Rad, Hercules, USA) according to the manufacturer's protocols. Relative amounts of mRNA expression for the gene of interest and the β-actin were calculated using the ΔΔCT method.[17]

Western blot analysis of chondrocyte proteins

Chondrocyte monolayers were rinsed with PBS solution, whole cell proteins were extracted by incubation with lysis buffer (25 mM HEPES, pH 7.5, 1% Triton X-100, 5 mM $CaCl_2$, 2 mM DTT, 1 mM EGTA [all: Carl Roth] and

proteinase inhibitors [proteinase complete mini, Roche]) on ice for 5 min. Cell debris were removed by centrifugation (26,000 × g, 20 min, 4°C). Supernatants were stored at −80°C until use. Total protein concentration of whole cell extracts was normalized using Bradford protein assay (Roti-Nanoquant, Carl Roth) using bovine serum albumin as a standard. Samples were separated by Tris-glycine SDS-PAGE (10% acrylamide) under reducing conditions before being transferred to a PVDF membrane (0.45 µm, Carl Roth), using a Trans-Blot apparatus (Bio-Rad Laboratories Inc., Hercules, USA). 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 (Roti-Block, Carl Roth) for 1 h at room temperature (RT) and incubated overnight at 4°C with primary antibodies (mouse anti-human β1-integrin [Millipore Corporation/Chemicon International, Billerica, USA], rabbit anti-human Sox9 antibody [EMD Millipore Co.] or monoclonal mouse anti-β-actin [Sigma-Aldrich]) diluted 1:1000 in blocking buffer. Membranes were washed with washing buffer (PBS/0.05% Tween 20) before and after incubation with horseradish peroxidase conjugated secondary goat-anti-mouse IgG antibodies (1:5000, 1 µg/mL Dako, Glostrup, Denmark) for 2 h at RT. For detection of the antibody binding, 600 µL of substrate luminol reagent and 600 µL of HRP substrate peroxide solution (both: Immobilon Western, Millipore Corporation) were added to the membrane. The membranes were covered for 30 s with the X-ray film in an X-ray-film cassette. Protein bands were semi-quantified by densitometric scanning (AlphaDigiDoc, Innotech, USA).

Type II, I and X collagen immunolabelling

Chondrocytes grown on cover slides (~900 cells/cm^2, 6 d) or paraffin sections were fixed with 4% paraformaldehyde for 15 min and were rinsed in Tris-buffered saline (TBS: 0.05 M Tris, 0.015 M NaCl, pH 7.6). Chondrocytes were subsequently blocked with protease-free donkey serum (5% diluted in TBS) for 30 min at RT, rinsed and incubated with polyclonal rabbit anti-type II collagen, respectively, anti-type I collagen antibody (27.5 µg/mL Acris Antibodies, Herford, Germany) or mouse anti-type X collagen (Abcam), in a humidifier chamber overnight at 4°C. Chondrocytes were subsequently washed with TBS before incubating with donkey-anti-rabbit or mouse-Alexa-Fluor®488 (10 mg/mL, Invitrogen, Darmstadt, Germany) secondary antibody for 30 min at RT. For negative controls, the primary antibodies were omitted during the staining procedure. Cell nuclei were counterstained using 4',6-diamidino-2-phenylindole (0.1 µg/mL, Roche). Labelled chondrocytes were rinsed several times with TBS, embedded with Fluoromount G (Southern Biotech, Biozol Diagnostica, Birmingham, USA) and examined using fluorescence microscopy (Axioskop 40, Carl Zeiss). Images were taken using a XC30 camera (Olympus, Soft Imaging Solution). The determination of fluorescence intensity was carried out with the program ImageJ. The photos were changed in a grey scale picture and in each picture five representative cells were evaluated. The integrated density (product of area and mean grey value) was calculated for each encircled cell (area). Five representative cells of each picture and pictures of three different pigs were included. In addition, the mean average value was deduced from five background intensities per picture (mean grey value$_{background}$). The average fluorescence intensity (mean grey value$_{cells}$) was calculated (=CFCT: integrated fluorescence density) whereby the total cell area multiplied with mean grey value$_{background}$ was subtracted from the average fluorescence intensity and summarized as the corrected total cell fluorescence.

Statistical analysis

All values were expressed as mean with standard deviation. Data were analysed using unpaired two-tailed student's t-test or as stated in each figure legend. The Kolmogorov–Smirnov test was used to analyse the data for the presence of a Gaussian distribution. In the cases where it was not possible due to few data, a Gaussian distribution was assumed. F-test was used for testing the homogeneity of variances (only not-normalized data sets). In the case of inhomogeneous variances, the Welch's correction preceded the unpaired two-tailed t-test (GraphPad Prism 5, GraphPad software inc, San Diego, USA). Statistical significance was set at a P value of ≤ 0.05.

Results

Histological joint cartilage organization in adult pig versus humans

The typical histological organization of mature joint cartilage consisting of four zones could be found in the adult minipig as well as in human joint cartilage. The highest concentration of proteoglycans was observed in zones II and III. Zone I contained only few sulphated glycosaminoglycans; this zone with poor glycosaminoglycan content was larger in human cartilage. Cell distribution was very similar. Cellularity was slightly lower in human cartilage. Proteoglycans in zone IV of adult pig and human joint cartilage remained mainly cell associated in the pericellular extracellular matrix (Figure 1(a) to (c)).

Macroscopical picture of the lateral and medial femur condyles of a hybrid pig and Göttingen minipig

The femur of the Göttingen minipigs revealed a higher intercondylar space and at all a higher inter-individual variation in condylar shape compared to the hybrid pig. The hybrid pig joint was larger and the cartilage was milky-bluish coloured. In contrast, the cartilage of the Göttingen minipig shimmered rose coloured. However, the lateral part of the trochlea appeared white suggesting a higher cartilage thickness (Figure 2(a_1) and (a_2)).

