A target for biomimetics and synthetic biology: the notochord of the Atlantic hagfish, *Myxine glutinosa*.

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As the promising fields of biomimetics and synthetic biology (SB) foment[3], engineers and biologists are scrambling to find target systems that will expose design principles of tissues and organs that map isomorphically from functional macromolecules (FM) to mechanical behavior (MB). Keys to the pursuit are knowing (1) the physiological function of the target tissue or organ, (2) the FM involved, and (3) the causal linkage — the isomorphic design principle — between FM and MB. Using the notochord of hagfish, we propose a design principle, the **cellular-hydrostat network (CHN)**, that links the notochord's bending functions during swimming and the intermediate filament (IF) bounded, polyelectrolyte-filled vacuoles in the large cells of the notochord's lumen. According to the CHN, the notochord's network of vacuolated cells compartmentalizes and pressurizes fluid intracellularly, creating elastic-solid mechanical behavior in the absence of an extracellular matrix. We test the CHN model by disrupting the cellular network, retaining the now-extracellular fluid inside the notochord, and comparing the flexural stiffness, EI (in Nm^2) of intact and altered notochords under physiological bending. If accurate, the magnitude of the CHN effect should be proportional to its contribution to I (in m^4), the second moment of area, a factor that accounts for the presence and spatial arrangement of solid material in a bending section (Fig. 1).

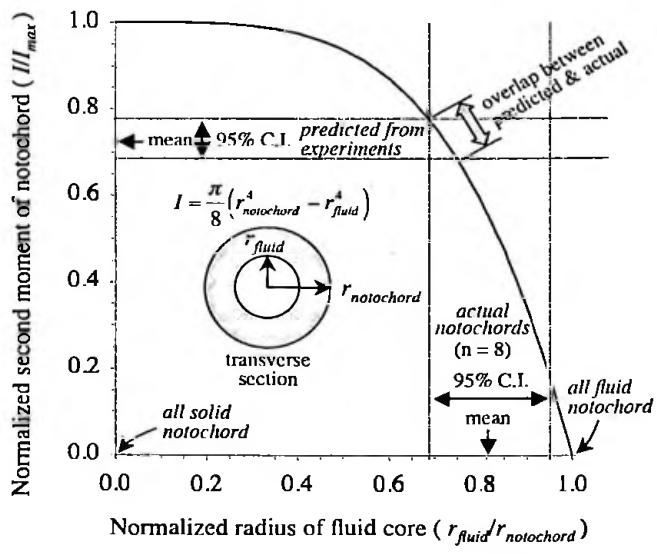

Figure 1. Cellular-hydrostat network (CHN) model for the notochord, where the vacuolated cell network in the core acts as an elastic solid by compartmentalizing and pressurizing the intracellular fluid. According to the CHN model, as the radius, r_{fluid}, of any non-vacuolated fluid portion of the notochord increases with relation to the total radius of the notochord, $r_{notochord}$, it decreases the second moment of area, I, a measure of the structural contribution (transverse shape and distribution of solid material) to flexural stiffness, EI. Because of the precipitous decline in I/I_{max} as r_{fluid} increases to more than 50% of the total radius, $r_{notochord}$, we predict that the network of vacuolated cells functions to produce, from the large potential fluid volume of the core, an elastic, solid-like notochord. That prediction is supported if the experimental destruction of the vacuolated cells, which produces a non-compartmentalized fluid core, reduces the flexural stiffness, EI, of the notochord by reducing I. Since we normalize all mechanical data by I assuming a solid notochord (Fig. 2), the apparent E should, with destruction of cells and the CHN, change in proportion to the actual I, as indicated here by the change in I/I_{max} predicted from bending experiments (see Fig. 2). The mean and range for the normalized radius of the fluid core from actual notochords is shown. The overlap in predicted and actual values on the curve support CHN. C.I. = confidence interval.

We tested the notochords from three wild-caught adult Atlantic hagfish, *Myxine glutinosa* (Huntsman Marine Laboratory, New Brunswick, Canada), that were kept for six weeks in running seawater at temperatures ranging from 13 to 15°C and fed daily on squid. The three fish weighed 68, 70, and 90 g and measured in total length 36.0, 39.0, and 40.0 cm, respectively. Each animal was

euthanized with an overdose of propylene phenoxetal. Immediately following, notochords were dissected from the animals and quartered axially in-between 00-silk ligatures that sealed the sections. The two middle quarters were used for experiments and randomly assigned to either the control or freeze-thaw treatment category. Thus each individual contributed a single control and treatment sample to the experiment. Notochord sections were equilibrated in pH 7.8, 906 mOsm hagfish Ringer solution[6] for 30 minutes before freeze-thawing. Control sections were left in Ringer on an ice bath. Treated sections were placed in a tube with enough Ringer to cover the sample and were completely frozen at -74°C in 16 to 24 minutes. Once frozen, samples were placed in a 13° C sea water table, thawing in 10 to 17 minutes. This freeze-thaw cycle was repeated three times. Thawed treated samples were equilibrated to the same temperature as the controls. To verify that this process lysed the cells, we glutaraldehyde-fixed and sectioned treated and control samples after running experiments. In frozen-thawed sections thus produced, fluid drained from the specimen; no fluid was lost from the control specimens when sectioned. Visual inspection showed an intact extracellular fibrous sheath while the vacuolated cells in the notochordal lumen were completely eliminated (Fig. 2).

