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NUTRIENT CHANNELS AND STIRRING ENHANCED THE COMPOSITION AND STIFFNESS OF LARGE CARTILAGE CONSTRUCTS

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Abstract

A significant challenge in cartilage tissue engineering is to successfully culture functional tissues that are sufficiently large to treat osteoarthritic joints. Transport limitations due to nutrient consumption by peripheral cells produce heterogeneous constructs with matrix-deficient centers. Incorporation of nutrient channels into large constructs is a promising technique for alleviating transport limitations, in conjunction with simple yet effective methods for enhancing media flow through channels. Cultivation of cylindrical channeled constructs flat in culture dishes, with or without orbital shaking, produced asymmetric constructs with poor tissue properties. We therefore explored a method for exposing the entire construct surface to the culture media, while promoting flow through the channels. To this end, chondrocyte-seeded agarose constructs (Ø10 mm, 2.34 mm thick), with zero or three nutrient channels (Ø1 mm), were suspended on their sides in custom culture racks and subjected to three media stirring modes for 56 days: uniaxial rocking, orbital shaking, or static control. Orbital shaking led to the highest construct E_Y , glycosaminoglycan (GAG), and collagen contents, whereas rocking had detrimental effects on GAG and collagen versus static control. Nutrient channels increased E_Y as well as GAG homogeneity, and the beneficial effects of channels were most marked in orbitally shaken samples. Under these conditions, the constructs developed symmetrically and reached or exceeded native levels of E_Y (~400 kPa) and glycosaminoglycans (GAG; ~9%/ww). These results suggest that the cultivation of channeled constructs in culture racks with orbital shaking is a promising method for engineering mechanically competent large cartilage constructs.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest associated with this study.

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Keywords

Cartilage; Tissue Engineering; Nutrient Transport; Chondrocytes; Agarose

INTRODUCTION

Osteoarthritis (OA) is a debilitating disease characterized by damage to the articular cartilage layers lining diarthrodial joints. In advanced cases, lesions span the full thickness of cartilage, leading to bone-on-bone contact during joint articulation. With its relatively low cell density and avascular nature, adult cartilage lacks the ability to regenerate when injured, leading to irreversible degeneration. Currently, there is no treatment available to fully restore durable articular surfaces.

A potential approach to the treatment of OA is by culturing cells within a scaffold under suitable conditions *in vitro* in order to produce viable replacement cartilage (Johnstone et al., 2013; Langer and Vacanti, 1993; Vacanti and Langer, 1999). Culture of chondrocytes within agarose scaffolds is a well-established technique which stabilizes chondrocyte phenotype and encourages the elaboration of sulfated glycosaminoglycans (sGAG) which are similar to those found in native cartilage (Benya and Shaffer, 1982; Buschmann et al., 1992; Mauck et al., 2000; Mouw et al., 2005; O'Connor et al., 2014; Rahfoth et al., 1998). These chondrocyte-agarose constructs have had some success in reaching native values of compressive Young's modulus (E_y) and sGAG content when their dimensions are small (~Ø4 mm) (Bian et al., 2009; Byers et al., 2008; Cigan et al., 2013a; Nims et al., 2014); however, constructs of this size may not be sufficiently relevant clinically, as OA symptoms often manifest themselves when cartilage defects become larger, e.g., Ø25 mm (Curl et al., 1997; Moisio et al., 2009). Previous attempts to engineer cartilage constructs of this size have been met with transport limitations; the consumption of nutrients by cells at the construct periphery deprives its center of these nutrients, establishing concentration gradients throughout the depth of the construct. Depletion of glucose beneath a threshold level within constructs results in little to no local matrix elaboration by chondrocytes (Cigan et al., 2013a; Nims et al., 2014) and dissolved oxygen plays a prominent role in chondrocyte metabolism and forms gradients within tissue engineered cartilage (Obradovic et al., 1999; Zhou et al., 2008). Furthermore, interplay between glucose, oxygen, and other nutrients elicit a broad range of metabolic behaviors that will vary with nutrient concentrations throughout the depth of the tissue (Heywood et al., 2006a; Heywood et al., 2006b; Sengers et al., 2005; Zhou et al., 2008). Thus as construct scale is increased, the general result is a heterogeneous construct with a soft, matrix-deficient center, with poor functional properties, that would be unsuitable for implantation (Hung et al., 2003; Hung et al., 2004).

