

# Human Annulus Progenitor Cells: Analyses of This Viable Endogenous Cell Population

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**ABSTRACT:** Back pain and intervertebral disc degeneration have growing socioeconomic/health care impacts. Increasing research efforts address use of stem and progenitor cell-based replacement therapies to repopulate and regenerate the disc. Data presented here on the innate human annulus progenitor cells: (i) assessed osteogenic, chondrogenic and adipogenic potentials of cultured human annulus cells; and (ii) defined progenitor-cell related gene expression patterns. Verification of the presence of progenitor cells within primary human disc tissue also used immunohistochemical identification of cell surface markers and microarray analyses. Differentiation analysis in cell cultures demonstrated a viable progenitor cell pool within Thompson grades III–IV discs. Osteogenesis was present in 8 out of 11 cultures (73%), chondrogenesis in 8 of 11 (73%), and adipogenesis in 6 of 6 (100%). Immunolocalization was positive for CD29, CD44, CD105, and CD14 (mean values 80.2%, 81.5%, 85.1%, and 88.6%, respectively); localization of CD45 and CD34 was negative in disc tissue. Compared to controls, surgical discs showed significantly downregulated genes with recognized progenitor cell functions: TCF7L2 (2.7 fold), BMI1 (3.8 fold), FGF receptor 2 (2 fold), PAFAH1B1 (2.3 fold), and GSTP1 (9 fold). Compared to healthier grade I/II discs, grade III/IV discs showed significantly upregulated XRCC5 (3.6 fold), TCF7L2 (6 fold), GSTP1 (3.7 fold), and BMI1 (3 fold). Additional significant cell marker analyses showed expression of platelet-derived growth factor receptor alpha, CD90, CD73, and STRO-1. Statement of Clinical Significance: Findings provide the first identification of progenitor cells in annulus specimens from older, more degenerate discs (in contrast to earlier studies of healthier discs or nondegenerative specimens from teenagers). Findings also increase knowledge on progenitor cells present in the disc and suggest their value in potential future utilization for regeneration and disc cell therapy. © 2016 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. *J Orthop Res* 34:1351–1360, 2016.

**Keywords:** intervertebral disc; annulus fibrosus; progenitor cells; progenitor cell differentiation; progenitor disc cells

Disc degeneration and back pain have an increasing socioeconomic health care impact; the lifetime prevalence of disc degeneration, and its associated low back pain, afflicts ~80% of the population, with estimated health-care costs exceeding \$100–200 billion per year. Efforts in many labs, including our own, have explored the utility stem or progenitor cells (primarily using mesenchymal stem cells (MSC)) for novel applications in disc therapy and regeneration (see refs.<sup>1–5</sup> for recent review articles).

Less well-studied and understood, however, are stem cells present within the aging and degenerating human disc. Stem/progenitor cells reside in local microenvironments, containing the stem cells and other cells, extracellular matrix and critical molecular signals that act to maintain stem cell biology<sup>6</sup> and which have specialized metabolic requirements for maintenance.<sup>7</sup> Within stem cell microenvironments, cells receive cues that direct their behavior and self-renewal<sup>8</sup>; such cues or signals often function only over a short range, keeping cells in a “self-renewal” mode and simultaneously allowing nearby daughter cells to differentiate.<sup>9</sup>

The concept of stem or progenitor cells present within the disc offers exciting possibilities for disc regeneration. Nelson et al. have reported on a similar viable stem cell pool within human osteoarthritic cartilage,<sup>10</sup> and researchers have recently activated

the endogenous cardiac stem cell compartment to optimize cardiac repair and regeneration.<sup>3</sup>

In the present study we investigate progenitor cells within the aging/degenerating human disc by: (i) assessment of the osteogenic, chondrogenic, and adipogenic potentials of cultured human annulus cells; and (ii) definition of stem-progenitor cell related gene expression patterns. Studies were carried out using cells derived from the human annulus, not only because of the role it plays in contributing tensile strength to the disc, but also because a healthy annulus cell population acts as a bulwark against annular tears and nerve ingrowth and serves to protect the inner nucleus region. We also felt it was important to investigate the potential presence of progenitor cells in annulus specimens from older, more degenerate discs compared to the cervical discs from grades II to III discs previously studied by Risbud et al.<sup>11</sup> or the “nondegenerative” teenage population studied by Feng et al.<sup>12</sup>

## METHODS

### Clinical Study Population

Study of human disc specimens was approved prospectively by the Human Subjects Institutional Review Board at Carolinas Medical Center. The need for informed consent was waived by the ethical board since disc tissue was removed as part of routine surgical practice (and discarded). Scoring of disc degeneration utilized a modification of the Thompson scoring system<sup>13</sup> which incorporated author ENH’s radiologic, MRI and surgical findings. The Thompson system scores disc degeneration over the spectrum from a healthy disc (Thompson grade I) to discs with advanced degeneration (grade V, the most advanced stage of degeneration). Patient

Conflict of interest: None.

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specimens were derived from surgical disc procedures performed on individuals with degenerative disc disease. Surgical specimens were transported to the laboratory in sterile tissue culture medium. Annulus cells were established in monolayer culture, and expanded for use in as previously described.<sup>14,15</sup> During primary culture and expansion, cells were maintained in a 37°C incubator with 5% CO<sub>2</sub> and 95% relative humidity. Characterization and stability of human annulus cells have previously been described for our lab methods.<sup>16,17</sup>

#### Verification of the Presence of Progenitor Cells in Cultured Annulus Cells

Osteogenesis, chondrogenesis, and adipogenesis assays used annulus cells from 4 Thompson grade IV, and 7 grade V surgical discs (mean age 54.9 years, range 33–74). Annulus cells were tested for osteogenic, chondrogenic and adipogenic progenitor cell potential using well-accepted differentiation endpoint criteria<sup>18</sup> using methods previously reported by our lab.<sup>19,20</sup> Digital images were taken to document alizarin-stained osteogenic, chondrogenic micromass cultures, and adipocytes.

