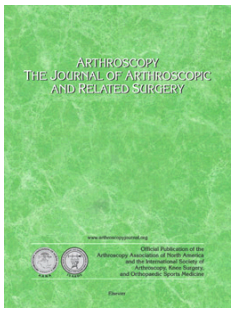
Journal Pre-proof



Assessing the Resident Progenitor Cell Population and the Vascularity of the Adult Human Meniscus

Jorge Chahla, MD PhD, Angela Papalamprou, PhD, Virginia Chan, BS, Yasaman Arabi, BS, Khosrawdad Salehi, BS, Trevor J. Nelson, BS, Orr Limpisvasti, MD, Bert R. Mandelbaum, MD DHL, Wafa Tawackoli, PhD, Melodie F. Metzger, PhD, Dmitriy Sheyn, PhD

PII: S0749-8063(20)30754-4

DOI: https://doi.org/10.1016/j.arthro.2020.09.021

Reference: YJARS 57135

To appear in: *Arthroscopy: The Journal of Arthroscopic and Related Surgery*

Received Date: 10 March 2020

Revised Date: 9 September 2020

Accepted Date: 10 September 2020

Please cite this article as: Chahla J, Papalamprou A, Chan V, Arabi Y, Salehi K, Nelson TJ, Limpisvasti O, Mandelbaum BR, Tawackoli W, Metzger MF, Sheyn D, Assessing the Resident Progenitor Cell Population and the Vascularity of the Adult Human Meniscus, *Arthroscopy: The Journal of Arthroscopic and Related Surgery* (2020), doi: https://doi.org/10.1016/j.arthro.2020.09.021.

Assessing the Resident Progenitor Cell Population and the Vascularity of the Adult Human Meniscus

Jorge Chahla, MD PhD[1*], Angela Papalamprou, PhD[2,3*], Virginia Chan, BS[,2,3] , Yasaman Arabi, BS[2,3], Khosrawdad Salehi, BS[,2,3], Trevor J. Nelson, BS[4], Orr Limpisvasti, MD[1] Bert R Mandelbaum, MD DHL[1], Wafa Tawackoli, PhD,[2,3,5,6] Melodie F. Metzger, PhD[4], Dmitriy Sheyn, PhD[1,2,4,5,6#]

[1]Kerlan Jobe Institute, [2]Orthopedic Stem Cell Research Laboratory, [3]Board of Governors Regenerative Medicine Institute, [4]Department of Orthopedics, [5]Department of Surgery, [6]Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, CA.

*JC and AP contributed equally to this study.

To whom the correspondence should be addressed

Dmitriy Sheyn, PhD
Assistant Professor
Orthopedic Stem Cell Research Lab
Board of Governors Regenerative Medicine Institute
Department of Orthopedics
Department of Surgery
Department of Biomedical Sciences
Cedars-Sinai Medical Center
8700 Beverly Blvd. AHSP A8308
Los Angeles, CA, 90048
Office: 424-3154579

ACKNOWLEDGMENTS

This study was funded by The American Orthopaedic Society for Sports Medicine 2018 Young Investigator Grant # YIG-2018-1 to (JC). The authors acknowledge the Joint Restoration Foundation (JRF, Centennial, CO), Musculoskeletal Transplant Foundation (MTF, Edison, NJ) and Biosource Medical (Lakeland, FL) for generously providing the allografts used in this study, the biobank and translational research core for the histological analysis. The authors wish to acknowledge Cedars-Sinai Biobank and Translational Research Core for performing the histological analysis and scanning the slides. The authors acknowledge NIH/NIAMS K01AR071512 funding to DS. Authors also wish to thank Julia Sheyn for the help with image processing.

Abstract

Purpose: To identify, characterize, and compare the resident progenitor cell populations within the red-red (RR), red-white (RW) and white-white (WW) zones of freshly harvested human cadaver menisci and to characterize the vascularity of human menisci using immunofluorescence and three dimensional (3D) imaging.

Methods: Fresh adult human menisci were harvested from healthy donors. Menisci were enzymatically digested, mononuclear cells isolated and characterized using flow cytometry with antibodies against MSCs surface markers (CD105, CD90, CD44 and CD29). Cells were expanded in culture, characterized and compared to bone marrow-derived mesenchymal stem cells (BM-MSCs). Trilineage differentiation potential of cultured cells was determined. Vasculature of menisci was mapped in 3D using a modified uDisco clearing and immunofluorescence against vascular markers CD31, lectin and alpha smooth muscle actin (αSMA).

Results: There were no significant differences in the clonogenicity of isolated cells between the three zones. Flow cytometry showed presence of $CD44^{+}CD105^{+}CD29^{+}CD90^{+}$ cells in all three zones with high prevalence in the WW zone. Progenitors from all zones were found to be potent to differentiate to mesenchymal lineages. Larger vessels in the RR zone of meniscus were observed spanning towards RW, sprouting to smaller arterioles and venules. $CD31^{+}$ cells were identified in all zones using the 3D imaging and co-localization of additional markers of vasculature (lectin and αSMA) was observed.

Conclusion: The presence of resident mesenchymal progenitors was evident in all three meniscal zones of healthy adult donors without injury. Additionally, our results demonstrate the presence of vascularization in the WW zone.

Clinical Relevance: The existence of progenitors and presence of microvasculature in the WW zone of the meniscus suggests the potential for repair and biologic augmentation strategies in that zone of the meniscus in young healthy adults. Further research is necessary to fully define the functionality of the meniscal blood supply and its implications for repair.

INTRODUCTION

Prognosis following meniscal injuries is highly variable depending upon the size and location of the tear. Some reports state that if the lesion communicates with the peripheral one-third of the meniscus, increased vascularity may help it heal and therefore are more amenable for repairs.[1] Conversely, injuries in the "avascular" zone are almost always resected due to their low potential success rate. Until recently it was believed that resecting a small percentage of meniscus would not significantly impact joint longevity. However, a direct relationship between the amount of meniscus resected and the presence/severity of chondral lesions in the ipsilateral knee compartment in prospective NFL players with a previous medial and/or lateral meniscectomy has previously been reported.[2] Meniscectomies have been reported to significantly reduce the career lengths of professional athletes,[3] while repairs carry high success rates at long-term follow-up. [4] Yet, since there are no randomized controlled trials to compare meniscal repair to resection, it is not entirely clear whether repair or resection would be favorable on a case-by-case basis.[5] The resident stromal progenitor cell population and the vascularization of the inner meniscus are not defined precisely in the literature and therefore strategies for repair might be better informed if a more consistent approach was used to characterize them.

Unlike highly vascularized bone tissue, fibrocartilaginous tissue has relatively limited self-repair capacity. Previous studies suggest healing of inner meniscal tears can be enhanced through progenitor cell mobilization[6] and recruitment from the synovium, followed by formation of an intermediate fibrous integration and cartilaginous remodeling.[7] Kobayashi *et al*[8] utilized an *in vitro* organ culture model of freshly prepared defects to investigate the healing potential of the rabbit meniscus without the influence of vascular supply. The authors found that grafts integrated better in the peripheral outer region of the meniscus, suggesting that the endogenous cellular composition of the meniscus may play a role in the local healing response. Progenitor cells have been identified in the menisci of goats, rabbits and more recently in humans,[9] which suggests an inherent healing capacity. Mauck *et al* reported that resident meniscal fibrochondrocytes from all regions of the meniscus possess a multilineage differentiation capability, particularly toward chondrogenesis and adipogenesis in calf menisci.[10] Since it is well established that mechanical cues affect the development and maturation of the cellular milieu,[11] it is important to better understand the cellular content of human menisci. The progenitor

content between the different meniscal zones has not been investigated in adult human menisci, most likely because of the scarcity of fresh human grafts made available for research purposes.

The intrinsic healing capacity of the meniscus is considered limited due to a poor blood supply that only reaches the periphery of the meniscus.[12] King[13] was the first to suggest that tears extending to the vascular periphery undergo spontaneous repair, whereas tears limited to the inner region do not. Seminal anatomical studies performed on human cadaveric menisci in the 1980s using injection techniques have established the current paradigm of meniscus vascularity.[1] However, there is controversy in the literature regarding the specific topology of meniscus vasculature as well as the specific timeline when the vasculature undergoes developmental changes.[12, 14] Therefore, the purpose of this study was to identify, characterize, and compare the resident progenitor cell populations within the red-red (RR), red-white (RW) and white-white (WW) zones of freshly harvested human cadaver menisci and to characterize the vascularity of human menisci using immunofluorescence and three dimensional (3D) imaging. We hypothesized that microvessels and resident progenitor cells in the inner zone of meniscus would be more prominent than previously reported.