A number of techniques have been implemented in attempts to improve nutrient transport in large engineered cartilage constructs, such as incorporation of nutrient channels to reduce the necessary path length along which nutrients must travel to reach the construct center (Bian et al., 2009; Buckley et al., 2009), stirring or perfusion of culture media to prevent stagnation around the construct (Davisson et al., 2002; Vunjak-Novakovic et al., 1999), and dynamic loading to pump solutes into the tissue (Albro et al., 2008; Chahine et al., 2009;

Mauck et al., 2003a; Mesallati et al., 2011). In statically cultured Ø10 mm chondrocyte-agarose constructs, the incorporation of nutrient channels has yielded higher E_Y , approaching native values (Bian et al., 2009); in Ø6 mm constructs, microchannels combined with rotational culture improved sGAG distribution, though sGAG content and E_Y fell short of native levels (Buckley et al., 2009). In chondrocyte-seeded Ø10 mm PGA scaffolds cultured flat in Petri dishes, orbital shaking at ~0.8 Hz increased collagen but not sGAG content versus static control, though sGAG and collagen contents were below that of the native tissue (Vunjak-Novakovic et al., 1996).

In our studies, Ø10 mm chondrocyte-agarose constructs cultured flat in Petri dishes with 0.8 Hz orbital shaking swelled asymmetrically due to extreme heterogeneity of sGAG deposition, rendering unconfined compression testing infeasible (Figure 1). Peripheral regions in some specimens reached twice the thickness of the central region, and many samples experienced warping due to the occlusion of fresh media to the downward-facing sides of the constructs. In channeled constructs, the direction of media flow produced by orbital shaking was perpendicular to the nutrient channels, and channels were not found to alleviate matrix heterogeneities nor did they increase matrix content.

Therefore, we sought to overcome the matrix heterogeneities observed in large constructs by manipulating the culture system to maximize the benefits of nutrient channels. Specifically, it was hypothesized that introducing media stirring and nutrient channels aligned with the direction of media flow could produce large, symmetric, and homogeneous constructs with functional properties consistent with those of our previous studies on smaller constructs (Bian et al., 2009; Cigan et al., 2013a; Kelly et al., 2009; Kelly et al., 2006; Kelly et al., 2008; Lima et al., 2007; Ng et al., 2009; Nims et al., 2014). To this end, we recently developed a simple and novel technique to reorient large constructs and maximize their surface area available for nutrient transport in culture dishes, and media stirring was introduced to the culture system to improve nutrient channel efficacy (Cigan et al., 2013b). Constructs were evaluated both quantitatively for their mechanical properties and content of the major matrix constituents sGAG and collagen, and qualitatively by histological staining for distribution of these matrix components.

METHODS

Mold fabrication

To produce constructs with precise arrangements of channels, a half-mold was fabricated (Figure 2A). A 6.35 mm thick PTFE (polytetrafluoroethylene) sheet (McMaster-Carr) was milled to size (76 mm × 102 mm), and a CNC mill was used to drill an array of Ø0.94 mm holes with 2.165 mm center-to-center distance in a triangular packing configuration. Stainless steel pins (Ø1 mm × 10 mm, McMaster-Carr) were press-fitted into the holes. By suppressing certain pins, several channel arrangements could be produced. For this study, equilateral triangles of pins spaced 4.33 mm center-to-center were deployed to produce three Ø1 mm channels per construct. This triangular pattern was selected such that, when imposed upon a Ø10 mm circle, the distance from the circle's center to the margin of the nearest channel (2 mm) matched the distance from the circle's edge to the channel margin. The PTFE half-mold was separated from a glass slide by a 2.34 mm-thick silicone rubber spacer.