#### A. Osteogenic Differentiation

Osteogenic differentiation of progenitor cells was performed using an Osteogenesis Kit (Lonza, Basel, Switzerland)<sup>21</sup> and employed positive alizarin red (Sigma, St. Louis, MO) staining of mineralized matrix following 21–28 days of culture. Annulus cells were seeded at 50,000 cells/well in a 24-well tissue culture plates, established in culture for 1–9 days, and then fed with the Osteogenic Differentiation Media. Control cultures were fed MSCBM (Mesenchymal Stem Cell Basal Medium) (Lonza) basal media only. Control and osteogenic differentiation cultures were fed three times/week.

#### B. Chondrogenic Differentiation

Chondrogenic differentiation was based on micromass formation by cells grown from 2 to 18 days in Chondrogenic Induction Medium (Lonza, Basel, Switzerland) supplemented with 5% FBS and 10 ng/ml transforming growth factor  $\beta$ 3. Cells were seeded at 200,000 cells/well in a 24-well tissue culture plates and fed three times/week. Proteoglycan production in the extracellular matrix of paraffin-embedded micromass cultures was verified with alcian blue and toluidine blue staining, and types I and II collagen and chondroitin sulfate immunolocalization. Immunohistochemical localization was carried out on paraffin-embedded sections of micromass cultures fixed in 10% neutral buffered formalin to identify chondroitin sulphate using a primary anti-chondroitin sulfate antibody from ICN Biomedicals (Costa Mesa, CA) at a concentration of 20  $\mu$ g/ml. The negative control consisted of mouse IgG (Dako, Carpinteria, CA) used at the same concentration as the primary antibody. The secondary antibody employed the LSAB2 Biotinylated Secondary kit (Dako) (10 min.). Negative controls were processed minus primary antibodies.

#### C. Adipocyte Differentiation

Cells were differentiated to adipogenic cells using the Lonza Mesenchymal Stem Cell Adipogenic Differentiation medium; differentiated cells were stained with oil red O (Sigma, St. Louis, MO) to demonstrate fat droplets. Per the Lonza kit directions, testing consisted of feeding cells for 3 days with the adipogenesis induction medium; this was followed by 3

days of culture in. Control cultures were fed with the supplemented adipogenic maintenance medium on the same schedule.

#### Immunolocalization of Mesenchymal Progenitor Cell markers

Seven annulus tissue specimens had been embedded in paraffin for future studies from the same tissues used for the above cell differentiation analyses; these seven subjects are identified in Table 1. Verification of progenitor cell isolation using cell surface markers<sup>19</sup> using immunohistochemistry was performed on paraffin embedded specimens for seven specimens whose demographic features are described in Table 1 using the following antibodies: Positive localization for stemness was verified by positive localization of CD105 (Thermo Scientific, Fremont, CA) pretreated with protease XXV (Thermo Scientific) and used at a concentration of 8  $\mu$ g/ml, CD44 (Thermo Scientific) required heat-induced epitope retrieval with citrate buffer (Biogenex, Fremont, CA) and was used at an 8  $\mu$ g/ml concentration. CD29 (Thermo Scientific) was pretreated with protease XXV (Thermo Scientific) and antibody was used at a concentration of 8  $\mu$ g/ml. Localization of CD 14 used anti-CD14 (Aviva Systems Biology, San Diego, CA) at a 1:50 dilution. Negative localization of CD45 (Dako North America, Carpinteria, Ca) required heat-induced epitope retrieval with citrate buffer (Biogenex,) at a 3.5  $\mu$ g/ml concentration, and CD34 (Dako) used pretreated with proteinase K (Sigma, 2  $\mu$ g/ml) used at a 1.5  $\mu$ g/ml concentration. All antibodies except CD14 used human tonsil as a positive control and mouse IgG (Dako) in place of primary antibody as a negative control. CD14 localization utilized human small bowel as the control tissue. Immunolocalizations were performed using the Vector ImmPRESS Reagent Kit (Vector Laboratories, Burlingame, CA); the ImmPRESS anti-mouse Ig reagent (Vector) was applied for 30 min followed by DAB (Dako) for 5 min for visualization. Slides were counterstained with light green, dehydrated, cleared and mounted with resinous mounting media.

These immunohistochemical slides from the seven subjects were scored for the presence or absence of positive cells. Computer-assisted cell counting was performed using OsteoMeasure software (OsteoMetrics, Inc., Decatur, GA). Adjacent sections in histology blocks were sectioned, used for immunohistochemistry, and cells counted as follows: The mean number of cells counted for CD29 analysis was  $440 \pm 42$  (7) (mean  $\pm$  S.D. (n)), for CD44  $337 \pm 48$  (7); for CD105  $392 \pm 40$  (7), and for CD14  $382 \pm 114$  (7). Localization for CD34 and CD45 was negative.

