The Effect Of Osmotic Stress On The Structure And Properties Of The Cell Nucleus

by

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Dissertation submitted in partial fulfillment of the requirements for the degree Doctor of Philosophy in the Department of Biomedical Engineering in the Graduate School of Duke University

2010
ABSTRACT

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Abstract

Chondrocytes maintain cartilage by transducing joint load into appropriate biosynthetic activity, a process commonly known as mechanotransduction. Malfunctioning mechanotransduction leads to cartilage degradation and osteoarthritis. The mechanism of mechanotransduction is only partially understood but osmotic stresses are thought to play an important role. This study shows that the chondrocyte nucleus shrinks and wrinkles under hyper-osmotic stress. It shrinks because the chromatin inside the nucleus contracts as the macromolecules in the cell become more crowded. It wrinkles because the nuclear lamina buckles as the nucleus contracts. These morphological changes accelerate transport across the nuclear envelope. Many cells have organized actin caps around their nuclei that constrain the nucleus from contracting under hyper-osmotic stress. Agents exist that can reverse this loss of osmotic sensitivity in vitro without damaging the cell.
Dedication

This work is dedicated to my beloved wife, Loren, who is always on my side.
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1. Background and Significance

1.1 Mechanotransduction and Osmotic Stress

Mechanotransduction is the process by which a cell generates a biological response to a mechanical load. It is a vital function in many cell types. The ability to respond to mechanical stresses allows homeostatic cells to adjust the properties of tissues to applied loading. For example, osteoclasts and osteoblasts can perceive increased loading of a bone and shift the balance of anabolic and catabolic activities to strengthen the bone (1). Solid stress is far from being the only physical signal that cells sense. The extracellular matrix frequently has complex properties that couple mechanical and electrochemical stresses so that the matrix may convert the applied physical signal into another signal that affects cell function. For example, articular cartilage is a triphasic material consisting of a porous solid matrix permeated by a solution of water and ions (2). Aggrecan molecules embedded in the solid matrix contain immobilized, negatively charged sulfate and carboxyl groups that attract dissolved ions. Fluid is drawn into these local accumulations of ions by diffusive effects that tend to eliminate spatial gradients of concentration (3). This pressurization of fluid by diffusive effects is known as osmotic pressure. Robust collagen fibers that run through the solid matrix resist extension and constrain fluid absorption (4). Cartilage equilibrium is defined by the balance between the osmotic pressure drawing water into the tissue and the tensile stress in the collagen fibers squeezing water out of the tissue.
When the cartilage is loaded, fluid is exuded as the balance between osmotic pressure and solid stress shifts. However, dissolved ions remain trapped by the fixed charges in the matrix. The result is that the applied compressive stress causes a change in the osmotic environment of the chondrocytes that perform homeostatic functions within the cartilage. Such changes in extracellular osmolarity stimulate a range of biological responses in chondrocytes including gene expression, protein synthesis, calcium signaling and reorganization of the actin cytoskeleton, making osmotic stress a strong candidate as a mechanism of mechanotransduction in cartilage. Failure of this mechanism may cause failed homeostasis that in turn leads to osteoarthritis, a widespread and debilitating disease.

The multi-faceted biological response elicited by osmotic stress has attracted a great deal of study and much progress has been made in understanding the process at a cellular level. The plasma membrane that envelopes mammalian cells allows free passage of water but blocks passage of almost all solute molecules. Any imbalance between the ion concentration inside and outside the cell creates osmotic pressure that drives water across the membrane to correct the imbalance so the cell swells when extracellular osmolarity falls and shrinks when extracellular osmolarity rises. This inverse proportionality between volume and osmolarity is stated mathematically by the Boyle Van’t Hoff relation, which normalizes volume and osmolarity to the iso-osmotic condition and takes the following form:
\[
\frac{V}{V_0} = A \left( \frac{c_0}{c} \right) + (1 - A)
\]

where \( V \) is the cell volume, \( c \) is extracellular osmolarity, subscript \( 0 \) denotes the iso-osmotic condition and \( A \) is a constant known as the Ponder's value. The Ponder's value is defined as the chemical activity of the intracellular water relative to the iso-osmotic state. It can also be understood as the fraction of the cell volume that is osmotically active. The remainder of the cell volume is osmotically inactive and is not diminished even when the osmolarity becomes infinite and all available water has been drawn out of the cell. A system that obeys the Boyle Van't Hoff relation is known as a perfect osmometer (Figure 1).
Figure 1: Behavior of a perfect osmometer. (A) When solute is added to the beaker on the left, water is drawn out of the osmometer but no solute is exchanged. (B) The volume versus osmolarity (denoted as c) curve becomes linear when osmolarity is inverted.

Several investigators have found that the perfect osmometer is an excellent model for mammalian cells when biological activity is suppressed. Chondrocytes(10), neutrophils(16), pancreatic islet cells(17) and lymphocytes(18) all agree well with the model. The volume change inherent in the perfect osmometer behavior of the cell would have severe consequences for cell functions if it were not controlled because the change in water content would alter the concentrations of molecules in the cytoplasm. Cells cannot resist osmotic pressure through mechanical stress because the mechanical stiffness of the cell is insignificant on the scale of osmotic pressures (19). However, cells
can control their volume by pumping ions across their membranes. This causes a flow of osmotically obliged water across the membrane and allows the cell to regulate its volume when the osmotic environment changes. For example, annulus fibrosus cells maintained at body temperature swell with a time constant of 58 seconds after a step hypo-osmotic load. However, they then begin pumping ions across the cell membrane and partially correct their volume with a slower time constant of 330 seconds (20).