METHODS

Study Design

The cadaveric studies were conducted according to the approved Institutional Review Board protocol (Pro00052234). Menisci from fresh human cadaveric knees (mean donor age: 21 ± 6.1 years) were donated by three tissue banks [Joint Restoration Foundation (JRF, Centennial, CO), Musculoskeletal Transplant Foundation (MTF, Edison, NJ) and Biosource Medical (Lakeland, FL)] for medical research purposes as they were deemed non-compatible with the recipient at the time of matching. Grafts were stored at 4ºC at the tissue banks until shipment according to standard procedures and were shipped under sterile conditions on ice, following the same procedure for allografts used in the clinic. Allografts for meniscal transplantation have a 21-day window, thus all donated grafts were between 1 and 21 days post-harvest.[15] Specimens were assessed by at least two investigators (initials blinded for review). In our preliminary studies we have determined that the viability of mononuclear cells significantly declined after 7 days post-harvest (*data not shown*), thus grafts greater than 7 days post-harvest were allocated to histological analysis and uDisco experiments, while only fresh grafts were allocated to mononuclear cell isolations and characterization. A total of 34 allografts from

17 different donors (including medial and lateral) were used in this study. Tibial plateaus were dissected to harvest medial and lateral menisci along with their entire length preserving 1mm of their peripheral capsular attachments (Fig.1). Fourteen menisci from 7 different donors were used for cell isolation and characterization. Twenty meniscal allografts from 10 different donors were used for assessing vascularity using histology and the modified uDisco 3D staining approach.[16]In this work, meniscal zones on cadaveric menisci were identified using the Cooper classification system.[14] For the cell isolation/characterization experiments, zones were identified on cadavers and sectioned, whereas for the 2D/3D immunolabeling experiments meniscal slices were sectioned, imaged as whole slide scans or 3D images and software was used to establish the distance between each zone (Supplementary Fig.1).

Mesenchymal Stromal Progenitor Cell Prevalence: Identification, Quantification and Characterization

Cell Isolation from fresh meniscus grafts

The red-red (RR), red-white (RW), and white-white (WW) zones were dissected and sectioned into equal thirds as measured by a caliper from the inner aspect to the marginal border of the meniscus by cutting in the radial direction, to replicate the clinical setting and to aid in a more standardized sectioning technique (Fig. 2A). Sterile gauge was used to remove excess media. Meniscal tissue was then placed in sterile tubes and wet weights were recorded. Tissue was manually minced to ~1mm^2 pieces in a sterile environment and then enzymatically digested. Since it is well established that meniscus tissue cellular content varies between each zone,[17, 18] several dissociation procedures were tested and the procedure yielding the highest cellular content for all zones was chosen. Highest yields were obtained from dissociation of meniscus tissue in 0.02% pronase (Millipore, Temecula, CA) for 1h at 37°C, followed by 18h 0.02% collagenase II (LS004205, Worthington Biochemical Corporation) at 37°C[19], therefore this procedure was chosen for meniscal cell content characterizations. Isolated cells were plated in culture-treated plates in approximately 2x10^3 cells/cm^2 density and incubated overnight at 37°C in 5% CO_2. Adherent cells were cultured at 37°C/5% CO_2 in growth medium containing 1mM L-glutamine (Invitrogen, Carlsbad, CA), 1% antibiotic antimycotic solution (Hyclone, Marlborough, MA) in Dulbecco's modified eagle medium (DMEM, GIBCO, Carlsbad, CA) and 10% fetal bovine serum (FBS, Gemini Bioproducts, West Sacramento, CA). Following the recommendations regarding nomenclature and mesenchymal stem/progenitor cell characterization from the International Society for Cell Therapy (ISCT), progenitor cells from the

meniscus are referred to as mesenchymal stromal cells and cultured meniscal cells (CMCs) in this manuscript and they are clearly distinguished from Bone Marrow-derived Mesenchymal Stromal Cells (BM-MSCs) that were used as controls for the various *in vitro* assays. [20, 21] BM-MSC were isolated from human bone marrow aspirate (Lonza, Benicia, CA) as previously described.[22-24]

Self-Renewal assessment (Colony Forming Unit-Fibroblast Assay)

Self-renewal of cultured meniscus cells (CMCs) was assessed using a standard CFU-F assay as previously described.[25] Briefly, cells isolated from different zones of the meniscus were separately plated onto 6-well plates at 10^4 cells/well in culture media, with media changed twice per week. Between 7 and 14 days medium was removed, and cells were washed with PBS. Afterward, cells were fixed with 4% formaldehyde, stained with hematoxylin, and aggregates of 50 cells or more were scored as CFUs.

Meniscus Stromal Progenitor Cell Characterization and Expansion (Surface markers)

Meniscus stromal cells were characterized immediately after isolation using flow cytometry with antibodies against the following Mesenchymal Stromal Cell (MSC) surface markers: CD105 (326-050, Ancell Corporation, Stillwater, MN), CD90 (MCA90F; Biorad, Hercules, CA), CD44 (559942;BD Pharmigen, San Diego, CA), CD29 (PB-219-T100; Abcore, Ramona, CA) as well as respective isotype controls.[20, 21] Afterward, meniscal cells were cultured, split twice when confluence was reached, characterized at passage 2 (P2), and compared to cultured bone marrow-derived MSCs (BM-MSCs, ≤P5, Lonza, Benicia, CA) using the same markers.

In vitro Differentiation to Mesenchymal Linages

Osteogenesis: For assessment of osteogenic differentiation, CMCs and BM-MSCs at low passage were plated in 24 well plates ($6x10^4$ per well) in triplicates and cultured in complete high glucose DMEM (Life Technologies). Once confluence was reached, the medium was supplemented with 100nM dexamethasone (D4902, Sigma, St Louis, MO), 10mM b-glycerophosphate (G9422, Sigma) and 50µg/ml L-ascorbic acid (A4544, Sigma) for 7 days or 21 days, with media changes every other day. Alkaline phosphatase (ALP) activity assay was performed to determine osteogenic differentiation after 7 days according to the manufacturer's protocol (ab83369, Abcam, Cambridge, MA). Production of pNP, was determined by measuring

absorbance at 405nm using a microplate reader (Bio-Rad). ALP activity (U/ml) in the test samples was calculated based on the equation: ALP activity = (B/ΔT *V)*D, where B = amount of pNP in sample wells calculated from standard curve (μmol). ΔT = reaction time (minutes), V = original sample volume added into the reaction well (mL), and D = sample dilution factor. ALP activity was normalized to total protein content quantified using BCA assay (Promega, San Luis Obispo, CA) performed on parallel wells. Each sample was run in technical triplicates. Lastly, cells were cultured in differentiation media for three weeks and gene expression of osteogenic marker Collagen-1 was evaluated. In order to evaluate osteogenic gene expression, cells were harvested and RNA isolated. For RNA isolation, media were aspirated, and cells lysed with RLT buffer (Qiagen, Valencia, CA) containing β-mercaptoethanol (M3148, Sigma). Lysates were transferred to 1.5 ml tubes and a handheld pestle and mortar was used to fully homogenize the cells in the RLT buffer and RNA was isolated with the RNeasy mini kit (Qiagen) following the manufacturer's recommendations. RNA yields were determined spectrophotometrically using a Nanodrop system (Thermofisher, Waltham, MA), and RNA was reverse-transcribed using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Thermofisher). Gene expression analysis was conducted using Taqman gene expression assay for Collagen-1 (Hs00164004_m1, Thermofisher). Target gene mRNA levels were quantified using FAM-MBG technology (Bio-RAD). The threshold cycle (Ct) value of 18S rRNA was used as an internal control using the Taqman gene expression FAM/MGB probe system (4333760F, Thermofisher). The Livak method was used to calculate ΔΔCt values and fold change was calculated as $2^{-\Delta\Delta Ct}$ as previously described.[26]

***Adipogenesis**:* For assessment of adipogenic potential, CMCs and BM-MSCs at low passage were plated in 24 well plates ($6x10^4$ cells per well) in triplicates and cultured in complete high glucose DMEM (Life Technologies) until they were fully confluent. Adipogenic differentiation was induced as previously described.[27, 28] Briefly, medium was changed to "induction medium" composed of complete high glucose DMEM and supplemented with 1μm dexamethasone (D4902, Sigma), 10uM insulin (I6634, Sigma), 0.5mM 3-isobutyl-1-methylxanthine (IBMX, I7018, Sigma) and 200μm indomethacin (I8280, Sigma) for three days, followed by 2-3 days of "maintenance medium", composed of complete high glucose DMEM and 10μM insulin. Cells were inspected daily for presence of adipogenic vacuoles. Five full cycles of induction followed by maintenance were performed. Finally, wells were fixed in ice-cold formalin and washed and stained with Oil-Red-O (O0625, Sigma) for 15 minutes. Cells were then washed three times with double distilled water and microphotographs were taken at 20x magnification using an EVOS XL Core imaging system (Thermo scientific).

