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Regional Multilineage Differentiation Potential of Meniscal Fibrochondrocytes: Implications for Meniscus Repair

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ABSTRACT

The knee menisci are wedge-shaped semilunar fibrocartilaginous structures that reside between the femur and tibia and function to transmit and distribute load. These structures have characteristics of both fibrous and cartilaginous tissues. The cartilage-like inner region and the fibrous vascularized outer region each has a distinct extracellular matrix, and resident meniscal fibrochondrocytes (MFCs) with distinct morphologies dependent on their location. Damage to the meniscus is common, and disruption of tissue structure and function result in erosion of the underlying articular cartilage. It has been observed that damage in the vascular periphery undergoes spontaneous repair, whereas damage of the inner region does not heal. While vascularity of the peripheral region plays a role in healing, recent findings have also suggested that local cellular composition influences local healing capacity. This study examined the variation in multipotential characteristics of cell populations isolated from different regions of the bovine meniscus. MFCs were isolated from the outer (vascular), inner (avascular), and horn (mixed) regions and induced toward chondrogenic, adipogenic, and osteogenic lineages. The results of this study suggest that MFCs from all regions of the meniscus possess a multilineage differentiation capability, particularly toward chondrogenesis and adipogenesis. MFCs from the outer region were most plastic, differentiating along all three mesenchymal lineages. These findings may underlie the experimental observation of improved integration of meniscus grafts from the outer zone and may have implications for developing strategies of cell-based meniscus repair. Anat Rec, 290:48–58, 2007.

Key words: meniscus; fibrochondrocyte; multipotential mesenchymal stem cells; adipogenesis; osteogenesis; chondrogenesis

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The knee menisci are wedge-shaped semilunar fibrocartilaginous structures that reside between the femur and tibia (Rath and Richmond, 2000). The main function of the meniscus is to transmit and distribute load, increase joint congruency, and stabilize the joint (Ghosh and Taylor, 1987; Arnoczky et al., 1988a). As a fibrocartilaginous structure, the meniscus has characteristics of both fibrous and cartilaginous tissues, with the inner region more cartilage-like (O'Connor, 1976) and the outer region more fibrous (Petersen and Tillmann, 1998). The meniscus is predominantly composed of collagen (85–95% of the dry weight), of which > 90% is collagen type I, with the remaining 1–2% consisting of collagen types II, III, V, and VI (Eyre and Wu, 1983; Cheung, 1987; McDevitt and Webber, 1990; Kambic and McDevitt, 2005). Proteoglycans make up less than 2–3% of the dry weight (Fithian et al., 1990; McDevitt and Webber, 1990; Adams and Hukins, 1992; Buma et al., 2004) and are concentrated in the inner cartilaginous zones. In the outer fibrous region of the meniscus, collagen fibers are circumferentially oriented (Bullough et al., 1970; Arnoczky et al., 1988a; Mow et al., 1992; Petersen and Tillmann, 1998), resulting in highly anisotropic material properties that are greatest in the fiber direction (Bullough et al., 1970; Setton et al., 1999). These properties are essential for the mechanical function of the tissue.

The cellular component of the meniscus reflects its fibrocartilaginous nature. The main cell type in the structure is the meniscal fibrochondrocyte (MFC), which sparsely populates the tissue (McDevitt and Webber, 1990; Adams and Hukins, 1992; Setton et al., 1999; Benjamin and Ralphs, 2004). The appearance and phenotypic characteristics of these MFCs vary according to their position in the meniscus (Hellio Le Graverand et al., 2001a). For example, MFCs located in the inner avascular region resemble articular chondrocytes; they are rounded and well separated from one another by their surrounding extracellular matrix (ECM). Conversely, MFCs in the outer fibrous region are spindle-shaped, with numerous projections that form gap junctions with neighboring cells (Hellio Le Graverand et al., 2001a). Collectively, MFCs function to maintain and remodel the meniscus ECM (McDevitt and Webber, 1990; Adams and Hukins, 1992) and alter their biosynthetic activities in response to a changing mechanical environment (Shin et al., 2003; Upton et al., 2003) and/or onset of joint instability (Hellio Le Graverand et al., 2001b, 2001c). MFCs can be isolated directly from the meniscus via digestion of the ECM, or coaxed to migrate out of meniscus pieces in tissue culture (Webber et al., 1985, 1989; Webber, 1990).

It has long been appreciated that damage to the meniscus is a common occurrence (MacAusland, 1931; Cravener and MacElroy, 1941), with indications of scarring (indicating past insult) in most patients over the age of 45. Damage can be of many forms, including flap and horn tears, longitudinal tears, or transverse or oblique tears (MacAusland, 1931; Cravener and MacElroy, 1941). Not all meniscus tears are considered treatable, and partial resection is more common than repair (Rath and Richmond, 2000). Mechanical compromise of the meniscus decreases tibiofemoral contact area and thereby increases stress concentrations on the underlying cartilage (Krause et al., 1976; Baratz et al., 1986; Ahmed, 1992; Rath and Richmond, 2000) and changes joint motion (Levy et al., 1982; Lanzer and Komenda, 1990). In several animal models (Hoch et al., 1983; Elliott et al., 1999; LeRoux et al., 2000), meniscectomy led to a decrease in the mechanical properties of the articular cartilage, with eventual degenerative changes, such as osteophyte formation and joint space narrowing (Petrosini and Sherman, 1996; Rath and Richmond, 2000).