Cartilage thickness and histological structure of hybrid pig and Göttingen minipig cartilage

The cartilage thickness of the medial and lateral femur condyles of both pig lines was measured. The thickness of the cartilage layer was significantly lower in the Göttingen minipig compared to the hybrid pig knee joint cartilage (Figure 2(b), $P = 0.0001$). Cartilage thickness was higher in the medial compared to the lateral femur condyles in the hybrid pig; however, the differences did not reach the significance level (Figure

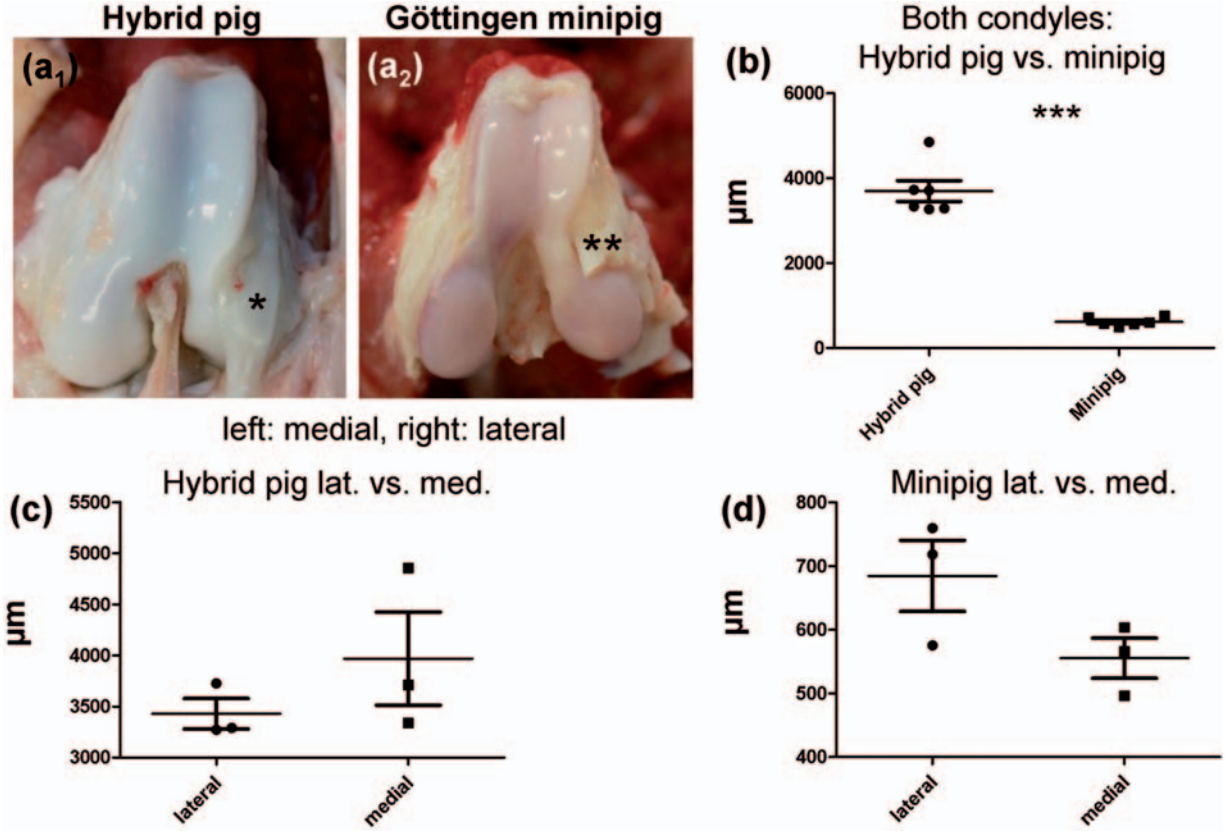

Figure 2 Femur condyles and caudal trochlea groove of the hybrid pig and Göttingen minipig. The caudal end of the trochlea groove and both femur condyles (medial and lateral) are shown. (a_1) Hybrid pig, (a_2) Minipig, left: medial; right: lateral condyle. *Tendon of the *Musculus extensor digitorum superficialis* and **stump of the same tendon. (b) Comparison of joint cartilage thickness (data of medial and lateral femur condyles were pooled) in the hybrid compared to the minipig. Statistics (a) Kolmogorov–Smirnov test positive (Gaussian distribution), F-test (inhomogenous variance), Welch's correction and unpaired two-tailed *t*-test. ***$P = 0.0001$. (c, d) Comparison of the cartilage thickness in the lateral versus the medial femur condyles in the hybrid (c) and minipig (d). Statistics (b to d) with the assumption of a Gaussian distribution a two-tailed *t*-test was performed. Cartilage samples of both condyles of three pigs of each pig line were used. (A color version of this figure is available in the online journal)

2(c)). Insignificant differences could also be detected in the minipig knee joint cartilage whereby the cartilage of the lateral condyle was thicker than that derived from the medial condyle (Figure 2(d)).

The Safranin-O staining was used to visualize and localize the negatively charged groups of sulphated glycosaminoglycans. The cartilage of the lateral condyle in both pig lines showed a more intensive staining compared to that of the medial condyle. The sulphated proteoglycans were more homogenously distributed in the hybrid pig cartilage zones II–IV compared to the Göttingen minipig (Figure 3(a) and (b1–4)). The hybrid pig cartilage revealed no major sulphated proteoglycan deposition in zone I, and in zones II–IV the sulphated proteoglycans were more or less homogenously distributed. The Göttingen minipig had the highest accumulation of sulphated proteoglycans in zone IV, whereas the sulphated proteoglycans were less intensely stained in zones I–III within the extracellular cartilage matrix. A slightly more ordered cell distribution, e.g. the beginning of a formation of vertical rows in zone III was evident in the Göttingen minipig but not the hybrid pig. The cartilage from the hybrid pig and the Göttingen minipig did not show the classical zonation in both femur condyles. The chondrocytes in the cartilage of the Göttingen minipig showed a more homogenous distribution with larger areas containing no cells in both condyles (Figure 4). The tidemark could not be detected in both pig lines (Figure 3a1–4). The mineralized cartilage zone contained a higher number of highly hypertrophic chondrocytes in the hybrid pig samples than the same zone in the minipig cartilage. The lateral and medial femur condyles of the hybrid pigs contained some singular blood vessels in zones II–IV (Figure 5). The vessels were surrounded by some mesenchymal endost-like tissue and the chondrocytes in the neighborhood showed features of hypertrophy. A faint staining for type X collagen was detectable. In contrast, no blood vessels could be detected in the cartilage of Göttingen minipigs.