***Chondrogenesis**:* The chondrogenic potential of CMCs was assessed as previously described.[27] Cells were trypsinized, neutralized with serum-containing low glucose DMEM and counted. Cell aliquots of $5x10^5$ meniscal cells or BM-MSCs at low passage were span at 240g for 5min and all media carefully removed. Pellets were resuspended in serum-free low glucose media and span again at 240g for 5min to ensure complete removal of serum-containing medium. Then, cells were resuspended in 100µl chondrogenic differentiation medium composed of low glucose DMEM, 1xITS (I2521, Sigma), 0.1µM dexamethasone (D4902, Sigma), 40µg/ml proline (Sigma), 50µg/ml ascorbic acid (Sigma) and 10ng/ml TGFβ1 (240B002, R&D Systems, Minneapolis, MN). The 100µl cell suspension was placed in transwells in order to induce disc-shaped 3D formation. The transwell plate was centrifuged at 200g for 5 minutes, and then filter inserts were transferred into a 24-well plate containing differentiation medium. Chondrogenic media were changed every two days. After 21 days, discs were fixed in formalin for 1 hour and dehydrated in passing through an increasing-grade series of ethanol baths. Afterward, discs were embedded in paraffin blocks, cut into 5µm sections and stained with Alcian Blue to identify chondrocytes. Whole slide scans were attained and imaged using QuPath software.

Vascularity Assessment using standard histology, immunofluorescent labeling and 3D imaging

Vascular Tree Histology

Samples were fixed in 10% buffered formalin. Following fixation, samples were dehydrated by passing through an increasing-grade series of ethanol baths, paraffin embedded, sectioned (4µm thick), deparaffinized and histological stains performed according to standard procedures. Hematoxylin and Eosin (H&E) staining was used for morphological evaluation. Masson's trichrome (MTC) stain was performed to further evaluate structure of the extracellular matrix and visualization of the larger vessels. QuPath quantitative pathology and image analysis software was used for imaging H&E and MTC full slide scans as well as quantifying the distance between zones.

Characterization of Microvasculature via Immunofluorescence

Immunofluorescence was performed on formalin-fixed (7 days in formalin), paraffin-embedded tissue sections. Briefly, sections were deparaffinized, rehydrated in PBS with 0.025% Triton-X (PBS-T), treated with antigen retrieval solution at 98°C for 1hr (pH 6.1; Dako #S1699, Agilent

Technologies, Carpinteria, CA) and blocked with 10% normal donkey serum in PBS-T. Endothelial cells were detected on meniscus cross sections by CD31 (1:50 dilution; ab28364, Abcam), αSMA (1:250 dilution, Abcam, Cat# ab21027) and Alexa488-conjugated lectin antibody (1:200 dilution, Dylight,Cat# DL-1174, Vector Labs, Burlingame, CA) in blocking solution, overnight at 4°C. To control for unspecific background labeling, primary antibody was omitted on background labeling controls. Then, sections were washed three times in PBS-T, followed by incubation with Cy5 (donkey anti-rabbit), Cy3 (donkey anti-goat) secondary antibody (1:200 dilutions, all from Jackson ImmunoResearch, Westgrove, PA) for 2 hours at room temperature. Subsequently, sections were washed three times in PBS-T and mounted with Prolong Gold with DAPI (Life Technologies, Carlsbad, CA). Vascularity was assessed stereologically based on the morphology and topology of endothelial cell arrangement in the tissue. Vessels were considered structures with lumen and colocalization of all vascular markers. Microvessels with one to three endothelial cells (CD31-positive cells) spanning the vessel circumference were classified as capillaries as previously described.[29] Whole slide scans were taken using a Leica DMi8 fluorescence microscope and imaged using Imaris Core 9.3 (Oxford Instruments, Concord, MA). Higher magnification images (40x) of selected areas were taken with a Nikon Eclipse Ti-2 fluorescence microscope (Melville, NY).

Qualitative Three-Dimensional Imaging using Light Sheet Microscopy

Meniscal allografts from cadavers were fixed in formalin. Meniscus tissue was cleared using a modified uDisco passive clearing and staining procedure for whole organs.[16] This multi-step procedure allows for staining of entire pieces of tissue (or whole organs) with antibodies without the requirement for sectioning of the tissues. Tissues are made fully transparent, labeled using the same antibodies required for immunofluorescence and then imaged using light sheet laser fluorescence microscopy. The ultimate 3D imaging of solvent-cleared organs (uDisco) protocol previously published in mice[16] was optimized and adjusted for human cadaveric menisci. Meniscus tissue was segmented into quarters and 2mm thin slices and fixed in formalin for up to four weeks, similar to the procedure followed in mouse tissues. Then tissue was incubated for 1 week in wash/permeabilization solution (0.4% v/v Triton-X, 0.3M glycine w/v, 20% DMSO v/v all from Sigma), followed by 8 days in primary antibody (anti-CD31, 1:50 dilution; ab28364, Abcam) diluted in wash/permeabilization solution at 37°C. Tissue was washed overnight with permeabilization solution and incubated for 6 days with secondary antibody (Alexa Fluor® 488-conjugated AffiniPure Donkey Anti-Rabbit IgG secondary antibody; Jackson Immunoresearch),

followed by a second wash with permeabilization solution, 37°C. Afterward, all cells were labeled with TO-PRO®-3 nuclear stain (0.1% v/v, Thermofisher Scientific) for 4 days at 4°C.[30, 31] and gradient dehydrated in *tert*-butanol (Sigma, 360538). Specifically, tissue pieces were incubated in ascending grades of *tert*-butanol solution diluted in distilled water (30%, 50%, 70%, 90% and 96%) for 1-3 days each and 100% *tert*-butanol at 37°C in the dark for 1 day, delipidated using dichloromethane (DCM, Sigma, 270997) for one day, and finally cleared with dibenzyl ether (DBE) at RT in the dark for at least 3 days. A lightsheet fluorescent microscope (LaVision Biotec Ultravision II, Miltenyi Biotec, Auburn, CA) was used for imaging and Imaris 9.3 was used for 3D reconstruction of the acquired images.

Statistical Analyses

All data are presented as mean ± standard deviation unless otherwise stated. Non-repeated measures analysis of variance and Tukey–Kramer post hoc analysis were performed on sample means for each analysis. For the changes in gene expression following three weeks of osteogenic induction one-way ANOVA was performed using Sidak post hoc analysis for multiple comparisons between the induced and non-induced controls of each group (BM-MSCs, RR, RW, and WW respectively). For flow cytometry marker analysis, two-way ANOVA was performed using Dunnett's post hoc analysis for comparisons between markers detected in different zones, using the WW zone as the control group. Statistical significance was set at $p<0.05$. GraphPad Prism 8 software (Irvine, CA) was used to analyze the data.

RESULTS

PROGENITOR CELL PREVALENCE

Cell Isolation from meniscal grafts, cell yields and self-renewal potential in vitro

The enzymatic digestion protocol produced comparable results between medial or lateral menisci in all three zones (n=6 donors, $p>0.05$; Figure 2B). Therefore, data from medial and lateral menisci were pooled and analyzed per zone. Clonogenic potential of isolated cells from each zone was confirmed using low CFU-F assay. Colony counts showed that freshly isolated cells were clonogenic in culture. Further, there were no significant differences in clonogenicity of the cells isolated from the three meniscal zones ($p>0.05$, Figure 2C).

Phenotypic analysis; Cell surface marker expression and assessment of differentiation potential in vitro

Flow cytometry analysis of cells from the three meniscal zones displayed presence of two distinct subpopulations of cells immediately after isolation. One subpopulation was $CD44^{+}CD105^{+}CD29^{+}CD90^{+}$ and the other one was $CD44^{-}CD105^{-}CD29^{-}CD90^{-}$ (Figure 3A top panel). Additionally, flow cytometry of cultured meniscus cells (CMC) at passage 2 displayed a shift of all four markers expression to the right (Figure 3A, bottom panel). Surface marker expression analysis showed differential marker expression patterns between different zones (Figure 3B). The WW zone contained a larger proportion of cells that express all four MSC markers (45.07±20.36%) compared to RR and RW zones (17.75 ±10.17 % and 23.47±13.62 % respectively $p<0.05$, Figure 3C).

CMCs were induced toward the three mesenchymal lineages (osteogenic, chondrogenic, and adipogenic) commonly used to assess MSC stem/progenitor cell potential.[20, 21] After one week of induction with osteogenic media, CMCs from all zones displayed increased ALP activity compared to non-induced respective controls (Figure 4A). ALP activity of BM-MSCs that were treated under the same conditions were significantly higher ($p<0.05$), although after three weeks of osteogenic induction all groups displayed increased Collagen type I expression similar to BM-MSCs ($p>0.05$ between groups, Figure 4B). CMCs from all zones were successfully induced towards the adipogenic lineage after 5 weeks of induction. CMCs from the RR-zone displayed higher prevalence of fully developed adipocytes with lipid droplets similar to those observed in induced BM-MSCs under the same conditions. CMCs from all zones were successfully induced to the chondrogenic lineage after 3 weeks in 3D culture in transwells, even though RW pellets showed less chondrogenic differentiation potential compared to BM-MSCs controls as can be observed in less Alcian Blue-stained extracellular matrix (Figure 4C).

