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Isolation and Characterization of Adult Spinal Disc Stem Cells from Healthy Human Spinal Disc Tissues

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Abstract

This report details the isolation, culture, and characterization of spinal disc stem cells derived from human adult spinal disc tissue specimens. Using stem cell suspension culture methods and biology, human adult spinal disc stem cells were isolated and monoclonally cultured into multicellular sphere-like clusters (discospheres). Discospheres from the first culture series were collected, processed, and replated as single stem cells for serial expansion studies using suspension culture, demonstrating linear expansion was possible. Discospheres and adult spinal disc stem cells were plated on matrix coated culture surfaces in stem cell media for several hours to allow fixation, and assayed for the stem cell biomarkers. Discospheres and adult spinal disc stem cells were plated on laminin-coated culture surfaces in chondrogenic media and culture conditions for 14 days to differentiate them into NP cells. NP cells cultured from these experiments demonstrated NP morphology and phenotype; NP biomarker expression, secretion of extracellular matrix, and the ability to be serially passaged with large volume expansion possible. Tissue engineering studies using the "burst kinetic assay", demonstrated that discospheres have remarkable intrinsic developmental and tissue engineering biology that is robust and organized. In summary, adult disc stem cells and NP cells have been isolated, cultured, and characterized, from healthy spinal disc tissues. These findings demonstrate the important potential to be explored for using stem cell based tissue engineering for the treatment of degenerative disc disease (DDD).

Keywords: Intervertebral disc tissue; Nucleus pulpous; Degenerative disc disease; Adult spinal disc stem cells; Adult stem cell biology; Matrix biology; Tissue engineering; Regenerative medicine

Introduction

Adult intervertebral spinal discs are the primary joints of the human spine, providing the critical stability, flexibility, and biomechanical support needed by bipedal animals [1-4]. The intervertebral spinal disc joint consists of three major anatomical structures: an outer capsule, the annulus fibrosis; and the nucleus pulposus; and the vertebral endplates. The spinal disc of mammals begins to develop early in fetal gestation, and originates from notochord cells (NC) that coalesce into cellular clusters containing large vacuolations [5-7]. Early NC cells are the primary cell population of the developing fetal and neonatal spinal disc. They have a driving role in disc tissue development and disc biology. For example, they actively direct the ordered assembly of disc tissue extracellular matrix, and they secrete connective tissue growth factors and extracellular matrix that is low in collagen and high in proteoglycan content [5-7]. In humans and some species the NC population classically is thought to rapidly disappear around age 3. The cell population that replaces notochordal cells with aging is nucleus pulpous cells [NP].

Most adult organs and tissues have some capacity to repair and regenerate cells, tissues, and their structural and functional biologic properties. Stem cells and stem cell biology have been demonstrated in recent years to play a significant role in repair and regeneration in adult human tissues. Conversely, the adult tissues and cells of the intervertebral spinal disc are unable to repair and regenerate in a similar manner in the face of spinal trauma, DDD, and aging. The tissue biology of the spinal disc is unlike any other in the human body. The spinal disc is a closed environment that is cell poor, harsh, avascular, and biologically formidable. Spinal disc tissue is hypoglycemic, hypoxic, acidic, and hypometabolic. Very few cell types can thrive in this environment; much less survive [8-13]. Spinal disc tissue is susceptible to the early onset of degenerative disc disease (DDD), which is arthritic in nature, and continues to progress in humans as they age. DDD is poorly understood, ubiquitous in humans, and has no boundaries, including gender, ethnicity, and demographics. DDD is one of the most common and morbid human diseases of the last several decades [14-18]. Indeed, DDD is one of the most common reasons to see a physician, to be admitted to a hospital, and to have a spinal surgical procedure [15]. The large and aging patient population represented, the impact on the work force and quality of life, combined with the costs of medical care, make DDD a medical and economic issue on a global scale [15].

In humans and some species the NC population classically was thought to rapidly disappear around age 3, as above. However, it is clear at this time that there are likely several different cell types comprising intervertebral disc tissue. For the purposes of this report, spinal disc tissue and spinal disc cells are refers to nucleus pulposus tissue and nucleus pulposus cells, respectively. Some animals do not lose their notochordal cell population as they age, and do not develop DDD (for example, nonchondrodystrophic dogs, rats, rabbits, and mice) [16-19]. This has led to some concern about which animal models are appropriate for studies of the intervertebral spinal disc, and the validity of the results of past studies with these models [15,20,21]. The issue at hand is not the quality or validity of the research in

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general. Instead, it relates to how relevant these models and studies are to the human condition. Conversely, other animal species (as described above for humans) including steadily lose their notochordal cell population early in life [for example, bovine and porcine], and subsequently develop DDD as they age [21-24]. Recently, it has been reported that notochordal cells may persist in adult human spinal tissues [so called notochordal remnants]. These cells are smaller, have different morphology than their precursors, and otherwise appear to lack notochordal phenotype *in vivo* [15,21,22]. Interestingly, MCann et al. using an elegant transgenic and genetic experimental approach demonstrated that all adult spinal disc tissue cells may by derived originally from notochordal cells [17]. This has significance from a developmental biology and functional biology perspective, given the role of notochordal cells in the development of the fetal spinal disc, and their ability to drive healthy NP biology and function in adult spinal tissue studies *in vitro* [18-20,25].