#### Microarray Analysis of Progenitor Cell-Related Gene Expression in Disc Tissue

##### Molecular Analysis of Human Disc Tissue

Affymetrix gene expression studies on disc tissue were performed using 8 control annulus lumbar (Cooperative Human Tissue Network) (4 females, 1 male; mean subject age 40.2 years) and 12 surgical lumbar annulus sites (2 grade II, 3 grade III, 4 grade IV, 2 grade V disc; 6 female, 6 male patients; mean age 46.6 years). Disc tissue was snap frozen in liquid nitrogen, pulverized (BioPulverizer, BioSpec Products, Inc., Bartlesville, OK), and homogenized via the FastPrep-24 instrument (MP Biomedicals L.L.C., Santa Ana, CA). Total RNA was isolated via a modified version of TRIzol Reagent (Life Technologies: Invitrogen, Carlsbad, CA), and prepared for microarray hybridization using the GeneChip 3' IVT Express Kit (Affymetrix, Santa Clara, CA). In brief, total

**Table 1.** Demographic Features and Differentiation Outcomes<sup>a</sup>

Donor Information					Stem Cell Differentiation				CD Marker
Age	Gender	Grade	Herniated	Site	Total Days in Culture	Osteogenesis	Chondrogenesis <sup>b</sup>	Adipogenesis	Immuno-Localization Analysis
53	Female	III	Yes	L <sub>5</sub> -S <sub>1</sub>	85	+	+(36 Days)	N/A	X
69	Female	III	No	L <sub>4</sub> -L <sub>5</sub>	75	+	+(24 Days)	N/A	
68	Female	IV	No	L <sub>4</sub> -L <sub>5</sub>	78	+	+(22 Days)	N/A	X
69	Female	IV	No	L <sub>5</sub> -S <sub>1</sub>	92	+	+(20 Days)	N/A	
42	Male	IV	Yes	L <sub>5</sub> -S <sub>1</sub>	59	+	-(36 Days)	N/A	X
53	Male	IV	Yes	L <sub>3</sub> -L <sub>4</sub>	30	+	+(16 Days)	+	X
36	Male	IV	Yes	L <sub>2</sub> -L <sub>3</sub>	38	+	+(33 Days)	+	X
55	Female	III	Yes	L <sub>4</sub> -L <sub>5</sub>	31	+	-(35 Days)	+	X
74	Female	IV	No	L <sub>4</sub> -L <sub>5</sub>	39	-	+(10 Days)	+	
66	Female	IV	Yes	L <sub>4</sub> -L <sub>5</sub>	24	-	-(31 Days)	+	
33	Male	III	Yes	L <sub>5</sub> -S <sub>1</sub>	34	-	+(14 Days)	+	X

<sup>a</sup>L, lumbar; S, sacral; N/A, not available; +, positive outcome; -, negative outcome. <sup>b</sup>Days shown are culture days on which micromass was first observed.

RNA was reverse transcribed to synthesize cDNA, converted to double stranded DNA, subjected to transcription generating biotin-labeled amplified RNA (cRNA) and hybridized to the DNA microarray in the Affymetrix Fluidics Station 400. Affymetrix human U133 X3P arrays were used. The GCOS Affymetrix GeneChip Operating System (version 1.2, Affymetrix, Santa Clara, CA 95051) was used for determining gene expression levels. mRNA from annulus tissue from each subject was analyzed separately (i.e., samples were not pooled).

Gene expression analysis used ontology stem cells searches and the GCOS Affymetrix GeneChip Operating System. Data were normalized and GeneSifter<sup>TM</sup> web-based software used for analysis. Statistical analysis used the student *t*-test (2 tailed, unpaired; *p* < 0.05 was considered significant).

**Measurement of Stem Cell Factor Levels in Conditioned Media**

Annulus cells were cultured in monolayer for four days, and media harvested and frozen for subsequent assay of stem cell factor (KIT Ligand) levels using the Quantibody Human Growth Factor Array I (RayBiotech, Inc., Norcross, GA) read on a Molecular Devices GenePix 4100A Scanner; assay lower sensitivity levels were 1.4–7.3 pg/ml. Extracted densitometry values were analyzed with an Excel-based software tool for the array from RayBiotech, Inc. (cat# QAH-GF-1-SW) for protein quantification. On the array, each antibody for its target protein was spotted in quadruplicate and results averaged.

**Statistical Analyses**

Additional standard statistical analyses were performed with InStat (GraphPad Software, Inc., San Diego, CA) using unpaired *t*-tests. Means ± S.D. were calculated; *p* > 0.05 was considered significant. Unpaired *t*-tests for nonparametric data were performed using the Mann–Whitney test.

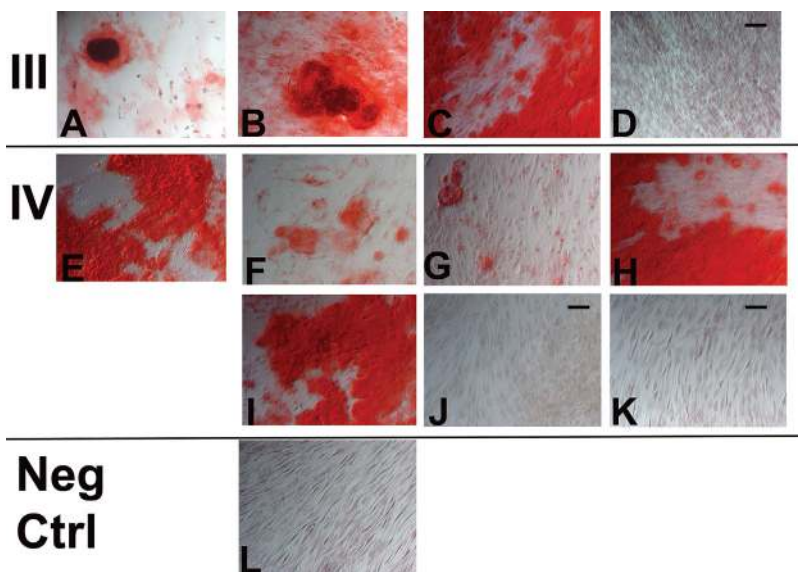
**RESULTS**

In vitro studies were carried out using annulus cells derived from human disc surgical specimens, Thompson grades III and IV (discs with moderate to more

advanced degeneration). Subject demographic data and differentiation results for each individual are presented in Table 1. Using well-accepted differentiation procedures, data showed the presence of a viable progenitor cell pool: Positive osteogenic ability was present in 8 of 11 cultures (73%; 3 of 4 grade III and 5 of 7 grade IV discs), chondrogenic ability in 8 of 11 cultures (73%), and adipogenic ability in 6 of 6 cultures (100%) (Figs. 1–3). Development of micromass cultures indicative of chondrogenesis varied from 10 to 36 days. Micromass cultures examined histologically showed abundant extracellular matrix (shown with alcian blue staining, Fig. 1C) and cells with plump, rounded cells (Fig. 1D). Immunolocalization showed strong presence of type II collagen (Fig. 1E), but minimal presence of type I collagen (Fig. 1F). (Fig. 1G shows a representative negative control image).

