The regenerative capacity of the notochordal cell: tissue constructs generated in vitro under hypoxic conditions

### Laboratory investigation

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**Object.** The intervertebral disc (IVD) is a highly avascular structure that is occupied by highly specialized cells (nucleus pulposus [NP] cells) that have adapted to survive within an O₂ concentration of 2–5%. The object of this study was to investigate the effects of long-term hypoxic and normoxic tissue cultures of nonchondrodystrophic canine notochordal cells—cells that appear to protect the disc NP from degenerative change.

**Methods.** The authors obtained notochordal cells from nonchondrodystrophic canines according to their established methods and placed them into monolayer and 3D culture using sodium alginate globules under either hypoxic (3.5% O₂) or normoxic (21% O₂) conditions. Histological, immunohistochemical, scanning electron microscopy, and histomorphometric methods were used to evaluate the cells within the globules after 5 months in culture.

**Results.** Notochordal cells under in vitro hypoxic tissue culture conditions produced a highly complex, organized, 3D cellular construct that was strikingly similar to that observed in vivo. In contrast, traditional normoxic tissue culture conditions resulted in notochordal cells that failed to produce an organized matrix. Hypoxia resulted in a matrix rich in aggrecan and collagen II, whereas normoxic cultured cells did not produce any observable aggrecan or collagen II after 5 months of culture.

**Conclusions.** Hypoxia induces notochordal cells to organize a complex 3D cellular/extracellular matrix without an external scaffold other than suspension within sodium alginate. These cells produce an extracellular matrix and large construct that shares exactly the same characteristics as the in vivo condition—robust aggrecan, and type II collagen production. Normoxic tissue culture conditions, however, lead to a failure of these cells to thrive and a lack of extracellular matrix production and significantly smaller cells. The authors suggest that future studies of NP cells and, in particular, notochordal cells should utilize hypoxic tissue culture conditions to derive meaningful, biologically relevant conclusions concerning possible biological/molecular interventions. (DOI: 10.3171/2009.2.SPINE08578)

### Key Words
- notochordal cell
- intervertebral disc
- tissue construct
- hypoxia

**Degenerative** disc disease is an extremely common and expensive ailment. While there have been advances in biological therapies for the management of fractures and disease-modifying drugs for the treatment of various arthropathies, there is no curative strategy for DDD that attenuates or reverses the degenerative cascade. With respect to the pursuit of a biological intervention, the study of the NCD canine model is noteworthy; these animals are unique among the canine subspecies in that the dogs preserve their notochordal cell populations and are protected from the development of DDD. We have previously determined that NCD canine intervertebral disc–derived notochordal cells secrete connective tissue growth factor (CTGF/CCN-2) and that conditioned medium obtained from these cells upregulates important matrix gene expression, cell proliferation, and proteoglycan production in NP cells. Connec-

tive tissue growth factor is known to be an important regulatory molecule during development that works in conjunction with a number of transcription factors and developmental genes such as *jun; sox5, -6, and -9; hedgehog; and noggin*—important genes in disc formation and chondrogenesis.

The IVD is a highly avascular compartment that is almost totally devoid of vascular supply in the mature adult. Therefore, nutrient balance in the IVD is likely to be tightly regulated by both the cells within and the structural aspects of the disc, including the vertebral endplates. The importance of nutrient and gas diffusion to the disc (a structure otherwise devoid of vasculature) has been highlighted by a number of studies. It has been reported that the capillary networks present at the vertebral endplates are up to 4 times more dense directly over the NP compared with the annulus—suggestive of the vital supply of such nutrients to disc cells in the ab-

Abbreviations used in this paper: DDD = degenerative disc disease; FCS = fetal calf serum; IVD = intervertebral disc; NCD = nonchondrodystrophic; NP = nucleus pulposus; PSF = penicillin, streptomycin, and Fungizone; SEM = scanning electron microscopy.

This article contains some figures that are displayed in color online but in black and white in the print edition.
sence of direct vascular supply. Nucleus pulposus cells have necessarily adapted to survive within a hypoxic and relatively nutrient-poor environment, the concentration of O2 within this compartment reportedly being between 2 and 5%. However, most studies concerning IVD metabolism within the in vitro setting have investigated these cells cultured under classical tissue culture conditions of 21% O2. A number of these studies have reported effective restoration of disc height in vivo and/or increased expression of desirable genes such as aggrecan and collagen, but the in vitro studies performed used tissue culture periods that were often only a matter of days and the cultures were often maintained within monolayer. Nucleus pulposus cells have been reported to assume a fibroblastic phenotype in monolayer culture—very different from the phenotype found in the in vivo setting.

