

Mesenchymal Stem Cells Isolated from the Anterior Cruciate Ligament: Characterization and Comparison of Cells from Young and Old Donors

Patrick Prager, MD¹, Manuela Kunz¹, Regina Ebert, PhD¹, Ludger Klein-Hitpass, PhD², Jakob Sieker, MD³, Thomas Barthel, MD¹, Franz Jakob, MD¹, Christian Konrads, MD^{1,4,*}, and Andre Steinert, MD^{1,5,*}

¹Department of Orthopaedic Surgery, Koenig-Ludwig-Haus, Center for Musculoskeletal Research, Julius-Maximilians-University Wuerzburg, Wuerzburg; ²BioChip Laboratory, Center for Medical Biotechnology, University of Duisburg-Essen, Essen, Germany; ³Department of Orthopaedic Surgery, Harvard Medical School, Boston, MA, USA; ⁴Department of Orthopaedic Trauma, Center for Trauma Research, BG Trauma Center Tuebingen, University of Tuebingen, Tuebingen; ⁵Department of Orthopaedics and Traumatology, Agatharied Hospital, Teaching Hospital of the University of Munich, Hausham, Germany

Purpose: Mesenchymal stem cells (MSCs) isolated from the anterior cruciate ligament (ACL) share multiple characteristics of bone marrow-derived mesenchymal stem cells (BMSCs), allowing their use for regenerative therapies. Injuries to the ACL can affect people of all ages. This study assesses whether the regenerative potential of ACL-derived MSCs (ACL-MSCs) from old donors is as high as the potential of ACL-MSCs from young donors.

Materials and Methods: ACL-MSCs were isolated from ACL tissues obtained from young and old donors at the time of ACL reconstruction or arthroplasty. Proliferative capacity, multilineage differentiation potential (chondrogenic, osteogenic, and adipogenic lineages), and transcriptome-wide gene expression were assessed and compared between young and old donors. BMSCs of middle-aged donors served as an additional comparator.

Results: No substantial differences between ACL-MSCs from young and old donors were observed in their proliferative capacity and multilineage differentiation potential. The latter did not substantially differ between both ACL-MSC groups and BMSCs. Differential expression of genes related to the cytoskeleton and to protein dephosphorylation amongst other pathways was detected between ACL-MSCs from young and old donors.

Conclusions: Regenerative potential of ACL-MSCs from old donors was not substantially lower than that from young donors, suggesting that regenerative therapies of ACL tears are feasible in both age groups. *In vivo* studies of the effect of age on the efficacy of such therapies are needed.

Keywords: Knee, Anterior cruciate ligament, Stem cell, Differentiation, Donor age

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Correspondence to: Christian Konrads, MD
Department of Orthopaedic Surgery, Koenig-Ludwig-Haus, Center for
Musculoskeletal Research, Julius-Maximilians-University Wuerzburg,
Wuerzburg, Germany

Tel: +49-931-803-3136, Fax: +49-931-903-1209

E-mail: christian.konrads@gmail.com

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*These authors contributed equally to this study.

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Introduction

A tear of the anterior cruciate ligament (ACL) is a common injury, which often requires surgical reconstruction using an autologous tendon graft, e.g., the semitendinosus tendon¹. In general, successful ligament healing is achieved when the sequence of blood clot formation, invasion by leukocytes and fibroblasts, proliferation, extracellular matrix (ECM) molecule secretion, and tissue remodeling take place without disturbances². Following an ACL injury, the high intra-articular activation of the fibrinolytic system disintegrates the blood clot too early to allow for invasion and ECM production of cells; and thereby permanently impedes successful healing³.

When untreated, tears of the ACL can result in severe joint instability and facilitate the development of secondary meniscal tears and osteoarthritis, which necessitates the functional restoration

of the ligament⁴). ACL reconstruction, the surgical replacement of the torn ligament by an autologous tendon graft (e.g., semitendinosus or patella tendon) is the current standard treatment⁵). Recent high level evidence suggests that current techniques of ACL reconstruction do not substantially improve patient-reported outcomes⁶). On average, patients that received joint surgery due to an ACL injury are subject to total knee replacement 15 years earlier than those with previous knee surgery not related to an ACL injury⁷). In order to improve outcomes after an ACL injury, novel regenerative therapies are being explored, which might allow restoration of the proprioceptive nerve functions of the native ACL obviating the harvest of an autologous tendon graft.

Tissue engineering approaches for the restoration of the ACL have been described, many of which use multipotent mesenchymal stem cells (MSCs) for several reasons. MSCs have been isolated from a variety of different tissues such as bone marrow, adipose tissue, trabecular bone, and tendon⁸). Research showed that cells isolated from torn ACLs of young donors possess a surface antigen expression profile highly similar to that of bone marrow-derived mesenchymal stem cells (BMSCs) and they were able to undergo multilineage differentiation, which qualifies them as MSC types⁹). Thus, the use of ACL-derived MSCs (ACL-MSCs) for regenerative therapies of the injured ACL appears attractive. However, several studies have reported age-dependent differences in the regenerative capacities of BMSCs of differently aged donors¹⁰). Since injuries to the ACL can occur at both young and old age, it is necessary to define whether such regenerative approaches can be beneficial to both age groups. The objective of this study was to assess whether the regenerative potential of ACL-MSCs from old donors (>60 years) is as high as the potential of ACL-MSCs from young donors (<30 years). We specifically tested the hypothesis that ACL-MSCs from old and young donors would be different regarding transcriptome-wide gene expression, proliferative capacity (colony forming unit [CFU] assay, cumulative population doubling times), and multilineage differentiation potential (chondrogenic, osteogenic, and adipogenic lineages). BMSCs of middle-aged donors served as an additional comparator in the cell culture assays.

Materials and Methods

This study was approved by our University's Ethics Committee. Informed consent was obtained from all individual participants included in the study.

1. Patient Characteristics and Tissue Collection

ACL tissue was obtained from 5 young (23.2 years; range, 17 to

27 years) and 6 old (73.7 years; range, 69 to 79 years) ACL donors at the time of ACL reconstruction or joint replacement surgery.

2. Isolation and Culture of ACL-MSCs

ACL-MSCs were isolated using collagenase digestion⁹). Tissue specimens were minced into smaller pieces and digested in 12.5 U/ μ L collagenase NB4 (Serva Electrophoresis GmbH, Heidelberg, Germany) in complete medium consisting of Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 (PAA Laboratories GmbH, Coelbe, Germany), 10% fetal bovine serum (FBS, PAA Laboratories GmbH), 1% penicillin/streptomycin (PAA Laboratories GmbH) and 50 mg/mL L-ascorbic acid 2-phosphate (Sigma Aldrich GmbH, Taufkirchen, Germany) for 18 hours. The released cells were separated from tissue debris using a cell strainer, pelleted at 1,200 g for 5 minutes, re-suspended in complete medium, and seeded in 75 cm² flasks. For all subsequent differentiation protocols, cells were cultivated in an incubator at 37°C and 5% CO₂ in a humidified atmosphere with media changes every 2–3 days. Cells were passaged using 1% trypsin (PAA Laboratories GmbH) for 5 minutes for detachment, an equal volume of complete medium to stop the reaction, centrifugation at 1,200 g for 5 minutes to pellet cells, and resuspension in complete medium, after which the cells were seeded at a minimal density of 5 \times 10³ per ml in cell culture flasks.