Adult stem cell biology is an established biologic paradigm those decades in the making, with the discovery of rare atypical cells in bone marrow with an extraordinary phenotype and array of cellular properties [8-13,25-27]. Hematopoietic Stem Cells (HSCs) can divide clonally to create identical stem cells, and maintain and/or expand the stem cell population in response to external biologic cues. Conversely, HSCs divide asymmetrically and give rise to stem cell progenitors and their respective terminal lineage blood cell types, as a part of their normal *in vivo* biology. HSCs have features of immortality, and can be serially passaged *in vitro* or *in vivo* several times. HSCs represent a small fraction of the bone marrow and blood cell population (1 in 10,000 cells, or 1 in 100,000 cells, respectively), and are quiescent relative to cell population and cell proliferation kinetics. HSCs express stem cell biomarkers that can be used to identify them in bone marrow aspirates, blood samples, and bone marrow cell cultures (i.e., CD34). Finally, HSCs are known to be maintained *in vivo* and *in vitro* with specific stem cell growth factors, receptors, and signal transduction cascades. Neural stem cells (NSC) were isolated and characterized from murine brain tissue by Reynolds and Weiss, and by Snyder et al. in the early 1990s [28,29]. NSCs were found to be rare and atypical cells, with a remarkable phenotype and array of cellular properties (similar to HSCs) [30-33]. NSCs can divide clonally and/or asymmetrically, and both are influenced by external environmental cues. NSCs can be serially passaged and differentiated into neural progenitors and terminally differentiated lineages of the central nervous system (i.e., neurons, astrocytes, oligodendrocytes, and ependymal cells). NSCs express known biomarkers (i.e., Nestin), represent a small fraction of neural tissue cells, (<0.1%), and are typically in quiescence, rarely undergoing cell division [33].

Over the last several decades, stem cell research for the treatment of DDD has focused on cell and stem cell based therapies derived from: 1) nonspinal tissue niches (i.e., chondrocytes, adipose stem cells, mesenchymal stem cells, etc.); or 2) tissue niches in close proximity spinal disc tissue (i.e., hyaline cartilage, annulus, etc.). Cells and stem cells from these tissue niches were delivered directly to spine disease models, or combined with tissue engineering biology and then used for therapeutic studies (in some examples, reprograming cell biology, to induce a more NP-like phenotype in the cells being delivered, was included) [34-38]. Most of these studies demonstrated some degree of safety and/or efficacy, although the data and results are typically limited to biochemistry, cell biology, and molecular biology assays [34-38]. Conversely, Risbud et al., in 2007, reported for the first time, the presence of a rare cell population that could be isolated from spinal disc tissue that was characterized as having a mesenchymal stem cell (MSC) phenotype [39]. In 2010, Blanco et al. demonstrated the ability to isolate and characterize cells from hyaline cartilage of the vertebral end plates with a mesenchymal stem cell phenotype [40]. In these studies, they assayed this cell population in parallel with bone marrow derived MSCs, and found that both were similar in phenotype and cellular properties. The conclusion from these studies was that BM MSCs could be a source of stem cells for treating DDD, given their abundance and the less invasive aspects of a bone marrow aspirate. In 2012, Navone et al. demonstrated that NP cells isolated from degenerated disc tissue could express neural and neurotrophic markers, suggesting there was more (stem) cell plasticity to NP cells and spinal disc tissue than previously thought [41]. In 2013, Erwin et al. isolated, cultured, and characterized a "stem/progenitor" cell population from the spinal disc tissue of the adult nonchondrodystrophic canine model, using stem cell suspension culture methodology and biologics [42].

Methods

Collection of healthy adult human spinal disc tissue specimens, and preparation of adult spinal disc tissue single cell suspensions for stem cell culture

Spinal disc tissue specimens were collected from young adults by a spine surgeon as part of a surgical procedure that involved lumbar discectomy. All studies were done according to approved IRB protocols at the Methodist University Hospital (Memphis, TN). The target patient population of this first research effort was young adults with healthy spinal disc tissue. Lumbar discectomy in young adults is rare and most commonly done in the context of lumbar trauma, requiring surgical fixation with lumbar interbody fusion and instrumentation. At the time of surgery, a disc tissue specimen was collected by the surgeon in a sterile manner, transferred to a 50 ml falcon containing 10 ml of cold PBS, and transported to the lab for processing. Disc tissue was placed in a 10 cm dish with sterile saline, and carefully dissected with surgical instruments under magnification with surgical microscopy. This step was done over several hours to carefully remove cell and debris contaminant (i.e., fat, ligament, annulus, muscle, etc.). The remaining disc tissue was washed, weighed, and placed in 10 cm dish culture flasks supplemented with media and 0.125% trypsin EDTA (0.25 mM/ml). The culture plate was placed on a slow rotating culture apparatus for gentle mechanical stimulation, and digested for 3 hours in a tissue culture incubator at 37°C, with 95% air, 5% CO_2, and 100% humidity. The single cell adult spinal disc tissue suspension resulting from digestion was washed, triturated with a fire polished Pasteur pipette, and resuspended in basic media cell for counting with Trypan Blue. After staining, aliquots were transferred to a heamocytometer, the cells were visualized with an inverted microscope, and counted (in triplicate; the average used for plating); to ensure accurate cell counts, and to assess for the presence of cell debris, apoptotic bodies, and dead cells.