To develop effective future biological therapeutics that may involve gene transfer, the injection of growth factors, and/or stem cell--based therapy, it is vital that such interventions be designed and studied in a biologically relevant system. Therefore, it is critical to determine the tissue culture conditions under which such critical applications must be performed. To our knowledge, these conditions have never before been examined in detail, particularly with respect to notochordal cells, which are vital to the developmental organization of the disc nucleus.

The realities of the hypoxic, ischemic environment within the disc nucleus create a unique challenge to the investigator in attempting to draw conclusions from in vitro experimentation that are biologically credible and relevant. There are a number of potential biologically based therapies currently under development and reported in the literature. However, the studies that reflect in vitro experimentation have been largely performed under classical tissue-culture O2 saturation—a condition that does not mirror the in vivo condition. Furthermore, the development of a biological therapy to treat DDD requires that preliminary studies must be performed within the context of biologically relevant experimental approaches with an emphasis upon primary cells. Primary cells reflect realities that may not be present when using cell lines obtained by immortalization. We are particularly interested in the biology of primary disc-derived notochordal cells since it is these cells that exert an anabolic/matrix protective function upon NP cells. The healthy disc nucleus maintains a range of O2 concentrations between 2 and 5%; therefore, for our hypoxic studies we chose a midrange value of 3.5%. During pilot studies, we found that maintaining 2% O2 levels, although certainly possible, required an inordinate use of nitrogen gas to displace the O2. Hypoxia-responsive genes are active below 5% O2; therefore, a concentration of 3.5% satisfied the requirements of hypoxic culture and did not consume nitrogen at a prodigious rate. Therefore, in this study, we chose to evaluate the ability of primary notochordal cells to survive long-term in culture, to examine the quality of the matrix that they produce, and to compare these variables in hypoxic and traditional normoxic tissue cultures. Here for the first time we report that primary notochordal cells cultured under hypoxic conditions thrive and form complex 3D tissue and remain viable for at least 5 months.

Methods

Nonchondrodystrophic Canine Disc Notochordal Cell Cultures

Notochordal cells were obtained by meticulous dissection of the NP from the lumbar spines of 5 freshly killed NCD canines (unclaimed animals that were not successfully adopted). Within 2 hours of death, the lumbar spines were removed, the spinal soft tissues were dissected away, and the spines were washed with DH2O, Cldox, and then liberally rinsed with povidone-iodine and cooled to 4°C. The discs were placed into a 70-mm2 Petri dish and suspended within advanced DMEM/F-12 supplemented with PSF and 5% FCS and placed into 3.5% O2 and 5% CO2. The following day, the NPs were meticulously separated from any remaining annulus and washed 3 times with medium. Next the isolated NP tissues were sequentially enzymatically digested according to our established methods. The following day the cells were rinsed, filtered through 70-μm cell striainers (Falcon, BD Biosciences), and examined microscopically. Nucleus pulposus cells have been reported to assume a fibroblastic phenotype in monolayer culture—very different from the phenotype found in the in vivo setting. The following day the cultures were sequentially enzymatically digested according to our established methods. The following day the cells were rinsed, filtered through 70-μm cell striainers (Falcon, BD Biosciences), and examined microscopically. Nucleus pulposus cells have been reported to assume a fibroblastic phenotype in monolayer culture—very different from the phenotype found in the in vivo setting.
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Histological Analysis of Notochordal Cells

At the appropriate time points some of the alginate globules were removed, fixed and embedded in paraffin, sectioned, and stained. Briefly, fixation of the globules consisted of using 0.1 M cacodylate (Sigma) in 10 mM CaCl₂ and 4% paraformaldehyde pH 7.4. The globules were left at room temperature for 8 hours and then switched to 0.1 M cacodylate buffer containing 50 mM BaCl₂ pH 7.4, at 4°C for 16 hours (during which period the buffer solution was changed 4 times). The globules were then removed from the buffer and embedded in paraffin, sectioned, dehydrated through the use of alcohols and xylenes, stained, and coverslipped. We chose 5 months of culture as our end point to ascertain the behavior of the cultures over long-term conditions. At 5 months, the alginate globules containing notochordal cells were fixed using the cacodylate buffer protocol described above modified from Petit et al., processed for histological examination, and stained with Toluidine Blue, Safranin-O, and for immunoreactivity to collagen Type II (Chemicon MAB1330) and aggrecan (Abcam BC-13 clone, ab3775).