Recently, an alternative to the perfect osmometer model has been proposed (21). Gels are porous solids permeated by fluid. They contain tortuous spaces that are too small to admit certain large solute molecules. Water can pass freely in and out of such a space but solute molecules cannot, blocked in this case not by a semi-permeable membrane but by steric exclusion effects. The concentration of solute in the pore fluid of the gel is lower than the concentration in the bath surrounding the gel as a result. The ratio of the concentration within the gel to the concentration in the surrounding fluid is defined as the partition coefficient and is determined by the porosity of the gel and the size of the solute molecule. For small solutes like ions, the steric exclusion effect is negligible and the partition coefficient is one. When the solute is larger, steric exclusion effects keep solute out of a significant fraction of the pore fluid and the partition coefficient becomes less than one (Figure 2). In this approach, the reference condition is when the gel is surrounded by a fluid with a solute concentration of zero since this is the only condition in which the inaccessible spaces within the gel are at equilibrium with the
rest of the solution. When the solute concentration is greater than zero, diffusive effects
suck water out of the inaccessible spaces in the gel to equilibrate them with the rest of
the solution. The cumulative suction is proportional to the difference in the
concentration inside and outside the gel. Since the two concentrations are related by the
partition coefficient, the following equation emerges:

\[ P = RT(1 - \kappa)c \quad (2) \]

where \( P \) is the osmotic pressure drawing water out of the gel, \( R \) is the universal
gas constant, \( T \) is the temperature, \( \kappa \) is the partition coefficient and \( c \) is the concentration
of the surrounding fluid. Drawing fluid out of the gel causes it to contract and imposes
a volumetric strain on the solid phase. Equilibrium is reached when the stress in the
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the osmotic pressure increases, the solid stress must also increase and the volumetric
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Figure 2: Schematic showing osmotic pressurization of a gel in the absence of a semi-permeable membrane due to steric exclusion effects. The circle represents a bead of a gel immersed in a solution of a macromolecule, colored green. The shade of green is lighter in the bead to represent the fact that the solute concentration is lower in the pore fluid of the gel. The concentration is lower because small pores and channels in the gel are inaccessible to the solute molecule, as shown in the close-up view of a portion of the gel.

1.2 The Cell Nucleus

The cell nucleus is a large, intracellular organelle that usually occupies about 10% of the cell volume. Its function is to contain the genome and control access to it. While
the genome is frequently thought of abstractly as a one-dimensional sequence of base pairs, its physical form is a series of long DNA strands coiled around histones and packaged in a hierarchical structure inside the cell nucleus. The nucleus is surrounded by the nuclear envelope, which consists of two lipid bilayers called the inner and outer nuclear membrane. The nuclear envelope is punctuated by nuclear pore complexes. Each nuclear pore complex contains more than 50 proteins organized into an octagonal structure around a central channel. The central channel is partially obstructed by flexible, unstructured nucleoporins that extend into the lumen (Figure 3) and block passage of molecules larger than 9nm in diameter (22). However, larger molecules can pass through the channel if they can bind to phenylalanine-glycine repeats in these extended nucleoporins (23). In this way, the nuclear pore complexes form a selective sieve that allows passage of small solute molecules such as ions but blocks passage of larger molecules unless they are bound to a nuclear transport receptor. The nuclear envelope is supported by a mesh of cytoskeletal proteins called the nuclear lamina. At interphase, the chromosomes are anchored to the lamina and occupy irregular, non-overlapping spaces inside the nucleus called chromosome territories (24). The nucleolus is small dense body near the center of the nucleus that produces ribosomes (25). Smaller bodies known as Cajal bodies and speckles exist throughout the nucleus. Their exact function is a subject of ongoing investigation but they appear to play a role in RNA
processing. All the subnuclear bodies, including the nucleolus, are macromolecular aggregates, not membrane bound vesicles (26).

Figure 3: Top view of nuclear pore complex. The subunits of the complex form an octagonal ring around a central channel. The channel is partially occupied by flexible, extended nucleoporins that bind to nuclear transport receptors and exclude other molecules, creating a selective sieve.

It is not trivial to extend our understanding of the osmotic response of the cell to the nucleus because the physical properties of the two structures are very different. The nucleus is several times stiffer than the cytoplasm (27, 28). While the plasma membrane effectively blocks the passage of dissolved ions, the nuclear envelope allows them to cross in both directions. In a study of chondrocytes in articular cartilage, the nucleus
changed shape and volume in response to bulk compression of the surrounding tissue (29). The change in shape was abolished by disruption of the actin cytoskeleton but the change in volume was not. The goal of disrupting the actin cytoskeleton in this experiment was to break the mechanical connection between the extracellular matrix and the nucleus. Therefore, the findings show that nuclear shape change is due to transmission of mechanical stress through the cytoskeleton but nuclear volume change depends on another mechanism. Osmotic stress is a plausible mechanism for nuclear volume change. Compressive stress in articular cartilage increases the osmolality of the interstitial fluid in the cartilage (see above), regardless of actin organization. In a study of isolated chondrocytes, osmotic loading caused volume change in the nucleus and the volume change was insensitive to disruption of the actin cytoskeleton (30). Thus, there is evidence that cell dilatation is not transmitted to the nucleus through the actin cytoskeleton. The actual mechanism of nuclear dilatation remains unknown.

