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Mesenchymal Stem Cells from the Retropatellar Fat Pad and Peripheral Blood Stimulate ACL Fibroblast Migration, Proliferation, and Collagen Gene Expression

B. L. Proffen¹, C. M. Haslauer¹, C. E. Harris², and M. M. Murray¹

¹Department of Orthopaedic Surgery, Children's Hospital Boston, Boston, MA, USA

²Department of Hematology/Oncology, Children's Hospital Boston, Boston, MA, USA

Abstract

Mesenchymal stem cells (MSCs) have been of recent interest as adjuncts for ligament repair. However, the effect of these cells on the resident ligament fibroblasts has not yet been defined. In this study, we hypothesized that co-culture of MSCs and ligament fibroblasts would result in increases in the proliferative rate of the ligament fibroblasts and their expression of collagen-related genes, as well as differentiation of the MSCs down a fibroblastic pathway. In addition, we hypothesized that these effects would be dependent on the source of the MSCs. Porcine MSCs were isolated from both the retro-patellar fat pad (ADSCs) and the peripheral blood (PBMCs) and co-cultured with porcine anterior cruciate ligament (ACL) fibroblasts. Fibroblast migration, proliferation, and collagen gene expression were evaluated at time points up to 14 days. ADSCs had a greater effect on stimulating ACL-fibroblast proliferation and procollagen production, while PBMCs were more effective in stimulating ligament fibroblast migration. In addition, co-culture with the ACL fibroblasts led to significant increases in collagen gene expression for ADSCs, suggesting a differentiation of these cells down a fibroblastic pathway during the co-culture period. This was not seen for the PBMCs. Thus, the effects of MSCs on in situ ACL fibroblasts were found to be source dependent, and the choice of MSC source should take into account the different performance characteristic of each type of MSC.

Keywords

mesenchymal stem cells; retropatellar fat-pad; peripheral blood mononuclear cells; ACL fibroblasts; co-cultureand

Introduction

Recent studies from our lab have suggested that bioenhanced repair, in which a scaffold capable of releasing growth factors is implanted in the wound site, can potentiate functional healing of the anterior cruciate ligament (ACL). This functional repair is mediated by the migration of fibroblasts into the scaffold, where they proliferate, produce collagen, and remodel the scaffold into a strong fibrovascular scar [1–3].

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Address correspondence to Benedikt L. Proffen, MD, Department of Orthopaedic Surgery, Children's Hospital Boston, Enders 270.4, 300 Longwood Avenue, Boston, MA 02115, USA. benedikt.proffen@childrens.harvard.edu.

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Other investigators have shown that mesenchymal stem cells (MSCs) can both contribute to wound healing and can also enhance the performance of dermal and pulmonary fibroblasts [4,5]. Therefore, MSCs may have a performance-enhancing effect on the resident fibroblasts in the ACL as well.

Originally described as cells derived from the bone marrow which would undergo bone formation after a heterotopic transplantation [6], MSCs have since been isolated from a variety of tissues including peripheral blood [7–10], adipose tissue [11,12], and even the ACL [13,14] itself. Although the International Society for Cell Therapy adapted three main criteria characterizing MSCs independent of their source, including plastic adherence, expression as well as lack of specific cell surface markers, and the ability to differentiate into the osteogenic, adipogenic, and chondrogenic pathway, results from recent studies show some differences between cells from different sources. Not only are there differences in yield between the different sources but also in immunophenotype, proliferative capacity, differentiation potential, gene expression profile, immunomodulatory capability, and the utility for specific medical applications [8,15–22]. Therefore, MSCs from different sources might have effects on resident ACL fibroblasts on a different scale. Also, studies have shown that MSCs, when in contact with another cell type, were able to adapt and differentiate into the same cell type [23,24]. This implies that MSCs would be able to differentiate into ACL fibroblasts as well and contribute to the ligament healing in a direct way.