Results of cell counting and cell diameter

Cell numbers were higher in the lateral condyles but lower in the medial condyles of the hybrid pigs compared to the respective condyles of the Göttingen minipigs (Table 3). The cell diameters were comparable in cartilage of both pig lines (zones II–III) being around 9.1 µm (±0.79 µm) irrespective of

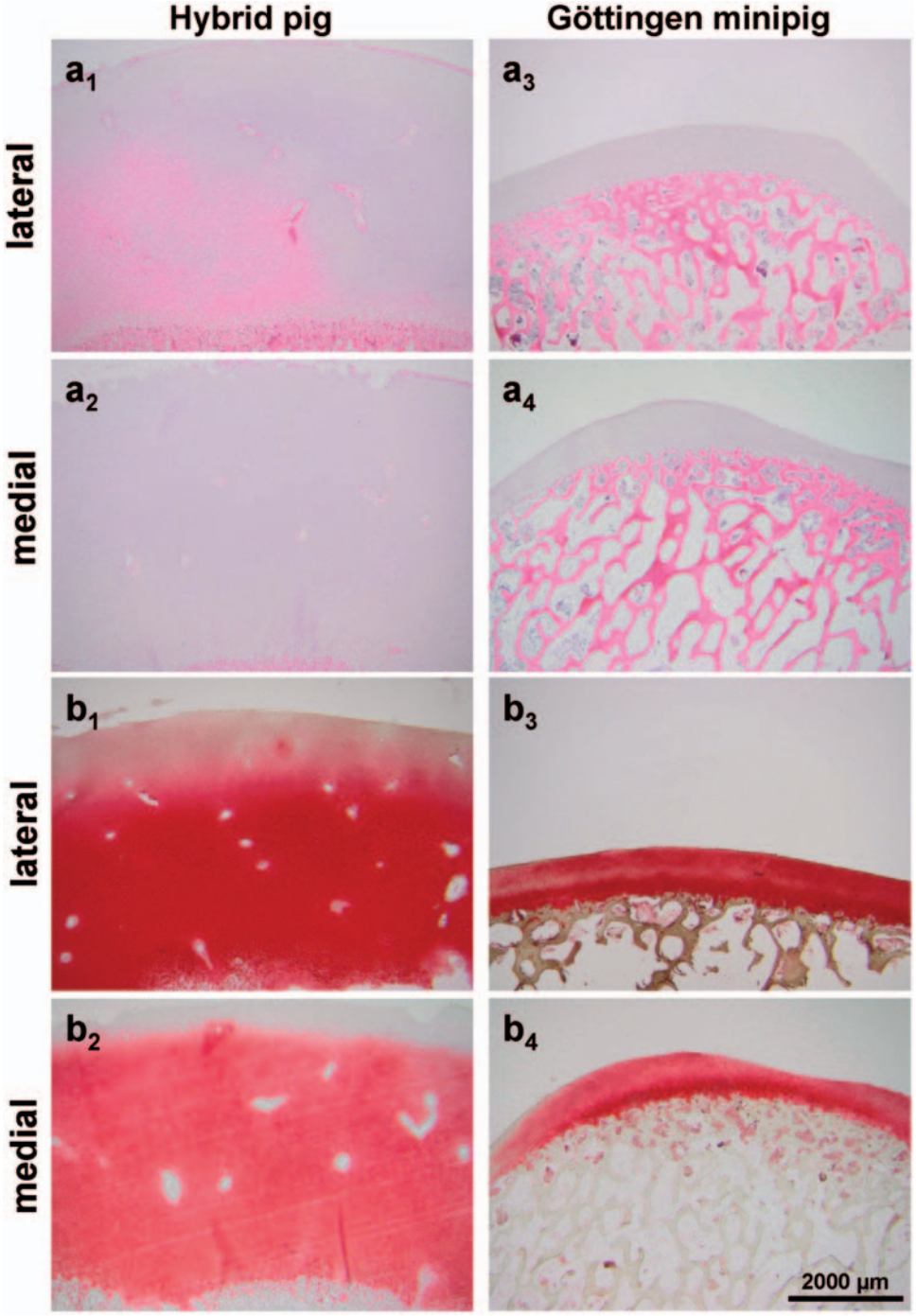

Figure 3 Cartilage thickness and proteoglycan distribution in hybrid pig and Göttingen minipig cartilage. Histological staining of both the lateral (a_1,a_3 and b_1,b_3) and the medial (a_2,a_4 and b_2,b_4) condyles of a representative hybrid pig (left) and Göttingen minipig (right) are shown. HE (a_1 to a_4), Safranin-O (b1-b4) stainings. Sulphated proteoglycans are more prominent in the deeper joint cartilage layer and immediate pericellular ECM and more pronounced in the hybrid pig whereby the superficial layer in the hybrid pig is lacking proteoglycans. Cartilage samples of both condyles of three pigs of each pig line were analysed. Scale bars: 2000 µm. (A color version of this figure is available in the online journal)

the condyles. Chondrocytes in the superficial zone were mostly singularly distributed. In zones II–III of the Göttingen minipig cartilage, isogenic groups consisting of two chondrocytes could be detected and in zone IV, there were isogenic groups predominantly containing two to four chondrocytes. There were a lower number of chondrocytes forming an isogenic group consisting of at least two chondrocytes in zones II and III in the hybrid pig compared to the minipig cartilage. In zone IV, the cell numbers in the isogenic groups increased by up to six chondrocytes with a less-defined columnar arrangement in the hybrid pig compared to the minipig.