VASCULARITY ANALYSIS

Histologic features and triple immunofluorescence colocalization analysis

Histological analysis and standard H&E staining confirmed the presence of larger vessels in the RR and RW zones (Figure 5A-B). Masson's Trichrome staining, which can differentiate between smooth muscle and extracellular matrix, confirmed the presence of a network of arteries and veins in the RR and RW zones (Figure 5B). H&E and Masson's Trichrome staining utilize dyes to differentiate between cellular structures based on their generic physicochemical properties

but cannot detect finer elements within a tissue including individual cells or smaller vessels, such as capillaries, that are composed of a single layer of endothelial cells and do not possess a smooth muscle actin lining (Figure 5C). Therefore, a more detailed immunofluorescence analysis was employed, which uses antibodies that detect specific antigens within the tissue of interest. To this end, triple immunostaining with αSMA and endothelial markers CD31 and lectin revealed the presence of endothelial cells in all three zones, including the WW zone, especially closer to the perimeter of the meniscus (Figure 6). The scan of entire IF-stained slides showed that when dividing the meniscus into three zones using the most conservative approach, that is ≤ 1/3 of total area, blood vessels were found in the WW zone. (Supplemental Figure 1 presents the full slide scan of the images shown in Figure 6).

Three-dimensional Imaging of Vessels in the Meniscus

$CD31^+$ staining demonstrated presence of endothelial cells, visualizing the vascular tree in RR towards RW and positive colocalization of green and red staining in the WW zone, especially on the periphery of the meniscus, confirming the findings in 2D immunofluorescence (Figure 7 and Supplementary Video 1).

DISCUSSION

The main finding of this study was that multipotent mesenchymal stromal progenitor cells and blood vessels were observed in all three zones of the meniscus including the WW zone. Isolated meniscus cells were clonogenic *in vitro* with no significant differences in the self-renewal potential between the three meniscal zones. Flow cytometry analysis demonstrated that these progenitor cells were expressing consensus MSC surface markers (CD44, CD105, CD29 and CD90). Meniscus stromal progenitor cells were enriched after *in vitro* culture, due to the plastic adherence that filters out non-adherent cells. Lastly, CMCs from all three meniscal zones were able to successfully differentiate into the three mesenchymal lineages (osteogenic, adipogenic and chondrogenic), and found slightly inferior to BM-MSCs in their osteogenic and adipogenic potential. Bigger vessels were observed in the RR and RW zones, and smaller vessels were identified in the WW zone using 2D immunofluorescence co-localization of endothelial markers and alpha smooth muscle actin in combination with a modified uDISCO 3D imaging approach.

Biologic augmentation approaches are currently being investigated to promote chemotaxis, cellular proliferation, and/or matrix production at the site of meniscal repair. These approaches include mechanical stimulation, marrow venting procedures, fibrin clots, injection of platelet-rich plasma, and stem cell-based therapies that involve injection of autologous MSCs.[32, 33] These approaches may augment an inherent meniscus healing capacity if resident progenitor/stem cells are present, as demonstrated in rabbits and humans.[9, 34, 35] The main role of resident mesenchymal progenitor cells in connective tissues is to maintain homeostasis and contribute to tissue repair when needed.[36] Hennerbichler *et al* demonstrated that punch defects directly filled with the removed punches, showed no significant difference in healing potential between the vascularized and avascular meniscus zone.[37] Furthermore, Croutze *et al* reported equivalent differentiation potential toward chondrogenic phenotype and extracellular matrix production of human meniscus cells isolated from the inner and outer zones.[38] Mauck *et al* reported similar differentiation potential of cells isolated from different zones of calf meniscus.[10]

Mesenchymal stromal cells (MSCs) are a heterogeneous cell population that consists of a mixture of multipotent and more committed progenitors. MSCs isolated from the bone marrow (BM-MSCs) have been shown to comply to the established stem cell criteria, that is they are multipotent, clonogenic *in vitro* and able to produce skeletal tissues via serial transplantations *in vivo.*[25] Stromal mesenchymal progenitors are found in multiple other tissues (adipose tissue, umbilical cord, etc), are clonogenic in culture, can be induced to differentiate into multiple lineages *in vitro* beyond skeletal tissues and express similar markers with BM-MSCs.[25] Both terms are sometimes used interchangeably in the literature, resulting in ISCT issuing a set of criteria in a position statement to assist comparisons between different studies.[20, 21] Based on ISCT criteria for mesenchymal stromal progenitor identification, we characterized CMCs on the basis of positive selection using plastic adherence and multipotentiality. We also assessed the expression of MSC consensus markers and compared them to freshly isolated and cultured BM-MSCs.

It is known that the cells of the meniscus differ in their morphology and their *in vitro* properties depending on their location.[12] At least four different meniscus cell types have been identified in the rabbit meniscus using fluorescent and scanning electron microscopy imaging.[18] In the present study we demonstrated a higher prevalence of isolated $CD44^{+}CD105^{+}CD29^{+}CD90^{+}$ meniscus cells in the WW zone compared to the other zones. Surface marker expression of cells freshly isolated from the WW zone was significantly greater than from the other two zones using standard flow cytometry analysis. The higher proportion of $CD44^{+}CD105^{+}CD29^{+}CD90^{+}$

cells in the WW zone suggests that the WW zone hosts a more homogeneous progenitor population compared to the other zones as defined by expression of those four markers, CFU-F clonogenicity and positive selection through plastic adherence. Interestingly, our results demonstrate that cells isolated from the RR zone have similar potential to be induced to the adipogenic lineage as BM-MSCs, whereas the cells from RW and WW could not be efficiently induced towards the adipogenic phenotype *in vitro*. Additionally, RW cells might not be as potent towards induction to the chondrogenic phenotype compared to RR and WW. This suggests that some of the cells in the RW and WW zones cannot differentiate towards the chondrogenic adipogenic lineages, so they are more specialized toward the fibrocartilage phenotype of the meniscus. However, the presence of other cell types in the RR as well as the plasticity of the resident $CD44^{+}CD105^{+}CD29^{+}CD90^{+}$ CMCs identified in RR zone might be an important factor that contributes to the regenerative potential of that zone.[8] Future research is needed to determine whether this phenomenon is age-dependent or related to other factors. It is unknown if the sole presence of progenitor cells can facilitate improved healing and therefore future studies are warranted. Also, recruitment mechanisms of these cells to the injury site needs to be addressed using *in vivo* models. Vascularization and progenitor cell availability will play an important role in recruitment of the cells and their contribution to the healing process.

Tears in the avascular zone have historically been treated with debridement, given the lower likelihood of successful healing of a repair in avascular tissue.[39] Although repair of meniscal tears in the avascular white-white zone have recently been reported to yield satisfactory outcomes.[40, 41] Cinque *et al*. reported low meniscal repair failure rates in avascular zone with only 3% of patients requiring a second surgery for a failed meniscus repair.[42] Noyes *et al*.[43] reported that 62% of red-white zone inside-out meniscal repairs had normal or nearly normal characteristics for pain, swelling, jumping and their Cincinnati score. In theory, meniscal tears require vascularization to deliver the biologic factors necessary for tissue repair. However, some animal studies demonstrate that meniscal tissue may heal without significant vascular contributions.[37, 44] In the present study, following identification of primary tissue features with standard histology (H&E and MTC stains), a comprehensive combined 2D and 3D immunofluorescence analysis was used to define vessels using cytological features and identify the presence of vessel-specific markers in cadaveric menisci. H&E and MTC staining revealed larger vessels in the RR zone of meniscus spanning towards the RW zone and sprouting to smaller arterioles. Small vessels and endothelial cells were found also in the WW zone, mainly in the periphery, suggesting that the WW zone might not be completely devoid of vasculature. However, it is possible that these are remnants of previously vascularized fetal tissues that

regressed after birth, as has been shown previously.[14] Thus, further research using functional imaging *in vivo* or cell tracking in animal models is needed to determine whether there is functional vasculature in the WW zone.[45, 46]

Large blood vessels were not found within the WW zone. However, around the perimeter of the tissue small vessels and endothelial cells were spotted, suggesting that they might be part of microvessels or remnants from vasculature that regressed in adulthood and that could potentially be revived with appropriate angiogenic stimulation. Schrepfer *et al.* reported that MSCs injected into the lungs get trapped in the pulmonary capillary network because their mean size is greater than the pulmonary capillary lumen.[47] The use of vasodilation agents has been proposed in combination with such treatments to improve perfusion and stem cell migration.[47] Importantly, recent evidence suggests that vasculature could increase in the case of meniscal tears, upregulating angiogenic factors, such as vascular endothelial growth factor (VEGF).[48] This could modify the affluence of progenitor cells not only by chemotaxis but as a *de novo* source of pericytes.[49] The specific topology of the capillary network in the WW zone of healthy adult donors is yet to be mapped. The vessel distribution may be affected by age, health or fitness level, and other factors that are yet to be determined. In this study we demonstrate that there is a small amount of vasculature and a significant population of progenitor cells in the WW zone of the meniscus. Future studies that investigate the healing potential of these resident stem/progenitor cells are warranted.