It is difficult to identify a single mechanical model that explains all the observed behaviors of the nucleus. Stiffness seems to depend on the length scale at which it is measured (31). When a 100 μm bead perturbed only by Brownian forces was tracked after microinjection into the nucleus, the subsequent analysis described the nucleoplasm as a Maxwell fluid with a Young’s modulus of 36 Pa (32). When a 500 μm bead was microinjected into the nucleus and loaded magnetically, the nucleoplasm was reported to be a Maxwell fluid with a Young’s modulus of 250 Pa (33). Micropipette aspiration
(27, 34) and unconfined compression of whole isolated nuclei (35) lead to Young’s moduli in the kPa range. Creep of nuclei obeys power-law rheology (31), which states that no single time constant dominates the response. This behavior is typical of gels and glasses, materials that exist near the transition between liquid and solid behavior (36). In these experiments, the nucleoplasm is more viscous than the lamina. The nuclear lamina is a two-dimensional viscoelastic solid (37) and the lipid bilayers in the inner and outer nuclear membrane are two-dimensional viscoelastic fluids (38). The distinction is that an elastic solid such as the lamina resists shear and so buckles under compressive strain. The lamina buckles in this way during micropipette aspiration experiments (39, 40). While our understanding of nuclear mechanics is incomplete, the available data points towards a model of the nucleus as a viscoelastic gel contained in a viscoelastic membrane.

The nuclear lamina is a two-dimensional mesh of intermediate filaments called lamins that supports the nuclear envelope (41). Lamins can be divided into A-type and B-type. The A-type lamins are lamin A and lamin C, which are produced by alternate splicing of the LMNA gene (42) and are commonly referred to together under the generic title lamin A/C. B-type lamins are encoded by two separate genes, LMNB1 and LMNB2 (43). Lamin B is expressed in all cells (44) but does not influence nuclear mechanics (45). Lamin A/C is not expressed in embryonic or adult stem cells and expression of these proteins increases as the cell differentiates (46). As the expression of
lamin A/C increases, the stiffness of the nuclear lamina increases, creating an intriguing parallel between the mechanical flexibility of the nucleus and the flexibility of cell fate(47). Although lamin A/C is primarily concentrated in the nuclear lamina, there is a population at much lower concentration distributed throughout the nucleoplasm and FRAP experiments show that a portion of the population in the nuclear lamina is continuously exchanged with the population in the nucleoplasm (48). Lamin connects to chromatin via proteins of the nuclear envelope such as emerin (44).

Mutations in the LMNA gene are responsible for a group of diseases known as laminopathies that includes Emery Dreifuss muscular dystrophy (49), dilated cardiomyopathy (50), familial partial lipodystrophy (51) and limb girdle muscular dystrophy (52). Symptoms include wasting in certain muscles, redistribution of fat, tendon contractures and cardiac conduction problems (44). The most devastating disease associated with an LMNA mutation is Hutchinson Gilford Progeria Syndrome (HGPS) (53). HGPS is a rare disorder that causes accelerated aging of certain tissues. The brain matures normally but all the load bearing tissues (heart, skin, bone, cartilage etc.) show symptoms of old age in juvenile subjects. Patients typically die of coronary artery disease in their teens. The apparently selective effects of the disease on mechanically loaded tissues lead some to suggest that it is caused by the inability of the mutant lamins to form an effective protective sheath around the genome while others suggest that lamins play a crucial role in mechanotransduction of extracellular signals.
into the nucleus and that this function is defective in mutant cells. The two hypotheses are not mutually exclusive and may in fact be closely related. Experiments on \textit{Lmna}-/- cells showed that nuclei stretch and rupture more easily and transcription of NF-κB in response to mechanical strain was also inhibited (54). The nuclei of cells from HGPS patients have an irregular shape in contrast to the typical smooth profile of healthy nuclei (55). They are not more fragile than healthy nuclei but it appears that it is more difficult for lamins to rearrange themselves within the lamina (48). Interest is growing in these mutations as a model for normal aging following the discovery that lamin A/C expression declines with age and some symptoms of laminopathies such as irregularly shaped nuclei are seen in cells from healthy, geriatric subjects (56). This raises the possibility that lamins may play a role in diseases associated with malfunctioning mechanotransduction and old age such as osteoarthritis.

\section*{1.3 Genome Organization}

The human genome would extend for over two meters if stretched out straight yet it is packed into a nucleus that typically measures about 6 μm across. This dramatic compaction is achieved without obstructing transcription factors that must have access to genes. The organization of the genome is hierarchical and historically, the investigation of genome organization began at the smallest level and has proceeded up to larger length scales as new tools have become available. The familiar DNA helix is
coiled 1.65 times around a protein core to create a fundamental organizational unit called a nucleosome (57). The protein core consists of two each of the histones H2A, H2B, H3 and H4. Multiple nucleosomes are arranged along a long strand of DNA in this way to make a molecule called chromatin. When chromatin is isolated and exposed to very low salinity, it extends due to repulsion between negative charges on the DNA. Electromicrographs of these samples have the appearance of beads on a string and the fiber is typically about 11 nm wide at its widest point (58). At more physiologic salinity, dissolved ions shield the negative charges on the DNA and the chromatin fiber compacts into a tighter configuration to create a fiber that is about 30 nm wide (58). In situ, this 30 nm fiber is further folded and condensed with small regions unpacked to allow them to ‘loop out’ from the rest of the chromosome. These looping out events are associated with transcription of genes in that region.