One of the interesting features of meniscus healing arises from the finding that the tissue is differentially vascularized, with a vascular periphery (outer one-third) and an avascular (cartilaginous) inner region (Arnoczky and Warren, 1983). King (1936a, 1936b) was the first to suggest that tears connected to the vascular periphery undergo spontaneous repair, whereas those limited to the inner region do not (Petrosini and Sherman, 1996). This observation has led to the belief that vascularity is essential for healing, and consequently, surgical techniques have been designed to improve vascular access to sites of meniscus damage (Arnoczky and Warren, 1983). These methods include the introduction of vascular channels or the implantation of fibrin clots (Arnoczky et al., 1985, 1988b; Arnoczky, 1992). More recently, it has been observed that different zones of the meniscus have differing healing potentials, even in the absence of an active blood supply (Kobayashi et al., 2004). In these studies of whole meniscus explants, portions of the vascular region were transplanted to freshly prepared defects in the avascular region and found to integrate better than grafts derived from the avascular region. These findings suggest that the endogenous cellular composition of the meniscus may play a role in the local healing response.

It is generally believed that endogenous tissue repair is driven by progenitor cells located within the tissue that migrate to injury sites, proliferate, and deposit new matrix (Barry, 2003). Indeed, an increasing number of musculoskeletal tissues have been found to harbor cells that retain some multipotential characteristics, including adipose tissue (Zuk et al., 2001; Erickson et al., 2002), articular cartilage (Barbero et al., 2003; Tallheden et al., 2003; Alsalameh et al., 2004), trabecular bone (Osyczka et al., 2002; Tuli et al., 2003), synovium (Nishimura et al., 1999; De Bari et al., 2003), heart and skeletal muscle (Warejcka et al., 1996; Williams et al., 1999; Young et al., 2001), and periodontal ligaments (Lekic and McCulloch, 1996; Seo et al., 2005). In this study, we examined the ability of MFCs to undergo multipotential lineage differentiation (chondrogenesis, adipogenesis, and osteogenesis). These differentiation lineages were assessed in comparison to bone marrow-derived MSCs, a cell source known to exhibit such multipotential characteristics (Caplan, 1991; Prockop, 1997; Pittenger et al., 1999). Furthermore, to determine if there exists a regional variation in this cellular component of endogenous meniscus repair, this study examined the potentiality of MFCs isolated from different regions: the outer (vascular), inner (avascular), and horn (mixed) regions. We hypothesized that MFCs derived from the outer vascular region would possess a multilineage differentiation potential, while those from the avascular inner zones would not, with inherent cell plasticity reflecting the overall healing capacity of each region.

MATERIALS AND METHODS

Meniscal Fibrochondrocyte Isolation and Expansion

MFCs were isolated from the medial and lateral menisci of 3- to 6-month-old bovine calves within 36 hr of slaughter (Fresh Farms Beef, Rutland, VT). For each of

four replicate experiments, the menisci of 3–5 donors were isolated and combined. After removal of any extraneous soft capsular tissue, menisci were sectioned into an inner (I; inner one-third of central region), outer (O; outer one-third of central region), and horn (H; anterior and posterior full thickness) regions. These sections were manually diced into 1–2 mm^3 pieces and plated in 10 cm tissue culture dishes with 40 mL of a basal medium consisting of high-glucose (hg) DMEM containing 1 × penicillin/streptomycin/fungizone (PSF) and 10% fetal bovine serum (FBS). MFCs were observed to emerge from meniscus pieces over a 1- to 2-week period, after which they were trypsinized, replated, and expanded through passage 2 (p2). Human bone marrow-derived mesenchymal stem cells (MSCs; p3 and p4) were isolated and expanded as described previously (Song and Tuan, 2004) according to an institutional review board-approved procedure (George Washington University) and used as a positive control for differentiation assays. For all studies, media were changed twice weekly over the 21-day culture period.

Multilineage Differentiation Culture Conditions and Analysis

Chondrogenesis. To assess the ability of meniscus-derived cells to undergo chondrogenesis, isolated MFCs and human MSCs were formed into pellets containing 250,000 cells in 96-well polypropylene plates as described previously (Mauck et al., 2006). In a preliminary study, cells from the entirety of the meniscus were isolated, while in subsequent studies cells from distinct regions were utilized. For long-term culture, pellets were maintained in either basal medium (BM) supplemented with 50 μg/mL ascorbate 2-phosphate or a chemically defined chondrogenic medium consisting of hgDMEM supplemented with 1 × PSF, 0.1 μM dexamethasone, 50 μg/mL ascorbate 2-phosphate, 40 μg/mL L-proline, 100 μg/mL sodium pyruvate, 1 × insulin/transferrin/selenium $(ITS)^+$ Premix (Becton Dickinson, Franklin Lakes, NJ) with (CM^+) or without (CM^-) 10 ng/mL transforming growth factor β3 (TGF-β3) (R&D Systems, Minneapolis, MN). At weekly intervals through day 21, several pellets from each group were removed from culture and either suspended in TRIzol reagent (Invitrogen, Carlsbad, CA) and stored at −80°C for RNA isolation or embedded in 2% low-melting-temperature agarose and fixed overnight at 4°C in 4% phosphate-buffered paraformaldehyde. These pellet-laden agarose blocks were paraffin-embedded and sectioned to 8 μm thickness. Sections were stained with hematoxylin and eosin (H&E), Picrosirius red, or Alcian blue (pH 1.0; Rowley Biochemicals, Danvers, MA) to visualize cells, bulk collagen, and sulfated proteoglycan, respectively. Additionally, sulfated glycosaminoglycan (sGAG) content was determined using the DMMB dye-binding assay (Farndale et al., 1986) on a per-pellet basis for 6–8 pellets/replicate on papain-digested samples (as in Mauck et al., 2003).