3. Isolation and Culture of Human BMSCs

BMSCs were isolated from 3 donors undergoing total hip replacement surgery (mean age 50 years). Trabecular bone was harvested from the femoral diaphysis and transferred to 50 mL canonical tubes containing 20 mL complete medium, vortexed and centrifuged at 1,200 g for 5 minutes. The fatty supernatant was carefully removed and pelleted cells were washed for five times in fresh complete medium. The washing medium was collected, vortexed, and separated from remaining bony fragments using a cell strainer. Following centrifugation at 1,200 g for 5 minutes, the cells were re-suspended in complete medium and plated at a density of at least 10⁶ cells per mL in complete medium in 175 cm² flasks. After three days, the adherent MSCs were washed with pre-warmed phosphate buffer solution to remove non-adherent blood cells from the flasks. Subsequent culture was performed similarly to the culture of ACL-MSCs.

4. Colony Forming Units-Fibroblasts Assay

A modified protocol of the CFU-fibroblasts (CFU-f) assay was used¹¹). First passage ACL-MSCs were seeded at a density of 200 cells per cm² in complete medium in 6 well plates. Medium was

changed every 2–3 days for a total time of 10 days followed by the fixation of the cell layer with cold methanol for 10 minutes. CFU-f colonies were detected by crystal violet staining for 5 minutes followed by three washing steps with phosphate buffer solution. The colonies were counted manually by four independent observers with an accumulation of at least 25 cells being counted as a single colony.

5. Cumulative Population Doublings

Cumulative population doublings were investigated in a long-term culture system. Therefore, third passage cells were seeded at a density of 5×10^3 cells per cm^2 in three 25 cm^2 cell culture flasks for each cell donor and cultured in 5 mL complete medium. Once the monolayer became subconfluent, the cells were detached from the flasks using trypsin digestion, counted, and reseeded as described above. The procedure was repeated until no repeated increase in cell number could be detected or until a total time of 100 days in culture.

6. Chondrogenic Differentiation

Chondrogenic differentiation was performed in a pellet culture system, as previously described¹²⁾. Third passage cells were detached from the plastic surface of culture flasks using a trypsin digestion and counted, and 2.5×10^5 cells were transferred in 50 mL canonical tubes containing 500 μL of either chondrogenic or control media, followed by centrifugation at 1,500 g. Pellets were cultured in DMEM high glucose medium supplemented with 1% penicillin/streptomycin, 40 ng/mL L-proline, 50 ng/mL L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate, 100 ng/mL pyruvate, 100 nM dexamethasone, 1% ITS (all Sigma-Aldrich, Schnelldorf, Germany), and human transforming growth factor (TGF)- β 1 (R&D Systems) for 28 days with the caps slightly opened to enable gas exchange. Control pellets were cultured in the absence of TGF- β 1. Reverse transcription polymerase chain reaction (RT-PCR) of the chondrocyte-specific marker genes aggrecan core protein (*ACAN*), biglycan (*BGN*), collagen alpha-1(II) chain (*COL2A1*), cartilage oligomeric matrix protein (*COMP*), and fibromodulin (*FMOD*) were used to gauge the degree of chondrogenic differentiation. Collagen alpha-1(X) chain (*COL10A1*) was used to determine the degree of chondrocyte hypertrophy. Glycosaminoglycan (GAG) deposition was quantified using the Blyscan Glycosaminoglycan Assay kit (Tebu-bio, Offenbach, Germany) and histomorphometric quantification of alcian blue positive areas. Further, qualitative type II collagen immunohistochemistry was performed.

7. Osteogenic Differentiation

Osteogenic differentiation was performed in a monolayer culture system as previously described with slight modifications¹²⁾. Confluent passage four donor cells were cultured in DMEM high glucose medium containing 10% FBS, 1% penicillin/streptomycin, 10 ng/mL bone morphogenetic protein-2 (kindly provided by Prof. Dr. Walter Sebald, Emeritus Chair of Physiological Chemistry, Faculty of Medicine, University of Wuerzburg, Germany), 10 mM β -glycerophosphate, 50 ng/mL L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate, and 100 nM dexamethasone (all Sigma Aldrich GmbH) for 28 days. Control cells were cultured in DMEM high glucose containing 10% FBS and 1% penicillin/streptomycin only. RT-PCR of the osteocyte-specific marker gene alkaline phosphatase, tissue-nonspecific isozyme (*ALPL*) and histomorphometric quantification of alkaline phosphatase (*ALP*) and alizarin red positive areas were performed to gauge the degree of osteogenic differentiation.

8. Adipogenic Differentiation

Adipogenic differentiation was performed in a monolayer culture system according to a modified protocol described before¹²⁾. Therefore, confluent fourth passage cells were incubated with DMEM high glucose containing 10% FBS, 1% penicillin/streptomycin, 1 $\mu\text{g}/\text{mL}$ insulin, 100 μM indomethacin, 0.5 mM 3-isobutyl-1-methylxanthine, and 1 μM dexamethasone (all Sigma Aldrich GmbH) for a total time of 21 days. Control cells were cultured in DMEM high glucose containing 10% FBS and 1% penicillin/streptomycin only. RT-PCR of the adipocyte-specific marker genes lipoprotein lipase (*LPL*) and peroxisome proliferator-activated receptor γ (*PPARG*), as well as the histomorphometric quantification of oil red O positive areas were performed to gauge the degree of adipogenic differentiation.

9. RNA Isolation and RT-PCR

RNA from ACL-derived cells was isolated using the NucleoSpin RNA II kit (Machery-Nagel GmbH, Dueren, Germany) according to the manufacturer's instructions. The pellets of chondrogenic cultures were previously homogenized in the first buffer of the respective kit using a pestle. Then, 1 μg of isolated RNA was transcribed into cDNA using the BioScript kit (Bioline GmbH, Luckenwalde, Germany) with random hexamer primers (Life Technologies GmbH, Darmstadt, Germany) according to the manufacturer's protocols. Gene expression was analyzed using RT-PCR with reaction mixtures containing 3 μL 10 \times reaction buffer (shipped with the MangoTaq from Bioline GmbH), 1 μL 10 mM dNTP mixture (Bioline GmbH), 1 μL of each forward