The chromatin in a given nucleus is not all maintained at the same level of condensation. Instead, there are distinct regions which are tightly packed and other regions that are more diffuse. Condensed chromatin, also known as heterochromatin, is associated with gene silencing whereas diffuse chromatin, also known as euchromatin, is associated with gene activation (59), although this is probably not due to the physical accessibility of the chromatin to transcription factors (60, 61). In most cases, the chromatin near the nuclear periphery is dense, gene-poor heterochromatin while the chromatin in the interior of the nucleus is more open, gene-rich, euchromatin (62).
During interphase, the shape of the chromosomes is irregular but each occupies a distinct chromosome territory with little overlap between adjacent territories (63). Cremer et al. coined the term inter-chromatin domain to describe the spaces between chromosome territories that appear to be empty in fluorescent images (24). These voids form an interconnected network of pores inside the nucleoplasm that allows Cajal bodies and speckles to circulate and access regions near the edges of the chromosome territories. This arrangement of chromatin around a network of pores is described as the architecture of the genome. Extracellular osmotic stress changes genome architecture (64). At high osmolarities, chromatin becomes more condensed and the interchromatin domain is enlarged. However, permeabilization of the plasma membrane abolishes this response, indicating that elevated intracellular osmolarity is not the mechanism.

The nucleoplasm differs from the cytoplasm in that it is organized into macromolecular aggregates rather than membrane-bound vesicles. Macromolecule concentration has a powerful influence these on aggregates due to a phenomenon known as the excluded volume effect. Macromolecules by definition have finite radius. This means that the center of the molecule is excluded not only from space occupied by another molecule but also from a region one radius deep surrounding that other molecule. This region is the excluded volume. As two molecules approach one another, their excluded volumes overlap, creating an attractive force that can be understood in entropic or osmotic terms. From an entropic perspective, overlap of excluded volumes
reduces the total excluded volume in the system. This increases the volume available to other solute molecules, allowing them to occupy a greater number of position states and become more disordered (Figure 4A and B). This gain in entropy outweighs the loss of entropy due to ordering of the aggregate. The attractive force between molecules in a crowded solution can equivalently be modeled as an osmotic pressure. There is an inaccessible region around the contact between two spherical molecules that can be thought of as an osmometer (Figure 4C). The concentration is zero in this region because solute molecules are too large to enter it so osmotic pressure tends to draw water out of it to equilibrate it with the rest of the solution. This pressure creates an attractive force at the site of contact. Osmotically-induced changes in cell volume change the concentration of macromolecules and influence aggregation. For example, the nucleolus shrinks under hypo-osmotic stress (65) and dissolves in permeabilized cells bathed in dilute buffer only to spontaneously reform when those cells are bathed in crowded solution of macromolecules (Hancock, 2004). Excluded volume effects influence aggregation of long coiled molecules and chromatin condenses when the concentration of macromolecules increases (66). Under hyper-osmotic stress, the intracellular concentration of macromolecules increases causing chromatin to condense within the nucleus (67), enlarging the inter-chromatin domain (64). The geometry of the inter-chromatin domain is important because gene transcription occurs at the edge of
the inter-chromatin domain and mRNA processing occurs at sub-nuclear bodies within the inter-chromatin domain.
Figure 4: The effects of molecular aggregation on excluded volume and osmotic pressure. The diagram depicts large molecules (green) in solution with another smaller but not infinitesimal species (red) contained in a finite volume (the black box). The area in yellow is the excluded volume. The scenario on the left (A) is less favored entropically than the scenario on the right (B) because molecular crowding minimizes the excluded volume and maximizes the number of position states available to the red molecules. (C) A region shaped like a concave lens around the contact between the two spherical, green molecules is inaccessible to the smaller red molecules in solution. The concentration in this region is zero, while the concentration in the solution is finite so there is osmotic pressure drawing water out of this region. This pressure thus creates an attractive force between the two green molecules.

There are also biological processes that affect chromatin condensation. The histone proteins that form the core of the nucleosome have tails that extend out to the surface of the chromatin molecule (57). Certain amino acids in these tails can be modified by covalent attachment of acetyl, methyl, phosphate groups among others(68). These modifications change the physical and biological properties of the genome and are epigenetic, meaning that they do not alter the DNA sequence but are inherited by daughter cells after mitosis. For example, histone acetylation shifts chromatin towards a
more open conformation and encourages activation of the encoded genes (69, 70) while histone methylation condenses chromatin and tends to silences the encoded genes (71). Patterns of histone modifications on genes regulating cell fate are involved in establishing and maintaining cell differentiation (72). There are two models for the activation of genes by histone acetylation. The first is that neutralization of the positive charge on the acetylated lysine residue frees negatively charged DNA from the histone. The second is that acetylated histones are recognized by regulatory proteins (73).

Histone acetylation is controlled by enzymes that function as histone deacetylases and histone acetyl transferases. There are at least 18 known histone deacetylases (HDACs) (74). Trichostatin-A is a streptomyces metabolite that acts as a potent and specific inhibitor of histone deacetylases (75). This specificity along with the capacity to induce expression of genes that regulate the cell cycle has inspired great interest in trichostatin A as a cancer therapy (76). It is also being studied as a possible therapy for osteoarthritis (77), rheumatoid arthritis (78, 79), ischemic brain injury (80) and Huntington's disease (81). Histone acetylation neutralizes positive charges on lysine and arginine groups and exposes negative charges on DNA. This causes the chromatin molecule to assume a more extended conformation due to self repulsion and results in a less compact genome architecture (82). One member of the histone deacetylase family, HDAC 6, does not act on histones at all. Instead it is confined to the cytoskeleton where it modulates cell motility via substrates including α-tubulin (83) and cortactin (84).
1.4 The Actin Cytoskeleton and the Nucleus

The cytoskeleton is composed of three families of proteins: microtubules, intermediate filaments and actin. Microtubules are long, relatively straight structures that spread out radially across the cell from a microtubule organizing center located next to the nucleus. They are used by the cell to organize intracellular transport and the location of sub-cellular organelles (85). Intermediate filaments form rope-like structures that are flexible but difficult to break. Their primary contribution is to make the cell tougher and stiffer (86). Actin forms dynamic structures that can expand or contract with sufficient force to enable the cell to crawl, exert tension or extend projections (87). Cytoskeletal structures are polymeric and exist in dynamic equilibrium with a population of their constituent monomers dissolved in the cytoplasm. This dynamic equilibrium means that there is little energetic difference between maintaining a structure in place and disassembling it to rebuild it elsewhere so the cytoskeleton is at once stable and easily reconfigured.