In our study, we chose to concentrate on two sources of translational MSCs, which could potentially be readily available to surgeons performing arthroscopic ACL surgery. We studied MSCs isolated Hoffa's fat-pad of the knee [25] and peripheral blood [7,26]. We hypothesized that MSCs would enhance the performance of ACL fibroblasts, specifically by increasing their ability to migrate, proliferate, and express collagen genes. In addition, we hypothesized that these changes would be source dependent. We also hypothesized that the co-culture of ACL fibroblasts with MSCs would encourage the MSCs down a fibroblastic pathway, as evidenced by upregulation of collagen type I gene expression by the MSCs.

Methods

For this IACUC approved study, all cells were retrieved from tissue or blood of skeletally mature 20- to 22-month-old male Yucatan pigs at Animal Resources at Children's Hospital (ARCH, Boston, MA, USA) undergoing another IACUC-approved study for ACL repair. All animals were shipped to the facility and housed there 3 days prior to the tissue and blood collection, exposing them to the same stress level and diet.

ACL Fibroblast Isolation

The mid-section of the porcine ACLs was harvested and the surrounding synovial and epiligamentous tissue was carefully resected. The remaining ligamentous tissue was then minced into 1-mm³ pieces and digested with 0.1% (w/v) collagenase type I solution (Worthington Biochemical, Lakewood, NJ, USA) for 18 hr. The digest was then filtered through a 40 µm-pore-size nylon mesh cell strainer (BD Falcon, Franklin Lakes, NJ, USA) and the cells counted. Cells were resuspended in the in-growth medium containing DMEM and Ham's F-12, 50/50 medium (Mediatech, Manassas, VA, USA), 10% fetal calf serum (FCS; Sigma, St. Louis, MO, USA), 0.2-mM L-glutamine (Sigma, St. Louis, MO, USA), 100-IU/ml penicillin, and 100-µg/ml streptomycin (Sigma, St. Louis, MO, USA) and seeded at a density of $3 \times 10^5/\text{cm}^2$ in a 150-cm² cell culture flask (Greiner Bio-One, Monroe, NC, USA). Nonplastic-adherent cells were taken off after 48 hr, adherent cells were washed twice with phosphate-buffered saline (PBS; Mediatech, Manassas, VA, USA) and expanded in growth medium as mentioned above.

Retropatellar Adipose Tissue-Derived Stromal Cells

Adipose tissue from the central part of the retropatellar fat pad was minced into 1-mm³ pieces and digested with 0.075% collagenase type I under gentle agitation for 45 min at 37°C, and centrifuged at 300 *g* for 10 min. The pellet was filtered through a 40-μm-pore-size nylon mesh cell strainer (BD Falcon, Franklin Lakes, NJ, USA) and the cells counted. Cells were resuspended in the growth medium described above and seeded at a density of 3×10^5 /cm² in a 150-cm² cell culture flask. Nonplastic-adherent cells were taken off after 48 hr, adherent cells were washed twice with PBS and expanded in growth medium as mentioned above.

Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated as previously described [27]. Briefly, anticoagulated peripheral blood was placed on Percoll-Paque (1.077 g/ml; GE Healthcare Biosciences, Upsalla, Sweden) and centrifuged at 500 *g* for 40 min. The cells in the interface layer were centrifuged and resuspended in growth medium mentioned above and seeded at a density of 3×10^6 /cm² in a 150-cm² cell culture flask. Nonplastic-adherent cells were taken off after 48 hr, adherent cells were washed twice with PBS and expanded in growth medium as noted above.