Of special interest were three cultures which failed the osteogenesis differentiation test, (possibly the most stringent of the differentiation procedures); these cultures were derived from one grade III and two grade IV discs (Table 1). There was no significant difference in these failed cultures in terms of subject age versus age of successful differentiation (57. 6 ± 3.0 (3) mean ± S.D. (n) years versus 55.6 ± 8.0 (8) years (*p* = 0.84), or in terms of the total number of days cells had been in culture: 32.3 ± 7.6 (3) versus 61.0 ± 25.1 (8) days (*p* = 0.09). One of the subjects whose cells failed the osteogenesis differentiation test also failed the chondrogenesis test (Table 1) (a Grade IV disc from a 66-year-old female).

Annulus tissue from seven of the subjects whose cells were used in the differentiation analyses described above were also used for immunohistochemical analysis of well-recognized positive stem cell markers CD29, CD44, and CD105; localization of CD34 and CD45 was negative.<sup>18</sup> Representative histologic images are presented in Figure 4 showing positive localization

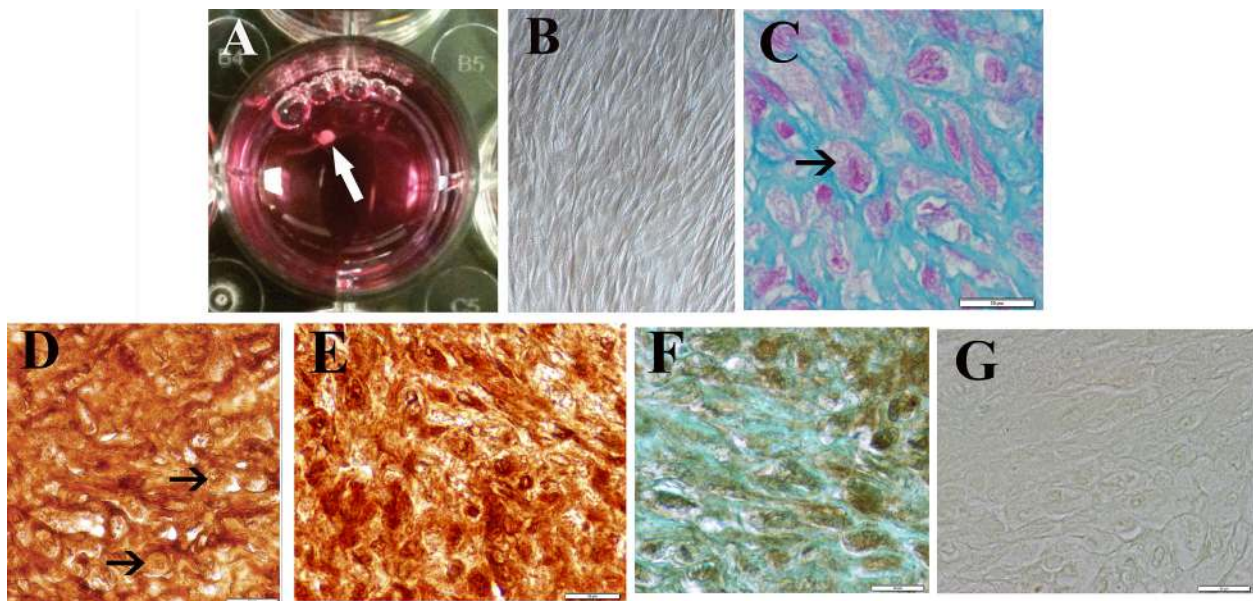


**Figure 1.** Alizarin red staining of mineralized nodules verifies osteogenesis induced in annulus cells from Thompson grades III (A–C) and IV (E–I) discs. D, J, and K show cultures which failed to form mineralized nodules. L shows a representative negative control grown without the induction media. (Alizarin-red staining; original magnification  $\times 100$ ).

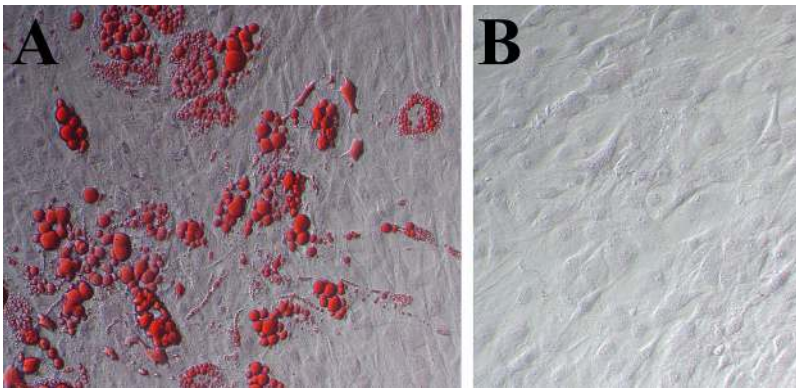
in the annulus for CD29 (present in 80.2% of cells), CD44 (present in 81.5% of cells), CD105 (present in 85.1% of cells), and CD14 (present in 88.6% of cells) (Fig. 5). These immunohistochemical findings provide additional verification of the presence of progenitor cells in the annulus.