The actin cytoskeleton can be assembled and disassembled in ways that allow the cell to apply force. The cytoplasmic population of actin monomers is maintained far in excess of the critical concentration required to extend actin filaments. Rampant assembly is prevented by thymosin (which binds and sequesters actin monomers) so the actin cytoskeleton is primed for assembly at all times. Expression of profilin, a protein
that replaces thymosin in the actin binding site and permits assembly, initiates a rapid
burst of actin filament polymerization (88). Actin filaments assemble into one of two
structures: bundled stress fibers (89) or cross-linked filament networks (90). Actin cross-
linking proteins influence the nature of the actin structure assembled. Some, such as α-
actinin, join actin filaments in a parallel orientation to form thick bundles that can exert
tension when acted upon by myosin motor proteins (91). Others, such as filamin, join
actin filaments at an angle to form a cross-linked gel (92). Physical signals influence the
organization of the actin cytoskeleton. Compression of agarose constructs containing
articular chondrocytes results in depolymerization of the cortically arranged actin (93).
Hypo-osmotic stress has a similar effect on isolated chondrocytes cultured in monolayer
(10). The physical properties of the cell environment also affect the actin cytoskeleton.
Adherent cells pull on their substrate with actin-myosin bundles. As the stiffness of the
substrate increases, so does the tension and attendant bundling of the actin cytoskeleton
(94). This phenomenon affects cell shape and differentiation (95).

The mechanical properties of the cell depend on the integrity of the cytoskeletal
structures within it and the connections between them. There is evidence that the
nuclear lamina contributes to the overall mechanical stiffness of the cell through its
connections to the rest of the cytoskeleton (54, 96). The mechanical connections between
the nuclear lamina and the wider cell cytoskeleton are termed LINC (linker of nucleus
and cytoplasm) complexes. SUN (Sad-UNC 84) proteins bind to the nuclear lamina and
traverse the inner nuclear membrane(97). Large nesprin proteins bind to the SUN proteins and stretch across the lumen between the inner and outer membrane and through the outer membrane into the cytoplasm where they bind directly to actin and intermediate filaments (43). These connections imply that changes in nuclear morphology may be influenced by the cytoskeleton.

In addition to its well-established role in the cytoskeleton, actin is emerging as an important player in chromatin modification. The SWI/SNF-like complexes are a family of protein complexes found in the nucleus that remodel chromatin and, in some cases, acetylate histones (98). Actin or actin related protein is a component of these complexes and, in some cases, their activity is diminished by agents that sequester monomeric actin (99). Cytochalasin D, a drug that disrupts the actin cytoskeleton, has been shown to decrease histone acetylation in human mammary epithelial cells (100). This is likely because histone acetylation is involved in a signaling feedback loop that controls contractility of the actin cytoskeleton (101). The mechanism of this feedback loop is only partially understood. CaMK, a signal involved in actin contractility (102), also phosphorylates HDAC 5, leading to its export from the nucleus (103). HDAC 8 has been shown to associate with actin, potentially sequestering it away from the nucleus (104). Whatever the mechanism, it is clear that actin plays a biochemical role in acetylation of chromatin in addition to the physical role it plays in anchoring the surface of the nucleus.
1.5 Hypotheses and Aims

**Hypothesis 1:** The volume of the chondrocyte nucleus is inversely proportional to extracellular osmolarity.

**Specific Aim 1:** Cell volume is typically inversely proportional to extracellular osmolarity. The nucleus is expected to be unaffected by osmotic changes because it lacks a semi-permeable membrane. Nevertheless, extracellular osmotic load changes nucleus volume (30). I will quantify the effect of osmolarity on nuclear volume, explore the underlying mechanism and develop a mathematical model of the relationship. I will also study the relationship between osmotic stress and nuclear shape.

**Hypothesis 2:** Osmotic stress alters nucleocytoplasmic transport.

**Specific Aim 2:** Many important biochemical processes are regulated by the passive and active transport of molecules across the nuclear envelope. These transport processes may be sensitive to macromolecule crowding effects or changes in nuclear morphology. I will quantify the rate of passive diffusion between the nucleoplasm and the cytoplasm at a range of osmolalities to test this hypothesis.

**Hypothesis 3:** Actin organization inhibits contraction of the nucleus under hyper-osmotic stress.
Specific Aim 3: Actin organization is modest in healthy chondrocytes, particularly around the nucleus. However, many cells have highly organized actin cytoskeletons that are mechanically connected to the nucleus. These mechanical connections may be strong enough to prevent the nucleus from shrinking under hyper-osmotic stress. I will measure hyper-osmotically induced contraction of the nucleus in cells with organized actin and in cells without it, before and after treatment to disrupt actin. I will also measure changes in genome architecture in these experiments.
2. Nonlinear osmotic properties of the cell nucleus

2.1 Introduction

The ability to perceive and respond to biophysical factors, such as mechanical or osmotic stresses, is critical for normal cell behavior. For example, many cell types exhibit biological responses to physical stimuli such as mechanical deformation, hydrostatic pressure, fluid shear stress, or osmotic stresses. These physical parameters are often coupled to one another in tissues that possess a charged, hydrated, extracellular matrix, such as articular cartilage(2, 105-112). Such stresses may alter cell behavior by initiating traditional cell signaling processes involving the activation of ion channels (113-116) or other membrane-bound receptors(117, 118). It has also been hypothesized that physical stresses propagate through the cell and act directly on the nucleus and other intracellular organelles, exerting a biological influence at a subcellular level(119). In this respect, the physical conformation of the nucleus is associated with many important biological phenomena. For example, the nuclei of epithelial cells enlarge dramatically when they become cancerous (120). Chromatin condensation is one of the first changes to occur in apoptosis (121). Nuclear lamins A/C accumulate as stem cells differentiate (122-124) and the stiffness of the lamina network increases as a result (47). Interestingly, lamin B1, the only sub-type that does not affect the stiffness of

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the lamina network (45), is unchanged during differentiation. The significance, if any, of these physical changes can be better interpreted through more detailed knowledge of the biophysical properties of the cell nucleus.