Adipogenesis and osteogenesis. To test for adipogenesis and osteogenesis, p2 MFCs derived from each region as well as human MSCs were plated at 20,000 cells/cm^2 in tissue culture-treated six-well plates and maintained in lineage-specific differentiation media (3 mL/well). Freshly prepared differentiation medium was changed twice weekly. Osteogenic medium (OS) consisted of DMEM supplemented with 10% FBS, 1 × PSF, 10 nM dexamethasone, 10 mM β-glycerophosphate, 50 μg/ml ascorbate 2-phosphate, and 10 nM 1,25-dihydroxyvitamin D3. Adipogenic medium (AS) consisted of DMEM supplemented with 10% FBS, 1 × PSF, 1 μM dexamethasone, 1 μg/ml insulin, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX). Control cultures were maintained in basal medium.

On days 1 and 21, relative cell numbers in OS, AS, and control cultures were determined using the MTT Cell Proliferation Assay (Molecular Probes, Eugene, OR). A further sampling for each condition was collected in 1 mL of TRIzol reagent (Invitrogen) and stored at −80°C for RNA extraction. On day 21, OS and control samples were fixed with 60% isopropanol and stained with Alizarin red (2%; Rowley Biochemicals) for mineralized matrix. Day 21 AS and control samples were fixed with 4% phosphate-buffered paraformaldehyde (FD Neurotechnologies, Catonsville, MD) and stained with Oil Red O (Sigma Chemicals, St. Louis, MO; dissolved at 0.18% in 60% isopropanol) to visualize lipid droplets. Oil Red O content was further quantified by elution with isopropanol and absorbance measurements at OD 540 nm (Sen et al., 2001). For each replicate study, 1–2 wells from Oil Red O-stained cultures were counterstained with hematoxylin (Sigma Chemicals; to visual cell nuclei) and photographed using an inverted microscope.

One-Step Reverse Transcriptase and Real-Time PCR

For undifferentiated MFC monolayers cultures, as well as those maintained in adipogenic or osteogenic culture, total RNA was extracted via mechanical disruption in TRIzol reagent followed by phenol-chloroform phase separation. For chondrogenic pellets, total RNA was extracted from pellets by mechanical dissociation with a motorized pestle (Kimble/Kontes, Vineland, NJ) in 1 mL of TRIzol reagent. RNA purity and quantity was assessed spectrophotometrically on the basis of $A_{260/280}$. In preliminary chondrogenesis studies using cells from the entirety of the meniscus, eight pellets were harvested on days 7 and 21. These samples were analyzed for transcript levels of *aggrecan* (*AGN*) and *cartilage oligomeric matrix protein* (*COMP*) using the One-Step Reverse Transcriptase PCR kit (Invitrogen) with primers and amplification conditions as defined previously (Li et al., 2003). For assessment of regional variation in chondrogenesis, 12–16 pellets were harvested per replicate study on day 21. Reverse transcription was performed using the First Strand cDNA Synthesis kit (Invitrogen) with 1 μg of total RNA using random hexamers according to the manufacturer's instructions. cDNA amplification was carried out using an iCycler real-time PCR system (Bio-Rad, Hercules, CA) using SYBR Green PCR Reaction Mix (Bio-Rad). Starting quantities of target gene transcripts in unknowns were derived from corresponding standard curves for each primer pair, with overall expression normalized to *GAPDH* in each sample. For AS and OS cultures and controls, expression of *fatty acid binding protein* (*FABP*) and *alkaline phosphatase* (*ALP*) were assessed (Baksh et al., 2004), respectively. For chondrogenic conditions, the expression of the ECM genes, *AGN, collagen types I and II*, were assessed (Fitzgerald et al., 2004).

Statistical Analysis

Analysis of variance (ANOVA) was performed using Statistica (Statsoft, Tulsa, OK), with Fisher's LSD post-