Molecular microarray analysis of annulus tissue (described in Table 2) was also utilized to provide additional confirmation of the presence of additional recognized stem cell markers: Platelet-derived growth factor receptor alpha, CD105 (Endoglin), CD73 (5-nucleotidase ecto, CD 90 (thy-1 cell surface antigen), STRO-1 (chemokine (C-X-C motif) ligand 12), or

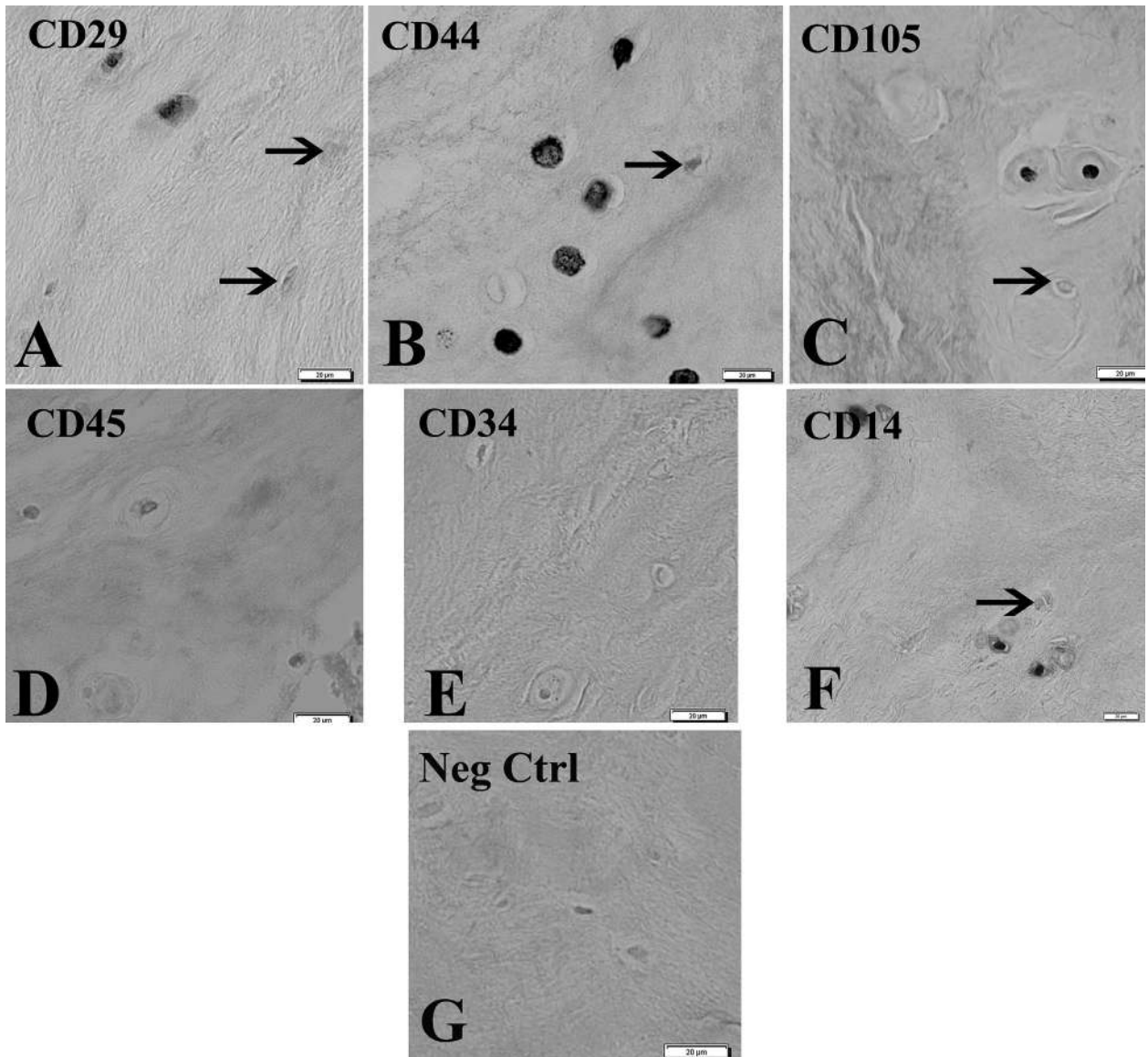
stromal cell-derived factor 1). Analyses revealed that platelet-derived growth factor receptor alpha was significantly upregulated in more degenerated tissue from grades III, IV, and V discs when compared to healthier grades I and II disc (5.3 fold upregulation,  $p = 0.04$ ). (This cell marker was also upregulated when degenerated disc tissues from grades IV and V discs were compared to grades I, II, and III discs (5.7 fold upregulation,  $p = 0.02$ ), and when this marker was tested for degenerated surgical disc specimens versus control discs obtained from control discs (obtained from the Cooperative Human Tissue Network) (6.6 fold upregulation,  $p = 0.01$ ). CD90 showed a 3.5 fold