It is now apparent that extracellular matrix interacts with the cytoplasm and other intracellular organelles such as the nucleus via a solid-state connection to the cytoskeleton(125-128). There has been considerable speculation that this connection provides a mechanism by which extracellular stresses can be transmitted intracellularly in a manner that can alter cell signaling directly at the level of the nucleus(127, 129). Osmotic stress, on the other hand, does not depend on solid connections, but rather is “transmitted” by the diffusion of ions and water through fluid compartments and is modulated by local fixed charge density. Since the cell membrane is permeable to water, osmotic stress propagates rapidly to the interior of the cell in the presence or absence of solid structures in the cytoplasm. However, the physical effects of extracellular osmotic stress on the cell nucleus, as well as the intrinsic physicochemical properties of the nucleus, are not well understood.

A few previous studies have examined the physical and biomechanical properties of the cell nucleus. The nucleus is stiffer and more viscous than the cytoplasm(27, 28, 33), potentially contributing to the stiffness of the cell as a structure (35). Furthermore, defective nuclear mechanics due to genetic alterations in nuclear cytoskeletal proteins compromise the mechanical integrity of the whole cell (96).
nuclear envelope is supported by a network of intermediate filaments called lamins and filled with chromatin (44). The stiffest part of the nucleus is the lamin network, while the most viscous part is the chromatin. Both appear to show power law dynamics after the application of a step load (31). Computational studies have shown that the mechanical response of the whole nucleus is highly sensitive to the stiffness of the nuclear lamina (130). Chromatin contains many charged groups that limit its compaction by repelling one another and cause it to condense or expand in response to changes in ion concentration (58, 131, 132). Small molecules and ions diffuse freely into and out of the nucleus through the nuclear pore complexes that populate the nuclear envelope (22). In charged hydrated tissues such as cartilage, bulk compression of the tissue changes the shape and volume of the chondrocyte and its nucleus in a coordinated manner (29, 133, 134). However, if the F-actin cytoskeleton is disrupted with cytochalasin D, the effects of tissue compression on nuclear shape disappear, but the effects on volume remain (29).

Osmotic loading affects a range of biological processes in articular chondrocytes including calcium signaling (7), protein synthesis (6), gene expression (5) and cytoskeletal reorganization (8-10). The physical response that accompanies these biological responses is well-understood at the whole-cell level. When active responses are suppressed, the reciprocal of osmolarity exhibits a linear relationship with cell volume, as predicted by the Boyle Van’t Hoff relation (135). Many mammalian cells
obey the Boyle Van’t Hoff relation, including chondrocytes (10, 136), neutrophils (16) and human islet cells (17). Changes in the extracellular osmotic environment cause intracellular changes such as alterations in genome architecture (64) due to an increase of macromolecular crowding in the cytoplasm that causes histone aggregation through an excluded volume effect (66). The volume of the chondrocyte nucleus changes with changes in osmolarity (30) but it is not known if the osmotic response of the nucleus shares the linear character of the cell response. We hypothesized that the nucleus, similar to the cell, would behave as an ideal osmometer, following the Boyle Van’t Hoff relation. The goal of this study was to examine this hypothesis by systematically quantifying the dynamic and equilibrium morphologic response of the cell nucleus to changes in extracellular osmolarity using two independent methods, confocal microscopy and angle-resolved low coherence interferometry (a/LCI). Further experiments examined the mechanisms of the observed behavior.

2.2 Methods

2.2.1 Mathematical Model

The equilibrium volume response of the nuclei was modeled using an extension of an existing model of the swelling of porous gels (21) to estimate the stiffness of the nuclear lamina. The original model describes a spherical gel without a surface membrane. The permeation of the solute molecule into the pore fluid is limited by steric exclusion. This is quantified using the partition coefficient, κ, which establishes the
following relationship (eqn. 19 in Albro et al.(21)) between the concentration within the gel at equilibrium, \( c_{eq} \), and the concentration in the surrounding bath at equilibrium, \( c_{eq}^\ast \).

\[ c_{eq} = \kappa c_{eq}^\ast \quad (1) \]

The partition coefficient is determined by the pore size distribution in the gel and the size of solute molecule. The partition coefficient only departs from unity in the case of large solute molecules for which steric exclusion effects are non-negligible. In the case of the cell nucleus, the surrounding bath is the cytoplasm and it contains many solute species, each of which has a different partition coefficient between the cytoplasm and the nucleoplasm so it is necessary to define an apparent partition coefficient, \( \kappa_a \), such that

\[ c_{nuc} = \kappa_a c_{cyt} \]

where \( c_{nuc} \) is the concentration in the nucleoplasm and \( c_{cyt} \) is the concentration in the cytoplasm. Note that:

\[ c_{cyt} = c_{eq}^\ast \quad (2) \]

for a cell that behaves as a perfect osmometer so that:

\[ c_{nuc} = \kappa_a c_{eq}^\ast \quad (3) \]