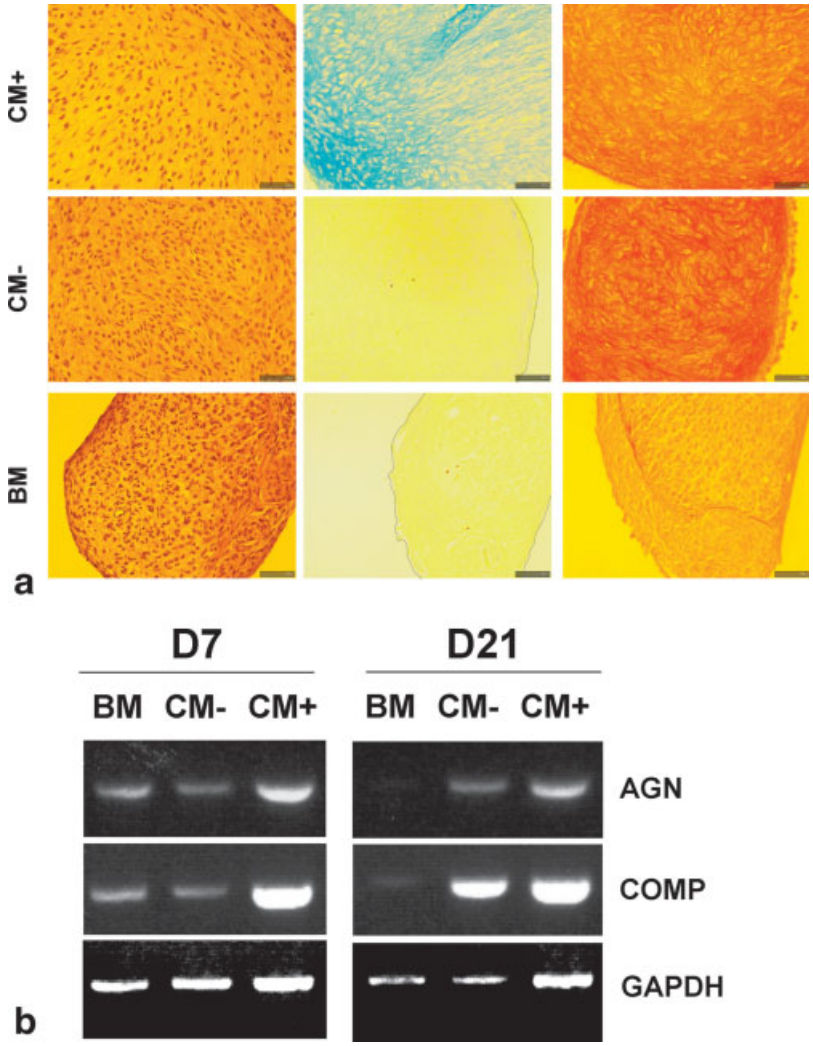

Fig. 1. Chondrogenic differentiation of day 21 pellet cultures of MFCs isolated from the entirety of the bovine meniscus. Medium composition: BM, basal medium; CM^+, chondrogenic medium with TGF-β3; CM^-, chondrogenic medium without TGF-β3. **A:** Histology: staining with H&E (left), Alcian blue (middle), and Picrosirius red (right). CM^+ cultures showed a dense collagenous matrix (right) that is also rich in proteoglycans (middle). In the absence of TGF-β3 (CM^-), collagenous matrix (right) is deposited with little proteoglycan (middle). In BM, small pellets formed with little ECM deposition. Dashed line highlights pellet periphery in CM^- and BM conditions. Scale bar = 50 μm. **B:** RT-PCR analysis of cartilaginous matrix gene expression. An enhanced chondrogenic phenotype is seen, with higher *aggrecan* and *COMP* expression, in pellet cultures maintained in CM^+ compared to either CM^- or BM on days 7 and 21.

hoc testing between groups. Independent variables were meniscus zone (inner, outer, or horns) and culture condition (CM^-/CM^+, AS, OS, and control), with dependent variables including wet weight, s-GAG content, cell activity (MTT), and Oil Red O content. All data are reported as the mean and standard deviation of 4–15 samples derived from a minimum of three replicate studies, depending on the assay, with significance set at $P < 0.05$.

RESULTS

Chondrogenesis

In preliminary studies, culture of MFCs derived from the entirety of the meniscus in pellet format resulted in the accumulation of a proteoglycan-and-collagen-rich ECM in CM^+ medium. In CM^-, a dense collagenous matrix was formed that was generally devoid of Alcian blue staining, indicating a lack of cartilage matrix accumulation. When maintained in BM for 21 days, small cellular pellets formed with little ECM (Fig. 1A). Gene expression corroborated these observations showing that CM^+ medium sustained and/or enhanced expression of *aggrecan* and *COMP*, two markers of the chondrocyte phenotype, while BM and CM^- conditions did not (Fig. 1B).

We next examined MFCs derived from distinct regions of the meniscus. The initial morphologies of these cells were different; after 5 days of outgrowth from meniscus pieces, inner zone cells remained slightly rounded while those from the outer zone took on a spindle shape (Fig. 2). After monolayer expansion, culture of MFCs in pellets in CM^+ led to pronounced cartilaginous ECM production in

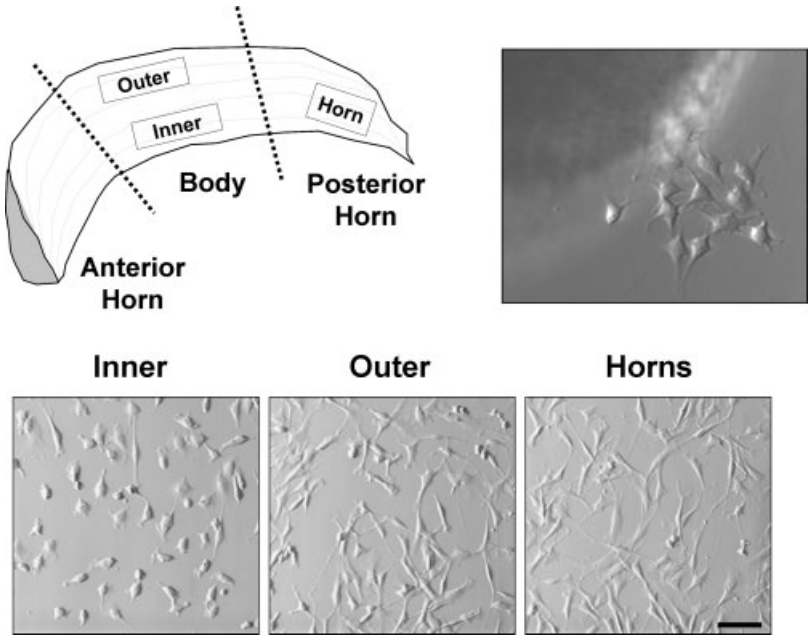

Fig. 2. Isolation of fibrochondrocytes from three distinct regions of the meniscus: the inner avascular body, the outer vascular body, and the mixed vascular horns. Cells were isolated via outgrowth from small pieces of each region (top right). Primary meniscal fibrochondrocytes in monolayer culture (day 5) showed distinct morphologies (bottom). Scale bar = 100 μm.