Culture rack fabrication

Custom culture racks were fabricated to suspend constructs, exposing nearly all surface area to media as well as orienting channels in the direction of media stirring. The racks were comprised of PTFE as this material has low binding affinity for TGF- β , as assessed in a preliminary test. Trapezoidal pieces of PTFE (30 mm base, 8 mm height) were fashioned from a 6.35 mm thick sheet with a laser-cutter, and a drill press was used to place twelve $\varnothing 0.99$ mm holes in two rows. Twelve PTFE-coated stainless steel wires ($\varnothing 1.00$ mm, 43 mm length, McMaster-Carr) were used to connect pairs of PTFE pieces. The spacing of wires allowed constructs to rest loosely upright in the culture dish without impeding media flow through the horizontally oriented nutrient channels (Figure 2B).

Harvest and cell isolation

Chondrocytes were harvested and isolated as described previously (Cigan et al., 2013a). Briefly, slices of articular cartilage were sterilely harvested from juvenile bovine calf wrists (local abattoir) and subsequently digested with collagenase (Sigma-Aldrich); the cartilage digest was then filtered through a 70 μ m mesh (McMaster-Carr) to remove debris before casting.

Construct preparation

Isolated chondrocytes were suspended in chondrogenic media (CM) (Cigan et al., 2013a) at a concentration of 120×10^6 cells/mL and combined 1:1 with molten 4% w/v type VII-A agarose (Sigma-Aldrich) to produce a 2% agarose gel with a nominal cell density of 60×10^6 cells/mL. The gel was cast into either the PTFE-glass mold (to produce channeled constructs) or a glass-glass mold (to produce constructs without channels) and incubated 20 minutes at room temperature to solidify. The molds were opened and a $\varnothing 10$ mm biopsy punch (Fisher Scientific) was utilized to create 2.34 mm-thick constructs with either three channels (3ch) or no channels (0ch).

Construct culture

The constructs were suspended in culture racks submerged in CM within deep Petri dishes ($\varnothing 63$ mm $\times$ 22 mm, Fisher Scientific). Dishes were either stirred throughout the culture duration on an orbital shaker (VWR International) at 0.8 Hz or on a rocker (Stovall Life Science) at 0.4 Hz, or were cultured statically (videos demonstrating shaking methods may be found in Supplementary Materials). The dishes were oriented on the rocker such that the direction of flow was normal to the flat faces of the cylindrical constructs, and therefore aligned with the channels.

In a preliminary study, acellular channeled 8% agarose gels in culture racks were subjected to boluses of 10 kDa fluorescein-conjugated dextran (Life Technologies) and imaged after 2 h by fluorescent microscopy (Figure 3); both 0.8 Hz orbital shaking and 0.4 Hz rocking improved the distribution of dextran to the regions surrounding channels, as compared to static controls.

All constructs were supplemented with 10 ng/mL human recombinant TGF- $\beta 3$ (R&D Systems) for the first 14 days of culture (Byers et al., 2008), a volume of 6 mL of CM for

each construct was implemented, and media were changed thrice weekly. Spent media samples were taken from each group at each media change.

Mechanical testing

At days 0, 35, and 56, constructs from each group ($n = 5$) were removed from culture. The diameters and thicknesses of constructs were measured, and the samples were subsequently tested in a custom unconfined compression apparatus under a creep tare load (0.23 N, 400 s) followed by stress-relaxation to 10% strain (0.58 $\mu\text{m/s}$ ramp, 11×10^3 s relaxation). The relaxation time was scaled proportionally with construct cross-sectional area based upon testing protocols for smaller constructs ($\varnothing 4$ mm, 1.8×10^3 s). E_Y was calculated from the construct cross-sectional area and the equilibrium load and displacement.

Biochemical composition

Mechanically tested samples were bisected and weighed, and half of each construct was lyophilized and digested in 0.5 mg/mL proteinase K solution (Fisher Scientific) overnight at 56 °C (Hollander et al., 1994; Kelly et al., 2006). Sulfated GAG content was determined by dimethylmethylene blue spectrophotometric assay (Farndale et al., 1986; Kelly et al., 2006), collagen content was assessed by orthohydroxyproline assay of acid-hydrolysates of proteinase K digests (Kelly et al., 2006) assuming a 1:7.64 OHP-to-collagen mass ratio (Hollander et al., 1994), and DNA content was determined by PicoGreen dsDNA quantitation assay (Life Technologies) using lambda bacteriophage standard. sGAG and collagen contents were expressed as a percentage of the constructs' final wet weights (%/ww), and DNA content was used to estimate cellularity assuming 7.7 pg DNA/cell (Kim et al., 1988; McGowan et al., 2002). Aliquots of spent media, taken at the time of each media change, were also assayed for sGAG content. Cumulative masses of media sGAG over the culture period were calculated and normalized to the day-0 volume of the constructs (mg/mL). Additionally, sGAG retention fractions were computed based upon the formula:

$$R.F. = \frac{\text{mass}(sGAG)_{\text{construct}}}{\text{mass}(sGAG)_{\text{construct}} + \text{mass}(sGAG)_{\text{media}}} \times 100\%$$