Limitations

This study is not without limitations. The sample size was limited and both medial and lateral meniscus were included. The donated cadaveric menisci were never frozen (received within 21 days) and stored at 4°C increasing the potential of cell death immediately post-harvest compared to the *in vivo* setting. The mean age of the specimens was 21 years old, which is not representative of an older population. Thus, further studies exploring the cellular content and vascularity of the older population is warranted. Only three zones were selected for the analysis: inner, middle and marginal, each representing a third of the width of the tissue which might not represent a true division of the vascular tree density throughout the meniscus tissue. Vessel distribution might be different between medial and lateral menisci and could also be affected by age and/or health status. It is known that in every cell isolation procedure from a

tissue cell yields inherently vary. Since the cellular composition of meniscus is heterogeneous, the current cell isolation approach was optimized for our work because it was shown as the most efficient for all three zones for our grafts. Additionally, no significant differences were observed between medial and lateral menisci. However, it might not be pertinent for grafts with different characteristics, such as fetal and/or neonatal menisci, or menisci from aged and diseased populations. Finally, the presence of blood vessels does not necessarily indicate functional vascularization, which requires further analysis. These vessels might be remnants of retreating vasculature from the fetal meniscus.

CONCLUSIONS

In conclusion, our results demonstrate the presence of resident mesenchymal progenitors in all three meniscal zones of healthy adult donors without injury. Additionally, our results demonstrate the presence of vascularization in the WW zone.

Figure Legends

Figure 1. Research Design. (1) *The first aim of this study was to characterize and identify the resident stromal progenitor cell population in all three zones in freshly harvested human cadaveric menisci.* ***(2)*** *The second aim was to characterize the vascularity of the menisci, using histology, immunofluorescence (IF), and three-dimensional (3D) light sheet fluorescence microscopy.*

Figure 2. Evidence of resident stromal progenitor cells in the WW of human meniscus*.* ***(A)*** *Dissection of meniscal zones for cell isolation.* ***(B)*** *Box and Whisker plots of cell yields of medial or lateral menisci normalized to tissue wet weight. Lines display median values.* ***(C)*** *Colony formation of isolated cells in vitro. Meniscal cells from all zones were clonogenic in culture. No significant differences were found between zones when assessed by CFU-assays (p>0.05).*

Figure 3*.* ***Identification of freshly isolated meniscus cells vs. cultured controls using flow cytometry (A)*** *Flow cytometry analysis of cells from the three meniscal zones displayed presence of two distinct subpopulations of cells immediately after isolation (top panel). One subpopulation was positive to MSC surface markers and the other population was negative. Meniscal cells that were selected using plastic adherence and cultured to P2 were all positive*

for all MSC markers similarly to BM-MSCs (bottom panel). ***(B)*** *Quantification of individual cell surface markers showing the proportion of each marker per zone. All four markers showed higher expression in the WW zone compared to RR and RW (p <0.05).* ***(C)*** *Proportion of* $CD105^{+}CD44^{+}CD29^{+}CD90^{+}$ *cells per zone, p<0.05.*

Figure 4. Multilineage differentiation potential of meniscal stromal cells. (A). *ALP activity after 1 week of osteogenic induction. Controls were cultured for one week without osteogenic media (*p <0.05, ***p<0.001)* ***(B).*** *Col1 expression after 3 weeks of osteogenic induction (p<0.05).* ***(C)*** *. Adipogenesis was induced for 5 weeks with adipogenic supplements. BM-MSCs were treated under the same conditions and were used as assay positive controls. Red-Oil O stains lipid droplets in red. Bars represent 50 µm* ***(D).*** *Chondrogenesis was induced in transwells for 3 weeks resulting in disk formation. Disks were processed histologically, sectioned and whole slide scans attained. Alcian blue was used to determine presence of proteoglycans in the disks. BM-MSCs were treated under the same conditions and were used as assay controls. Bars represent 50µm.*

Figure 5. Limitations of simple 2D histology approaches to visualize the vasculature of the human meniscus. *(****A****): Schematic Illustration of the three meniscal zones: red-red (RR), red-white (RW), white-white (WW). (****B****): Full thickness cut of the meniscus stained with Hematoxylin & Eosin (H&E) [left] and Masson's Trichome stain [right], and higher magnification (bottom panels) of boxed areas shows relatively low cellularity throughout the RW and WW zones. Top bars represent 1mm, in bottom panels bars represent 100µm.*

Figure 6. Immunofluorescent triple staining with lectin (green), CD31 (orange), αSMA *(magenta) and DAPI (blue) in all three zones using fluorescent microscopy and imaging with two different microscopes. Left images from whole-slide scans were taken (20x scan, Leica). Right, orange boxes from left images were identified using a Nikon Eclipse microscope and imaged at higher magnification (40x). Whole-slide scans allow visualization of the exact region imaged (left images, top right; yellow arrows). Areas were assessed using Qupath software to accurately quantify the distances between regions. The RW snapshot shown is at the bottom border of the RW zone of the whole-slide scan and was chosen in order to show the continuity of the scan. Bigger vessels with* αSMA *lining and lectin/CD31 can be found in both RR and RW zones. However, in WW zone only lectin/CD31/DAPI positive staining was found, suggesting presence of relatively smaller vessels, such as capillaries in this area. RR red-red zone; RW, red-white zone; WW, white-white zone. Scales bars represent 50µm.*

Figure 7. Successful clearing of human meniscus with modified uDISCO procedure and nucleic staining with To-Pro®-3 and the endothelial marker CD31. (A). *Fully transparent slices of human meniscal tissue cleared with a modified uDisco approach. On the left a quarter of fully cleared meniscus. On the right, a slice before and after clearing (**B).** 2D slice of cleared meniscus tissue stained with only with TO-Pro®-3, displaying cells distributed in all 3 zones **(C).** Three-dimensional reconstruction of meniscus slices after imaging with light sheet fluorescence microscopy. Double-labeling with To-Pro®-3 (red, pan-nuclei marker) and CD-31 (green, endothelial marker) indicates presence of vessels of various sizes in all zones. Bigger vessels were found spanning the RR to RW zones, confirming 2D results. Blood Vessels are present in the WW zone. Upper and bottom panels display different views of the same slice. Left; merged channels (red and green), middle; red channel (To-Pro®-3), right; green channel (CD31). Arrows point to vessels. Bar represents 500µm.*

Supplementary Figure 1.

Full slide scans of the same block of meniscus tissue. A. H&E, B. MTC, C, IF staining against lectin, CD31, aSMA. QuPath and Imaris software programs were used to measure and classify the three meniscus regions using the most conservative approach, that is ≤1/3 of long axis (distance is displayed in the image).[50] The presence of smaller vessels in WW is evident.

Supplemental Video

Mp4 video created using Imaris 3D reconstruction software of a meniscus thin section stained with To-Pro®-3 and the endothelial marker CD31.

References

1. Arnoczky SP, Warren RF. The microvasculature of the meniscus and its response to injury. An experimental study in the dog. *Am J Sports Med.* 1983;11:131-141.

2. Chahla J, Cinque ME, Godin JA, et al. Meniscectomy and Resultant Articular Cartilage Lesions of the Knee Among Prospective National Football League Players: An Imaging and Performance Analysis. *Am J Sports Med.* 2018;46:200-207.

3. Dave LY, Caborn DN. Outside-in meniscus repair: the last 25 years. *Sports Med. Arthrosc.* 2012;20:77-85.

4. Stein T, Mehling AP, Welsch F, von Eisenhart-Rothe R, Jäger A. Long-term outcome after arthroscopic meniscal repair versus arthroscopic partial meniscectomy for traumatic meniscal tears. *The American journal of sports medicine.* 2010;38:1542-1548.

5. Rodeo SA, Monibi F, Dehghani B, Maher S. Biological and mechanical predictors of meniscus function: Basic science to clinical translation. *Journal of Orthopaedic Research®.* 2020;38:937-945.

6. Murakami T, Otsuki S, Okamoto Y, et al. Hyaluronic acid promotes proliferation and migration of human meniscus cells via a CD44-dependent mechanism. *Connect Tissue Res.* 2019;60:117-127.

7. Tarafder S, Gulko J, Sim KH, Yang J, Cook JL, Lee CH. Engineered Healing of Avascular Meniscus Tears by Stem Cell Recruitment. *Sci Rep.* 2018;8:8150.

8. Kobayashi K, Fujimoto E, Deie M, Sumen Y, Ikuta Y, Ochi M. Regional differences in the healing potential of the meniscus—an organ culture model to eliminate the influence of microvasculature and the synovium. *The Knee.* 2004;11:271-278.

9. Shen W, Chen J, Zhu T, et al. Intra-articular injection of human meniscus stem/progenitor cells promotes meniscus regeneration and ameliorates osteoarthritis through stromal cell-derived factor-1/CXCR4-mediated homing. *Stem cells translational medicine.* 2014;3:387-394.

10. Mauck RL, Martinez-Diaz GJ, Yuan X, Tuan RS. Regional multilineage differentiation potential of meniscal fibrochondrocytes: implications for meniscus repair. *Anat Rec (Hoboken).* 2007;290:48-58.

11. Humphrey JD, Dufresne ER, Schwartz MA. Mechanotransduction and extracellular matrix homeostasis. *Nature reviews Molecular cell biology.* 2014;15:802-812.