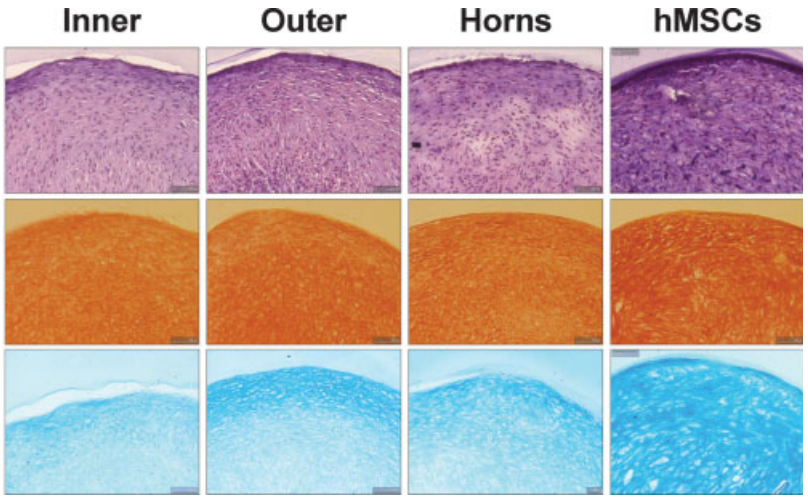

Fig. 3. Chondrogenic differentiation in day 21 pellet cultures of MFCs isolated from different regions of bovine meniscus compared to that in human MSCs maintained under identical CM^+ conditions. All cultures showed pronounced chondrogenic differentiation, with human MSCs showing more homogeneous distribution of cartilaginous ECM. H&E (top row), Picrosirius red (middle row), and Alcian blue (bottom row). Scale bar = 50 μm.

cells from all regions, particularly in the pellet periphery (Fig. 3). Real-time RT-PCR analysis of gene expression demonstrated a marked induction of *aggrecan* (2- to 20-fold) and *collagen type II* (8- to 180-fold) in all MFC populations compared to monolayers, with no distinct differences in cell response observed between groups (Fig. 4). *Collagen type I* gene expression was high for all populations, did not change in pellet cultures compared to monolayers with culture in CM^+, and remained much higher (1–2 orders of magnitude) than collagen type II expression levels for these same pellets. Over a 21-day period, pellet wet weights increased significantly in CM^+ compared to those in CM^- for cells from each zone of the meniscus ($P < 0.01$; n = 4). On day 21, s-GAG content increased with CM^+ for each group as well ($P < 0.001$; n = 4), with no significant differences observed between groups (I: 54 ± 8; O: 66 ± 5; H: 57 ± 7; MSC: 61 ± 3 μg s-GAG/pellet).

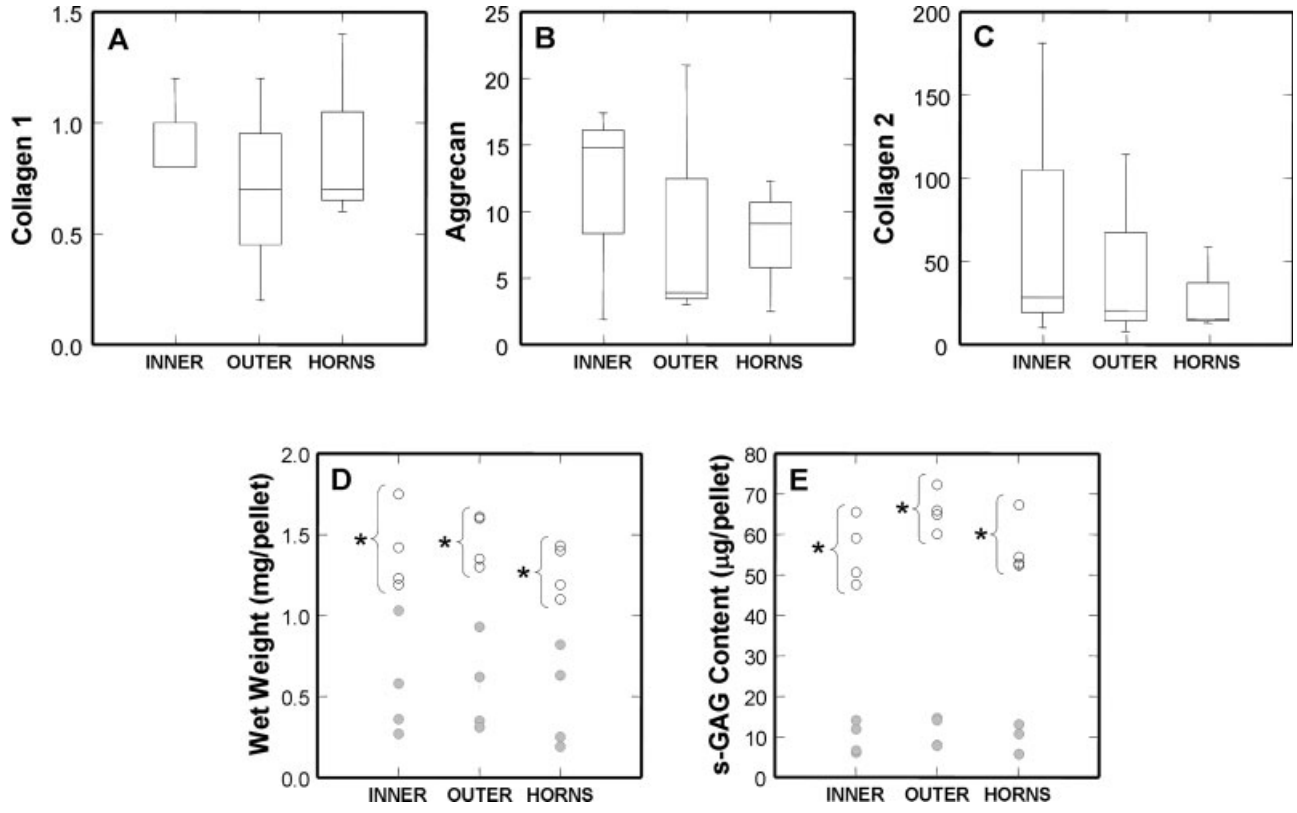

Fig. 4. Expression of cartilage matrix genes, and wet weight and s-GAG content of day 21 pellet cultures MFCs isolated from different regions of bovine meniscus. **A–C:** Real-time RT-PCR analysis showing fold change in expression in *collagen type I*, *aggrecan*, and *collagen type II* (compared to monolayer) for pellets maintained in chondrogenic (CM^+) conditions (data from three replicate experiments). Cartilage matrix genes, *collagen type II* and *aggrecan*, are both upregulated, whereas *collagen type I* levels remain unchanged. Wet weight (**D**) and s-GAG content (**E**; per pellet) of pellets from four replicate experiments cultured in either CM^+ (black circles) or CM^- (gray circles). All chondrogenic cultures showed increased weight and s-GAG content. Asterisk indicates $P < 0.05$ for CM^+ pellets vs. CM^- pellets.