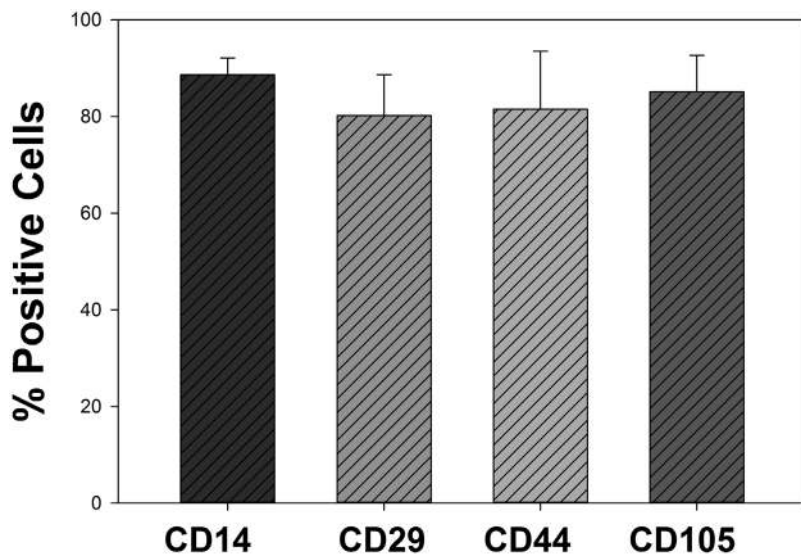
**Figure 2.** Chondrogenic differentiation of cultured annulus cells was indicated by micromass formation (A). B: Shows cultured cells under control conditions without the induction media; these monolayer cells did not form a micromass. C and D: Histologic studies utilizing alcian blue (C) and immunolocalization of chondroitin sulfate (D) further defined presence of rounded, plump cells (arrows) and cartilage-like matrix. E and F: Images illustrating abundant immunolocalization of type II collagen (E), but minimal localization of type I collagen (F) in the micromass. G: Shows a representative negative control. (A, diameter of the culture plate well is 16 mm. B, original magnification  $\times 100$ . Bars = 20  $\mu\text{m}$ ).



**Figure 3.** A: Adipogenesis differentiation is shown by formation of lipid droplets, stained here with oil red O. B: Control cells grown without the differentiation media. (Original magnification  $\times 100$ ).



**Figure 4.** Positive histologic immunolocalization at the protein level for progenitor cell markers CD29 (A), CD44 (B), CD105 (C), and CD14 (F) in human annulus tissue. Localization for CD45 (D) and CD34 (E) was negative. G: Shows a representative negative control for CD29. (Bars = 20  $\mu\text{m}$ ). Arrows mark nearby cells which did not show immunolocalization in A, B, C, and F.



**Figure 5.** Quantitative data measuring immunohistologic presence in human annulus tissue of positive progenitor cell markers CD14, CD29, CD44, and CD105. Immunohistochemical analysis also showed the predicted progenitor cell absence of localization for CD45 or CD34 (0% localization for both; data not shown). (Data are means  $\pm$  S.D.;  $n = 7$  as shown in Table 1).

upregulation in more degenerated grades IV and V discs versus grades I, II, and III discs ( $p = 0.006$ ), and also when surgical discs were compared to control discs (3.1 fold upregulation,  $p = 0.01$ ). CD73 and STRO-1 also showed highly significant differences in surgical versus control, and more degenerated versus less degenerated discs, but at lower fold changes (1.2 and 1.9, respectively, with  $p$  values of 0.004 and 0.02 respectively).

Compared to controls, downregulation was seen in surgical discs in a number of additional recognized stem cell markers which were identified using ontology microarray gene searches: TCF7L2 (2.7 fold,  $p = 0.02$ ), BMI1 (3.8 fold,  $p = 0.008$ ), FGF receptor 2

(2 fold,  $p = 0.04$ ), PAFAH1B1 (2.3 fold,  $p = 0.02$ ), and GSTP1 (9 fold,  $p = 0.004$ ). (GSTP1 showed an even greater fold increase when grade V discs were compared to grade I/II (15 fold,  $p = 0.02$ )). Compared to healthier grade I/II discs, grade III/IV discs showed upregulation in: XRCC5 (3.6 fold,  $p = 0.03$ ), TCF7L2 (6 fold,  $p = 0.02$ ), GSTP1 (3.7 fold,  $p = 0.04$ ), and BMI1 (3 fold,  $p = 0.04$ ).

Data were also obtained from analysis of media levels of stem cell factor (KIT ligand), a growth factor recognized as important in several cell lineages during embryogenesis as well as one with important actions on hematopoiesis in the adult.<sup>22–24</sup> Media were harvested from annulus cells cultured in monolayer for

**Table 2.** Demographic Features for Disc Tissue Utilized in Gene Expression Studies<sup>a</sup>

Subject #	Age (Years) and Gender	Thompson Grade	Site	Herniated?	Source
1	34/F	I	Lumbar	No	CTHN
2	21/M	II	L5-S1	Yes	OR
3	30/M	II	L3-4	No	CTHN
4	30/M	II	L3-4	No	CTHN
5	54/F	II	L4-5	No	OR
6	40/F	II	L4-5	Yes	OR
7	52/F	III	L3-4	No	CTHN
8 <sup>b</sup>	36/F	III	L4-5	Yes	OR
9 <sup>b</sup>	52/F	III	L3-4	No	CTHN
10 <sup>b</sup>	52/F	III	L3-4	No	CTHN
11	52/F	III	L3-4	No	CTHN
12	33/F	III	L2-3	No	CTHN
13	37/M	III	L5-S1	Yes	OR
14	43/M	III	L4-5	Yes	OR
15	63/F	IV	L4-5	No	OR
16	65/F	IV	L2-3	No	OR
17	45/M	IV	L5-S1	Yes	OR
18	43/M	IV	L5-S1	No	OR
19	72/F	V	L5-S1	Yes	OR
20	41/M	V	L4-5	No	OR

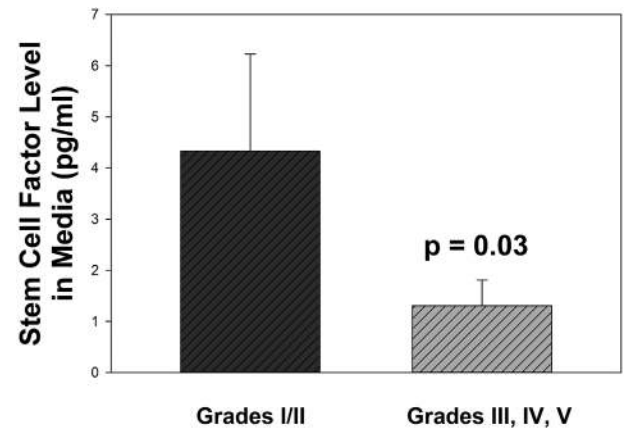
<sup>a</sup>CTHN, normal specimens obtained from the Cooperative Human Tissue Network. M, male; F, female. L, lumbar; S, sacral. <sup>b</sup>Denotes discs from the same individual. OR, surgical specimen.