Osteogenic, Adipogenic, and Chondrogenic Differentiation

ACL fibroblasts, adipose tissue-derived stromal cells (ADSCs), and PBMCs from each of the four donor pigs were subjected to osteogenic, adipogenic, and chondrogenic differentiation media. Osteogenic and adipogenic differentiation was assessed in monoculture, seeding the different cell types of each donor pig in individual wells of a six-well plate each and expanding the cells to 100% confluency. Osteogenesis was induced by supplementing the growth medium with 100-nM dexamethasone, 50-μg/ml L-ascorbic acid, and 10-mM β-glycerophosphate (all purchased from Thermo Fisher Scientific, Pittsburgh, PA, USA). After 3 weeks, cells were fixed in 4% paraformaldehyde (Sigma, St. Louis, MO, USA) and stained with Alizarin Red (Sigma, St. Louis, MO, USA). Adipogenic differentiation was induced by supplementing the growth medium with 1-μM dexamethasone, 1-μg/ml insulin, 0.5-mM 3-isobutyl-1-methylxanthine, and 100-μM indomethacin (each purchased from Sigma, St. Louis, MO, USA). After 3 weeks, cells were fixed in 4% paraformaldehyde and stained with Oil-red O solution (Sigma, St. Louis, MO, USA). Chondrogenesis was assessed by the pellet culture method as previously published [14]. A cell pellet was made by centrifugation of 3×10^5 cells at 400 *g* for 5 min and the pellet cultured with medium containing DMEM-high glucose, 1% antibiotic/antimycotic solution (both Mediatech, Manassas, VA, USA), 1% ITS + Premix, 40-μg/ml proline, 100-nM dexamethasone, 50-μg/ml L-ascorbic acid (all from Sigma, St. Louis, MO, USA), and 10-ng/ml recombinant human TGF-β1 (PeproTech, Rocky Hill, NJ, USA). After 3 weeks, aggregates were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. Representative sections were stained with 1.0% (w/v) Alcian blue (Sigma, St. Louis, MO, USA).

To evaluate the differentiation of each cell type, digital images were obtained from representative areas of the differentiated and stained cell monolayers or sections for each cell type and donor. In addition, the percentage of pixel area per field of view (1/cell type/donor) covered by stained calcium deposits, lipid vacuoles, or stained cartilage was determined by color-based image analysis using Adobe Photoshop (San Jose, CA, USA).

Fluorescent Labeling of ADSCs and PBMCs with Lentiviral Vector and FAC-Sorting

ADSCs and PBMCs were cultured with a lentiviral vector containing the DNA of yellow fluorescent protein (YFP) and polybrene. After labeling, the cells were sorted using a FACS Aria cell sorter (BD Biosciences, San Jose, CA, USA) to obtain 100% YFP expressing ADSC and PBMC cell lines. Cells were then resuspended in growth medium as mentioned above and seeded in 150-cm² cell-culture flasks for further expansion. When cells reached 80% confluence, they were frozen and stored at -80° C until use. Cells from passages 3 and 4 were used for all experiments since first passage cells were used for fluorescent labeling and the following passages were necessary to obtain a sufficient amount of cells for the subsequent experiments.

ACL Fibroblast Migration

Fibroblast migration was determined using a modified Boyden Chamber Assay (Millipore, Temecula, CA, USA) according to the manufacturer's instructions. ADSCs and PBMCs were seeded individually at a density of 3.3×10^4 cells/well in the lower chamber while ACL fibroblasts were cultured in serum-free DMEM high-glucose containing 5% BSA. After 24 hr, 5×10^4 ACL fibroblasts were added to each upper chamber. Migration was measured at 24 hr.

Co-Culture of ACL Fibroblasts with ADSCs or Plastic-Adherent PBMCs

For co-culture, ACL fibroblasts were mixed with YFP-labeled ADSCs or PBMCs at a ratio of 1:1. The co-cultures were then seeded on six-well cell culture plates at a density of 1×10^3 cells/cm² in growth medium with the addition of 0.25 mg/ml L-ascorbic acid. Monocultures of each cell type seeded at the same cell density were grown as controls. All experiments were conducted with samples from four different individuals ($n = 4$) and run in triplicates.

Cell Proliferation

Cell proliferation in the cultures was measured at 7 and 14 days using the alamarBlue® cell proliferation assay (AbD Serotec, Raleigh, NC, USA) according to the manufacturer's instructions.

Procollagen Synthesis

Procollagen synthesis at 7 and 14 days was determined using the commercially available Procollagen Type I C-Peptide EIA kit (Takara Bio, Inc., Otsu, Shiga, Japan) according to the manufacturer's protocol.