Table 1. Primers and Polymerase Chain Reaction Conditions

Gene	Forward and reverse primer	Product length (bp)	Annealing temperature (°C)	MgCl ₂ concentration (g/L)	Accession ID (NCBI)
Adipogenic markers					
<i>LPL</i>	F: 5'-CTGCAAATGAGACACTTTCTC-3' R: 5'-CATGTCCTGGCAGTCTCTGA-3'	276	51	1.0	NM_000237
<i>PPARG</i>	F: 5'-GCTGTTATGGGTGAAACTCTG-3' R: 5'-TAAGGTGGAGATGCAGGCTC-3'	350	51	1.0	NM_005037 NM_015869 NM_138711 NM_138712
Osteogenic marker					
<i>ALPL</i>	F: 5'-TGGAGCTTCAGAAAGCTCAACACCA-3' R: 5'-ATCTCGTTGTCTGAGTACCAGTCC-3'	454	51	1.0	NM_000478 NM_001127501 NM_001177520
Chondrogenic markers					
<i>ACAN</i>	F: 5'-GCCTTGAGCAGTTACCTTC-3' R: 5'-CTCTTCTACGGGACAGCAG-3'	392	54	1.0	NM_001135 NM_013227
<i>BGN</i>	F: 5'-AGTAGAGGCAGCTGCAGCCAC-3' R: 5'-GTGTCTGGACAGGTGCGGCAGC-3'	424	53	1.0	NM_001711
<i>COL2A1</i>	F: 5'-GGTATTGCTGGCTTCAAAGG-3' R: 5'-AGGACGACCATCTTCACCAG-3'	387	58	1.0	NM_001844 NM_033150
<i>COMP</i>	F: 5'-CAGGACGACTTTGATGCAGA-3' R: 5'-AGGCTGGAGCTGTCCTGGTA-3'	312	54	1.0	NM_000095
<i>FMOD</i>	F: 5'-ACCAGTGATAAGGTGGGCAG-3' R: 5'-AAGTAGCTATCGGGGACGGT-3'	368	54	1.0	NM_002023
Hypertrophic marker					
<i>COL10A1</i>	F: 5'-GGTATTGCTGGCTTCAAAGG-3' R: 5'-AGGACGACCATCTTCACCAG-3'	468	54	1.0	NM_000493
Housekeeping gene					
<i>EEF1A1</i>	F: 5'-AGGTGATTATCCTGAACCATCC-3' R: 5'-AAAGTGGATAGTCTGAGAAGC-3'	235	54	1.0	NM_001402

Forward and reverse primers to amplify specific gene products along with their associated product lengths (I_{Amplicon}), the annealing temperature (T_{Anneal}), the concentrations of MgCl₂ used in the reaction mixture (c_{MgCl_2}), and the accession ID at the Gene database of the National Center for Biotechnology Information (NCBI).

LPL: lipoprotein lipase, *PPARG*: peroxisome proliferator-activated receptor γ , *ALPL*: alkaline phosphatase, tissue-nonspecific isozyme, *ACAN*: aggrecan core protein, *BGN*: biglycan, *COL2A1*: collagen alpha-1(II) chain, *COMP*: cartilage oligomeric matrix protein, *FMOD*: fibromodulin, *COL10A1*: collagen alpha-1(X) chain, *EEF1A1*: expression analyses, elongation factor 1-alpha 1.

and reverse primer corresponding to a concentration of 5 μM (Table 1), and 3 μL MangoTaq polymerase (5,000 U/mL, Bioline GmbH), and 1 μL of cDNA in 21.9 μL distilled water. The PCR was started by an initial denaturation step at 94°C for 3 minutes followed by cycles of denaturation at 94°C for 45 seconds, primer annealing for 45 seconds with primer specific temperatures as illustrated in Table 1, and an elongation step at 72°C for 1 minute. Subsequent agarose gel electrophoresis was performed using 1.5% agarose gels (Merck) containing 0.5 $\mu\text{g/L}$ ethidium bromide (AppliChem GmbH, Darmstadt, Germany) and 0.5 \times TBE buffer

at 0.9 V/cm of gel length. Gels were documented photographically using the Bio Profile software (LTF, Wasserburg, Germany) and analyzed densitometrically using the GelAnalyzer software (<http://www.gelanalyzer.com>). For all targeted gene expression analyses, elongation factor 1-alpha 1 (*EEF1A1*) was used as housekeeping gene.

10. GAG Expression

Pellets were digested using Papain (Sigma Aldrich GmbH) for 24 hours and resulting solution was used to determine the GAG