Plating of spinal disc stem cells in ultra-low density suspension culture; culture of discospheres from monoclonally plated adult spinal disc cells; serial passaging of discospheres

Single cell suspensions were resuspended at a cell density of 20,000 cells per ml in 2 ml of 2X serum free N2 stem cell media supplemented with 20-ng/mL basic fibroblast growth factor (b-FGF, Sigma, USA), and 20-ng/mL epidermal growth factor (EGF, Sigma). The resuspended disc cells were mixed with 2% methylcellulose in a 1:1 ratio. After mixing the stem cell composite and methylcellulose, a final density of 10,000 cells per ml was achieved. The stem cell suspensions were then plated in precoated ultra low binding 6-well tissue culture

plates (Corning Bioscience) at 2 ml/well, and placed in tissue culture incubators at standard conditions. Fresh supernatant and growth factors were added to the cultures every 3 days. Plated stem cells were observed in culture over time with inverted microscopy to follow the development of stem cell sphere clusters. The number of spheres per well were counted at day 12-14 by defining the culture floor into grids and counting the grids under 20X magnification with an inverted microscope. To assess the capacity for discospheres from the first culture series to be serially passaged, 300 spheres were collected from several dishes, added to 50 ml falcon tubes with media and Trypsin EDTA (0.25 mM/ml). The tubes were incubated for digestion for 5 minutes in a tissue culture incubator at incubator at 37°C, with 95% air, 5% CO2, and 100% humidity. The single cell suspension from the enzymatic digestion was washed, triturated with a fire polished Pasteur pipette, resuspended in basic media for cell counting with Trypan Blue. After staining, aliquots were transferred to a heamocytometer. The cells were visualized with an inverted microscope and counted (in triplicate; the average used for plating); to ensure accurate cell counts, and to assess for the presence of cell debris, apoptotic bodies, and dead cells. Single cell suspensions were resuspended at a cell density of 20,000 cells per ml in 2 ml of 2X N2 stem cell media supplemented with growth factors 20 ng/mL basic fibroblast growth factor (b-FGF, Sigma, USA), and 20 ng/mL epidermal growth factor (EGF, Sigma). The resuspended disc cells were mixed with 2% methylcellulose in a 1:1 ratio. After mixing the stem cell composite and methylcellulose, a final density of 10,000 cells per ml was achieved. The stem cell suspensions were then plated in precoated ultra low binding 6-well tissue culture plates (Corning Bioscience) at 2 ml/well, and placed in tissue culture incubators at 37°C, with 95% air, 5% CO_2, and 100% humidity. Fresh supernatant and growth factors were added to the cultures every 3 days. Plated stem cells and emerging discospheres were observed in culture over time with inverted microscopy. The number of spheres per well were counted at day 12-14 by defining the culture floor into grids and counting the grids under 20X magnification with an inverted microscope. The spheres from the second culture series were again passaged as above two additional times (3 total passages).

Expression studies of stem cell and NP cell biomarkers

Adult spinal disc stem cells or discospheres were resuspended in N2 stem cell media supplemented with growth factors as above, and plated on cover slips coated with 50 µg/ml laminin (Life Technologies, Gaithersburg, MD) for 8 hours to allow attachment. To differentiate adult spinal disc stem cells into NP cells, the cells were plated on cover slips coated with 50 µg/ml laminin (Life Technologies, Gaithersburg, MD) as discospheres, or as single stem cell suspensions, and cultured in chondrogenic media and conditions supplemented with 10% fetal calf serum [29,36,41] for 14 days. Differentiated NP cells were assayed *in vitro* in culture for morphology, phenotype, expression of extracellular matrix, culture cell biology, and expression of biomarkers. Attached stem cells or spheres were processed, fixed in 4% paraformaldehyde (in PBS, pH 7.2, for 30 min at room temperature), followed by three washes [5 min each, in PBS, pH 7.2]. The cells were permeabilized for 5 min in PBS containing 0.3% Triton X-100, rinsed in PBS two times, and blocked for 20 min in PBS containing 10% normal goat serum (NGS). Rabbit monoclonal IgG1 anti-CD133 (Sigma-Aldrich, USA), mouse monoclonal IgG1 anti-Nestin (AbCam, UK), and mouse monoclonal IgG1 anti-Survivin (AbCam, UK) were diluted in PBS containing 10% NGS overnight at 4°C, at dilutions of 1/200, 1/200, and 1/100, respectively. Coverslips were rinsed in PBS three times, and incubated with mouse anti-rabbit (FITC, AbCam, UK) or rabbit anti-mouse (spectral red, AbCam, UK) secondary antibodies at dilutions of 1/1000. The coverslips were rinsed three times in PBS, mounted in Fluoro-mount (BDH laboratory, Poole, UK). The signal intensity and distribution was visualized using confocal microscopy with fluorescent filters, and the digital images were collected using standard confocal imaging software. NP cells were processed, fixed in 4% paraformaldehyde (in PBS, pH 7.2, for 30 min at room temperature), followed by three washes [5 min each, in PBS, pH 7.2]. The cells were permeabilized for 5 min in PBS containing 0.3% Triton X-100, rinsed in PBS two times, and blocked for 20 min in PBS containing 10% normal goat serum (NGS) for 30 min at room temperature, followed by three washes [5 min each] in PBS, pH 7.2. Rabbit monoclonal IgG Anti-Collagen2 and mouse monoclonal IgG anti-Vimentin primary antibodies (Boehringer Mannheim) were diluted at 1:200 in PBS containing 10% NGS overnight at 4°C. Coverslips were rinsed in PBS three times, and incubated with mouse anti-rabbit (FITC, AbCam, UK) or rabbit anti-mouse (Spectral red, AbCam, UK) secondary antibodies at dilutions of 1/1000. The coverslips were rinsed three times in PBS, mounted in Fluoro-mount (BDH laboratory, Poole, UK). The signal intensity and distribution was visualized using confocal microscopy with fluorescent filters, and the digital images were collected using standard confocal imaging software.