12. Athanasiou KA, Sanchez-Adams J. Engineering the knee meniscus. *Synthesis Lectures on Tissue Engineering.* 2009;1:1-97.

13. KING D. THE HEALING OF SEMILUNAR CARTILAGES. *JBJS.* 1936;18:333-342.

14. Karia M, Ghaly Y, Al-Hadithy N, Mordecai S, Gupte C. Current concepts in the techniques, indications and outcomes of meniscal repairs. *European Journal of Orthopaedic Surgery & Traumatology.* 2019;29:509-520.

15. Verdonk R, Kohn D. Harvest and conservation of meniscal allografts. *Scand J Med Sci Sports.* 1999;9:158-159.

16. Pan C, Cai R, Quacquarelli FP, et al. Shrinkage-mediated imaging of entire organs and organisms using uDISCO. *Nat Methods.* 2016;13:859-867.

17. Grogan SP, Duffy SF, Pauli C, Lotz MK, D'Lima DD. Gene expression profiles of the meniscus avascular phenotype: A guide for meniscus tissue engineering. *Journal of Orthopaedic Research®.* 2018;36:1947-1958.

18. LE GRAVERAND M-PH, Ou Y, Schield-Yee T, et al. The cells of the rabbit meniscus: their arrangement, interrelationship, morphological variations and cytoarchitecture. *The Journal of Anatomy.* 2000;198:525-535.

19. Sanchez-Adams J, Athanasiou KA. Regional effects of enzymatic digestion on knee meniscus cell yield and phenotype for tissue engineering. *Tissue Eng Part C Methods.* 2012;18:235-243.

20. Horwitz E, Le Blanc K, Dominici M, et al. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. *Cytotherapy.* 2005;7:393-395.

21. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy.* 2006;8:315-317.

22. Sheyn D, Shapiro G, Tawackoli W, et al. PTH Induces Systemically Administered Mesenchymal Stem Cells to Migrate to and Regenerate Spine Injuries. *Mol Ther.* 2016;24:318-330.

23. Sheyn D, Ben-David S, Shapiro G, et al. Human Induced Pluripotent Stem Cells Differentiate Into Functional Mesenchymal Stem Cells and Repair Bone Defects. *Stem cells translational medicine.* 2016;5:1447-1460.

24. Sheyn D, Pelled G, Netanely D, Domany E, Gazit D. The effect of simulated microgravity on human mesenchymal stem cells cultured in an osteogenic differentiation system: a bioinformatics study. *Tissue Eng Part A.* 2010;16:3403-3412.

25. Wilson A, Webster A, Genever P. Nomenclature and heterogeneity: consequences for the use of mesenchymal stem cells in regenerative medicine. *Regenerative medicine.* 2019;14:595-611.

26. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C T method. *Nature protocols.* 2008;3:1101.

27. Mizrahi O, Sheyn D, Tawackoli W, et al. Nucleus pulposus degeneration alters properties of resident progenitor cells. *Spine J.* 2013;13:803-814.

28. Sheyn D, Ben-David S, Shapiro G, et al. Human iPSCs Differentiate Into Functional MSCs and Repair Bone Defects. *Stem Cells Transl Med.* 2016.

29. Waring CD, Vicinanza C, Papalamprou A, et al. The adult heart responds to increased workload with physiologic hypertrophy, cardiac stem cell activation, and new myocyte formation. *Eur Heart J.* 2014;35:2722-2731.

30. Nehrhoff I, Ripoll J, Samaniego R, Desco M, Gomez-Gaviro MV. Looking inside the heart: a see-through view of the vascular tree. *Biomed Opt Express.* 2017;8:3110-3118.

31. Song E, Seo H, Choe K, et al. Optical clearing based cellular-level 3D visualization of intact lymph node cortex. *Biomed Opt Express.* 2015;6:4154-4164.

32. Murrell WD, Anz AW, Badsha H, Bennett WF, Boykin RE, Caplan AI. Regenerative treatments to enhance orthopedic surgical outcome. *PM&R.* 2015;7:S41-S52.

33. Woodmass JM, LaPrade RF, Sgaglione NA, Nakamura N, Krych AJ. Meniscal repair: reconsidering indications, techniques, and biologic augmentation. *JBJS.* 2017;99:1222-1231.

34. Segawa Y, Muneta T, Makino H, et al. Mesenchymal stem cells derived from synovium, meniscus, anterior cruciate ligament, and articular chondrocytes share similar gene expression profiles. *Journal of Orthopaedic Research.* 2009;27:435-441.

35. Shen W, Chen J, Zhu T, et al. Osteoarthritis prevention through meniscal regeneration induced by intra-articular injection of meniscus stem cells. *Stem cells and development.* 2013;22:2071-2082.

36. Mabuchi Y, Matsuzaki Y. Prospective isolation of resident adult human mesenchymal stem cell population from multiple organs. *Int J Hematol.* 2016;103:138-144.

37. Hennerbichler A, Moutos FT, Hennerbichler D, Weinberg JB, Guilak F. Repair Response of the Inner and Outer Regions of the Porcine Meniscus in Vitro. *The American Journal of Sports Medicine.* 2007;35:754-762.

38. Croutze R, Jomha N, Uludag H, Adesida A. Matrix forming characteristics of inner and outer human meniscus cells on 3D collagen scaffolds under normal and low oxygen tensions. *BMC Musculoskelet Disord.* 2013;14:353.

39. Grant JA, Wilde J, Miller BS, Bedi A. Comparison of inside-out and all-inside techniques for the repair of isolated meniscal tears: a systematic review. *The American journal of sports medicine.* 2012;40:459-468.

40. Longo UG, Campi S, Romeo G, Spiezia F, Maffulli N, Denaro V. Biological strategies to enhance healing of the avascular area of the meniscus. *Stem Cells Int.* 2012;2012:528359.

41. Dean CS, Chahla J, Mitchell JJ, LaPrade RF. Outcomes Following Biologically Augmented Isolated Meniscal Repairs with Marrow Venting are Comparable to Meniscal Repairs with Concomitant ACL Reconstruction. *American Journal of Sports Medicine.* 2016;In Press

42. Cinque ME, DePhillipo NN, Moatshe G, et al. Clinical Outcomes of Inside-Out Meniscal Repair According to Anatomic Zone of the Meniscal Tear. *Orthop J Sports Med.* 2019;7:2325967119860806.

43. Noyes FR, Chen RC, Barber-Westin SD, Potter HG. Greater than 10-year results of red-white longitudinal meniscal repairs in patients 20 years of age or younger. *Am J Sports Med.* 2011;39:1008-1017.

44. Huang TL, Lin GT, O'Connor S, Chen DY, Barmada R. Healing potential of experimental meniscal tears in the rabbit. Preliminary results. *Clin Orthop Relat Res.* 1991:299-305.

45. Baldwin J, Wagner F, Martine L, et al. Periosteum tissue engineering in an orthotopic in vivo platform. *Biomaterials.* 2017;121:193-204.

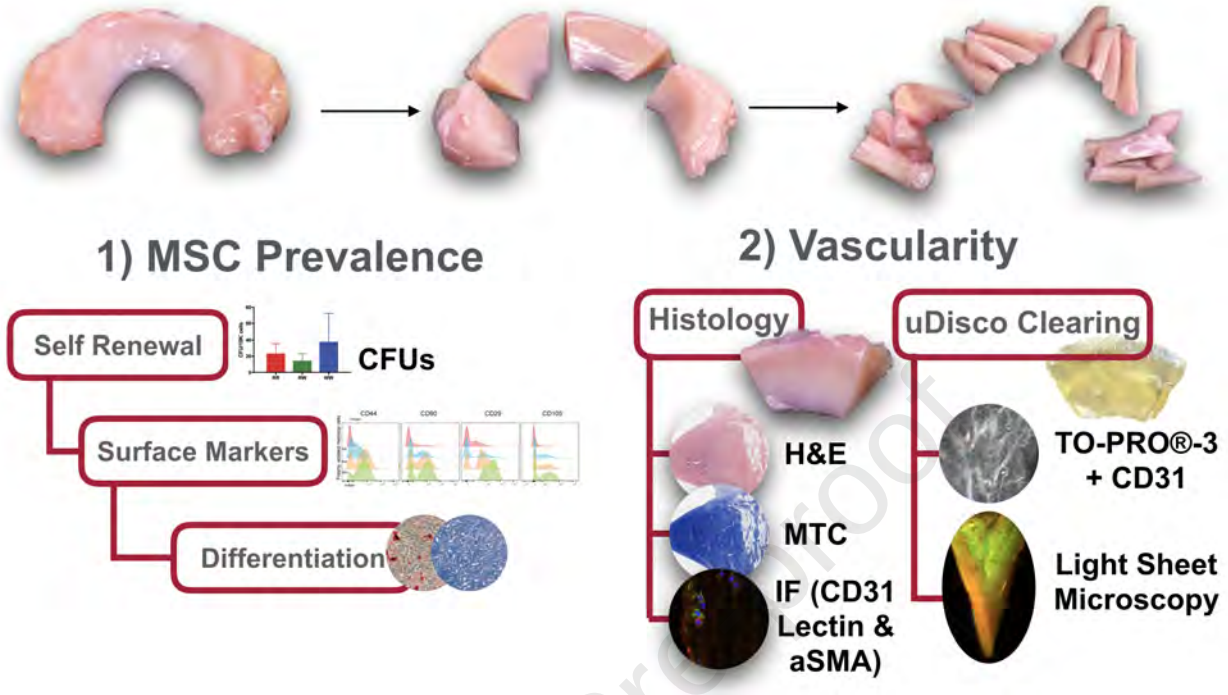
46. Histed SN, Lindenberg ML, Mena E, Turkbey B, Choyke PL, Kurdziel KA. Review of functional/anatomic imaging in oncology. *Nuclear medicine communications.* 2012;33:349.