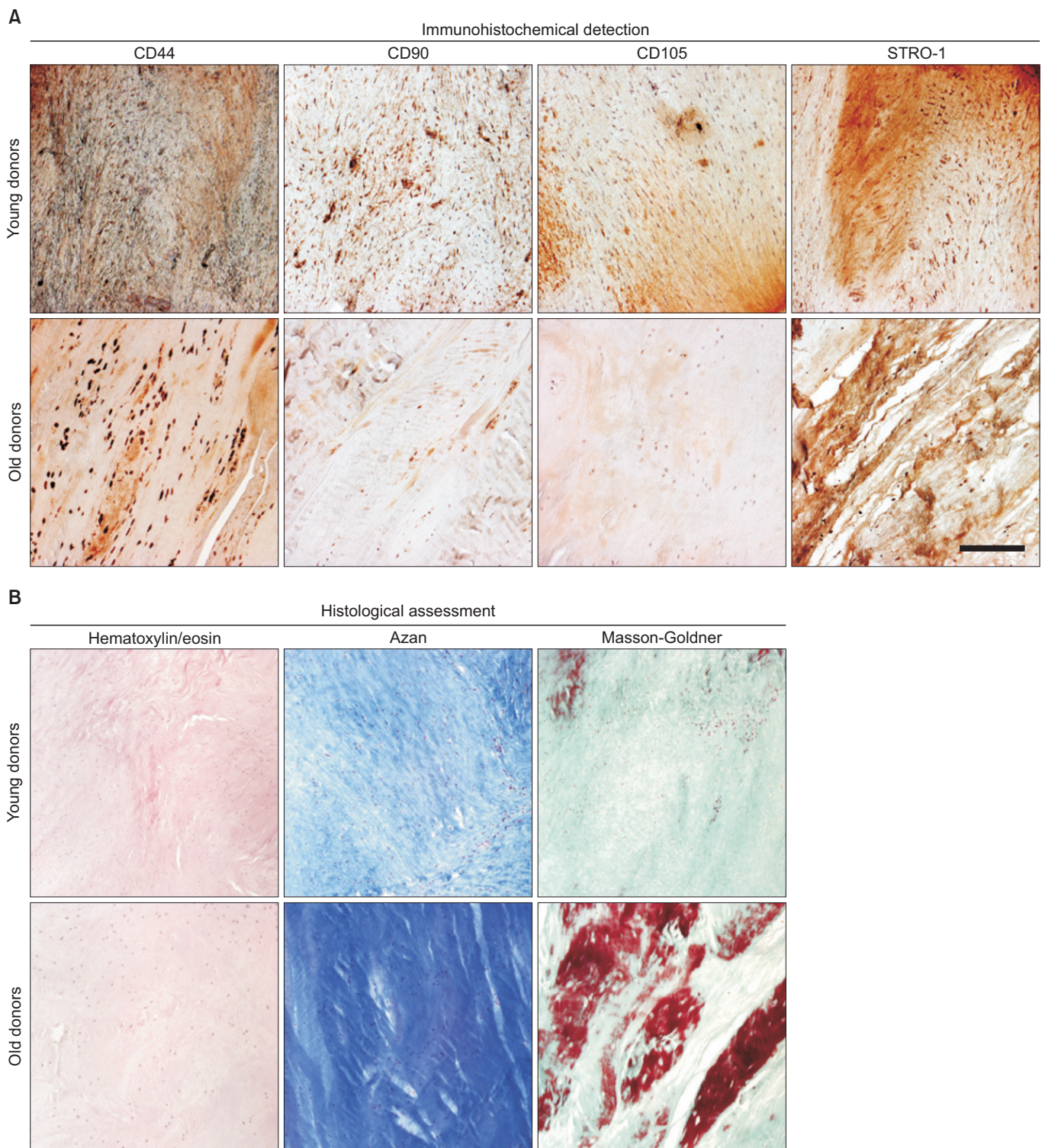


Fig. 1. Immunohistochemical and histological assessment of native anterior cruciate ligament (ACL) tissue. (A) The native ACL tissues from young and old donors were sectioned and investigated for the expression of the surface antigens CD44, CD90, CD105, and the STRO-1 antigen using immunohistochemistry. (B) Histological stainings for hematoxylin/eosin, azan, and Masson-Goldner were used to illustrate the cell and collagen distribution within the tissue specimens. Representative images are shown.

content using the Blyscan Glycosaminoglycan Assay kit (Tebubio) according to the manufacturer's instructions. GAG content was normalized to the DNA content, which was determined using the Quant-iT dsDNA Broad-Range Assay Kit (Life Technologies GmbH) following the manufacturer's protocol.

11. Microchip Hybridization Experiments

First passage cells were used for the analysis of transcriptome-wide gene expression. Denaturing agarose gel electrophoresis and photometrical methods were used to ensure the messenger RNA (mRNA) quality. Synthesis of complementary DNA, microchip hybridization, and signal detection were performed using the Affymetrix GeneChipR 3'IVT express kit, the Affymetrix GeneChip expression analysis protocol ver. 2, the GeneChip scanner 3000 with the operating software GeneChip 1.2 (all Affymetrix). Using the comprehensive R based microarray analysis web frontend CARMAweb, the generated raw data were normalized by the guanine cytosine robust multi-array analysis (GCRMA) algorithm^{13,14}. Afterwards, the values for the three biological replicates of each group were merged calculating the arithmetic mean for each probe followed by the determination of the relative fold change comparing the two age groups. Applying the moderated *t*-statistics included within the bioconductor package, linear models for microarray data (limma), and the Benjamini-Hochberg

method for correction for multiple hypothesis testing, the statistical significance of the computed data was tested^{15,16}. The resulting list of probesets was filtered by discarding those that were not associated with a specific gene, had a fold change between 0.5 to 2.0, or a $p > 0.05$. The remaining probesets and their corresponding genes were either grouped into positively or negatively regulated genes and used for pathway enrichment analysis using the gene ontology enrichment analysis and visualization tool Gorilla and the Gene Ontology (GO) Consortium's annotation and ontology toolkit AmiGO^{17,18}. A list of all detectable genes was used as a background list. We filtered the resulting GO terms, and we discarded GO terms with less than 5% differentially regulated genes or small GO terms being represented by < 3 .

12. Statistical Analysis

The Student *t*-test was utilized to test for significant differences in the CFU assay, the population doubling times, the histomorphometric analyses, densitometric RT-PCR analyses, GAG contents, DNA contents, and the ATP activities. *p*-values below 0.05 were considered statistically significant. Three independent samples from each group, each with technical triplicates, were used for all applicable assays; with exception to the CFU-f assay (young, $n=5$; old, $n=6$) and cumulative population doubling (young, $n=2$; old, $n=4$).

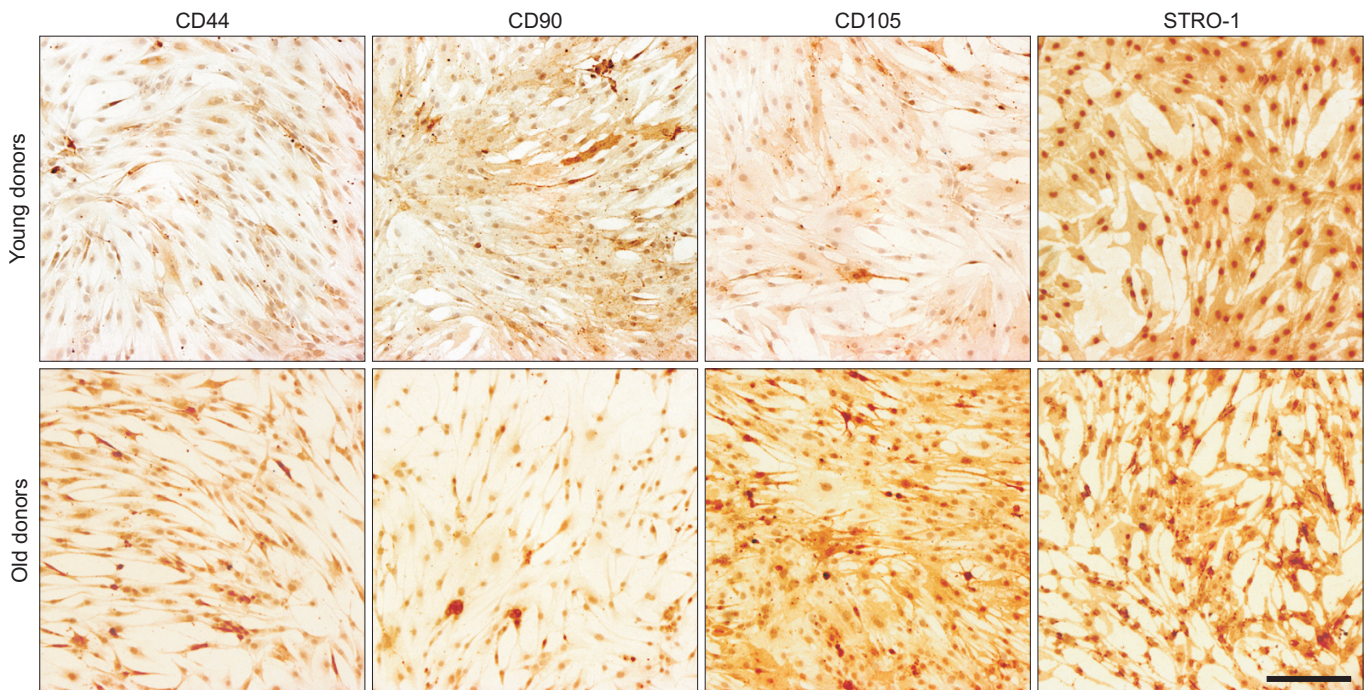


Fig. 2. Immunohistochemical detection of surface antigens on anterior cruciate ligament-derived mesenchymal stem cells (ACL-MSCs). Immunohistochemical detection showed positive signals for CD44, CD90, CD105, and the STRO-1 antigen on ACL-MSCs from young and old donors. Representative images are shown.