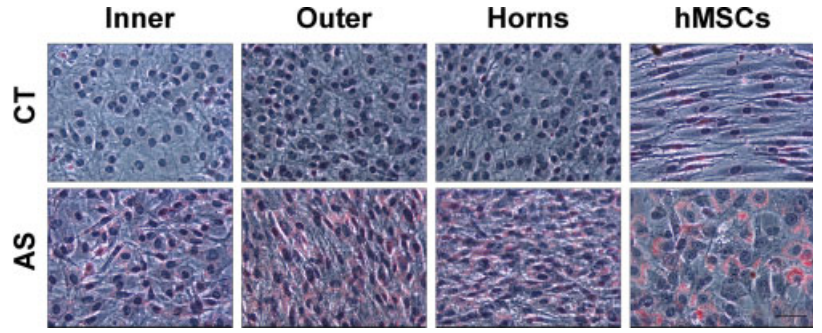

Fig. 5. Adipogenic differentiation of day 21 monolayer cultures of MFCs isolated from each region of the meniscus maintained in adipogenic medium and examined by histological staining. AS, adipogenic medium; CT, control in basal medium. AS cultures of all MFCs showed intracellular deposition of lipid droplets as shown by Oil Red O staining with hematoxylin counterstain. Bone marrow-derived human MSCs were also similarly cultured and stained as a positive control. Scale bar = 50 μm.

Adipogenesis

Monolayer cultures of MFCs derived from each region of the meniscus were treated with adipogenic supplements (AS) for 21 days. Compared to controls, MFCs in AS conditions increased their expression of *FABP* by 10- to 100-fold (data not shown), with no clear differences observed among cells from the different meniscus regions. Staining with Oil Red O showed positive lipid droplet accumulation in cells derived from all regions of the meniscus (Fig. 5). Quantification of bound dye demonstrated significant increases in AS cultures of cells from every region, with significantly higher levels in outer zone and horn MFCs maintained in AS cultures compared to inner MFCs similarly maintained ($P < 0.05$; n = 15; Fig. 6). In these cultures, cell number increased by four- to sevenfold in both

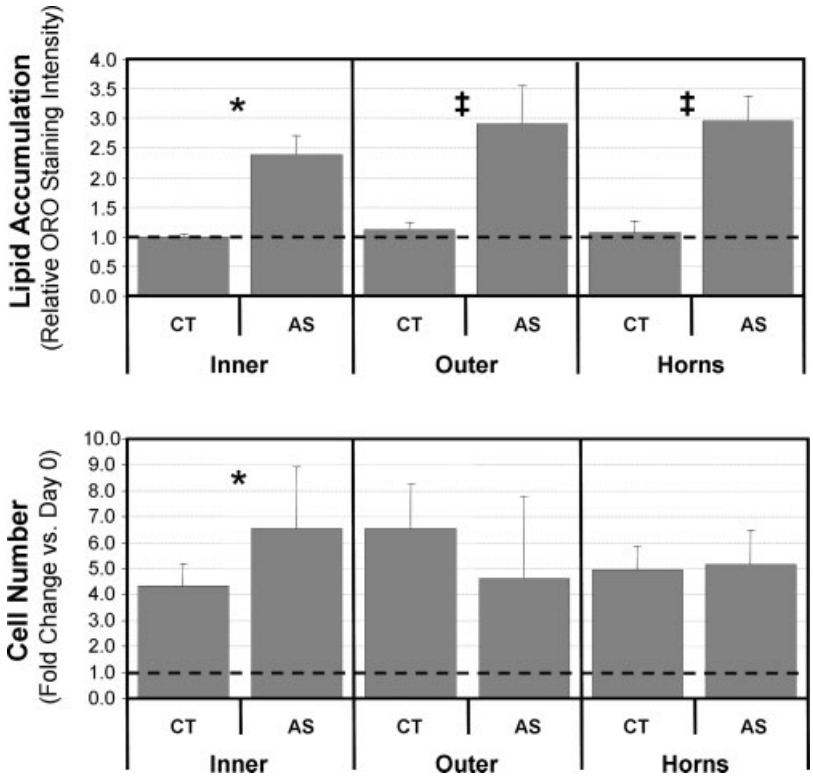

Fig. 6. Adipogenic differentiation of day 21 monolayer cultures of MFCs isolated from each region of the meniscus maintained in adipogenic medium. AS, adipogenic medium; CT, control in basal medium. Top: Quantification of Oil Red O-stained lipid droplet accumulation. AS cultures showed significantly higher dye content, particularly in outer and horn MFCs (asterisk, $P < 0.001$ compared to CT; double dagger, $P < 0.03$ compared to CT and inner AS; n = 9–15). Bottom: Day 21 cell numbers normalized to initial cell number (dotted line). In all cultures, cell numbers increased four- to sevenfold. Asterisk, $P < 0.05$ compared to CT (n = 6–9). Data are from three replicate studies.

media, with significantly more cells resulting from AS treatment of inner MFCs compared to its control ($P < 0.05$; n = 9).