4 days (Table 3 presents the demographic features of discs from which these cells were obtained), and levels assessed in samples from cells derived from healthier grades I and II discs versus cells derived from more degenerate grades III, IV, and V discs. Stem cell factor levels were significantly lower in media from grades III, IV, and V annulus cells compared to that from grade I/II cells (Fig. 6,  $p = 0.03$ ).

**DISCUSSION**

The presence of progenitor cells in cultured annulus cells was demonstrated by osteogenesis, chondrogenesis and adipogenesis, and molecular analyses which revealed genes expressed in vitro and in vivo with well-recognized progenitor cell associations related to proliferation and differentiation.

This work expands and extends a relatively small current body of literature on the stem cell population in the annulus. Risbud et al. studied five discs from which cells were cultured and examined for stem cells using analysis of cell surface markers and other techniques.<sup>11</sup> Their work utilized cervical grades II and III discs, and concluded that there was a heterogeneous population of cells which expressed cell surface proteins similar to the population characteristic of marrow stem cells. The studies of Risbud et al. used



**Figure 6.** Levels of stem cell factor (KIT ligand) were significantly lower in media from annulus cells derived from more degenerated grade III/IV/V discs compared to cells derived from healthier grade I/II discs ( $p = 0.03$ ; data are means  $\pm$  s.e.m.  $n = 8$  for grade I/II cells, and 18 for grade III/IV/V cells).

mainly healthy cervical grade II discs. Feng et al. have studied multi-potential differentiation in annulus cells from a 13- to 16-year-old scoliosis patients.<sup>12</sup> Disc grades were not provided, but the discs were described as “nondegenerative.” Cell surface markers in cultured cells were those associated with MSCs, although two

**Table 3.** Demographic Features of Discs From Which Annulus Cells Derived and Cultured for Stem Cell Factor Assay<sup>a</sup>

Subject #	Age (Years if Not Otherwise Listed)/Gender	Thompson Grade	Site	Diagnosis	Source
1	2 mo/F	I	L	Normal	CHTN
2	10 weeks/M	I	L	Normal	CHTN
3	Newborn/M	I	L	Normal	CHTN
4	27/M	II	L4-5	Herniated disc	OR
5	39/F	II	L5-S1	Herniated disc	OR
6	25/F	II	L5-S1	Herniated disc	OR
7	18/M	II	L4-5	Herniated disc	OR
8	58/F	II	L4-5	Herniated disc	OR
9	63/F	III	L5-S1	Herniated disc	OR
10	67/M	III	L3-4	Herniated disc	
11	40/M	III	L4-5	Herniated disc	
12	39/F	III	L5-S1	Lumbar spondylosis	OR
13	43/M	III	L3-4	Herniated disc	OR
14	44/M	III	L4-5	Lumbar spondylosis	OR
15	52/M	IV	L5-S1	Recurrent herniated disc	OR
16	21/M	IV	L5-S1	Recurrent herniated disc	OR
17	50/F	IV	L4-5	Lumbar spondylosis	OR
18	74/F	IV	L3-4	Lumbar spondylosis	OR
19	54/M	IV	L4-5	Herniated disc	OR
20	53/M	IV	L5-S1	Herniated disc	OR
21	62/F	IV	L3-4	Herniated disc	OR
22	47/F	IV	L5-S1	Spinal stenosis	OR
23	68/F	IV	L4-5	Lumbar spondylosis	OR
24	38/F	IV	L5-S1	Lumbar spondylosis	OR
25	60/F	IV	L5-S1	Herniated disc	OR
26	51/F	V	L5-S1	Herniated disc	OR

<sup>a</sup>CHTN, normal specimens obtained from the Cooperative Human Tissue Network. M, male; F, female. L, lumbar; S, sacral.

neuronal stem cells markers (nestin and neuron-specific enolase) were also identified.

In work presented here, more degenerated grades III and IV discs were chosen for study because discs of these grades are the probable future candidates which would be treated with either autologous disc progenitor cells or with selected techniques to amplify the regenerative capability of the disc.

Work by Henriksson et al. reported cell proliferation zones and progenitor cells in potential cell “niches” in discs from the rat, minipig, rabbit, and human.<sup>25</sup> 5-bromo-2-deoxyuridine (BrdU) labeling of dividing cells provide evidence of slow, ongoing cell division in many regions of the annulus, especially in the annulus border along the ligament zone and the perichondrium. Saraiya et al. have studied whether reversine could be used to enhance generation of progenitor-like cells of the rat annulus.<sup>26</sup> It did indeed induce cell plasticity and cell differentiation along mesenchymal lineages.