Results

1. ACL-MSCs from Young Donors Have a Greater Amount of Surface Antigens Characteristic for MSCs than ACL-MSCs from Old Donors

Characterization of ACL-MSCs from young and old donors by tissue properties and surface antigens show differences in histological and immunohistochemical examinations (Figs. 1 and 2).

2. The Transcriptome-Wide Analysis Reveals Differential Gene Expression Patterns between ACL-MSCs from Young and Old Donors

Comparing ACL-MSCs from young and old donors, 561 probesets were found to be upregulated in the cells from young donors (Top 10 genes in Fig. 3A). The corresponding genes encompassed ECM molecules (such as *COL1A1*, *COL5A1*, *COL6A1*, and *BGN* amongst others) or growth factors (such as *TGFBI*) and enriched 16 terms in the GO biological process, 1 term in the GO biological function and 7 terms in the GO cellular component ontologies.

These terms were mainly associated with protein dephosphorylation and components of the cytoskeleton (Fig. 3B). Further, 613 probesets were upregulated in cells from old donors, when compared to young donors. The corresponding genes encompassed genes encoding for proteins that are involved in ossification processes (such as *PDE1A*, *EREG*, and *SFRP1*) or osteoarthritis (such as *CTNNB1*) and enriched 8 terms in the GO biological process and 3 terms in the GO biological function ontologies. Those were mainly related to nuclear transport, protein kinase B activity, epithelial-mesenchymal cell signaling, and the activity of RNA polymerase II cofactor (Fig. 3B).

3. The Proliferative Capacity of ACL-MSCs Does Not Differ between Young and Old Donors

The CFU-f frequency ranged from 0.005 to 0.3 with no distinct age-dependent distribution pattern being detectable (Fig. 4A). Similarly, no distinct age-dependent differences in the cumulative population doublings were observed. However, ACL-MSCs from two old donors entered growth arrest after approximately 80 days

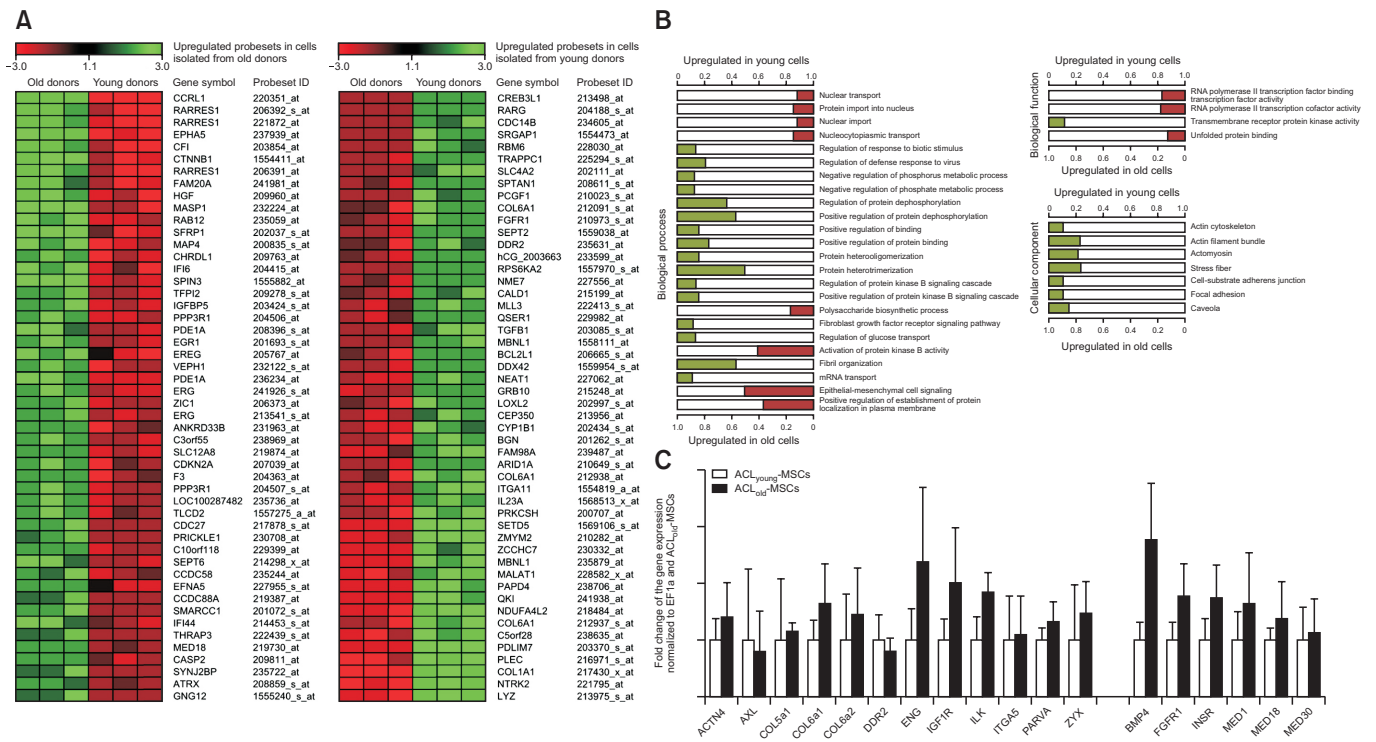


Fig. 3. Transcriptome-wide differential gene expression and pathway enrichment in the comparison of anterior cruciate ligament-mesenchymal stem cells (ACL-MSCs) from young and old donors. Using a microarray, 561 probesets were found to be upregulated in the cells from young donors, while 613 probesets were upregulated in cells from old donors. (A) The top 10 differentially expressed probesets are demonstrated as a heat map. (B) The corresponding genes enriched several terms in the GO biological process, biological function, and cellular component ontologies. Those enriched by the genes upregulated in ACL-MSCs from young donors mainly enriched pathways related to protein dephosphorylation and components of the cytoskeleton (B). Those enriched by the genes upregulated in ACL-MSCs from old donors were mainly related to nuclear transport, protein kinase B activity, epithelial-mesenchymal cell signaling, and the activity of RNA polymerase II cofactor. (C) Gene expression for BMP4 was higher in ACL-MSCs from old donors than in ACL-MSCs from young donors.