47. Schrepfer S, Deuse T, Reichenspurner H, Fischbein M, Robbins R, Pelletier M. Stem cell transplantation: the lung barrier. *Transplantation proceedings.* Vol 39: Elsevier; 2007:573-576.

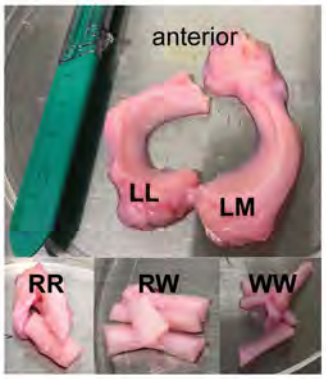
48. Lu Z, Furumatsu T, Fujii M, Maehara A, Ozaki T. The distribution of vascular endothelial growth factor in human meniscus and a meniscal injury model. *J Orthop Sci.* 2017;22:715-721.

49. Crisan M, Yap S, Casteilla L, et al. A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell.* 2008;3:301-313.

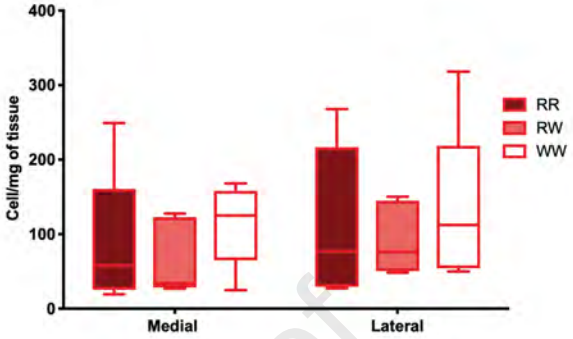
50. Anderson AF, Irrgang JJ, Dunn W, et al. Interobserver reliability of the International Society of Arthroscopy, Knee Surgery and Orthopaedic Sports Medicine (ISAKOS) classification of meniscal tears. *The American journal of sports medicine.* 2011;39:926-932.