Real-Time PCR

Total RNA was extracted from monoculture wells, co-culture wells, and co-cultured cells after FACS sorting using a PureLink RNA Mini Kit (Ambion, Austin, TX, USA). RNA was extracted after 7 and 14 days of culture. All samples were then lysed in 1-ml Trizol (Sigma, St. Louis, MO, USA) and transferred to the spin columns. After additional on-column DNase digestion, RNA concentration and purity was determined at 260 and 280 nm, respectively. The RNA samples were reverse-transcribed into cDNA using the RETROscript Kit (Ambion, Austin, TX, USA) following the supplier's instructions. Real-time PCR was performed in ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBRGreen PCR Master Mix Kit (Applied Biosystems). Targeted genes were types I and III procollagen (Col I and Col III) and GAPDH was selected as a reference gene (primers purchased at IDT, Newark, NJ, USA). The primer sequences of selected genes for real-time PCR are listed in Table 1. The transcript level of target genes

normalized to GAPDH and relative to time point zero was calculated using the 2^{-Ct} formula.

Statistical Analysis

Differences between groups were determined using an analysis of variance (ANOVA), with significance levels set at $p < 0.05$ for the overall ANOVA and Bonferroni adjustment for post-hoc testing to determine the significance between groups. All these calculations were performed using Intercooled STATA 10 (Statacorp LP, College Station, TX, USA). All data are presented as mean $\pm$ standard deviation.

Results

Qualitative Evaluation of Osteogenic, Adipogenic, and Chondrogenic Differentiation of ACL Fibroblasts, ADSCs, and PBMCs

All three cell types had the ability to express an osteogenic, adipogenic, and chondrogenic phenotype (Figure 1). In the osteogenic differentiation, mineralized matrix could be seen in all three cell lines after 21 days. Mineralization was significantly more prominent in the ADSC group than in the ACL or PBMC group (54.7 ± 18.2 vs. 21.3 ± 6.0 vs. 27.8 ± 7.4 ; $p < 0.05$), in which the mineralization was more localized. There was no significant difference in the mineralized area between the ACL and the PBMC group ($p = 1.0$). In the adipogenic differentiation, significantly fewer cells in the ACL fibroblast and PBMC group contained intracellular lipid droplets and had a less homogeneous distribution pattern than ADSCs, which had intracellular lipid droplets in almost all cells (22.0 ± 3.6 vs. 12.5 ± 2.4 vs. 10.9 ± 4.0 ; $p < 0.05$). For chondrogenic differentiation, the ADSC samples had on average a significantly larger area stained with Alcian blue stain. While positive staining was present in the samples from ACL fibroblasts and PBMCs, they also had a less intense hue than in the ADSC samples (54.4 ± 16.3 vs. 27.7 ± 13.4 vs. 21.3 ± 4.5 ; $p < 0.05$) (Figure 2).

Migration Assay

ACL fibroblasts migrated more vigorously toward the PBMCs than toward the ADSCs ($p < 0.05$, Figure 3). The migration of ACL fibroblasts toward PBMCs and ADSCs was significantly higher than toward the control of serum-free media ($p < 0.001$, Figure 3).

Cell Proliferation

Both ACL fibroblasts and ADSCs had similar proliferation rates, both of which were higher than the PBMCs at day 7 and 14 ($p < 0.001$, Figure 4). Co-culture of ACL fibroblasts with the ADSCs did result in a higher rate of cell proliferation than the monoculture of ACL fibroblasts at both the 7- and 14-day time points ($p < 0.05$). However, co-culture of ACL fibroblasts with PBMCs did not increase the cell proliferation rate at either time point ($p > 0.68$).

FACS Analysis

During the 2 weeks of co-culture of ACL fibroblasts with ADSCs, the percentage of ADSC cells in the co-culture dropped from 50% initially to 40% at the 2-week time point (Figure 5A, $p < 0.001$). The percentage of PBMCs in co-culture also decreased over the same period from 50% to approximately 10% (Figure 5B, $p < 0.001$).

Procollagen Synthesis

Procollagen production was greatest in the ACL/ADSC co-culture at both day 7 and day 14 ($p < 0.01$, Figure 6). Procollagen production was similar in the ACL fibroblast and ADSC monoculture ($p = 1.0$), but the PBMC monoculture had a significantly lower procollagen

production at all time points than all other groups ($p < 0.001$). Procollagen production in the ACL/PBMC co-culture did not differ significantly from the ACL monoculture at day 7 or 14 ($p = 0.657$ and 1.0 , respectively; Figure 6).