Osteogenesis

MFCs from each region of the meniscus were also maintained in monolayer culture and treated with osteogenic supplements (OS) for 21 days. In these cultures, cell number increased by five- to eightfold, with significantly more cells in OS-treated samples compared to control cultures for cells from all regions ($P < 0.04$; n = 6–9; Fig. 7), with outer zone and horn regions under OS condition having significantly more cells on day 21 compared to the inner region ($P < 0.05$). Osteogenesis under OS culture condition was indicated by increased *ALP* gene expression in both inner and outer MFCs, with a small decrease in horn MFCs (fold difference, I: 1.9; O: 3.5; H: 0.8; n = 3). Culture of MFCs under OS condition also showed a marked increase in mineralized matrix deposition, specifically in cells derived from the outer region of the meniscus. Minimal staining for matrix mineralization was observed in cultures of MFCs derived from the inner or horn regions (Fig. 7).

DISCUSSION

The results of this study demonstrate that culture-expanded meniscal fibrochondrocytes possess a marked multilineage differentiation potential, including the capacity to undergo chondrogenesis, adipogenesis, and osteogenesis. To better understand the origin of these multipotential cells, and their relationship to meniscus repair, we isolated MFCs from the inner avascular, outer vascular, and mixed horn region of the meniscus. We hypothesized that a multipotential characteristic would be apparent only in cells derived from the outer region, where some degree of endogenous meniscus repair occurs. Of the three regions investigated, cells from the outer vascular zone did show the greatest capacity to differentiate along all three lineages examined. These findings suggest that the cells of the outer meniscus have a wide phenotypic range, a potential explanation for the increased integration observed when explants from this region are transplanted to avascular regions of the tissue (Kobayashi et al., 2004). Counter to our initial hypothesis, MFCs from the inner avascular region of the meniscus also showed some multipotential capacity, particularly toward the adipogenic and chondrogenic lineages. These inner MFCs, which show a more cartilaginous phenotype in situ (Sweigart and Athanasiou, 2001), had a reduced osteogenic potential when compared to MFCs from the outer region. In light of the reduced capacity of the inner regions to mount a reparative response in vivo, this diminished multipotential capacity of inner MFCs may account for some of the limitations in endogenous repair.

Given the wide array of tissues found to house cells that possess a multilineage differentiation potential, some con-

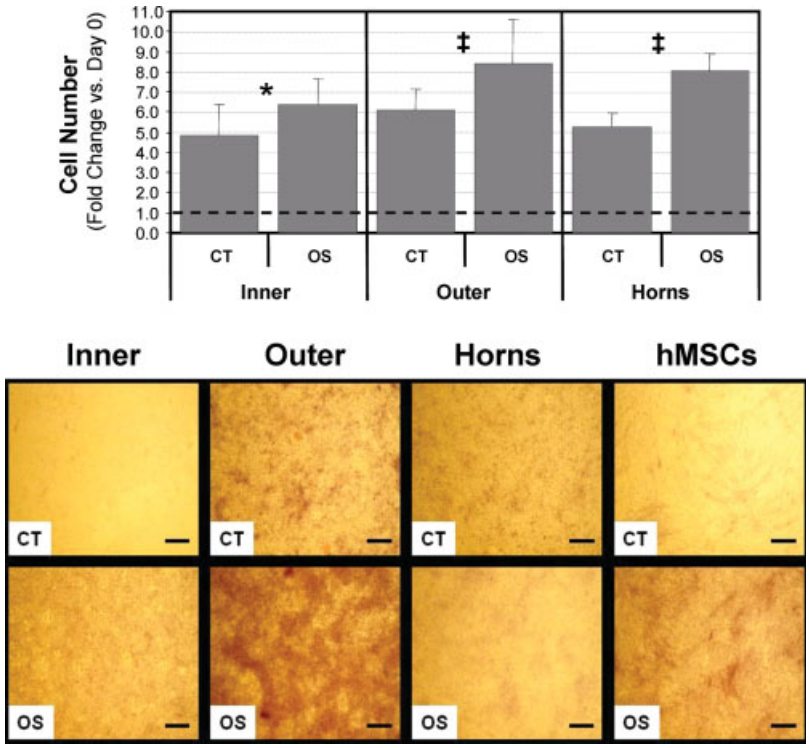

Fig. 7. Osteogenic differentiation of day 21 monolayer cultures of MFCs isolated from each region of the meniscus maintained in osteogenic medium. OS, osteogenic medium; CT, control in basal medium. Top: Day 21 cell numbers normalized to initial cell number (dotted line). In all cultures, cell numbers increased five- to eightfold, with higher levels seen in cells from the outer and horn regions (asterisk, $P < 0.05$ compared to CT; double dagger, $P < 0.05$ compared to CT and inner OS; n = 6–9). Bottom: Alizarin red staining of mineralized matrix in cultures of MFCs. High level of matrix mineralization is seen in OS cultures of MFCs isolated from the outer region of the meniscus. Bone marrow-derived human MSCs similarly cultured were also stained as positive control. Scale bar = 500 μm. Data are from three replicate studies.

sideration should be given to the nature of the adult stem cell. It has been suggested that the conceptualization of the a stem cell most accurately refers to a biological function, rather than a distinct cell type (Blau et al., 2001). It is further suggested that this functional capacity is not necessarily equal among all cells, but is likely present in most. In this study, MFCs from the meniscus were observed to possess sufficient plasticity to distinguish them as putative stem cells, i.e., they were capable of a minimum of three different lineage-specific differentiation profiles when presented with the appropriate culture and chemical environments. When compared to bone marrow-derived MSCs, differentiated MFCs retained a distinct appearance. For example, in chondrogenic pellets, Alcian blue staining filled the entirety of the MSC-laden pellets, while MFC-laden pellets showed pronounced staining only at the periphery. In adipogenic cultures, MSCs were generally larger than MFCs, and their lipid droplets were densely packed in individual cells compared to the wider but more diffuse distribution in MFCs. Under osteogenic conditions, only MFCs from the outer region showed pronounced mineralized matrix deposition, and they did so to a greater extent than the corresponding MSC cultures. The diminished capacity of MFCs from some regions to undergo osteogenesis may point to their distinct phenotype and/or origin. It should be noted that in this study a standard differentiation cocktail was used to induce each lineage. Recent work has shown that adult MSCs derived from different sources (bone marrow vs. adipose tissue) undergo more or less robust differentiation with different media formulations and growth factors (Estes et al., 2006). Thus, the induction of multiple lineage differentiation in MFCs derived from different regions of the meniscus may be further optimized.