2-Dimensional tissue engineering with 3-dimensional discospheres (the burst kinetic assay)

Discospheres were plated on coverslips coated with 0.1% gelatin, and cultured in chondrogenic media and culture conditions for 5 days. Imaging data was collected at 24 h intervals with inverted light microscopy at various magnifications, starting on day 1, ending on day 5 [28,35,40]. Discosphere cultured in the burst kinetic tissue-engineering assay were observed *in vitro* in sphere and cell morphology phenotype changes, expression of extracellular matrix, and several culture cell biology parameters were documented.

Results

Isolation, culture, and characterization of adult spinal disc stem cells from human spinal disc tissue

Human adult spinal disc tissue was collected from patients at the time of spine surgery according to approved hospital IRB protocols. The target patient population was restricted to healthy young adults with clinically normal spinal disc tissue at the time of presentation, and presumed normal spinal disc tissue biology and function prior to surgery. Spinal disc tissue specimens were processed through several steps in the lab to create single cell suspensions of adult spinal disc cells. Spinal disc cells were plated in suspension culture in supplemented stem cell media, at ultra-low clonal plating densities (10,000 cells per ml). Methylcellulose was mixed with the stem cell/media composite in a 1:1 ratio to restrict cell movement, and "suspend" cells in culture, followed by plating of on ultra-low binding tissue culture plates. Figure 1A demonstrates the appearance of human adult spinal disc cells plated in suspension culture at time 0 at ultra-low clonal densities. Several critical culture biology parameters and paradigms can be observed in Figure 1A: ultra-low density of cultured cells; relatively even distribution of cells in the culture; lack of dead cells or debris; and a space maintained between all cells and the tissue culture vessel walls and floor. Plated stem cells were observed in culture over time with inverted microscopy to follow the development of stem cell sphere clusters. The number of spheres per well were counted at day 12–14 post plating. Figure 1B demonstrates the appearance of monoclonally derived stem cell clusters with sphere-like morphology (discospheres), on day 10 post-plating. In the first experimental series, the sphere

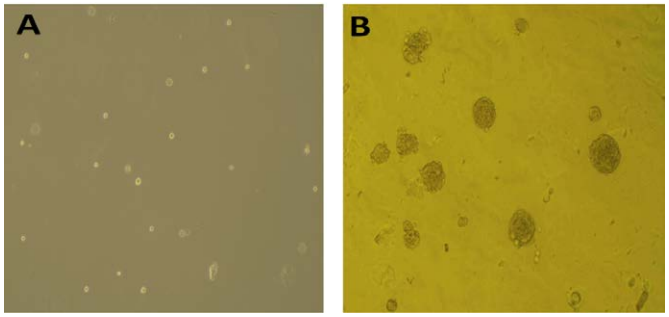

Figure 1: Isolation, culture, and characterization of adult spinal disc stem cells from human spinal disc tissue. A. Photomicrograph taken with light microscopy at 20X magnification, of the first plating of adult spinal disc cells as a single cell suspension, at ultra-low densities, in suspension culture (10,000 cells per ml). B. Photomicrograph taken with light microscopy at 20X magnification of monoclonally derived sphere-like cellular clusters (discospheres), comprised of stem cells and early progenitors. Shown are spheres at day 10 of growth in suspension culture.

yield was less than expected (~80 spheres per ml +/- 9; although the spheres were viable, healthy, and had typical diameters, cell volume, cell numbers upon further study).

Serially passaging of discospheres in suspension culture

To assess the capacity for discospheres from the first culture series to be serially passaged, 300 spheres were collected from several culture dishes, washed with PBS, and added to 50 ml falcon tubes with media and prepared enzymatic solutions. The tubes were incubated for 5 minutes in a tissue culture incubator to allow enzymatic digestion. The single cell suspension resulting from the enzymatic digestion was washed, triturated with a fire polished Pasteur pipette to ensure no clumps of cells remained, and resuspended in basic media for cell counting. Single cell suspensions were resuspended at ultra-low cell density in supplemented stem cell media. The resuspended single disc stem cell suspensions were then plated on precoated ultra low binding 6-well tissue culture plates and placed in the tissue incubator. Fresh supernatant and growth factors were added to the cultures every 3 days. Plated stem cells were observed in culture over time with inverted microscopy to follow the development of stem cell sphere clusters. The spheres from the second culture series were passaged twice more. At the end of each culture series, the number of spheres per well were counted at day 12–14 m as above. Thus, a total of 3 passages were made from the stem cell culture product of the first culture series, and this experiment demonstrated that linear expansion of the stem cell population was possible (Figure 2, black line graph). As shown in the figure, and discussed below and in the appendix, the overall yield of spheres initially, and with serial passaging was far less than expected. The initial yield was quite low when compared to other organ tissue results. Further, the stem cell and discosphere frequency did not begin to expand in suspension culture until after the discospheres or the first culture series had been passaged once. In a second experimental series, several revisions, additions, and changes to the suspension culture protocols and bioreagents were made. Again, 300 spheres were collected from culture plates, on day 14 post plating, washed, prepared as single cell suspensions, and plated in suspension culture media and conditions, as above. The spheres from the second culture series were passaged twice more. Thus, a total of 3 passages were made from the stem cell culture product of the first culture series, and demonstrated that linear expansion of the stem cell population was possible (Figure 2, red line graph). The second experimental series did not improve initial yields or early passages, but there was significant gain in disc stem cell and discosphere yields in the latter half of the culture experiment. The issues and challenges of the first experimental series are basic and technical and are described in detail in the discussion and the supplemental data section.