Quantitative PCR/RNA Expression

Collagen type I mRNA—Col I expression was highest in the ADSC/ACL (1.53 ± 0.11) and PBMC/ACL (1.43 ± 0.11) co-culture groups after 14 days in culture ($p < 0.05$ for all comparisons). The PBMC monoculture had the lowest expression of Col I of all the groups (0.61 ± 0.1 at day 7, 0.65 ± 0.02 at day 14; $p < 0.001$; Figure 7A). After FACS sorting, the Col I mRNA levels for the ACL fibroblasts co-cultured with ADSCs were higher than the ACL fibroblast monoculture at 14 days (Figure 7B). The ACL fibroblasts co-cultured with PBMCs also had a higher Col I expression than the monoculture ACL fibroblasts at day 14 (Figure 6). The co-culture of ADSCs with ACL fibroblasts also increased the Col I gene expression of the ADSCs (Figure 7C), but not the PBMCs (1.35 ± 0.15 vs. 1.16 ± 0.12 in the PBMC monoculture; $p = 0.14$).

Collagen type 3 mRNA—Col III mRNA expression was also highest in the two co-culture groups—a difference that was significant after 7 and 14 days in culture for the ACL/ADSC co-culture (0.43 ± 0.07 and 0.54 ± 0.11) and after 14 days for the ACL/PBMC co-culture (0.46 ± 0.1) when compared to the ACL-monoculture (0.15 ± 0.1 and 0.17 ± 0.08 ; $p < 0.05$ for all comparisons). The PBMC monoculture had the lowest expression of Col III of all the groups ($p < 0.001$) (Figure 8A).

After FACS sorting, the Col III expression of the ACL fibroblasts co-cultured with ADSCs were higher at day 7 and 14 (0.45 ± 0.1 and 0.43 ± 0.12) compared to the ACL fibroblast monoculture (0.15 ± 0.1 and 0.17 ± 0.08 ; $p < 0.05$ for each comparison). The ACL fibroblasts co-cultured with PBMCs also had a higher Col III expression than the monoculture ACL fibroblasts at day 14 (0.42 ± 0.1 ; $p < 0.05$) (Figure 8B). The co-culture of ADSCs with ACL fibroblasts also increased the Col III mRNA expression of the ADSCs after 14 days (1.59 ± 0.16) compared to the ADSC monoculture (1.16 ± 0.16 ; $p < 0.05$), but not the PBMCs (1.42 ± 0.32 vs. 1.23 ± 0.22 in the PBMC monoculture; $p = 0.27$) (Figure 8C).

Discussion

The results of this study supported the hypothesis that MSCs from either source enhance the migration, proliferation, and collagen gene expression of the ACL fibroblasts in vitro, but that they do so in different ways. While ADSCs had a greater effect on stimulating ACL fibroblast proliferation and procollagen production, PBMCs were more effective in stimulating ACL fibroblast migration. Both ADSCs and PBMCs significantly accelerated gene expression for both type I and type III collagen for the ACL fibroblasts. This suggests that ADSCs and PBMCs may contribute to wound healing not just through their own production of collagen but also by stimulating the in situ ACL fibroblasts. These findings are consistent with previous work with dermal fibroblasts, where Kim et al. [4] and Wang et al. [27] reported that media conditioned by culture of ADSCs [4] and plastic adherent PBMCs [27] (named fibrocytes by the authors) significantly increased the collagen gene expression of those cells as well as their migration in vitro. Our article builds on those findings by comparing ADSCs and PBMCs and also using co-culture of the three cell types and FACS separation after culture to begin to differentiate potential roles of the MSCs in influencing ACL fibroblast behavior. Even though the MSC cells tend to disappear over time in vivo, their effects in the early weeks of wound healing may be profound—a 100% or 300% increase in migration (seen in our migration assay for the co-culture with ADSCs or

PBMCs, respectively) of fibroblasts to the wound site or the 20% increase in early procollagen production in the ACL fibroblast/ADSC co-culture—findings that can be critical to stabilization of the provisional scaffold during the healing of the primarily repaired ACL.