These findings also raised another question: if cells that are sufficiently plastic to participate in repair processes do reside in all zones of the meniscus, then why is endogenous repair of this tissue limited to only the outer zones? This same question may be posed for many of the dense tissues of the musculoskeletal system, such as articular cartilage, ligament, and tendon, all of which have been shown to contain a similar cell population, and yet fail to heal. One explanation for these observations may simply be the density of these tissues. To attain their mature load-bearing capacity, these tissues sacrifice vascular and neural invaginations that are present during development to achieve the requisite mechanical properties for adult function (Clark and Ogden, 1983). Without a route for cellular mobility, this dense tissue may impede the progression of progenitor cells to the point of injury. Furthermore, as mechanical loading is typically present during the regenerative process, mechanical stress at the newly forming union may modulate differentiation toward a fibrous phenotype (Altman et al., 2002), or simply result in micro- and macroscopic fractures within the forming neo-tissue prior to its functional maturation. Finally, it has been demonstrated in tissue culture that MFCs within the meniscus are mobilized by the chemical

signals found in fibrin clots (Webber et al., 1989). In vitro studies have also demonstrated a number of cytokines that differentially enhance the migration of MFCs from different meniscus zones (Bhargava et al., 1999). In other systems, homing cytokines, such as stromal cell-derived factor 1, have been implicated in the recruitment of MSCs to sites of injury (Ceradini et al., 2004; Ji et al., 2004; Ma et al., 2005). Without exposure to gradients of these signals, multipotential cells embedded within the meniscus (particularly the avascular inner region) may not migrate toward the site of damage. Local administration of such signals, either by protein (Imler et al., 2004) or gene delivery (Goto et al., 1999; Hidaka et al., 2002), may improve the migration and localized matrix deposition by endogenous MFCs and thereby accelerate meniscus repair.

While improving migration of MFCs to sites of injury may hasten meniscus repair, the exogenous delivery of multipotential MSCs may be a more direct approach. This is particularly relevant considering the limited differentiation spectrum of MFCs from the horn and inner regions observed in this study. To this end, one recent study reported on the delivery of MSCs to meniscus defects within a fibrin clot. These cells were shown to maintain residence in the defect for up to 8 weeks after implantation (Izuta et al., 2005; Yamasaki et al., 2005). In another study, MSCs delivered in fibrin glue enhanced filling of an avascular defect (Ishimura et al., 1997), although a similar study showed no improvement in tensile properties across the defect site compared to repair with sutures alone (Port et al., 1996). Clearly, MSCs can play a role in the reparative process, although their administration (and carrier materials) requires further optimization.

While the findings of this study are intriguing, several issues bear further consideration. First, the MFC populations used in this study were derived from young healthy bovine donors. In both animals and humans, meniscus vascularity decreases substantially with increasing age (Gardner and O'Rahilly, 1968). In the inner meniscal tissue used in this study, vascular channels were not apparent, although the persistence of cells from these vascular remnants may have contributed to the observation of cells with multipotential characteristics in this region. In addition to aging effects, differences have been noted in the menisci between species. For example, anatomic form (size and shape) varies, and in some species (rat, for example), a central ossicle is observed within the meniscus substance (Messner and Gao, 1998). Therefore, extrapolation of our findings in a young animal model to that of aged human tissues warrants further study. Finally, it has recently been demonstrated via flow cytometry that digestion of whole menisci yields a heterogeneous population of cells, with a main MFC group that is $CD44^+/CD105^+$, and a mixed subgroup containing $CD34^+$ cells (Verdonk et al., 2005). It has been postulated that the $CD34^+$ group, derived primarily from the vascular outer zone and a thin synovial sublining, may contain an endothelial progenitor population. These authors reported that this subpopulation of cells disappeared within 2 weeks of monolayer expansion and therefore would not be expected to play a significant role in our differentiated populations, although their presence and contribution to the patterns observed in the present study cannot fully be discounted.

Taken together, the results of this study suggest that fibrochondrocytes from all regions of the meniscus possess a multilineage differentiation capability and may therefore contribute to endogenous repair processes. The finding that MFCs from the outer region are most plastic may underlie the recent experimental observation of improved integration of meniscus pieces from the outer zone transplanted to avascular defects in an organ culture model (Kobayashi et al., 2004). While multilineage differentiation is not per se a requirement for in vivo meniscus repair, these findings demonstrate the presence of a population of cells with a flexible phenotype throughout the tissue. Thus, in the inner region, where multipotential cells are present, a limitation in healing capacity is more likely due to the mechanical demands placed on the injured site or impediments to cellular migration within this dense fibrous structure rather than the local cellular composition. As such, more direct approaches involving the enhancement of MFC migration or the direct delivery of MSCs on scaffolds with the appropriate structural and mechanical properties (Mauck et al., 2005) will be required to improve meniscus repair. If successful, such cell-based tissue engineering therapies would rectify what is otherwise an untreatable and progressively debilitating orthopedic disorder.

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