Expression of stem cell biomarkers in adult spinal disc stem cells; expression of NP biomarkers in differentiated NP cells, derived from adult spinal disc stem cells cultured in chondrogenic media and conditions

Discospheres and single stem cells were resuspended in stem cell media, plated on laminin-coated cover slips, incubated for 8 hours to allow attachment, and then fixed and processed for biomarker expression studies. As shown in Figures 3A-E (and data not shown), Nestin and CD133 were expressed in discospheres in most cells at high levels (single stem cell suspensions plated in parallel also had uniform and high expression, data not shown). Survivin expression was ubiquitous and strongly expressed in stem cells plated from single cell suspensions (Figure 3F), but its expression in discospheres was much lower (data not shown). NP cells were differentiated by plating them on laminin-coated cover slips, in chondrogenic media and culture conditions for 14 days. Differentiated NP cells developed appropriate morphology and phenotypic changes (Figure 4), and secreted significant extracellular matrix (Figures 4C and D). Differentiated NP cells could be serially passaged and expanded exponentially in culture (Figure 5). Finally, differentiated NP cells and expressed NP biomarkers including Collagen 2 A, and Vimentin at strong levels (Figure 6).

2-Dimensional tissue engineering with 3-dimensional discospheres (the burst kinetic assay)

As functional proof of concept, a simple but very relevant tissue

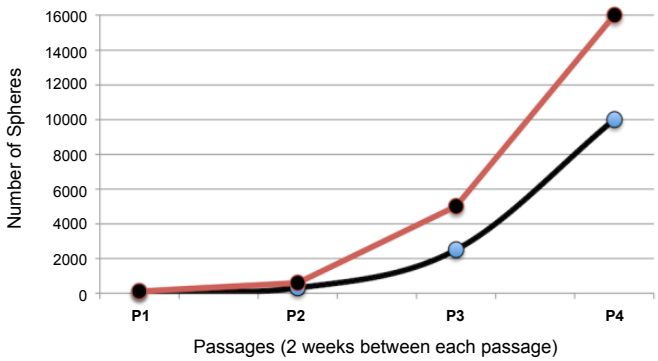

Figure 2: Linear graphs of serially passaging of discospheres in suspension culture in two experimental series. In the first experimental series, 300 spheres were collected from culture plates, on day 14 post plating, washed, prepared as single cell suspensions, and plated in suspension culture media and conditions, as above. The spheres from the second culture series were passaged twice more. Thus, a total of 3 passages were made from the stem cell culture product of the first culture series, demonstrated that linear expansion of the stem cell population was possible (Figure 2, black line graph). The black line graph represents the expansion of stem cells derived from the first culture series through serial passaging. In the second experimental series, several revisions, additions, and changes to the suspension culture protocols were made. Again, 300 spheres were collected from culture plates, on day 14 post plating, washed, prepared as single cell suspensions, and plated in suspension culture media and conditions, as above. The spheres from the second culture series were passaged twice more. Thus, a total of 3 passages were made from the stem cell culture product of the first culture series, and again demonstrated that linear expansion of the stem cell population was possible (Figure 2, red line graph).

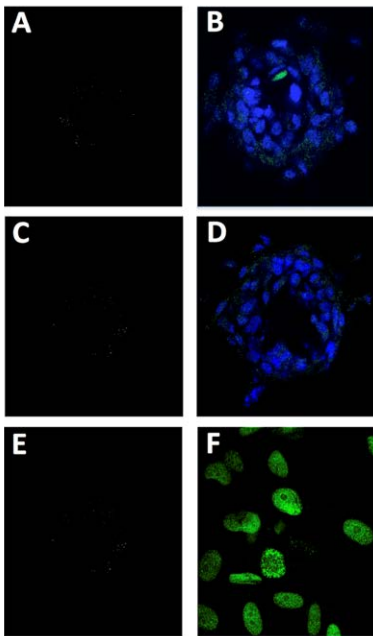

Figure 3: Immunohistochemical assays of stem cell biomarker expression in adult spinal disc stem cells. Discospheres and single stem cells were resuspended in stem cell media, plated on laminin-coated cover slips, incubated for 8 hours to allow attachment, and then fixed and processed for biomarker expression studies. A. C. E. Negative controls for immunostaining of CD133 (A), Nestin (C), and Survivin (E). B. D. F. Immunohistochemistry for the expression of these stem cell biomarkers. B and D. The expression of CD133 (B) and Nestin (D) in discospheres attached to laminin coated coverslips for 8 hours. Photomicrographs taken with fluorescent confocal microscopy at 40X magnification demonstrate uniform and high expression of both biomarkers throughout the sphere structure. F. The expression of Survivin in adult spinal disc stem cells plated on laminin coated culture surfaces as single cell suspensions, as shown in photomicrographs taken with fluorescent confocal microscopy at 40X magnification, demonstrate uniform and high expression of Survivin biomarkers in attached spinal disc stem cells.