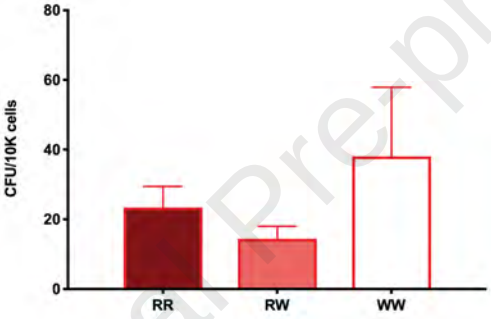
A

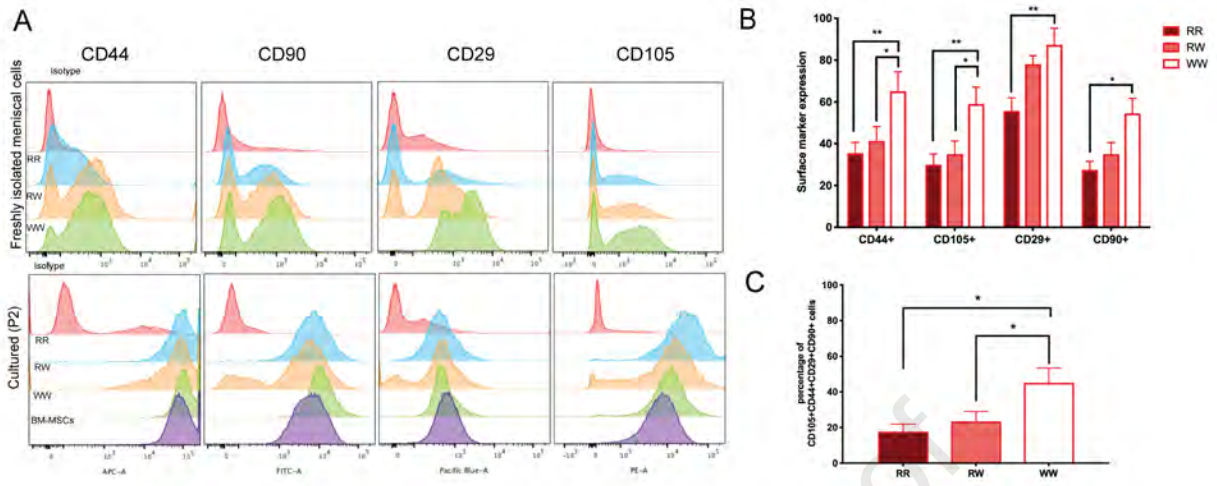


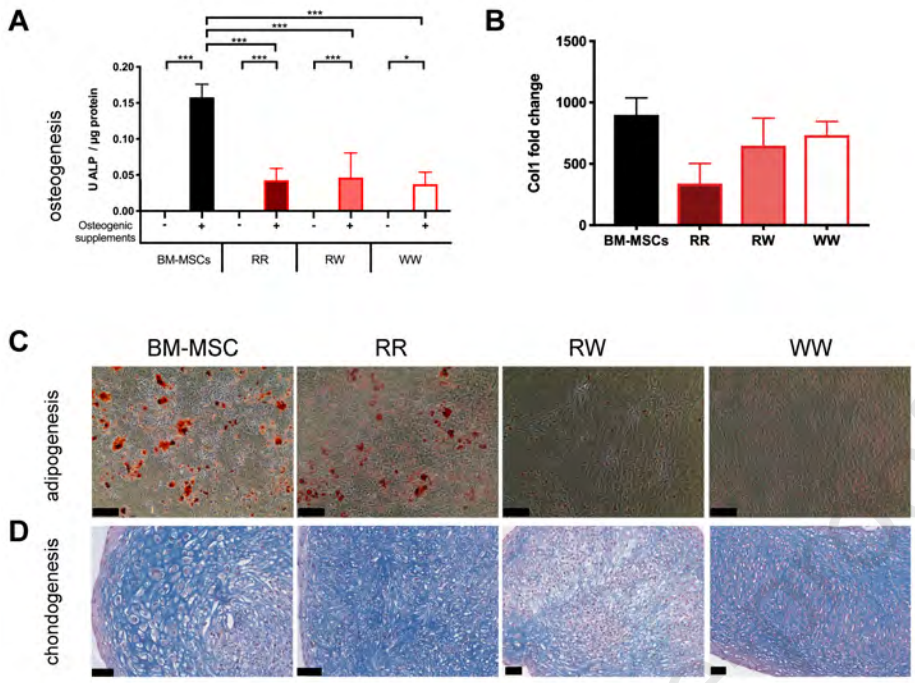
B

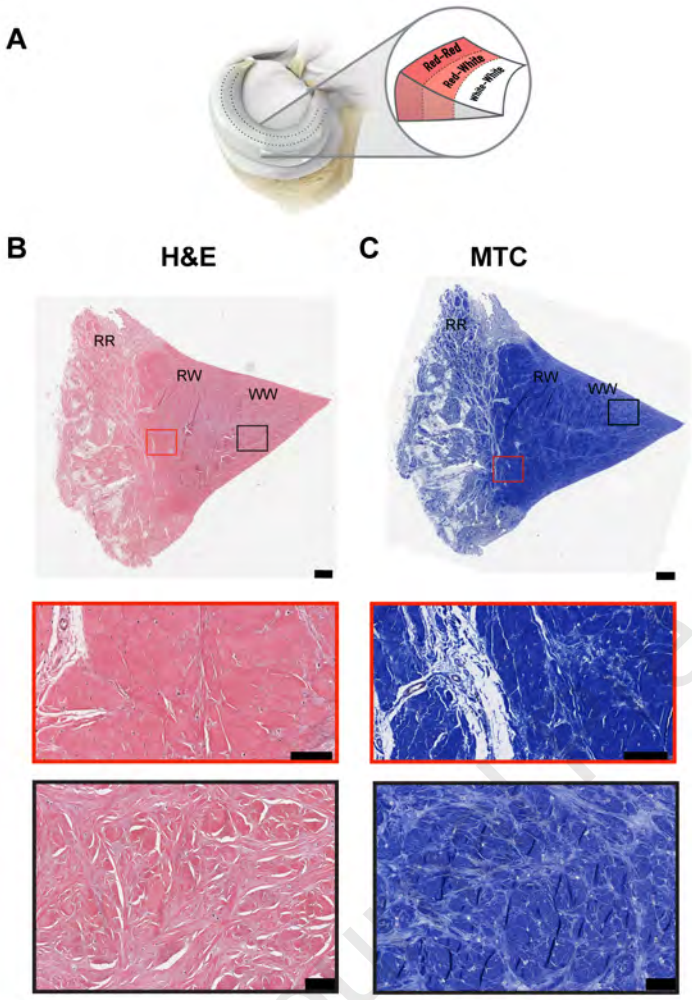


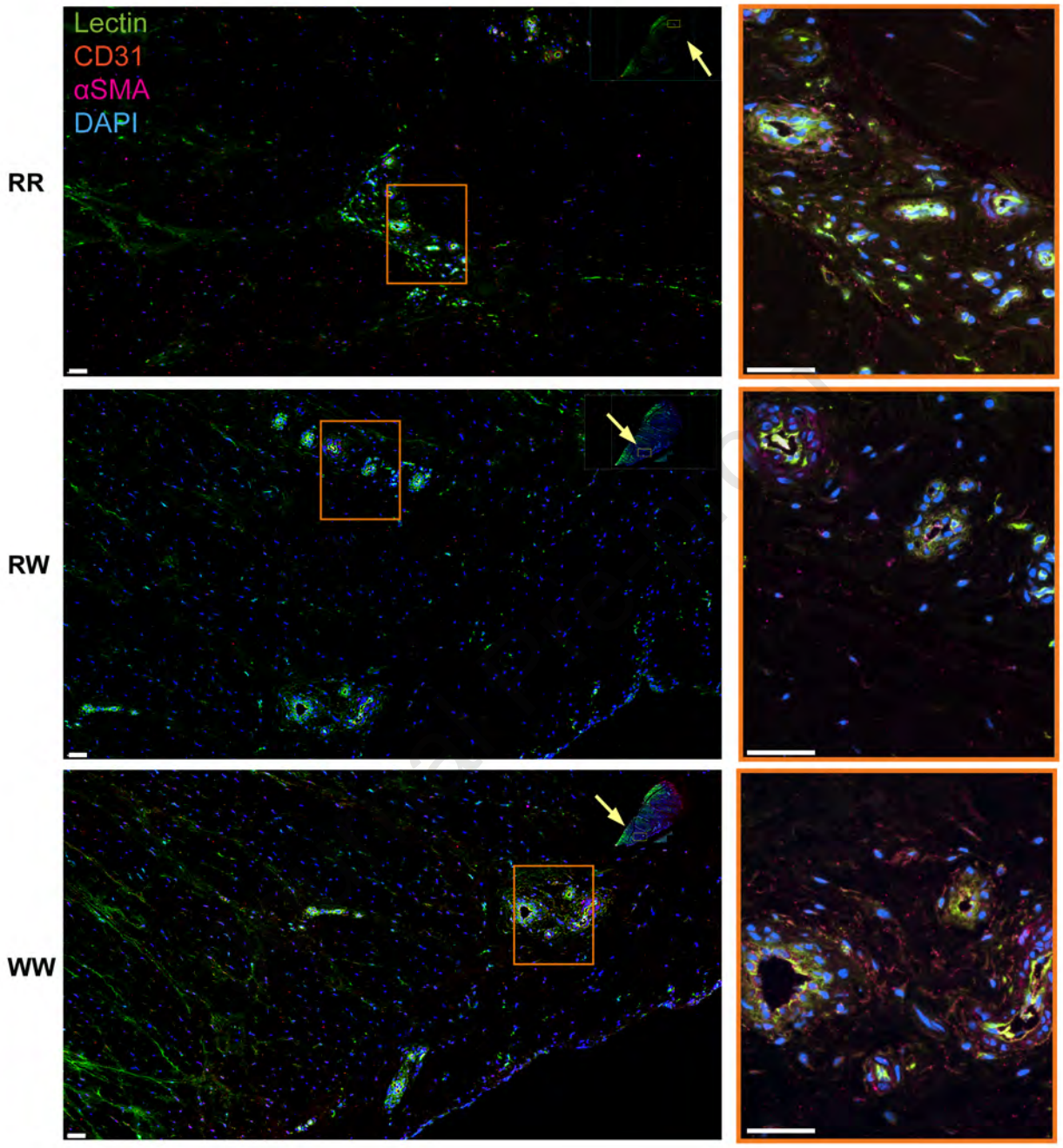
C

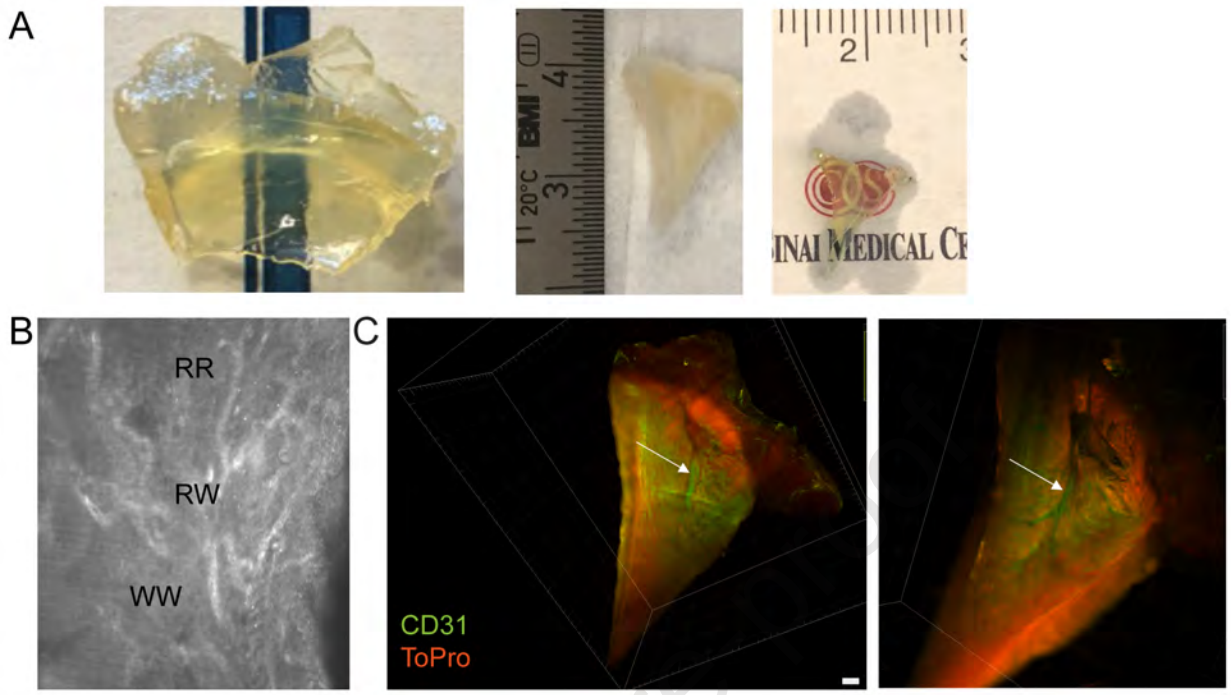









Lectin
CD31
αSMA
DAPI
RR
RW
WW



Authorship justification:

1. **Jorge Chahla, MD PhD**: Substantial contributions to the conception and design of the work, drafting the work for important intellectual content, interpretation of data for the work, final approval of the version to be published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
2. **Angela Papalamprou, PhD**: Substantial contributions to the conception and design of the work, acquisition, analysis, or interpretation of data for the work, drafting the work for important intellectual content, final approval of the version to be published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
3. **Virginia Chan, BS**: Substantial contributions to acquisition and analysis for the work, drafting the work for important intellectual content, final approval of the version to be published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
4. **Yasaman Arabi, BS**: Substantial contributions to acquisition and analysis for the work, drafting the work for important intellectual content, final approval of the version to be published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
5. **Khosrawdad Salehi, BS**: Substantial contributions to acquisition and analysis for the work, drafting the work for important intellectual content, final approval of the version to be published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
6. **Trevor J. Nelson, BS**: Substantial contributions to acquisition and analysis for the work, drafting the work for important intellectual content, final approval of the version to be published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
7. **Orr Limpisvasti, MD**: Substantial contributions to the conception of the work, final approval of the version to be published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
8. **Bert R Mandelbaum, MD DHL**: Substantial contributions to the conception of the work, final approval of the version to be published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
9. **Wafa Tawackoli, PhD**: Substantial contributions to the conception and design of the work, drafting the work or revising it critically for important intellectual content, final approval of the version to be published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
10. **Melodie F. Metzger**, PhD: Substantial contributions to the conception and design of the work, drafting the work or revising it critically for important intellectual content, final approval of the version to be published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
11. **Dmitriy Sheyn, PhD**: Substantial contributions to the conception and design of the work, acquisition, analysis, or interpretation of data for the work, drafting the work or revising it critically for important intellectual content, final approval of the version to be published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.