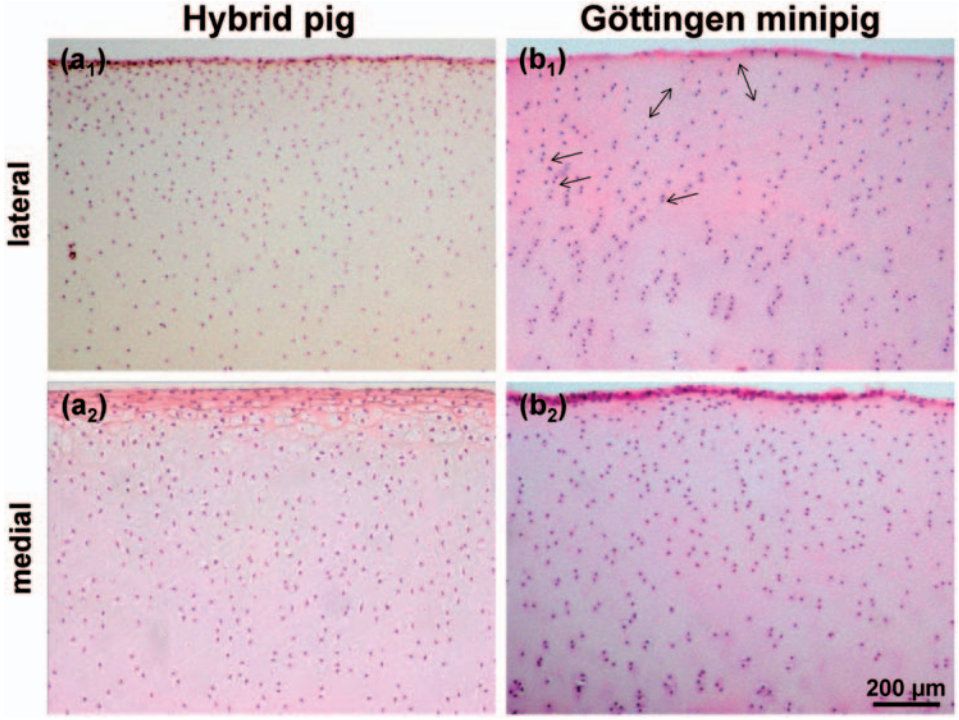

Figure 4 Articular cartilage of hybrid pig of zones I and II and Göttingen minipig cartilage of zones I–III. HE, in contrast to the hybrid pig (a_1, a_2), the Göttingen minipig (b_1,b_2) shows larger isogenic groups (arrows). In the minipig cartilage, cell-free matrix areas are detectable (double head arrows). Cartilage samples of both condyles of three pigs of each pig line were analysed. Scale bars: 200 µm. (A color version of this figure is available in the online journal)

Morphology of monolayer-cultured hybrid pig and Göttingen minipig chondrocytes

The chondrocytes of both pig lines showed a round phenotype in passage 0 (Figure 6). During the cultivation period, the cells developed a fibroblast-like morphology and formed multiple focal adhesion sites with their filo- and lamellipodia. The Göttingen minipig-derived chondrocytes showed more strongly developed multiple filo- and lamellipodia in passage 1 in comparison to hybrid pig chondrocytes. During the culturing period, the chondrocytes of both pig lines increased their cell diameters.

Gene expression profile of isolated articular chondrocytes derived from the hybrid pig or Göttingen minipig

The type II collagen and Sox9 genes were expressed at a significantly higher level in monolayer-cultured chondrocytes of the Göttingen minipig compared to the hybrid pig chondrocytes (Figure 7, $P = 0.0105$; $P = 0.0241$). Type II collagen was almost 20-fold up-regulated in the Göttingen minipig chondrocytes compared to the hybrid pig cartilage. Despite not reaching a significant level, type I collagen expression was also higher in the minipig than the hybrid pig-derived chondrocytes. Aggrecan gene expression did not significantly differ between chondrocytes of both pig lines (Figure 7).

Western blotting results

At the protein level, the chondrogenic transcription factor Sox9 was highly expressed in Göttingen minipigs compared to hybrid pig joint chondrocytes (Figure 8, $P = 0.0334$). The adhesion receptor β1-integrin revealed both the hypoglycosylated (biosynthetic precursor of β1-integrin subunit) as well as the hyperglycosylated form (mature β1-integrin subunit).[18] The hypoglycosylated form was expressed at a significantly higher level in the Göttingen minipig chondrocytes when compared to the hybrid pig (Figure 8; $P = 0.0144$).

Immunohistological results

Type II collagen expression and distribution was evaluated in cultured chondrocytes using immunofluorescence labelling. Göttingen minipig-derived chondrocytes expressed a higher level of type II collagen than chondrocytes isolated from hybrid pig knee cartilage. The expression and distribution of type I collagen was significantly higher in the minipig-derived chondrocytes (Figure 9; $P = 0.0076$).

Discussion

The pig is a frequently used orthopaedic large animal model for the testing of surgical techniques and therapies.[6,19–22] Laboratory parameters and cartilage thickness are more similar to humans' physiology and anatomy compared to other animal models.[23]

The rose colour of the Göttingen minipig cartilage suggests that the vascularized subchondral bone is shining through the rather thin cartilage layer. Adult porcine cartilage has been reported for several pig lines to possess a thickness of 1.5–2.0 mm in the medial femoral condyle.[4,7] In contrast to these studies with adult animals, we found a thicker cartilage in the knee joint cartilage of immature hybrid pigs (3.7 ± 0.25 mm). Our measurement in the immature minipig led to slightly thinner cartilage layers compared to the hybrid pig in the medial condyle of $0.62 \text{ mm} \pm 0.41$ mm (Figure 2(b)). The cartilage