engineering assay was used to study the developmental biology and regenerative potential of discospheres (the "burst kinetic assay"). Discospheres were plated on coverslips coated with gelatin, and cultured in chondrogenic media and culture conditions for 5 days. Imaging data was collected at 24 h intervals starting on day one. Figure 7A shows a discosphere plated on a gelatin coated surface, and attached at 24 hours post plating. Figures 7B and 7C show so called "burst kinetic" cell blastic activity that occurs at the periphery of the discospheres at 48 hours (with the appearance of spindle shaped elongated cells that radiate in near 90 degree angles from the discosphere). Most often, spindle cells were found to migrate from the sphere and change phenotype and morphology around 72 to 96 hours (Figures 7D and 7E), transforming into small, innocuous, rounded cells that were highly mobile, and highly proliferative. Long-term studies with these assays demonstrated that this cell population would continue to migrate and proliferate until the culture surface was crowded with cells. Cell crowding and cell contact with other cells and physical structures, resulted in yet another change in morphology and phenotype (Figure 7F). Mature NP cells began to appear, ceased proliferation and migration, and secreted significant extracellular matrix around the cell periphery. The culture product was a NP confluent culture bed, embedded in extracellular matrix, with local tissue remodeling (i.e., appearance, properties, and characteristics of prefabricated engineered tissue) (Figure 7F).

Discussion

The discovery and development of HSC biology, and subsequently NSC biology, has led to breakthroughs in understanding, sophistication of methods and biologics, and applications and approaches that can be directed at other solid organs and tissues. In recent years, pioneers in the field have pushed back against many fundamental paradigms of NSC biology [41-44]. Nonetheless, extensive investigation, review, and discussion, have led to new understanding, and the identification

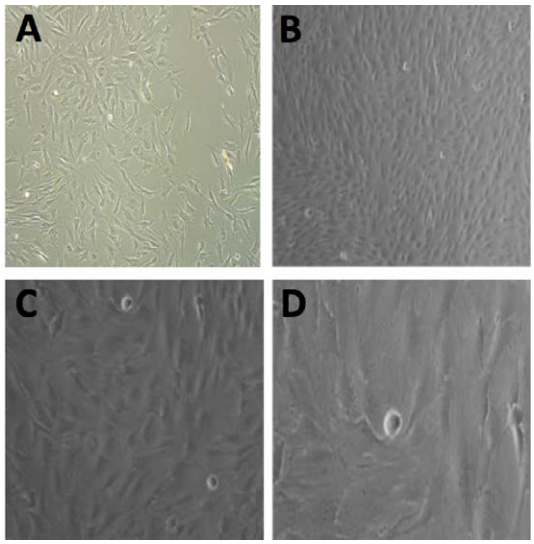

Figure 4: Differentiation of human adult spinal disc stem cells into NP cells, and characterization of their phenotype. Single stem cell suspensions derived from discospheres were plated on cover slips coated with 50 µg/ml laminin in chondrogenic media supplemented with 10% fetal calf serum, and chondrogenic culture conditions, for 14 days. A. Photomicrograph taken with light microscopy at 10X magnification of NP cells differentiated from spinal disc stem cells over 14 days, at 70% confluency. B. Photomicrograph taken with light microscopy at 10X magnification of NP cells differentiated from spinal disc stem cells over 14 days at 100% confluency. C. Photomicrograph taken with light microscopy at 20X magnification of NP cells differentiated from spinal disc stem cells over 14 days at 100% confluency. D. Photomicrograph taken with light microscopy at 40X magnification of NP cells differentiated from spinal disc stem cells over 14 days at 100% confluency. Escalating photomicroscopic magnifications are shown to demonstrate the significant amount of secreted extracellular matrix in differentiated mature confluent NP cells.

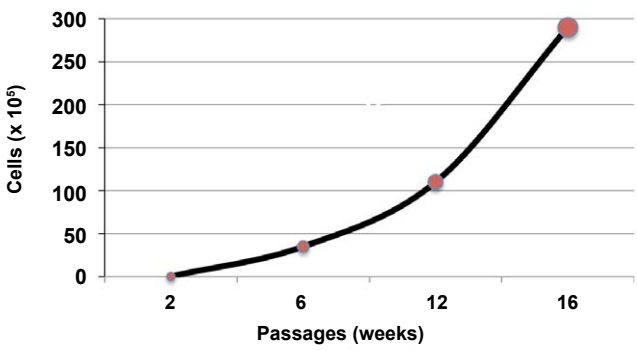

Figure 5: Linear graph of NP cells differentiated from spinal disc stem cells, and serially passaged for expansion. 40 discospheres were plated on a T25 tissue culture flask, coated with laminin, in chondrogenic media and culture conditions. The T25 culture was passage into a T75 culture flask at confluency. The T75 culture was expanded into 3 T75 culture flasks at confluency.