The apparent partition coefficient of the chondrocyte nucleus can be determined from the hyperosmotic contraction using equation 15 in Albro et al.

\[ H_a \left[ \frac{\partial u}{\partial r} \bigg|_{r=a} + \frac{2v}{1-v} \frac{u(a,t)}{a} \right] = -R \theta (1-\kappa_a) c^\ast(t) \quad (4) \]

where \( H_a \) is aggregate modulus, \( u \) is solid matrix displacement, \( r \) is the radial coordinate, \( a \) is the radius in a zero osmolarity bath, \( v \) is the Poisson’s ratio, \( t \) is time, \( R \) is the universal gas constant and \( \theta \) is the absolute temperature. Albro et al. show that...
the displacement solution for this system has the form \( u(r, t \to \infty) = \xi r \) where \( u \) is the radial displacement, \( r \) is the radial co-ordinate, \( t \) is time and \( \xi \) is a constant determined by the boundary conditions. Since the model is one-dimensional, all other deformation terms can be deduced from this constant \( \xi \). The bulk modulus \( K \) is related to the aggregate modulus by the following expression \( K = H_A (1 + \nu) / 3(1 - \nu) \). Substituting this expression and the displacement solution into equation 4 yields the following relation:

\[
3K\xi = -R\theta(1 - \kappa_s)\kappa^{eq}_q
\]  

(5)

Writing volume in terms of \( \xi \) and neglecting higher order terms because this is a small strain analysis leads to the following expression (20 in Albro et al.) relating the normalized volume to the extracellular osmolarity.

\[
\frac{V_{eq}}{V_r} = \frac{1 - R\theta(1 - \kappa_a)\kappa^{eq}_q / K}{1 - R\theta(1 - \kappa_a)\kappa^{eq}_r / K}
\]

(6)

where subscript \( r \) refers to the reference condition, 380 mOsm in this experiment. This allows determination of \( \kappa_q \) from the hyperosmotic response if \( K \) is specified. Most of the mechanical models of the cell nucleus in the literature model it as an incompressible material and therefore that data cannot be used to determine \( K \). However, one investigator reported large volumetric strains under pressure in micropipette experiments (40). While the data was not presented in a form that allows exact determination of \( K \), the range of pressures applied and strains measured suggests
that a value of 10 kPa is accurate to within one order of magnitude. More accurate measurements of this property in the future would increase the utility of this model. In this analysis, a single data point corresponding to an extracellular osmolarity of 482 mOsm was used to evaluate $\kappa_a$ on the grounds that the model was originally developed using small strain assumptions violated at higher osmolarities. The strain at 482 mOsm is the only hyperosmotic strain that is within the range for which the model was originally validated. The value of $\kappa_a$ determined by this approach was 0.9956.

The hypoosmotic response of this model with respect to inverse normalized osmolarity is non-linear. However, the non-linearity is not strong enough to fit the trend in the observed data. This, combined with the observed changes in shape during hypoosmotic expansion, supports the introduction of a membrane stress term. Equation 4 is a relation between volumetric strain and pressure. The membrane tension is represented by adding a pressure term to equation 4 using the Law of Laplace (137) to create the following equation:

$$3K \xi = -R \theta (1 - \kappa_a) \kappa_{eq} \xi - \frac{2T}{q}$$

(7)

where

$$T = \begin{cases} 
0, & A < A_{crit} \\
K_A \left( \frac{A}{A_{crit}} - 1 \right), & A > A_{crit}
\end{cases}$$

(8)
where $T$ is membrane tension, $q$ is the radius, $K_A$ is the dilatational modulus of the membrane, $A$ is the membrane area and $A_{\text{crit}}$ is the critical area at which the membrane is drawn taut. Fitting the governing equations 7 and 8 to the data presented in Figure 6A allows determination of the mechanical properties of the nuclear lamina, specifically the $K_A$ and $c_{\text{crit}}$, the osmolarity at which the nuclear membrane begins to resist further osmotic expansion (the critical value of the osmolarity is determined from the corresponding value of $\xi$ using equations 9 and 4). To solve these equations, the area must be written in terms of $\xi$ as follows:

$$A = 4\pi q^2 = 4\pi a^2 (1 + \xi)^2 \approx 4\pi a^2 (1 + 2\xi)$$

$$A_{\text{crit}} \approx 4\pi a^2 (1 + 2\xi_{\text{crit}})$$

(9)

and substituted into equation 7. For $A<A_{\text{crit}}$, $\xi$ is related to $c^*$ by equation 5. For $A>A_{\text{crit}}$, the resulting form of equation 7 was solved in Mathematica (Wolfram Research Inc., Champaign, IL) to yield an analytical expression for $\xi$. Albro et al.’s equation 17 can be used to give the normalized volume in terms of $\xi$ as follows

$$V_{eq} = \frac{1+3\xi}{1-R\theta(1-\kappa_a)\kappa_r/K}$$

(10)

This model was coded in Matlab (The MathWorks, Inc., Natick, MA) and fit to the hypoosmotic volume data numerically. Ponder’s plots for various values of the model properties are presented in Figure 5.
Figure 5: (A) Ponder’s plots predicted by the mathematical model when $K = 10$ kPa, $cc_{crit} = 300$ mOsm and $KA$ varies between 0 N/m and 0.1 N/m. (B) Ponder’s plot predicted by the mathematical model when $K = 10$ kPa, $K = 0.1$ N/m and $cc_{crit}$ varies between 350 and 230 m