Genome-wide gene analyses carried out in the present study pointed to a downregulation of the following genes in surgical discs versus control discs: TCF7L2, BMI1, FGF receptor 2, PAFAH1B1, and GSTP1. TCF7L2 (transcription factor 7-like 2) has been found to be necessary for the maintenance of the epithelial stem-cell compartment of the small intestine.<sup>27</sup> BMI1, a *Polycomb* group repressor, is critical for self-renewal of adult murine hematopoietic stem cells and neuronal stem cells (via repression of senescence-associated genes).<sup>28</sup> In vitro studies have shown that fibroblast growth factor (FGF) receptor 2 and IGF act cooperatively to establish the regulatory stem cell features of pluripotent human cells<sup>29</sup>; FGF-2 is also recognized for enhancing chondrogenesis in human MSC.<sup>28</sup> FGF signaling is related to stemness in a variety of stem cells (see ref.<sup>30</sup> for a recent review). FGF signaling can induce major changes in the transcriptome in cells,<sup>31</sup> important because cytokines influence neighboring cells via a paracrine route, a process which could influence annulus cells situated near the stem cells.

PAFAH1B1 plays a part in cell cycle adaptation to differentiation.<sup>32</sup> GSTP1 (glutathione S-transferase P) has been identified in the proteome of MSC<sup>33</sup> and to be involved as a mediator of cell proliferation/cell death.<sup>34</sup> Our studies also found that compared to healthier grade I/II discs, grade III/IV discs had upregulation of TCF7L2, BMI1 and XRCC5 (a DNA repair gene). In aging MSCs, XRCC5 was up-regulated after induction of oxidative stress.<sup>35</sup>

It is important to consider why the innate progenitor cell population within the human annulus fails to repopulate and regenerate the aging and degenerating disc. One explanation may be deleterious effects of the proinflammatory cytokine microenvironment in the degenerating disc. Growth and differentiation factor-5 (GDF-5, also called cartilage-derived morphogenetic protein 1) is a member of the TGF- $\beta$  superfamily which regulates cell division and differentiation. Early bone

studies showed the presence of GDF-5 at sites of morphogenesis.<sup>36</sup> GDF-5 previously attracted the interest of our laboratory because of its role in skeletal development. We found that GDF-5 expression was downregulated when human annulus cells were exposed to proinflammatory cytokines.<sup>37</sup> Therefore, one explanation might be that high proinflammatory cytokine levels in the microenvironment of the disc could limit cell recovery, at least in mechanisms utilizing a GDF-5 response mechanism.

Studies of the nucleus from human discs found that progenitor cells in patient discs decreased with age and disc degeneration stage, pointing to a possible exhaustion of key progenitor cells, and the role of Tie2-positive cells in the aging/degenerating disc.<sup>38</sup>

Literature on the microenvironment of the degenerating disc and its possible impact on bone marrow MSCs used in stem cell-based disc therapy has been reviewed by Huang et al.<sup>39</sup> Well-recognized in the complex disc milieu are degeneration-associated changes in cytokines, pH, and oxygen levels. It is interesting to note that hypoxia actually induced a metabolic shift and enhanced stemness and expansion of the stem cell population in cochlear spiral ganglion stem cells, indicating that there may be unique responses of stem cells in specific sites.<sup>9</sup> Wuertz et al. have studied the behavior of MSC in the disc environment; they found that low glucose enhanced matrix biosynthesis and maintained proliferation, but high osmolarity and low pH reduced biosynthesis and proliferation of young and mature MSCs.<sup>40</sup> Metabolic cues may also be important factors which can influence the regulation of stem cell self-renewal.<sup>41</sup>

Data shown here identified lower levels of stem cell factor (KIT ligand) in media harvested from cells derived from more degenerated discs versus levels in media from cells derived from healthier, grades I and II discs (Fig. 6). Ichii et al. found downregulated stem cell factor in cells which were acquiring osteoblastic features compared to levels seen in mesenchymal stem cells.<sup>23</sup> Stem cell factor may also be playing a role in the disc via its synergism with other growth factors which can enhance mobilization of progenitor cells.<sup>42</sup>

A comment should be made that the ability to proliferate is also often mentioned as a characteristic of stem cells. A previous study from our laboratory reported on 23 cultures of human annulus cells which were passaged for periods of 26–66 days.<sup>43</sup> That work showed good proliferative ability, utilizing Grades I, III, IV and V specimens, with subject age ranging from 37 to 78 years. Another comment might be appropriate regarding the variability that cultures showed in the ability to be positive for all three types of differentiation outcomes (osteogenesis, chondrogenesis, adipogenesis). One possible interpretation of these findings is that some annulus cells may retain some degree of metabolic plasticity.

In summary, work presented here verified progenitor cell potency in annulus cells cultured from grades



III–IV human discs (stages of degeneration which could be used for cell-based disc therapy), and molecular studies identified expression changes in disc tissue and in cultured annulus cells for genes with well-recognized stem cell roles. Future profitable research might focus upon a comparison of progenitor cells present in the aging degenerating disc and those in other sites such as bone marrow or adipose tissue, and should include qRT-PCR confirmation of progenitor cell markers. Additional research studies on the progenitor population within the disc would benefit from confocal simultaneous imaging of multiple CD markers, and flow cytometry characterization.

In addition, careful study of entire disc specimens (not only surgically removed segments as utilized here) would be highly informative. Optimization of progenitor cells in the adult annulus offers an exciting and important novel approach for potential disc regeneration. We suggest that the native progenitor cell population might be able to be enhanced and directed into functional disc cell populations (as shown in our previous work with adipose-derived mesenchymal stem cells<sup>19,20</sup>), thereby optimizing disc repair and regeneration through activation of the endogenous disc progenitor cells.

#### AUTHORS' CONTRIBUTIONS

Drs. HEG and ENH conceived of the project, Dr. HEG wrote the manuscript, FER performed the cell cultures, GIH performed the molecular work, LB assisted with stem cell factor analyses, and JAI performed the staining and immunohistochemistry.

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