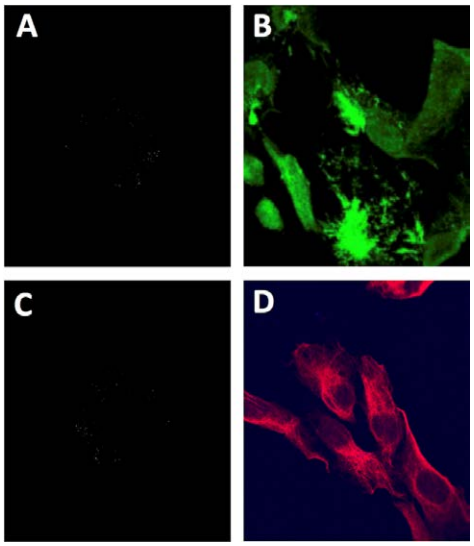

Figure 6: Immunhistochemical assays of stem cell biomarker expression in adult NP cells. Single stem cell suspensions derived from discospheres were plated on cover slips coated with 50-µg/ml laminin in chondrogenic media supplemented with 10% fetal calf serum and chondrogenic culture conditions for 14 days. A. and C. Negative controls for immunostaining of Collagen 2a (A) and Vimentin (C). B. and D. Immunohistochemistry for. B and D. The expression of the NP cell biomarkers Collagen2a (B) and Vimentin (D) in NP cells. Shown are photomicrographs taken with fluorescent confocal microscopy at 60X magnification.

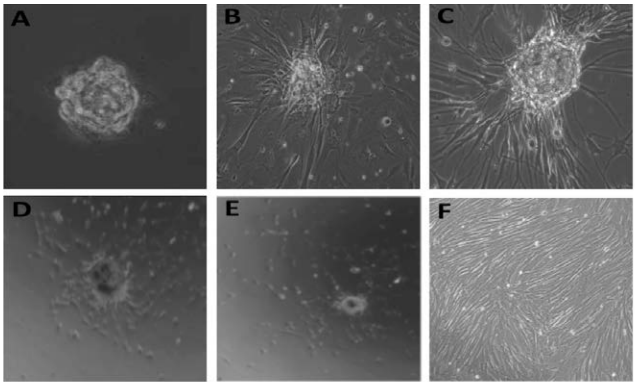

Figure 7: 2-Dimensional Tissue engineering with 3-dimensional discospheres (the burst kinetic assay) Discospheres were plated on coverslips coated with 0.1% gelatin, and cultured in chondrogenic media and culture conditions for 5 days. Imaging data was collected at 24 h intervals with inverted light microscopy at various magnifications, starting on day 1, ending on day 5. A. Photomicrograph taken with light microscopy at 60X magnification of a discosphere cultured in the burst kinetic tissue-engineering assay at 24 hours. B. and C. Photomicrographs taken with light microscopy at 60X magnification of a discosphere cultured in the burst kinetic tissue-engineering assay at 48 hours (B) and 72 hours (C) demonstrating a dramatic "blastic" phase. As shown in the figure, the cells at the sphere edge change dramatically in phenotype, and become elongated spindle shaped cells that migrate from the sphere and change phenotype and morphology. D. and E. Photomicrographs taken with light microscopy at 20X magnification at 96 hours (D) and 120 hours (E) of a discosphere cultured in the burst kinetic tissue engineering assay; demonstrating the appearance of small rounded cells that migrate out across the culture surface away from the sphere, and gain and cell migration and proliferation biology. F. Cultures allowed to continue to grow were followed over time. Long-term studies with these assays demonstrated that these cells continue to migrate and proliferate, until the culture surface was crowded with cells. At this time, the NP cells change morphology and phenotype, differentiated into mature NP cells, ceased migration and proliferation, and secreted significant extracellular matrix.

of several caveats of note. Most of these issues, caveats, and outliers, can be explained by one of three findings. First, not all adult tissue stem cells are the same. There is heterogeneity at the tissue level that carries forward in vitro with R&D and suspension culture methods and approaches. Second, there is significant biologic rigor and good research practice required for success with these approaches and it is not uncommon to find outliers that relate to technical errors and misconceptions by some researchers. Finally, there is still much stem cell biology that is simply unknown at this time. Regardless, with these caveats in mind, adult stem cell biology has stood the test of time, peer review, and independent challenges. Thus, it is a rationale and proven paradigm, in the present, to use this knowledge base and methodology as a platform and approach to new stem cell research programs. Proof of concept, can be found by noting the many adult stem cell discoveries over the last few years in various solid tissues, disease models, and solid cancers (i.e., breast, liver, prostate, neural crest, cardiac, etc.) [45-50].