Mechanical properties of each notochord section were determined using a custom-built dynamic bending machine. The bending machine consisted of a four-bar linkage driven by a DC motor (Bodine series 200) and controller (Minarik model SL52). At the hinge between the fixed and driver linkages one end of the notochord was mounted, allowing the motor to apply an end moment to small (0.5 cm long) portions of the samples. The resulting bending moment, M (in Nm), was transduced at the fixed end of the notochord, on the opposite side of the bending section, which was mounted to a cantilevered force transducer (two 120 Ω resistors in a half-bridge configuration on steel stock) excited and amplified by a bridge amplifier (Omega model DMD-520; 40 kHz response time). The curvature, κ (in m^{-1}), applied to the sample by the motor-linkage system was transduced using a rotary-variable-differential transducer (Shaevitz model DC) mounted rigidly to the driver linkage and coaxially to the linkage hinge. From the linkage hinge angle, θ (in rad), κ was calculated from analytical geometry:

$$\kappa = \frac{4\cos\left(\pi - \theta/2\right)}{l}$$

where l is the length (0.5 cm) of the test section. Moment and curvature signals were sampled at 200 Hz using an analog-to-digital converter system (Vernier model LabPro and LoggerPro 3.3).

The notochord sections were bent cyclically and sinusoidally at physiological tailbeat frequencies, f (1, 2, 3 Hz), and body curvature amplitudes, κ_0 (26, 54, 80 m^{-1}). For each of 6 samples (3 individuals, 2 samples each) all possible combinations (9) of frequencies and curvatures were tested in random order. For each test, flexural stiffness, EI, was calculated as the quotient of moment amplitude, M_0, and κ_0, averaging data from three bending cycles at least ten cycles past test initiation to allow for preconditioning. The apparent complex modulus, E (in Pa), was estimated as the quotient of EI and I, where I is determined from the radius of the notochord, assuming a liquid core of zero radius. The storage modulus, E' (in Pa), loss modulus, E'' (in Pa), and the tangent of δ (E''/E'), were calculated as the product of E and the cosine of δ, and E and the sine of δ, respectively, where δ is the phase lag (in rad) between the peak M_0 and κ_0. E' and E'' are measures of the purely elastic-solid and purely viscous-fluid stiffness properties, respectively. The CHN model predicts that the core will act as a fluid when the cell network is disrupted, reducing I (see Fig. 1); thus, if disruption of the cell network decreases E and E' under the all-solid I assumption then the CHN model is supported.

To determine if the disruption of the vacuolated cells by freeze-thawing decreased either E or E', we analyzed the bending data using a mixed-model, three-way, factorial ANOVA with individual as a randomized block (JMP software). The three main factors were (1) treatment (control, lysed cells), (2) f, and (3) κ_0. As predicted by the CHN model, the apparent E and E' decreased significantly (α = 0.05) with lysed cells (Fig. 2). Qualitatively, this result supports the general feature of CHN, that the network of vacuolated cells creates an elastic, solid-like core.

To examine the quantitative accuracy of CHN, we measured the outer radii and the inner luminal radii of eight notochord sections. The mean and 95% confidence interval are plotted on the abscissa in Figure 1 to show the range of actual fluid/solid radii. The lower end of that range of actual radii is predicted by the range of fluid/solid I values generated from the change in apparent E with the lysing of cells (Fig. 2). To convert changes in E to changes in I, we assumed that E remained constant and all changes in EI were caused by changes in I. The mean and range of fluid/solid I values predicted from the mechanical experiments are plotted on the ordinate (Fig. 1). The overlap of predicted and actual values (see Fig. 1) shows, at the very least, that the CHN model accurately predicts the mechanical behavior of the vacuolated cells of the notochord.