ACL fibroblasts and ADSC monocultures show a similar proliferation rate in monoculture and both cell types are significantly faster proliferating than the PBMC monoculture. However, the FACS analysis indicates a decrease of the ADSCs/ACL and PBMC/ACL ratio over time. There are several possible reasons for the observed changing ratio of ACL cells to ADSCs and PBMCs in co-culture. For the PBMCs, the significantly lower basic proliferation rate compared to the ACL fibroblasts could be the reason for a proportional decrease over time, since the ACL fibroblasts will divide at a higher rate until confluence is reached. This simple explanation fails in case of the ADSCs, which should proliferate at the same rate as the ACL fibroblasts. The higher proliferation rate of the ACL fibroblasts in co-culture with the ADSCs could be caused by the ADSCs' function as trophic mediators, inducing a higher proliferation rate in the ACL fibroblasts by excreting growth factors [28]. The same characteristic is known for the PBMCs [27] and might even have boosted the proportional difference in cell number between the PBMCs and the ACL fibroblasts during cell culture. Additionally, the stemness of the ADSCs and PBMCs could result in greater stimulation of fibroblast proliferation than ADSC or PBMC proliferation [12,29], which enabled them to undergo differentiation while in contact with the ACL fibroblasts. Another possibility is that the PBMCs and ADSCs begin to undergo apoptosis when co-cultured with the ACL fibroblasts. This would lead to an increased rate of cell death for these particular cells, and thus a change in the ratio of ACL/ADSC or ACL/PBMC in the culture. This would be supported by prior work by Jones et al. showed in their research that fibroblasts could have an antiproliferative effect on PBMCs [30]. Additional experiments are planned to investigate these hypotheses.

Both ADSC and PBMC co-culture increased the collagen gene expression of the ACL fibroblasts above the rate of that seen in the ACL fibroblasts cultured alone. This positive effect on collagen synthesis of fibroblasts is also consistent with previous reports where an indirect effect of media conditioned by exposure to MSCs stimulated collagen gene expression of fibroblasts [4,27]. The increase in mRNA expression by the ACL fibroblasts here is consistent with previous reports in the literature [4,27], but a new finding in this report is that the co-culture also resulted in an upregulation of the collagen mRNAs in the ADSCs and plastic adherent PBMCs themselves, as shown in the qPCR results after FACS sorting of the co-cultures. This supports our second hypothesis that MSC differentiation can be influenced by the surrounding microenvironment as suggested by Ball [31] and Karaoz [32]. Our findings suggest that MSC-like cells derived from various tissues might be able to adapt and differentiate depending on the surrounding tissue. This seems to be true for the fat pad-derived ADSCs, since they significantly altered their collagen mRNA levels when they were co-cultured with the ACL fibroblasts. However, our data did not support the same finding for the PBMCs.

In addition, we were also able to show that cells from the ACL, fat pad, and the peripheral blood would show MSC-like properties, a finding consistent with prior reports [7,9,14,25]. The cells used in this study were third or fourth passage following isolation as expansion was required both before and after lentiviral infection. Although no further experiments were conducted to test the influence of multiple passages on the MSCs, studies indicate that proliferation or differentiation capability seems not to be influenced until greater than the fifth passage [33].

We were able to confirm the findings of Steinert et al. [14], Jürgens et al. [25], Cao et al. [9], Faast et al. [7], and Chong et al. [10], who were able to differentiate ACL fibroblasts, fat pad-derived cells, and plastic adherent peripheral blood mononuclear cells along the three pathways. In our experiments, ADSCs showed the greatest ability to differentiate down all three pathways. Their ability to differentiate is well documented in the literature [12] and recent findings suggest a strong similarity of ADSCs and bone marrow-derived MSCs in their molecular signature [34], growth kinetics and differentiation potential [11], as well as the expression of different growth factor genes related to their trophic potential [34], suggesting that the fat pad may be a good source of multipotent MSCs. The superior differentiation and trophic potential could have had a positive influence on the ACL fibroblasts in co-culture as well, inducing a higher proliferation rate, upregulation of collagen mRNA, and stimulation of the collagen production compared to the PBMC co-culture.