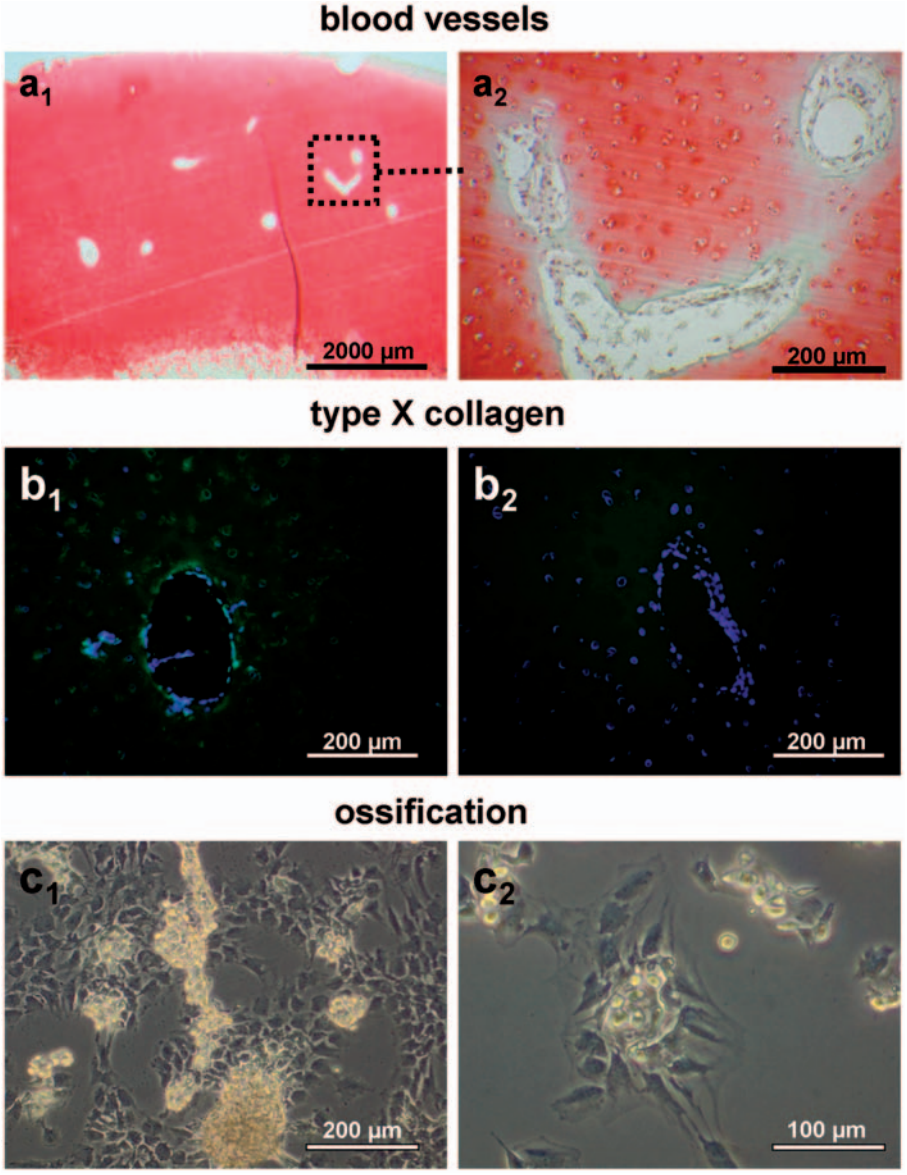

Figure 5 Blood vessels of the articular cartilage of the hybrid pig and ossificating hybrid pig-derived chondrocytes in culture. Histological paraffin sections were stained with Safranin-O. (a_1) Survey of all four zones of the articular cartilage of the hybrid pig shows vessels in zones II–IV. Scale bar: 2000 µm. (a_2) A blood vessel is depictured at higher magnification. It is surrounded by some mesenchymal tissue. Scale bar: 200 µm. (b_1) type X collagen immunolabelling revealed a faint reactivity in the neighborhood of a vessel. (b_2) Negative staining control. (c_1) Porcine chondrocytes ossificating in culture are shown. Cells form dense nodules. Scale bar: 200 µm. (c_2) At higher magnification. Scale bar: 100 µm. (A color version of this figure is available in the online journal)

Table 3 Results of cell counting in articular cartilage

	Hybrid pig		Göttingen minipig	
Condyles	**Lateral**	**Medial**	**Lateral**	**Medial**
Cell number/cm^2				
Zones I and II	1404 (±377)	1195 (± 97)	1054 (± 308)	1492 (± 504)
Zones III and IV	817 (± 132)	705 (± 33)	762 (± 284)	810 (± 94)
Cell diameter	9.1 (± 0.79)			

Cartilage was divided into four zones (Figure 1). Cells were counted in corresponding areas of the lateral and medial femur condyles of the hybrid pig and Göttingen minipig cartilage. Cell diameters were assessed from histological slides ($n = 3$, both condyles in µm).