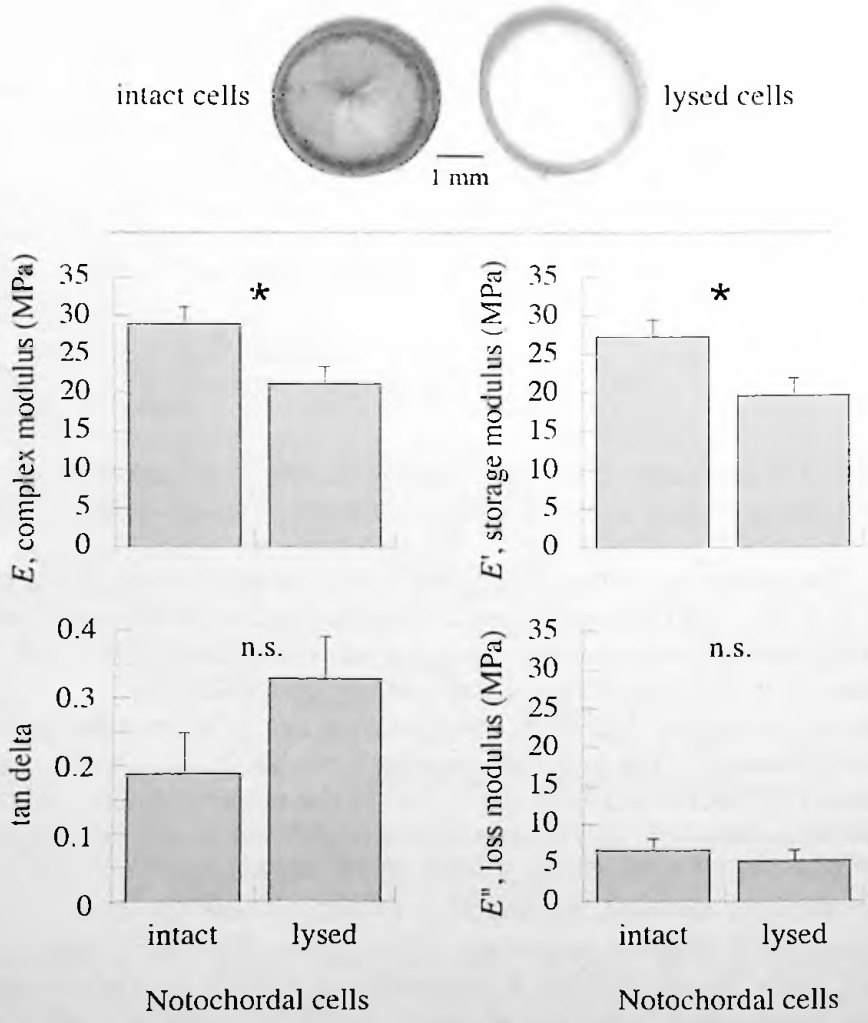

Figure 2. Test of the cellular-hydrostat network (CHN) model. Freeze-thawing lyses cells in the core (top panel: transverse sections). Assuming that the notochord in both conditions has a solid core, the apparent complex and storage moduli, E and E', decrease significantly, by an average of 27 and 28%, when the cells are lysed. According to the CHN model, however, this decrease in apparent E is predicted by a decrease in I if the notochord is assumed to have converted a solid-like core to a fluid one after the cells were lysed. This would reduce the ratio of the I to I_{max} as predicted by the change from a solid radius to one with a fluid core (Fig. 1). As plotted in Fig. 1, the change in I predicted from the range of experimental data depicted here overlaps the lower range of ratios of fluid radius, r_{fluid}, to total notochord radius, $r_{notochord}$. Thus CHN is supported. Moreover, the fact that the loss modulus, E'', remains unchanged suggests that CHN adds elasticity *per se* (E') rather than reducing viscocity (E''). Levels of statistical significance are $p < 0.05$, *, $0.05 < p < 0.10$, (*), and $0.10 < p$, "n.s." Statistical analysis described in text.

We contend that the notochord is an excellent system for biomimetics and SB, since, in addition to the CHN model provided here, recent genetic and developmental studies[9] have linked genes and gene products to notochord phenotype. Moreover, ultrastructural and immunocytochemical observations have identified FM and cellular structures[7] that potentially contribute to load-bearing in the notochord. IFs surround vacuoles that contain a polyelectrolyte and these vacuolated cells are extensively interconnected with desmosomes *via* the IFs. While attention has been given to the ultrastructure and composition of these giant vacuolated cells, their mechanical function remains unclear. Schmitz[7] suggested that the resulting desmosome-cell membrane-IF network is sufficient to give the notochord important functional properties. The results presented here indicate that the CHN compartmentalizes liquid resulting in an increase in the notochord's complex and storage moduli, E and E', respectively.

For bioengineers engaged in biomimetics or SB, this study should raise a red flag: why build a solid-like network of cells (the notochordal lumen), particularly when a portion of the structure is successfully composed of extracellular fibers (the notochordal sheath)? The engineering challenge presented by cells is that they require an exchange system for fuel, dissolved gases, amino acids, and wastes. The benefit — beyond the obvious ability to grow and repair — is that cellular systems can change, in response to external environmental stress, entire locomotor organ systems. Within a few weeks, for example, environmental stress induces changes in the notochord-containing tail of amphibian tadpoles; that such plasticity is heritable[5] and potentially mediated hormonally by corticosterone[1] suggests that the CHN has, in addition to its proximal mechanical function, the function of regulating the notochord's mechanical properties. While the osmotic activity of the notochord can alter its bending stiffness *in vitro*[8], and hydrostatic pressure within the CHN varies by individual and body position *in situ*[2], we do not yet know if hagfish use the CHN in life to modulate the locomotor function of their primary axial skeletal system[4].

We thank the following funding sources: the Shriners Hospitals for Children (8610) and the National Science Foundation (BCS-0320764 and DBI-0442269).

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