PBMCs were found to have almost similar molecular expression patterns [9,29] as bone marrow-derived MSCs or ADSCs. Nonetheless, there are varying results in the literature about their stemness and differentiation potential. Cao et al. [9] were able to differentiate PBMCs along all three pathways, and Chong et al. [10] showed similar chondrogenic potential for human peripheral blood and bone marrow-derived MSCs. However, our findings are more similar to prior reports for plastic adherent PBMCs from horse peripheral blood, which showed a no chondrogenic differentiation in the PBMC culture [35]. Also, in healthy donors, the number of mesenchymal progenitor cells as well as their trophic potential seems to be lower in the peripheral blood compared to the bone marrow or adipose tissue [36], but proliferation as well as growth factor excretion reached levels comparable to bone marrow-derived MSCs after inflammation, injury, or hypoxia [37,38]. Since healthy donor animals were used in our study, this might have contributed to the lower proliferative, differentiation, and trophic potential of the PBMC culture in our results. The lower trophic and differentiation potential of the PBMCs compared to the ADSCs might also have impaired the stimulatory effect of the PBMCs on the ACL fibroblasts in co-culture, ultimately leading to the observed inferior performance compared to the ADSC/ACL fibroblast co-culture.

In summary, our results suggest that ACL fibroblasts, ligament healing, and therefore ACL-repair, in general, might be enhanced by the addition of MSCs from the fat pad and the peripheral blood. The fat pad-derived cells showed not only a significant stimulatory effect on the ACL fibroblasts but were able to be coaxed into differentiating along the fibroblast pathway as evidenced by upregulation of collagen gene expression. On the other hand, cells obtained from the peripheral blood, PBMCs, showed a stimulatory effect on ACL fibroblasts but their potential to differentiate along a fibroblast pathway appeared more limited. Nonetheless, they might play an important role in the regulation of ACL healing because of their strong influence on the migration of the ACL fibroblasts, which could ultimately lead to a faster re-colonization of the ACL defect. This migratory stimulus could also potentially enhance the migration of ADSCs from the retro-patellar fat-pad into the ACL defect and bolster the healing process further. It might also be beneficial to combine ADSCs and PBMCs in vivo to have a synergistic effect of faster migration of the resident ACL fibroblasts into the defect promoted by the PBMCs and an enhanced proliferation and collagen production stimulated by the ADSCs.

Future studies evaluating the signaling mechanisms behind these primary observations and in vivo studies of the effects of implanting these cells in an ACL wound are planned.

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Figure 1.

Histology of ACL fibroblasts, ADSCs, and PBMCs after 21 days of incubation with osteogenic, adipogenic, and chondrogenic differentiation media. In the top row, matrix mineralization is seen in red (Alizarin Red where red is a positive stain) denoting osteogenic differentiation. In the middle row, adipogenic differentiation is noted by the presence of intracellular lipid droplets (red; Oil-red O solution histology) in each of the three cell types. The bottom row shows chondrogenic differentiation most strongly in the cell pellets from ADSCs (Alcian Blue Stain where blue is a positive stain for glycosaminoglycans). Scale bar represents 100 μm .

Figure 2.

Percentage of positively stained pixel area for ACL fibroblasts, ADSCs, and PBMCs after 21 days. Positively stained pixels were counted in an area of 1.4 mm² and divided by the overall pixel count. ADSCs show a significantly higher differentiation in all three pathways compared to ACL fibroblasts and PBMCs ($p < 0.05$). (ACL = ACL fibroblast monoculture; ADSC = ADSC monoculture; PBMC = PBMC = monoculture).

Figure 3.

ACL fibroblast migration with and without exposure to ADSCs or PBMCs. PBMCs were the strongest stimulator of ACL fibroblast migration ($p < 0.05$ vs. ADSCs and no cell control, indicated with a triangle). ADSCs stimulated ACL fibroblast migration when compared with culture with no cells ($p < 0.05$, indicated with a star).