As mentioned above in the introduction, Erwin et al. was able to isolate and culture disc stem cells from adult canine models. Stem cells were isolated that could survive harsh suspension culture conditions, and grow into sphere-like cellular clusters over 2 weeks. Isolated stem cells expressed a rich array of stem cell markers, and demonstrated significant pluripotency in MSC (mesoderm) and NSC (ectoderm) differentiation assays. This study is important and of note as a first report of its kind. Namely, the author reports the isolation of stem cells from adult mammalian spinal disc tissue, using stem cell suspension culture methodology and biologics. However, the study did have some limitations making the results difficult to interpret and apply to humans, especially the aging population with DDD. First, by using the nonchondrodystrophic canine model, the isolated stem cells were derived from notochordal rich and healthy spinal disc tissue. Second, the stem cell biomarker assays were done with semiquantitative PCR, but without other assays to validate, and to correlate the expression of mRNA with protein expression. Finally, despite excellent pluripotency in these studies, they did not include differentiation assays and biology of NP cells (the primary and terminal lineage cells of the tissue from which the stem cells were isolated). These are caveats that should be addressed and further investigated, given the potential and importance of novel stem cell based therapies for treating DDD in the aging human population.

This report endeavors only to present a new stem cell population, and to prove the phenotype using classic and proven approaches and criteria. The survival of adult spinal disc stem cells in these harsh culture conditions, and their growth over time into discospheres from adult disc stem cells and early progenitors is major proof of stem cell phenotype. These findings support the discovery and isolation of a spinal disc stem cell population in healthy adult spinal disc tissues. In particular, contact inhibition (an integral part of suspension culture), derived from 1) plating the cells at ultra-low density; 2) supplementing stem cell media with methylcellulose; and 3) using ultra low binding culture plates, is critical for stem cell selection and acts as a filter of sorts. Late stage progenitors, and adult differentiated spinal disc cells, cannot survive in these conditions, and undergo apoptosis. The ability to serial passage NP cells is another proof of stem cell phenotype. In this first report, only 3 passages were done, with the primary intent of determining if serial passaging was possible, and if stem cell and discosphere expansion was possible. Differentiation of adult spinal disc stem cells into NP cells with appropriate phenotype, and capacity for expansion is further proof of concept. Additionally, the expression of stem cell biomarkers in discospheres, and NP biomarkers in NP cells is strong evidence that a tissue specific stem cell has been isolated, that

can be give rise readily to the terminally differentiated lineage cell from which it was derived. Finally, the robust tissue engineering biology in the burst kinetic assay is functional evidence of value.

In summary, this report details the isolation, culture, and characterization of spinal disc stem cells derived from human adult spinal disc tissue specimens. Using stem cell suspension culture methods and biology, we were able to isolate spinal disc stem cells, and monoclonally grow them into multicellular sphere-like clusters (discospheres). Discospheres from the first culture series were collected, processed, and replated as single stem cells for serial expansion studies using suspension culture, demonstrating linear expansion was possible. Discospheres and adult spinal disc stem cells were plated on matrix coated culture surfaces in stem cell media for several hours to allow fixation, and assayed for the stem cell biomarkers. Discospheres and adult spinal disc stem cells were plated on laminin-coated culture surfaces in chondrogenic media and culture conditions for 14 days to differentiate them into NP cells. NP cells cultured from these experiments demonstrated NP morphology and phenotype; NP biomarker expression, secretion of extracellular matrix, and the ability to be serially passaged with large volume expansion possible. Tissue engineering studies using the "burst kinetic assay", demonstrated that discospheres have remarkable developmental biology potential, and *in vitro* tissue engineering biology that is robust and organized. In summary, adult disc stem cells and NP cells have been isolated, cultured, and characterized from healthy spinal disc tissues. These findings demonstrate the exciting potential to be explored for using stem cell based tissue engineering for the treatment of DDD.

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This is novel report and first in class, and an exciting time for our research program. This report is the first of its kind in the academic literature, and is the first in a series (of many publications to follow) that come from a research program that now is in its 8th year of activity (founded in 2005 at the University of Tennessee, Department of Neurosurgery.) This research effort, over the years, has been collaboration with faculty, neurosurgeons, residents, students, and the rest of the team. We would like to acknowledge the following for the contributions and efforts here and over the last many years. Jon Robertson, MD. Rano Chatterjee, MD. Zoran Pavecevic, MD. Zorica Janjetovic, MD, Mariya Nazarova, PhD., Qihong Zhou, MD, PhD, Bing Teng, MD, Terreia Jones, PharmD, Jeff Sorenson, MD, Julius Fernandez, MD. Valery Kukekov, PhD, Gala Dulatova, MD. Raisa Krukalina, MD.

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Disclosures and Conflicts of Interest

There are no conflicts of interest for this submission. I would add for clarification, that this research was done at the University of Tennessee, Department of Neurosurgery, in the laboratories of the senior author. Early results and proof of concept were deemed important for academic research, but also as having potential for clinical science as well. Thus, early patents were filed to protect the IP as recommended by leadership in the group. While this and subsequent studies led to a formal biotechnology group in 2009 (life sciences intellectual property and patents), the senior author of both patents and manuscripts, maintained at all times an academic track, and agreed to patent these findings before publishing, only after it was agreed that there would be no restriction by biotechnology in academic endeavors, including publishing this research. The senior author was a Professor in the department from 2000 – 2012, but was also the Chief Science Officer for the company (Discgenics, Inc.), from 2006–2011 by request. He has no executive role, no leadership responsibilities, and no control of the interests of this company at this time. The senior author has been assigned equity in this company, for collective efforts over 15 years that relate to this research program. However, all research reported here, was patented many years ago, and has been issued by the USPTO and by the WPTO. Thus, the effort to publish the same science, publically and in a peer review context, is a commitment to academics, and to the fundamental ideology that research and scientific results are public domain and should be shared.

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