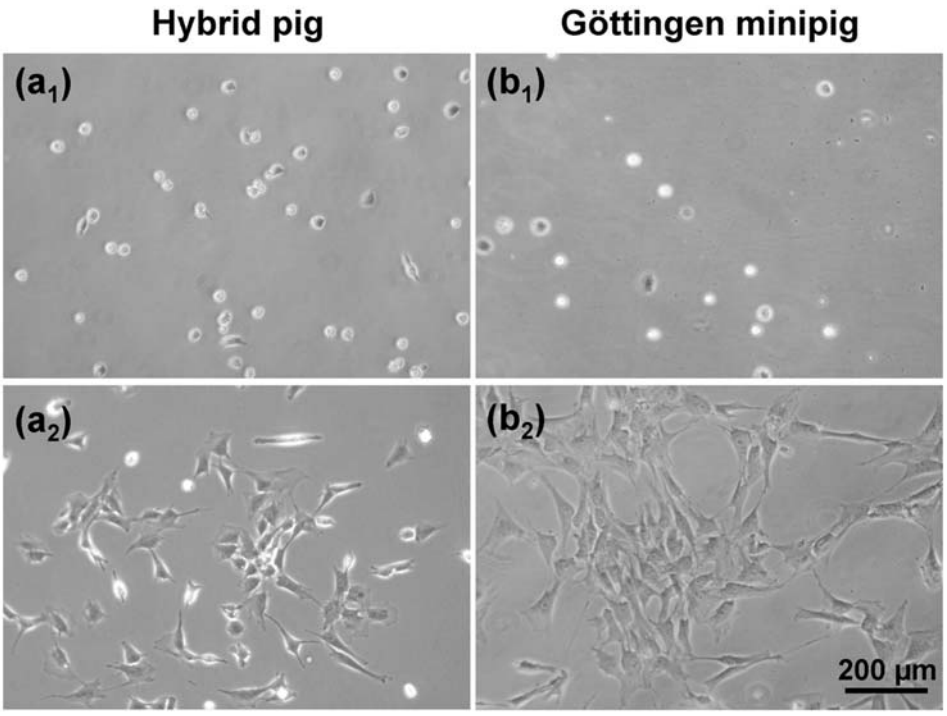

Figure 6 Monolayer-cultivated porcine articular chondrocytes of the hybrid pig and Göttingen minipig**.** Freshly isolated primary porcine articular chondrocytes of the hybrid pig (a_1,a_2) and Göttingen minipig (b_1,b_2) were seeded in monolayer culture in the passage 0 (a_1, b_1) and passage 1 (a_2, b_2). Chondrocytes of the medial and lateral femur condyle cartilages were pooled. A morphologic shift towards a more and more fibroblastic phenotype is evident in cells of both pig lines. Representative pictures are shown. Isolated chondrocytes of three hybrid and five Göttingen minipigs were analysed. Scale bars: 200 µm

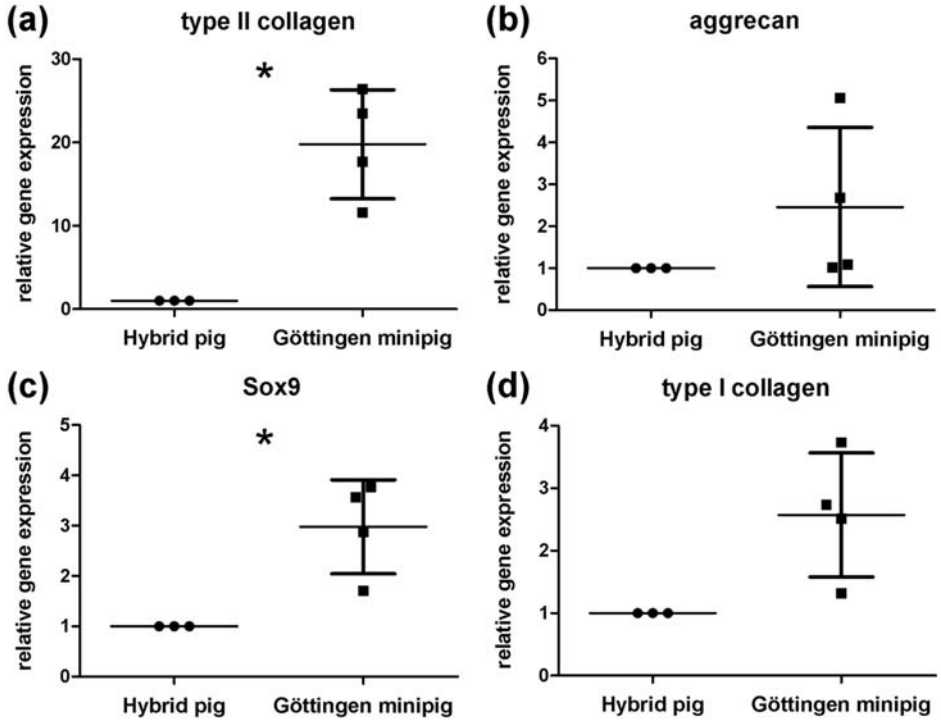

Figure 7 Relative gene expression of cartilage markers in monolayer-cultured chondrocytes of the hybrid pig and Göttingen minipig. Chondrocytes (passage 0, derived from cartilage of the medial and lateral femur condyles) were cultured for six days. Type II collagen (a), aggrecan (b), Sox9 (c) and type I collagen (d). Hybrid pig, $n = 3$, Göttingen minipig, $n = 4$, $P < 0.05$. Hybrid pig cartilage data were normalized (set 1). Statistics: two-tailed *t*-test (with the assumption of a Gaussian distribution). A: *$P = 0.0105$; *C: $P = 0.0241$. Isolated chondrocytes of three hybrid and four Göttingen minipigs were analysed