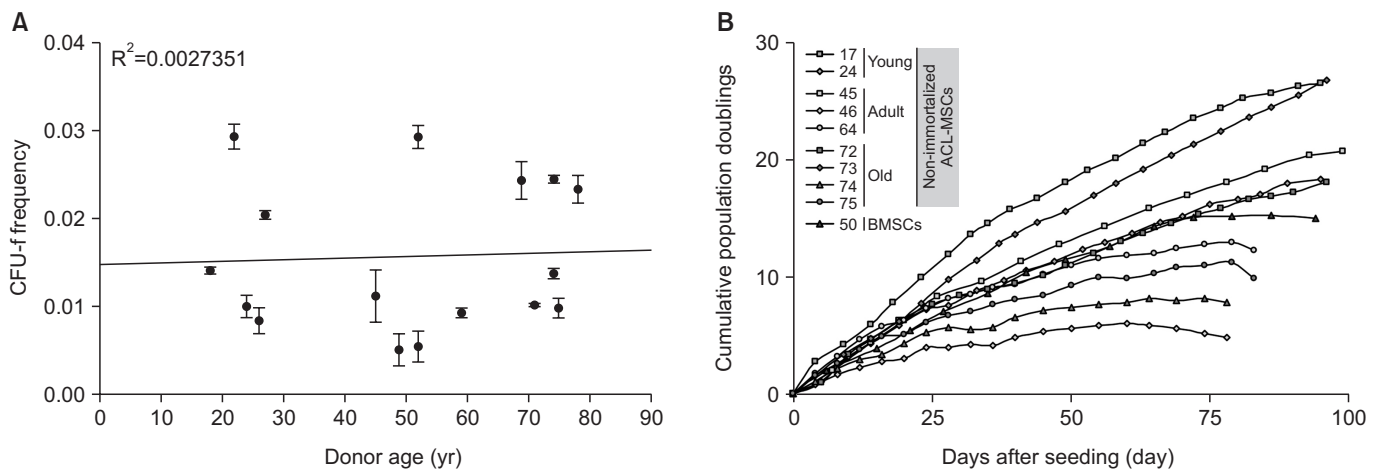


Fig. 4. Proliferative capacity of anterior cruciate ligament-mesenchymal stem cells (ACL-MSCs) from young and old donors. (A) The colony forming unit-fibroblast (CFU-f) frequencies of ACL-MSCs isolated from young ($n=5$) and old donors ($n=6$) were determined. No correlation with donor age was observed. (B) Cumulative population doublings for ACL-MSCs isolated from young ($n=2$) and old donors ($n=4$), as well as bone marrow-derived mesenchymal stem cells (BMSCs, $n=1$), did not demonstrate significant differences between the groups. The dots represent mean values of technical triplicates.

in culture (Fig. 4B). When compared to BMSCs, ACL-MSCs of both groups did not show a distinct difference (Fig. 4B).

4. ACL-MSCs from Young and Old Donors Do Not Differ in Their Potential for Chondrogenic Differentiation

ACL-MSCs from both age groups responded with a strong increase in alcian blue staining, when cultivated as pellets in the presence of TGF beta-1, comparable to BMSCs (Fig. 5A–C). Likewise, the GAG content normalized to the DNA content increased in both ACL-MSC groups, when comparing induced versus control pellets. However, the normalized GAG content was generally higher in control and induced ACL-MSCs from young donors than in those obtained from old donors (Fig. 5D). All groups expressed the articular cartilage marker *COL2A1*, as well as the hypertrophy marker *COL10A1* (Fig. 5E), resulting in the deposition of type II collagen in the superficial zones of the induced pellets (Fig. 5A). The staining intensity was highest in the ACL-MSCs from young donors, mildly reduced in ACL-MSCs from old donors, and even further reduced in BMSCs. These differences were not substantial (Fig. 5A). In the analysis of proteoglycan expression, the upregulation of *ACAN* expression after chondrogenic induction was lower in the ACL-MSCs from old donors than in those from young donors or in BMSCs, but this finding did not reach the level of statistical significance ($p>0.05$). No difference in the upregulation of *BGN* expression was observed (Fig. 5F).

5. ACL-MSCs from Young and Old Donors Do Not Differ in Their Potential for Osteogenic Differentiation

After osteogenic induction, ACL-MSCs from both age groups showed an increased *ALP* activity, comparable to BMSCs (Fig. 6A). Likewise, a strong production of mineralized ECM was detected, without any detectable difference between ACL-MSCs from young and old donors (Fig. 6A). Qualitatively, both ACL-MSC groups produced less mineralized ECM than BMSCs (Fig. 6A). However, no significant difference was detected after histomorphometric quantification (Fig. 6D). On the level of mRNA expression, a strong increase in *ALP* expression was detected in both ACL-MSCs from young and old donors (Fig. 6B). As BMSCs show a relatively high expression of *ALP* in uninduced control cells (Fig. 6A and B), the increase in gene expression after osteogenic induction is smaller than the increase observed in both ACL-MSCs ($p>0.05$).

6. ACL-MSCs from Young and Old Donors Do Not Differ in Their Potential for Adipogenic Differentiation

After adipogenic induction, ACL-MSCs from young and old donors synthesized lipid droplets (Fig. 7A). A slightly higher amount of lipid droplets was detected in ACL-MSCs from old donors when compared to young donors, but this effect was not considered significant ($p>0.05$) (Fig. 7B). Both ACL-MSC groups produced significantly less lipid droplets than the induced BMSCs. On the level of mRNA expression, the presence of adipogenic medium induced the expression of the adipocyte marker genes *LPL* and *PPARG* in all groups (Fig. 7C).