Cell Morphometry

We used histomorphometric methods to evaluate the cultured cells. Total cell area measurements were performed using a Visiopharm Integrator System (Viopharm, Inc.) with a Leica DM 4500 B microscope equipped with a motorized stage with 8-slide capacity and Olympus DP70 camera. We counted 100 cells from Safranin-O–stained sections of both normoxic and hypoxic cultured cells using specific inclusion criteria that required the cells to have an intact cell membrane, visible nucleus, and clear cytoplasm. Each cell was individually masked and image analysis was performed to obtain the total cell area using Visiopharm software.

Scanning Electron Microscopy

Parallel notochordal cell cultures grown in monolayer, and alginate globules were removed, fixed, and prepared for electron microscopy. Briefly, the monolayer cultured cells and alginate globule-containing cells were immersed in primary fixative containing 4% paraformaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 20 minutes. Next they were washed 3 times with 0.1 M phosphate buffer, pH 7.2, and post-fixation was performed using 1% osmium tetroxide buffered with 0.1 M phosphate, pH 7.2, for 20 minutes, dehydrated through immersion in graded alcohols, and dried using a critical point drier. The fixed monolayer cells and globules were examined using SEM (Hitachi S-3400 variable pressure scanning electron microscope) at various magnifications and stage angulations to obtain the best images of the specimens.

Immunohistochemistry

Specimens were sectioned at 4 µm, mounted on siliconized slides, cleared using xylenes and alcohols, and stained in parallel with positive and negative controls. The antibodies were optimized for concentration of primary antibody such that anticollagen II was stained at a dilution of 1:800. The antiaggrecan was obtained from a hybridoma supernatant and therefore was not as concentrated, and was used at a 1:10 dilution. The immunolike reactivity was evaluated using avidin-biotin–conjugated primary and secondary antibodies, visualized using diaminobenzidine methods (DAB kit, Vectastain) and counterstained with hematoxylin. For positive control, we stained articular cartilage, known to contain both aggrecan and collagen II (bovine tarsal joints); the negative control lacked the primary antibody.

Statistical Analysis

We plotted the data obtained from our histomorphometric analysis, which consisted of 200 separate measurements, and as suspected, found that it was not normally distributed. Therefore, we used nonparametric statistical methods to analyze for statistical significance (Mann-Whitney U test).

Results

Hypoxic and Normoxic Monolayer Cultures

Canine NCD notochordal cells thrive under hypoxic culture for at least 5 months, the time at which they were removed from culture and evaluated. There was no evidence of decline in cell growth within the hypoxic cultures at 5 months. The cultures were examined every 3 days as the medium was replenished and were photographed at least weekly throughout the 5 months of culture. Within 1 week, cells appeared on the bottom of the 6-well dishes, initially appearing spherical/void, bubbly-looking, or “physaliferous.” Within 2 weeks these cells had spread to become large complexes within both the normoxic and hypoxic conditions. In the normoxic cultures there was a completely confluent monolayer of cells by 4 weeks. The hypoxic cultures, however, exhibited a striking difference in appearance. Within 1 month large, macroscopic structures formed on the tissue culture plate surface, in stark contrast to the more uniform monolayer appearance typical of the cells cultured under normoxic conditions. The hypoxia-cultured cells spontaneously formed a circular structure that measured 2 mm in height and approximately 8 mm in diameter after 2 months in culture (Fig. 1A). The construct was examined after fixation and sectioning and found to demonstrate robust Safranin-O staining, indicative of a proteoglycan-rich matrix (Fig. 1B). During removal using forceps the construct demonstrated viscoelastic properties by resistance to compression and resumption of its oval shape after forceps compression was released.

After 4 weeks in culture, cells cultured under normoxia and hypoxia were evaluated using SEM and found to have dramatic differences in appearance. After 4 weeks the cells grown under hypoxic conditions had developed large, spanning “fibrils” throughout the culture plates, whereas the normoxic cultures had simply grown into a confluence of strictly monolayer fibroblastic-appearing cells (Fig. 1C and D).

Alginate Globules

Notochordal cells contained within alginate globules
demonstrated marked differences in behavior under culture in hypoxia compared with normoxia. The hypoxic cultures formed clusters of cells within 2 weeks that continued to develop over time. The hypoxic cultures formed dense cell clusters both within the alginate globule as well as many “buds” of cell clusters on the surface and within the alginate globule. During the 5-month observation period of this study, such behavior never occurred within globules of cells cultured under normoxia (Fig. 2A and B).