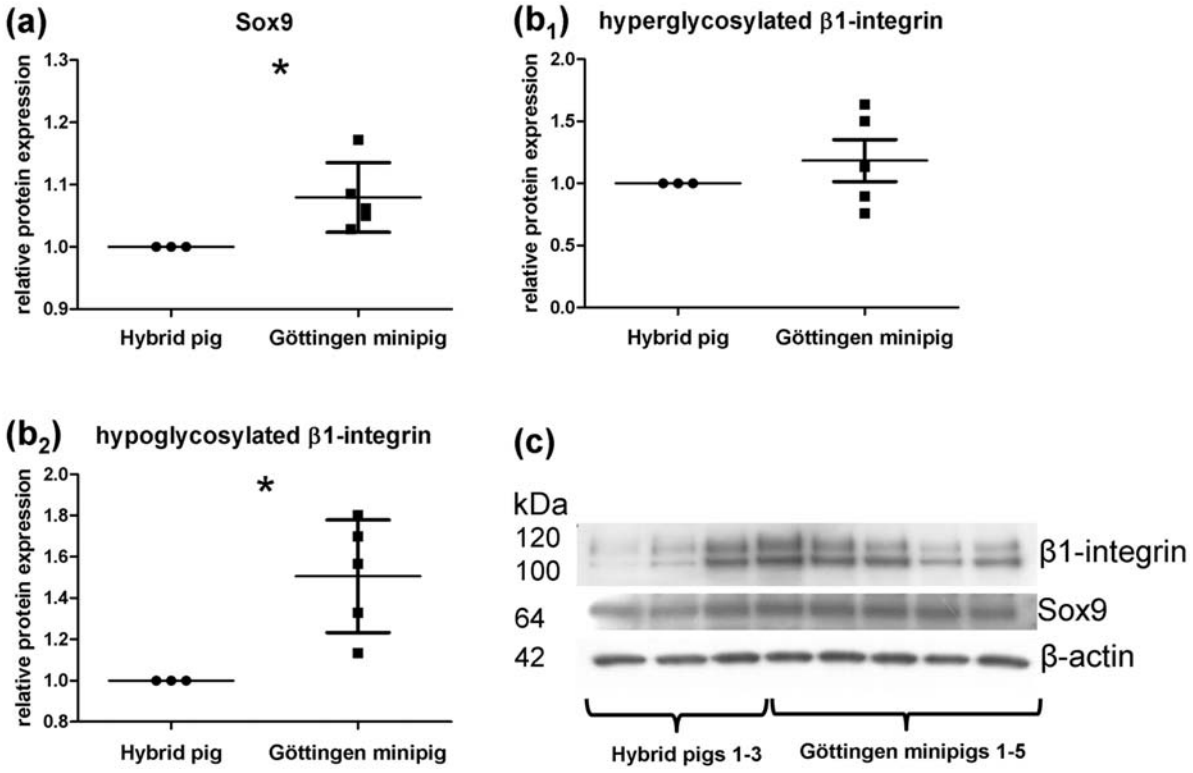

Figure 8 Relative protein expression of monolayer-cultured chondrocytes of the hybrid pig and Göttingen minipig. Chondrocytes (passage 0, derived from cartilage of the medial and lateral femur condyles) cultured for six days were used for western blotting followed by densitometrical analysis. Sox9 (a), hyperglycosylated and hypoglycosylated β1-integrin (b_1,b_2). Protein bands generated by western blotting are depicted (c). Hybrid pig, $n = 3$, Göttingen minipig $n = 5$, $P < 0.05$. Data of hybrid pigs were normalized. Statistics: Kolmogorov–Smirnov test positive (Gaussian distribution), F-test (homogenous variance), two-tailed *t*-test. (a) $^*P = 0.0334$; (b_2) $^*P = 0.0144$. Isolated chondrocytes of three hybrid and five Göttingen minipigs were analysed

layer was also thinner compared to the report of Chang et al.[7], which described that the knee joint cartilage of adult Göttingen minipigs is between 1 and 2 mm. The differences in the cartilage thickness between our study and the report of Chang et al.[7] could be due to the fact that Chang worked with another minipig line and with adult individuals. Our study revealed that the cartilage of the lateral condyle was thinner than that of the medial condyle in the hybrid pig, whereas the Göttingen minipig had thicker cartilage on the lateral condyle. Temple-Wong et al.[24] reported that the thickness of human joint cartilage covering the medial and lateral femur condyles was not significantly different in adults. This discrepancy in femur condyles cartilage thickness between immature individuals of both pig lines and adult humans might arise from the species- and maturation-dependent leg position and load exposure of both condyles, which can also differ between biped and quadruped mammals. It might be influenced by a higher joint loading caused by the genu valgus position of the legs in the knee joints of Göttingen minipigs. Using a minipig model for the analysis of chondral defect healing, the lateral condyle covered by a thicker cartilage layer might offer more favourable defect localization than the medial condyle; however, the tendon of Musculus extensor digitorum longus hinders surgical access. This tendon does not bridge the knee joints in humans.

The fact that the tidemark could not be detected in the tissue sections investigated in this study is typical for immature cartilage. The tidemark is developed only in the adulthood as reported by Simkin.[25] The deeper cartilage regions in the immature joint were transformed later into subchondral bone via enchondral ossification. Hence, some of the isolated cells from these deeper layers represent chondroblasts which can undergo hypertrophy and osteogenic differentiation. Accordingly, sometimes osteogenesis could be observed in chondrocyte cultures of immature hybrid pigs, as shown in Figure 5. The animals included in this study were immature, but immature pigs were usually used to isolate porcine chondrocytes and were therefore selected for this study since adult pigs are less abundantly available. The FDA states that minipigs reach skeletal maturity at an age of 10.5–13 months[4] (the minipig line was not mentioned in this report). Musculoskeletal maturity of the pigs is the prerequisite for their use as orthopaedic in vivo models. Immature animals reveal a high capacity for intrinsic cartilage repair.[5] Musculoskeletal maturity is usually based on epiphyseal closure of the long bones. However, cartilage maturity may differ from the bone growth completion. The presence of the blood vessels in the cartilage of juvenile hybrid pigs indicates that the cartilage maturity is not achieved. Therefore, the absence of blood vessels in the minipig joint cartilage suggests also a higher grade of cartilage maturity in this pig line at the same age. Blood vessels in the hybrid pig joint cartilage, which were detectable not only in the deeper but also in the upper cartilage layers, were surrounded by areas of hypertrophic chondrocytes. A weak immunoreactivity for type X collagen as an indicator of hypertrophy underlines this observation. This