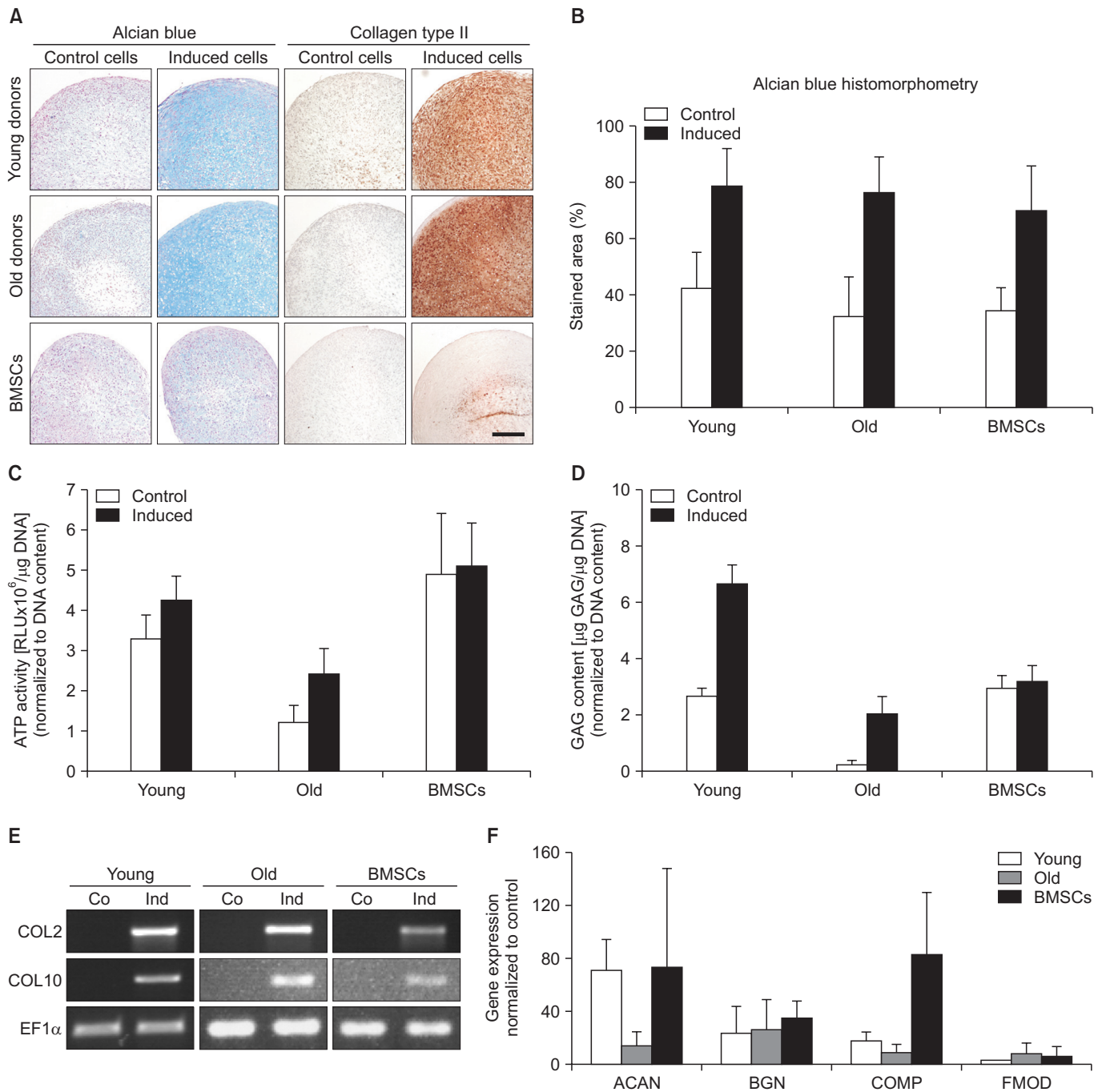


Fig. 5. Chondrogenic differentiation of anterior cruciate ligament-mesenchymal stem cells (ACL-MSCs) from young and old donors. (A–C) Increased glycosaminoglycan (GAG) staining was observed in cell pellets of all groups after induction of chondrogenic differentiation, which was quantified by histomorphometry. (D) Similarly, an increased GAG/DNA ratio was observed after induction in both ACL-MSCs from young and old donors. (E) Correspondingly, on the messenger RNA level, the expression of chondrocyte marker genes was increased in all groups after induction of chondrogenesis. (F) While the upregulation of *ACAN* expression was smaller in ACL-MSCs from old donors, this was not considered statistically significant. Upregulation of *BGN* showed no difference. Bars represent mean values of n=3 donors per group.

Discussion

The *in vitro* regenerative potential of ACL-MSCs from old do-

nors did not substantially differ from that from young donors, suggesting that regenerative therapies of this intra-articular ligament are feasible in both age groups. Specifically, no significant

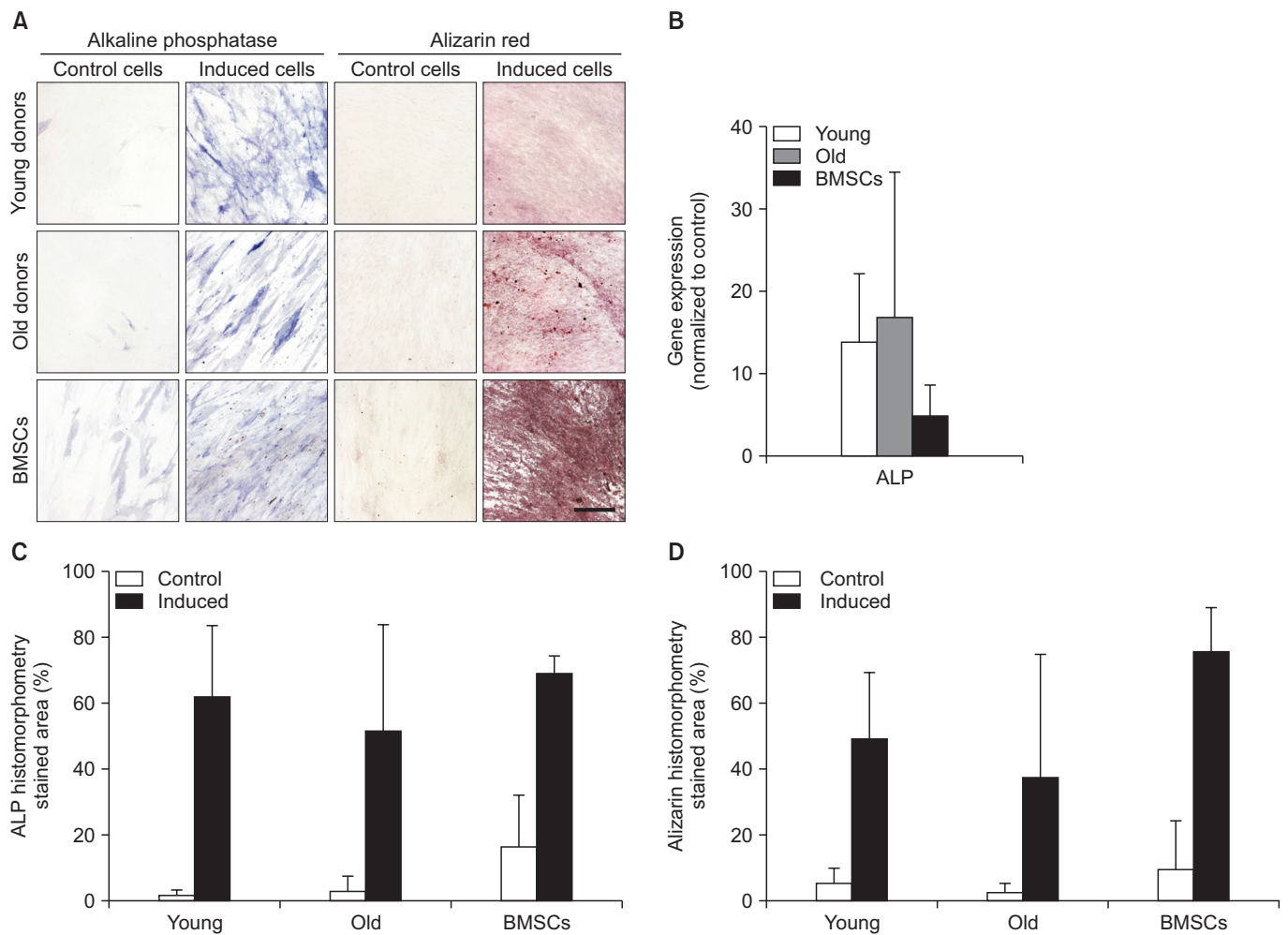


Fig. 6. Osteogenic differentiation of anterior cruciate ligament-mesenchymal stem cells (ACL-MSCs) from young and old donors. ACL-MSCs from both age groups showed an increased *ALP* activity and matrix mineralization (A) without significant differences between age groups or ACL-MSCs and bone marrow-derived mesenchymal stem cells (BMSCs) (C, D). A strong increase in *ALP* expression was detected in both ACL-MSCs from young and old donors, whereas the relative increase in BMSCs was lower due to the presumably high *ALP* expression in uninduced cells (B). Bars represent mean values of $n=3$ donors per group.

differences in proliferative capacity (assessed using the CFU-f assay and cumulative population doubling times) and potential for multilineage differentiation (chondrogenic, osteogenic, and adipogenic lineage) were observed. However, in the transcriptome-wide analysis of gene expression, the differential expression of several genes was detected in the comparison of ACL-MSCs from young and old donors, which enriched several pathways related to protein dephosphorylation, cytoskeleton, or protein kinase B activity. The different gene expression patterns in first passage ACL-MSCs of both age groups did not significantly impact the subsequently performed *in vitro* proliferation and multilineage differentiation.