Histological Analysis of Globule Cultures

The cells cultured under hypoxia demonstrated a robust cellular and extracellular matrix, with large, well-defined cells that demonstrated intense metachromatic staining when stained with toluidine blue; this appearance was in stark contrast to the normoxic cultures (Fig. 2C and D). Safranin-O staining was similar, confirming the superior histological properties of hypoxic culture as compared with normoxia; the hypoxic cultures preserved an organized matrix and large cells similar to those seen in the in vivo setting and in comparison with the normoxic cultures (Fig. 2E–G). The globules containing notochordal cells cultured under hypoxic conditions demonstrated strong immune-reactivity to collagen Type II and aggrecan that was very similar in both the in vivo disc NP as well as the hypoxic cultures (Figs. 3A–F). However, strikingly, at 5 months, the normoxic cells demonstrated no evidence of collagen II and limited, poor aggrecan immune reactivity.

Histomorphometry

In general, the cells cultured under hypoxic conditions were much larger and showed intact cytomediology (intact cell membrane, visible nucleus, and clear cytoplasm, as well as some cells with matrix within the cytoplasm). Normoxic cultures, on the other hand, had far
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smaller cells, and many did not show the features necessary to be counted according to the morphometry criteria specified (see Methods). In fact, many of the cells did not appear viable, as evidenced by a disorganized nucleus, fragmented cell membrane, and fragmented/fibrillary appearance on Safranin-O and toluidine blue staining. Moreover, the cells cultured under hypoxic conditions demonstrated significantly larger area than those cultured under normoxia (Figs. 4A and B, and 5). Our morphometric analysis revealed a strikingly statistically significant reduction in cell area for cells cultured under normoxic conditions compared with those under hypoxia. We found that the difference in mean cell area for hypoxic-cultured NC cells compared with normoxic-cultured cells was statistically significant (p = 0.0001).

**Scanning Electron Microscopy**

Scanning electron microscopy is an ideal technique with which to observe such structures from a morphological and topographical perspective; therefore, in parallel with the histological and immunohistochemical staining, SEM was also performed. Scanning electron microscopy is a high-resolution technique capable of providing clear morphological details of cells and tissues, and it is able to show a 3D representation not possible using histological section. We were interested in assessing the ability of notochordal cells to self-assemble and to assume complex 3D configurations and in determining whether such behavior occurred in both normoxic and hypoxic conditions. We found a striking difference in cellular behavior under our 2 different tissue culture conditions, with the normoxic cultures revealing nothing but fixed and denatured alginate and the hypoxic cultures forming a robust, complex 3D tissue construct with variable cellular formations, including fibrillary structures, complex cellular networks, and stiffening adhesions between cells of the construct (Fig. 6A–C).

**Discussion**

**Hypoxia and Notochordal Self-Assembly**

Here we demonstrate for the first time that notochordal cells can thrive under hypoxic tissue culture for at least 5 months and develop an organized, complex 3D
extracellular matrix rich in collagen Type II and aggrecan, which are vital to the function of the disc nucleus. Parallel notochordal cell cultures harvested from identical sources but cultured under normoxic conditions (21% O₂) reveal far smaller cells—many of which lack a cell membrane and nucleus—that do not, in general, appear to survive this length of time in culture. Further, the matrix produced by these cells appears to consist of remnants of cellular debris and is quite distinct from what was observed in the hypoxic cultures. In addition, normoxic cultured cells do not demonstrate either collagen II or aggrecan staining. Hypoxia appears to drive notochordal cells cultured in monolayer to form large fibrillar processes that interconnect cells growing on the tissue plate surface. This phenomenon was not seen under normoxia. The ability of notochordal cells to “self-assemble” under hypoxic tissue culture conditions in both monolayer and 3D constructs strongly suggests that notochordal cells retain their innate capacity to function as “organizers” as well as structurally supporting cells.
Developmental Aspects of Notochordal Cells

During development and morphogenesis, cells within the embryo migrate and differentiate along commitment pathways through a complex process termed convergence and extension (for example, during gastrulation and neurulation).12 This process of convergence and extension defines how cell populations narrow and lengthen during development and in so doing exert pressure within and around developing cells/tissues of the embryo. Tissues become “stiff” at pivotal times during early development in order for the embryo to properly form. Ordered cellular migration and differentiation are typified by the notochord and its sheath, which represent one of the earliest areas where tissue stiffness develops to provide the longitudinal axis of the developing embryo.