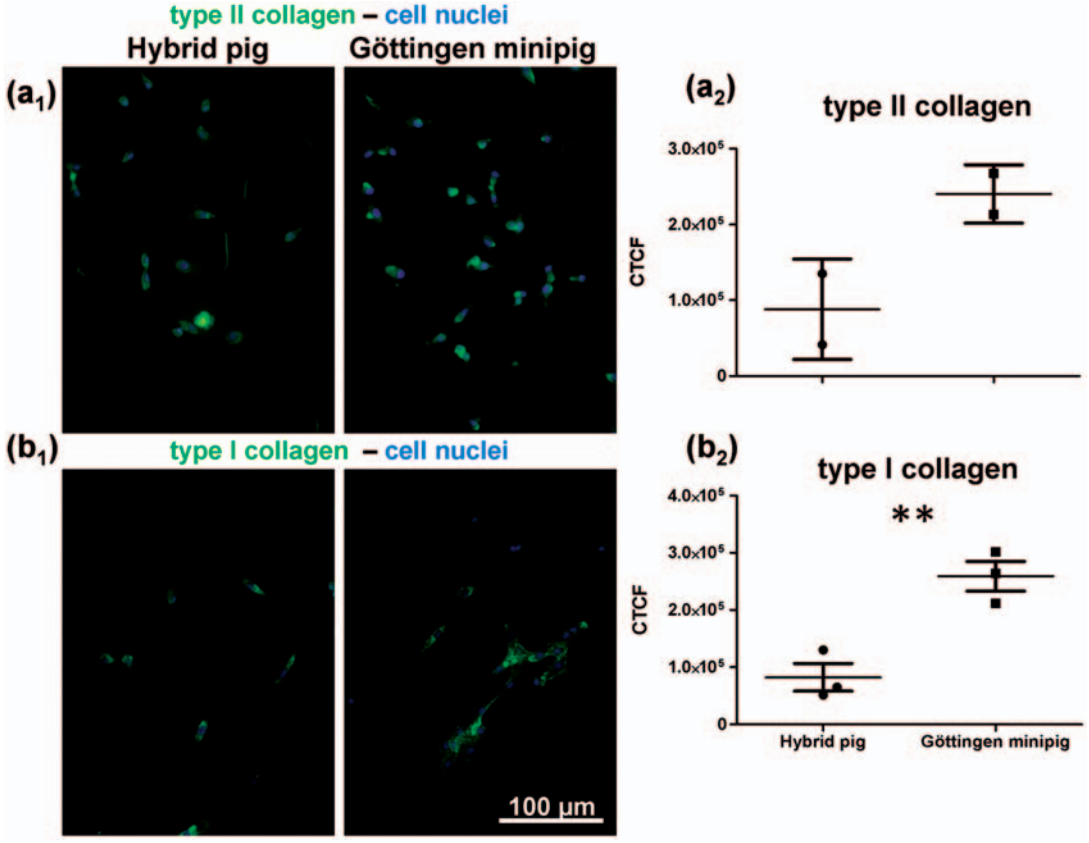

Figure 9 Immunofluorescence labelling of type II and I collagen in cultured chondrocytes of the hybrid pig and Göttingen minipig. Immunolabelling for type I and II collagen expression in chondrocytes isolated from the medial and lateral condyle cartilage of hybrid pig and minipig is shown. (a_1) type II collagen; (b_1) type I collagen. Scale bar: 100 µm. (a_2, b_2) densitometric evaluation. CTCF: corrected total cell fluorescence. Statistics: unpaired students *t*-test. $^{**}P = 0.0076$. (A color version of this figure is available in the online journal)

fact suggests that enchondral ossification expands from multiple centres within the immature joint cartilage and not only from the subchondral district. Apart from that observation, the diffusion of nutrients through the thick joint cartilage layer of the hybrid pig remains limited and may lead to some hypoxic stimuli, which induce vessel formation and maintenance.

The higher variability in the condyle shape of the Göttingen minipig compared to the hybrid pig could be influenced by a different loading on the cartilage due to deviations of the leg axis. One can consider that the leg axis might also differ between immature and adult individuals. The limited genetic pool in the closed pig population used for breeding the Göttingen minipig[15] could also influence it. However, no lameness or cartilage degeneration could be observed in adult Göttingen minipigs used for orthopaedic studies (own non-published results). The divergent grade of zonation and proteoglycan distribution can result from the different body weight development in both species. Mature minipig and human cartilage showed many similarities. As already mentioned, hybrid pigs show the classical sigmoid growth pattern, whereas the Göttingen minipig follows an almost linear growth curve. At the time of sampling, the hybrid pig was in the exponential phase of the growth, whereas the Göttingen minipig revealed a linear growth progress.[16] The start of cartilage zone formation in the minipig suggests the progression of its maturation. The higher number of cells within the isogenic groups of deeper cartilage zones in the hybrid pigs might be indicative for a higher proliferative activity of the chondrocytes to form a thicker cartilage layer. The total cell numbers were higher in zones I and II in both pig lines compared to that of the zones III and IV, a feature which can also be observed in mature bovine cartilage.[26] Higher expression of typical cartilage markers such as type II collagen and the master chondrogenic transcription factor Sox9 regulating its expression[27,28] could be detected in the minipig chondrocytes compared to those of the hybrid pig indicating a higher grade of maturity. Sox9 is a negative regulator of cartilage vascularization[29] possibly explaining the absence of blood vessels in the immature minipig but not the hybrid pig cartilage. The non-specific extracellular matrix protein type I collagen was also expressed more highly at the gene and protein level in the minipig chondrocytes, suggesting an overall higher synthetic capacity compared to the hybrid pig. β1-integrins play a role in chondrogenesis mediating cell–extracellular matrix (ECM) interactions.[30–32] The higher amount of integrin precursors in the minipig cartilage might suggest a more intense cell–ECM interaction and probably a higher ECM stability.

Outlook and limitations

Despite the present study is limited by the rather low animal number of each pig line that could be included, the following conclusions can be supposed: Compared to hybrid pig cartilage, the minipig cartilage revealed at the same juvenile age characteristics of a higher degree of cartilage maturity. This observation was confirmed by histological structure and chondrocyte gene expression profile. Therefore, the use of minipig instead of hybrid pig cartilage for chondrocyte isolation should be preferred for in vitro studies, especially in case of selecting a cartilage defect model in the pig. Orthopaedic cartilage repair models are usually conducted in the minipig. Since many studies based on pig-derived cartilage provide no further information about the particular pig line used, the results of the present study indicate that details concerning the pig line might have relevance and should be provided in orthopaedic in vitro and in vivo studies.

Author contributions: CM and GST wrote the manuscript and interpreted data; BK, UM, KES, AL and WE reviewed and proof read the manuscript; CM conducted most of the experiments and analysed the data; BK, UM, CM and KES performed some experiments and analysed some data; GST, UM, KES and AL established the study design; KES, AL, UM and GST were involved in sample acquisition.

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