Figure 4.

Cell proliferation of ACL fibroblasts, ADSCs, PBMCs, and co-cultured cells. ACL fibroblasts and ADSCs both proliferated at a greater rate than PBMCs ($p < 0.001$, denoted by a star). The ACL/ADSC co-culture had a significantly higher proliferation rate than the ACL monoculture ($p < 0.05$, denoted by a triangle). There was no significant difference between the ACL monoculture and the ACL/PBMC co-culture. (ACL = ACL fibroblast monoculture; ADSC = ADSC monoculture; PBMC = PBMC monoculture; ACL/ADSC = co-culture of ACL fibroblasts with ADSCs; ACL/PBMC = ACL fibroblast co-culture with PBMCs).

Figure 5.

Cell ratio of co-cultures at day 7 and 14 determined by FACS. During the 2 weeks of co-culture of ACL fibroblasts with ADSCs, the percentage of cells that were ADSCs dropped from 50% to 40% at the 2-week time point ($p < 0.001$, denoted by a star). The percentage of PBMCs decreased even more dramatically from 50% to approximately 10% ($p < 0.001$, denoted by a triangle).

Figure 6.

Procollagen production. The ACL/ADSC co-cultured cells had a significantly higher procollagen excretion over 24 hr than all other groups at day 7 and 14 ($p < 0.01$, indicated by a star). The PBMC monoculture had the lowest procollagen production at each time point ($p < 0.001$, indicated by a triangle). (ACL = ACL fibroblast monoculture; ADSC = ADSC monoculture; PBMC = PBMC monoculture; ACL/ADSC = co-culture of ACL fibroblasts with ADSCs; ACL/PBMC = ACL fibroblast co-culture with PBMCs).

Figure 7.

Collagen type I mRNA expression. ACL/ADSC and ACL/PBMC co-cultures showed a higher Col I expression at day 14 than the ACL monocultures ($p < 0.05$; indicated by a star; A). After FACS sorting ACL fibroblasts cultured with ADSCs and PBMCs had significantly higher gene expression for Col I than ACL fibroblasts in monoculture ($p < 0.05$; indicated by a star; B). ADSCs co-cultured with ACL fibroblasts also had significantly elevated Col I gene expression at day 14 compared to the monoculture ADSCs ($p < 0.01$; indicated by a star; C). (ACL = ACL fibroblast monoculture; ADSC = ADSC monoculture; PBMC = PBMC monoculture; ACL/ADSC = co-culture of ACL fibroblasts with ADSCs; ACL/PBMC = ACL fibroblast co-culture with PBMCs).

Figure 8.

Collagen type III mRNA expression. ACL/ADSC co-cultures showed a higher Col III expression from day 7 and ACL/PBMC co-cultures from day 14 than the ACL monocultures ($p < 0.05$; indicated by a star/triangle; A). After FACS sorting ACL fibroblasts cultured with ADSCs and PBMCs had significantly higher gene expression for Col III than ACL fibroblasts in monoculture ($p < 0.05$; indicated by a star/triangle; B). ADSCs co-cultured with ACL fibroblasts also had significantly elevated Col III gene expression at day 14 compared to the monoculture ADSCs ($p < 0.01$; indicated by a star; C). (ACL = ACL fibroblast monoculture; ADSC = ADSC monoculture; PBMC = PBMC monoculture; ACL/ADSC = co-culture of ACL fibroblasts with ADSCs; ACL/PBMC = ACL fibroblast co-culture with PBMCs).

Table 1

Gene sequences used in RT-PCR

	Gene forward primer sequences	Reverse primer sequences
Collagen I	5 -CAGAACGGCCTCAGGTACCA-3	5 -CAGATCACGTCATCGCACAAC-3
Collagen III	5 -CCTGGACTTCCTGGTATAGC-3	5 -TCCTCCTTCACCTTTCTCAC-3
GAPDH	5 -GGGCATGAACCATGAGAAGT-3	5 -GTCTTCTGGGTGGCAGTGAT-3