Histology and immunohistochemistry

The remaining halves of the samples were further bisected into quarter-cylinders, and fixed in an acid-formalin-ethanol solution at 4 °C for 48 hours (Kelly et al., 2006). The fixed samples were then dehydrated with ethanol, cleared, and infiltrated with paraffin wax (Fisher Scientific). For each sample, one quarter-cylinder was oriented axially within the mold, and the other quartercylinder was oriented so as to cut transverse sections. A sledge microtome was used to cut 7 μm sections, which were subsequently stained for sGAG (1% Safranin O, Sigma-Aldrich) or for collagen (0.1% Picrosirius Red, Sigma-Aldrich) and mounted in toluene solution (Permount, Fisher). Polarized light microscopy was used to examine collagen morphology and orientation in Picrosirius Red-stained samples. To further clarify collagen distribution and type, a subset of sectioned samples were processed for collagen-specific immunohistochemistry (types I and II), which was performed as described

previously (employing primary antibodies derived from rabbit in place of mouse)(Mauck et al., 2003b).

Statistics

A two-way ANOVA ($\alpha = 0.05$) was employed (STATISTICA) to determine significance, using presence of channels and mode of stirring as independent factors and E_Y , sGAG, collagen, and DNA as dependent variables. Once significance was established, Tukey's HSD post-hoc tests were run to examine differences between experimental groups. Because multiple constructs were cultured within each dish, values for media sGAG represent averages for their corresponding groups without standard deviations; thus, statistical comparisons among groups for media sGAG were not performed.

RESULTS

The constructs were evaluated qualitatively by inspection and by histological staining, and quantitatively for mechanical stiffness and biochemical content. Because similar trends between stirring and channel groups existed at days 35 and 56 and overall functional properties were highest at the latter time point, only the day 56 data are presented herein.

Construct appearance

Constructs grew symmetrically and little to no regional variations in opacity or appearance were discernible within individual constructs. Samples that had been rocked during culture were the most translucent, and those that were orbitally shaken were most opaque (Figure 4). Some channels had decreased from their original diameters, and some had become partially or completely occluded due to matrix elaboration and swelling.

Histology and immunohistochemistry

Safranin O staining for sGAG was visibly darker in orbitally shaken constructs as compared to static controls, whereas constructs from the rocker exhibited lighter staining (Figure 5A). Thin regions immediately encompassing the channel edges exhibited lighter staining, though generally beyond this region darker staining was seen in the interior of channeled constructs as compared to no-channel controls. Some channels were partially or fully filled with matrix. When stained for collagen, variations between samples were less apparent (Figure 5B); however, channeled constructs tended to exhibit darker staining throughout their depths (as seen in sagittal sections). Generally, throughout their depths the channeled, orbitally shaken samples stained darker than all other groups. A fibrous collagen ring was observed around the peripheries of orbitally shaken constructs, particularly those with channels (Figure 6A). Polarized light revealed the presence of thick collagen bundles in this region (Figure 6B). Collagen-specific immunohistochemistry detected primarily type II collagen at the periphery and at the construct interior (Figure 6C). Type I collagen staining was also found in the periphery and at the center, to a lesser extent, but was most prominently localized in a transitional region in between.

Cellularity

All groups increased in cell density over the course of the culture period (Day 0 ρ : 85×10^6 cells/mL; $p < 0.0001$). Mode of stirring and presence of channels influenced cell density ($p < 0.01$ and $p < 0.05$, respectively; Figure 7A). Orbital shaking resulted in the highest cellularities, at $\sim 143 \times 10^6$ cells/mL ($p < 0.05$). Channels elicited a slight, yet significant overall decrease in cell density ($p < 0.05$).