We examined the age-dependent regenerative capacity of ACL-MSCs. The obtained results indicate that ACL-MSCs can

be isolated from old donors (up to 79 years) with knee osteoarthritis and be used for regenerative therapies. A recent study also showed comparable phenotypes and multilineage potentials between ACL-MSCs derived from young and old donors, supporting our results¹⁷. In contrast to our study, they found a greater proportion of MSCs in young patients than in older patients at initial isolation of MSCs, while in further cell passages, the proportions of MSCs from young and older patients were without differences in both studies¹⁷. This study supports our findings that ACL-MSCs from young and old donors have similar potentials for multilineage differentiation, but differences in gene expression could be detected, which might contribute to a different cytoskeleton and ECM¹⁷. In contrast to Lee et al.¹⁷ and our presented study, Uefuji et al.¹⁹ found not only a higher

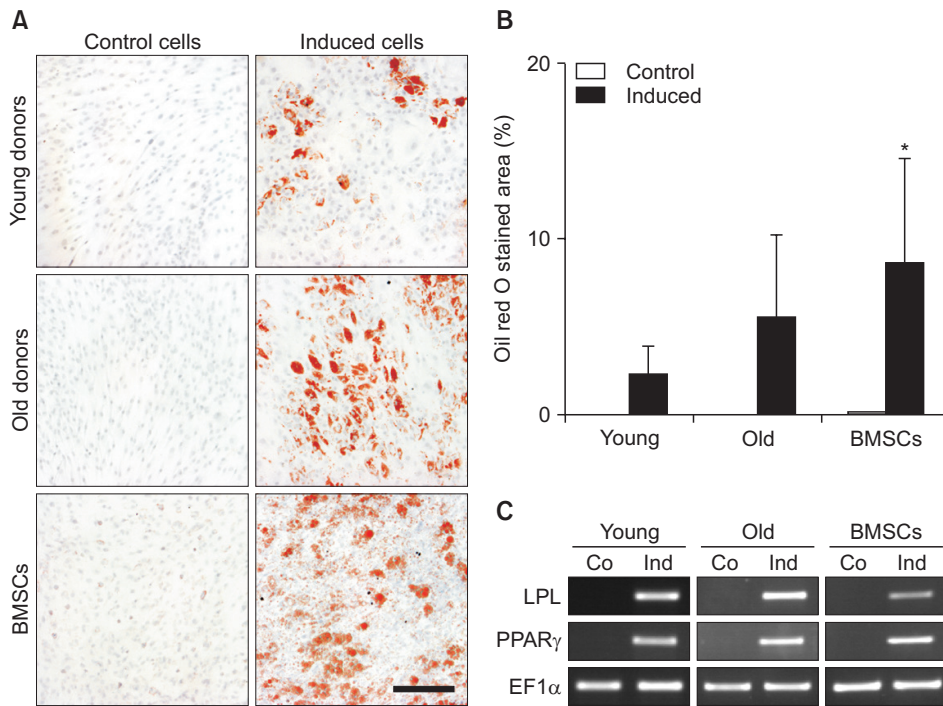


Fig. 7. (A) Adipogenic differentiation of anterior cruciate ligament-mesenchymal stem cells (ACL-MSCs) from young and old donors. (B) After adipogenic induction, a slightly higher increase in lipid production was detected in ACL-MSCs from old donors. When compared to young donors, this was not considered significant ($p > 0.05$). Both ACL-MSC groups produced significantly less lipid droplets than the induced bone marrow-derived mesenchymal stem cells (BMSCs). (C) Adipogenic induction resulted in the expression of adipocyte marker genes in all groups. Bars represent mean values of $n = 3$ donors per group.

proportion of MSCs but also a higher potential for proliferation and multilineage differentiation of MSCs from young donors than MSCs from older donors *in vitro*. These findings might be important for native tissue healing potential *in vivo*, but as we and others showed, MSCs from older donors probably can be used for the development of regenerative therapies as MSCs from younger donors¹⁷. However, our study has limitations. One such limitation is the use of *in vitro* assays, as it is currently not possible to define an effect size at which an observed difference would translate into a meaningful difference on *in vivo* application. It is likely that some of the slight differences observed here would reach the level of statistical significance when more independent samples were included. However, existing studies assessing tendon-derived MSCs, or BMSCs from healthy patients and patients with osteoarthritis did not document age-dependent differences in CFU-f frequencies either^{20,21}. Other studies showed slight age-dependent differences in CFU-f formation for BMSCs isolated from young and old donors, but none of them was statistically significant^{19,22}. Likewise, no significant age-dependent difference in the cumulative population doublings was observed, which is in accordance with some published characterizations of BMSCs^{23,24}.

High donor variability for human BMSCs was demonstrated previously, suggesting that other patient-specific factors might influence the regenerative potential of MSCs to a greater extent than age²⁵⁻²⁷. The present study cannot isolate age effects and the effects of acute ACL-injury and knee osteoarthritis, as all young

donors were subject to knee injury and all old donors received a total knee replacement due to osteoarthritis. This might explain the findings of the transcriptome-wide gene expression analysis obtained in the present study. Higher expression of ligament ECM molecules in first passage ACL-MSCs (such as *COL1A1*, *COL5A1*, *COL6A1*, and *BGN* amongst others) or growth factors (such as *TGF B1*) was observed in young donors, while old donors demonstrated the upregulation of genes related to ossification (such as *PDE1A*, *EREG*, and *SFRP1*) and osteoarthritis (such as *CTNNB1*)²⁸⁻³⁰. In both conditions, older age and osteoarthritis, a lower regenerative capacity of ACL-MSCs would be expected in *in vitro* assays. The finding that ACL-MSCs obtained from young donors without a chronic joint disease are not substantially more potent than ACL-MSCs obtained from old donors with chronic osteoarthritis makes the conclusion of this study more versatile. ACL-MSCs can be derived from ligaments of young and acutely injured, as well as from old patients with chronic osteoarthritis and be used for regenerative therapies that require capacity to proliferate and the potential to differentiate along multiple mesenchymal lineages. However, future *in vivo* studies need to assess whether age-dependent differences can be detected in the respective application of ACL-MSCs.

Conclusions

In the conducted examinations, the regenerative potential of

ACL-MSCs from old donors was not substantially lower than that from young donors, suggesting that the use of these cells for regenerative therapies is feasible in both age groups. These findings may be of considerable use for those developing regenerative therapies for the ACL or other knee joint tissues, which could be augmented with MSCs.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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