The densely packed cells of the notochord and somatic mesoderm interact with one another and/or with the extracellular matrix between the cells; through this process, they undergo critical and complex changes in spatial regulation called “cellular intercalation”—inclusive of “protrusion.”7 Protrusions occur in parallel with the development of stiffening adhesions between neighboring cells; these adhesions allow the cells to impose traction upon each other, which in turn allows them to produce forceful intercalation and tissue organization.21 The ability to resist deformation while at the same time resisting rearrangement is accomplished by virtue of the cells developing in a tightly knit configuration as well as the development of stiffening adhesions between the cells.12,22 Through the use of SEM, we found that notochordal cells organize into a 3D configuration and over time “bud” within and out of the alginate globule containing the cells as they form a large, interconnected construct. Further, the presence of stiffening adhesions between cells of the tissue construct indicates that, under hypoxic conditions, notochordal cells are able to self-assemble a complex 3D cellular and extracellular matrix that in many ways appears to recapitulate key aspects of development (Fig. 6).

Cellular and Molecular Aspects of Hypoxia and NP Cells

To survive within the hypoxic nucleus, NP cells have been reported to highly express GLUT-1 (glucose transporter protein), a protein that facilitates the entry and utilization of glucose in anaerobic metabolism.1,17 Another key factor in the hypoxic environment in many cells and tissues is the multifunctional and atypical growth factor “connective tissue growth factor” (the current accepted terminology is CCN2), which is upregulated by hypoxia through HIF-1(hypoxia inducible factor)-dependent and TGF-β1-independent pathways.10 The expression of CCN2 is regulated by HIF-1α and in many cells is increased under hypoxic conditions.10 It is HIF-1α that regulates such mechanisms as glucose transport and angiogenic factors.
(where vasculature is present) such as VEGF, as well as various genes involved in cell cycle regulation and apoptosis.\textsuperscript{19} A multifunctional molecule, CCN2 is known to be involved in a host of cellular processes, such as cell proliferation, differentiation, matrix deposition and remodeling, and acquisition of tensile strength in cartilage due to its importance in the production of aggregan and link protein; it may confer anti- or proapoptotic signaling, depending upon the cells and tissues involved. With respect to pathological conditions in human patients, it has been reported that HIF-1$\alpha$ is more strongly expressed by NP cells in herniated human discs than in normal discs, suggesting an attempt at recovery/repair, although there is currently no explanation for the mechanism at play under these circumstances.\textsuperscript{6,14}

It has been reported that rat NP cells increase their expression of the classic chondrogenic genes \textit{collagen II}, aggregan, and Sox9 when cultured for 36 hours under hypoxia, providing further evidence of the beneficial effect of hypoxia.\textsuperscript{18} In this study it was observed that hypoxia activates Akt signaling, a factor considered important in the maintenance of Glut-1 transcriptional activity and, therefore, anaerobic delivery of glucose to the cells. Furthermore, activation of MEK1/ERK pathways by hypoxia was reported to confer protection from apoptosis in NP cells induced by hypoxia, and the products of the activation of these pathways may represent one mechanism whereby NP cells adapt to their environment.\textsuperscript{18}

\textbf{Matrix Production}

It has been demonstrated that notochordal cells of the NCD canine secrete a form of aggregan that is superior to that formed by the notochordal cells of the chondrodystrophic canine, in that the aggregan formed by NCD notochordal cells assembles into aggregates farther away from the cell surface and in so doing allows a superior hydrophilic capacity.\textsuperscript{6} Owing to the resistance to DDD and greater biomechanical load-bearing capacity of the NCD IVD, it has been postulated that the IVD that is rich in notochordal cells may confer superior biomechanical properties compared with the more fibrocartilaginous disc.\textsuperscript{14}

The results of this study suggest that future investigations of strategies focused upon cell-based therapies, particularly those involving notochordal cells, should culture such cells under hypoxic conditions since normoxia over the long term does not support cell survival and matrix deposition. Furthermore, hypoxia seems to provide the single necessary component to activate notochordal cells to proliferate and produce an abundant, biologically relevant extracellular matrix and to undergo self-assembly—akin to that very process which occurs during development. Understanding the biological mechanisms underlying the robust responses of notochordal cells to hypoxic tissue culture, the capacity of notochordal cells to stimulate other NP cells and particularly the role(s) played by reactive oxygen species will allow future mechanistic investigations of the responses of these cells to low O$_2$ environments. It may be that notochordal cells act as a kind of regenerative reservoir capable, under the right conditions, of inducing restorative processes that could
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Disclosure

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