Biochemical composition

Stirring condition had a highly significant influence on sGAG ($p < 0.0001$, Figure 7B). Orbital shaking yielded approximately 9%/ww sGAG, significantly higher than other stirring conditions ($p < 0.0005$). Rocked constructs had less sGAG than static controls ($p < 0.0005$). A similar trend was found for collagen (Figure 7C), in which orbital shaking yielded the most collagen ($\sim 2\%/ww$; $p < 0.0005$) and rocking yielded the least ($p < 0.0005$). Channels had no significant influence on total sGAG or collagen content per construct.

The highest cumulative media sGAG was found in the orbitally shaken, channeled group (42.1 mg/mL); all other groups had similar levels (~ 30 mg/mL, data not shown). Retention of sGAG within constructs was highest in orbitally shaken constructs ($\sim 78\%$ with or without channels) and lowest in rocked groups (38% with channels, 53% without channels); static controls had $\sim 67\%$ retention.

Mechanical properties

At day 56, E_Y (Figure 7D) varied from near-day-0 values at the lowest (rocked groups) to nearly 400 kPa in the highest group (3ch, orbited). Stirring type had a significant effect ($p < 0.0001$), as did presence of channels ($p < 0.05$). Orbited constructs were significantly stiffer than their static and rocked counterparts ($p < 0.0005$), as were channeled constructs over their no-channel controls ($p < 0.01$).

DISCUSSION

This study demonstrated that it is possible to engineer high-quality cartilage constructs on the scale of $\varnothing 10$ mm, by refining our culture system with channeled scaffolds and stirring during the cultivation. By suspending constructs on their sides in culture racks, the surface area was increased for nutrient diffusion, enhancing nutrient availability within the tissue and effectively ameliorating the uneven swelling and warping observed in preliminary studies (Figure 1 versus Figure 4). The alignment of channels in the direction of media flow resulted in stiffer constructs as compared to no-channel controls (Figure 7D). With the incorporation of nutrient channels and introduction of orbital shaking, constructs attained values of E_Y comparable to native values (~ 400 kPa) and sGAG levels ($\sim 9\%/ww$, Figure 7B) exceeding native values ($\sim 4\%/ww$) (Basalo et al., 2004); to the best of our knowledge, these are the highest sGAG levels to be observed in agarose-chondrocyte constructs of this size to date. Collagen reached levels typically observed in our smaller constructs ($2\%/ww$, Figure 7C) though these levels remain below native values ($\sim 10\%$) for immature bovine cartilage (Basalo et al., 2004).

Orbital shaking of constructs was found to significantly increase E_Y , sGAG, collagen, and construct cellularity (Figure 7A–D); staining for sGAG was also intensified, as was staining for collagen in the presence of nutrient channels. Stains also revealed a fibrous ring comprised of thick collagen fibers encompassing constructs that were subjected to orbital shaking (Figure 6AB), which is consistent with effects seen in spinner flask culture (Vunjak-Novakovic et al., 1999). Immunohistochemistry detected collagens type I and II in this ring, though type II was more abundant (Figure 6C). Quantitatively, ELISAs have previously shown that constructs of this type (juvenile bovine chondrocyte-agarose) produce predominantly type II collagen (Ng et al., 2009). Orbitally shaken constructs also exhibited the highest sGAG retention rates. Conversely, uniaxial rocking resulted in significantly decreased matrix concentrations (Figure 7B–C) as compared to statically cultured constructs, an observation consistent with histological staining in constructs' peripheral regions (Figure 5). Rocked specimens did not stiffen throughout the culture period, and they experienced the lowest retention of sGAG. This disparity occurred despite both stirring methods demonstrating similar potential for improving solute transport in acellular, channeled agarose gels in a preliminary study (Figure 3). These data suggest that the media flow induced by rocking at this frequency (0.4 Hz) either adversely affected matrix production by cells, washed matrix macromolecules out into the media before they became entrapped by the scaffold, or elicited some combination thereof.

The disparities between the rocking and orbital shaking observed in this study strongly suggest that ample validation must be performed for any tissue engineering system that incorporates media stirring. The detrimental effects of rocking on these constructs were unanticipated and the underlying cause was not fully elucidated from the available measurements and observations. However, from a tissue engineering perspective, in-depth investigation of those causes is not a high priority, as the primary goal of this study was to improve our culture system, which was amply achieved with orbital shaking (Figure 7) in custom racks that expose virtually all the construct surface area to media (Figure 2B). This superior stirring method may thus be employed in future studies with larger constructs.

At the conclusion of the 56-day culture period, many of the channels remained unoccluded and had not been entirely filled by neocartilage, an observation consistent with a previous study which employed Ø1 mm channels (Bian et al., 2009). This has the benefit of improving nutrient transport throughout culture, yet channel filling would be desirable when cultivating tissue for implantation. Furthermore, in constructs with supraphysiological levels of sGAG (i.e. those with orbital shaking), channels led to slight warping of constructs (Figure 5). Since such warping was not exhibited at day 35 (data not shown), it is likely that shorter culture durations or techniques to retard or halt sGAG production could mitigate this effect. In light of these observations, experiments seeking to fine-tune parameters such as channel diameter and culture duration may eventually be required to ensure timely channel filling and isotropic tissue growth.

The constructs in this study were subjected to their respective stirring conditions for the entirety of the culture period, and temporal effects of these stirring conditions were not explored. A number of culture techniques have been shown to possess temporal effects on the properties of engineered cartilage, such as transient TGF- β supplementation (Byers et al.,

2008), delayed dynamic compressive loading (Lima et al., 2007), transient hypoxia (Yodmuang et al., 2013), and transient perfusion (Davisson et al., 2002). In light of these findings, the effects of transient or delayed media stirring in this culture system could prove to be a worthwhile investigation. Furthermore, the frequencies of orbital shaking and uniaxial rocking were not modulated in this study, and relationships between stirring frequency and construct properties were not established. It is possible that an optimum frequency exists for orbital shaking which could produce even higher-quality tissue, or that certain frequencies could reconcile the detrimental effects of uniaxial rocking; these notions could also merit further study.

Due to the beneficial effects of nutrient channels on functional properties of constructs in culture racks with orbital shaking, we believe that this culture system holds promise for further investigations with large channeled constructs. Future studies will employ finite element modeling of nutrient consumption and matrix synthesis (Ateshian et al., 2014; Maas et al., 2012) to anticipate the optimal placement of nutrient channels within large constructs, for the purpose of producing tissue that will be more clinically effective for treatment of OA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Abnormal swelling of Ø10 mm constructs cultured flat in Petri dishes for 56 days with orbital shaking; (A) perspectives of whole specimens, and (B–C) profiles of bisected specimens with or without channels (channel positions denoted by dotted lines, regions of excessive swelling denoted by arrows).

Figure 2.
Diagram of (A) custom mold used to cast 3-channel constructs, and (B) constructs resting within their culture rack.

Figure 3. Distribution of bolus of 10 kDa fluorescein-dextran to PBS bath of agarose gels suspended within culture racks, subjected to (A) 0.4 Hz rocking, (B) 0.8 Hz orbital shaking, (C) static condition, after 2 h.

Figure 4. Construct morphology, freshly cast (Day 0) and after 56 days in culture under various stirring conditions.

Figure 5. Effects of culture conditions on the amounts and distributions of cartilaginous tissue components: stains for (A) sGAG by Safranin O and (B) collagen by Picrosirius Red in sample quarter-cylinders after 56 days of culture (both sagittal and transverse sections). Arrows indicate filled-in channel margins.

Figure 6.

Portion of orbitally-shaken, 3-channeled construct sagittal section visualized with (A) Picrosirius Red histological staining, (B) polarized light microscopy of Picrosirius Red stain, (C) overlaid collagen-specific immunohistochemistry of sample sections, colorized such that purple indicates collagen type I and green indicates collagen type II (dashed line marks sample edge). Arrows indicate fibrous collagen ring.

Figure 7.

(A) Cell density, (B) sGAG, (C) Collagen, and (D) E_Y after 56 days. (*) indicates $p < 0.05$ for the effect of stirring condition, (#) indicates $p < 0.05$ for effect of channels. Dotted line indicates D0 cellularity.