2.2.2 Cell isolation and culture

Chondrocytes were isolated from articular cartilage of the lateral femoral condyle of 2-3 year old skeletally mature female pigs obtained from a local abbatoir.
Cells were isolated by sequential digestion in pronase and collagenase solution and seeded on glass coverslips in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum, 1.5% HEPES and 1% non-essential amino acids (all cell culture reagents were supplied by Gibco, Grand Island, NY). The medium was adjusted to pH 7.4, and its osmolarity was increased to 380 mOsm with sucrose and verified with a freezing point osmometer (Precision Systems Inc., Natick, MA). 380 mOsm is an approximate value for the extracellular osmolarity experienced by chondrocytes in situ (138). For confocal microscopy experiments, 0.2 ml of media containing approximately $2 \times 10^5$ cells was spread on the center of a 42mm diameter coverslip that was placed in a 60 mm diameter culture dish. Cells were incubated for 1 hour to allow attachment to the coverslip, and 5 ml of media was added to each dish before incubation was continued. For angle resolved low coherence interferometry (a/LCI) experiments, chondrocytes were seeded at a density of $7 \times 10^5$/cm$^2$ in chambered coverglasses. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO$_2$ for 18 hours. As a result, the chondrocytes were attached to the glass but maintained their rounded morphology at the time of the experiments (7).

### 2.2.3 Microscopic Imaging

Each coverslip was incubated in 380 mOsm saline with 10 μM acridine orange (Molecular Probes, Eugene, OR), a fluorescent label of nucleic acids, for 20 minutes to
label the nuclei. The cells and their nuclei were visualized using confocal laser scanning microscopy (LSM 510, Carl Zeiss, Thornwood NY). Images were obtained through an inverted fluorescent microscope (Axiovert 100M, Carl Zeiss) with a C-Apochromat, 63x, water immersion, 1.2-NA objective lens. Acridine orange fluorescence was excited by an argon-ion laser (488 nm) at 2% power and the stimulated emission was collected through a 505 – 550 nm filter on an 8-bit intensity scale. For DIC images, the sample was also illuminated with a helium-neon laser at 80% power. Images of 1024 X 1024 pixels were recorded with a scale length of 0.12 microns per pixel. The confocal pinhole was fully open, creating an optical slice thickness in excess of 7.5 μm. For 3 dimensional imaging of isolated nuclei, the optical slice thickness was 1 μm and step size between images was 0.5μm and samples were incubated with Syto 13 (Molecular Probes, Eugene, OR) for 30 minutes to fluorescently label nuclei acids. Other settings were identical to those used for two dimensional imaging.

2.2.4 Angle resolved low coherence interferometry (a/LCI) experiments

A/LCI is a non-perturbative optical technique that measures the average nuclear size of cells in a biological sample, such as tissues or, in the case of the present study, isolated cells. A/LCI combines the capabilities of low coherence interferometry with light scattering techniques to determine nuclear morphology with subwavelength accuracy (139, 140). Low coherence interferometry uses a wide bandwidth source for
the purposes of achieving high depth resolution and rejecting multiply scattered light in a signal, as in optical coherence tomography (141). This permits the ability to make sensitive measurements of light scattered from a single cell monolayer. Inverse light scattering analysis (ILSA) is predicated on the fact that elastically scattered light from an object yields a unique signature that is a function of the shape, size, and electromagnetic properties of the object. In the case of a/LCI, the angular light scattering distribution of a population of cells is processed to isolate the nuclear scattering contribution and compared to an appropriate model (142). Upon statistical comparison to the model, the characteristics of the scattering objects, in this case nuclear size, can be discerned. The nuclear size of a monolayer of chondrocytes cultured in a chambered coverglass was measured after equilibration to saline at a range of different osmolarities.

2.2.5 Equilibrium effects of osmolarity on cell and nuclear morphology

The coverslip was loaded into a perfusion chamber (PeCon GmbH, Kornhalde, Germany) and washed three times with saline at 380 mOsm. The chamber was mounted on the microscope stage and 9 consecutive sequences of images were recorded, each consisting of 25 DIC images at 5 second intervals followed by a single fluorescent image. At the beginning of each of these sequences, the saline in the chamber was withdrawn and replaced with saline at a different osmolarity, starting with the highest value and proceeding to the lowest. The osmolarities used are listed in Table 1.
2.2.6 Transient effects of osmolarity on cell and nuclear morphology

Samples were washed and mounted in the perfusion chamber on the microscope stage as described above. A single scan, including DIC and fluorescent images, was recorded. The saline in the chamber was then exchanged and 9 further scans were taken over the next 140 seconds at irregular time intervals so that imaging would be most frequent during the most dynamic portion of the response. The saline was exchanged with saline at 280 mOsm for hypo-osmotic transients and 480 mOsm for hyper-osmotic transients. In the transient experiments, size is quantified using cross-sectional area.

2.2.7 Calculation of time constants of cross-sectional area change

The following equation:
\[ A(t) = 1 + K(1 - e^{(-t/\tau)}) \]  \hspace{1cm} (13)

was fit to aggregated data points from multiple experiments using non-linear regression to calculate the time constant of the response. In this expression, \( A(t) \) is the normalized cross-sectional area, \( K \) is the amplitude of the response, \( t \) is time and \( \tau \) is the time constant. Non-linear regression was performed using Matlab (The Mathworks, Natick, MA) to determine values and 95% confidence intervals for the amplitude and time constant. Values are reported to be significantly different if the confidence intervals do not overlap.
2.2.8 Response of isolated nuclei to elevated concentrations of macromolecules and ions

Nuclei were chemically isolated from freshly isolated chondrocytes as described previously (27). Briefly, 10 million freshly isolated porcine chondrocytes were suspended in 0.1% IGEPAL CA-630 for 8 minutes at 4°C. The suspension was diluted 10:1 with 0.75% BSA in HBSS (both from Gibco, Grand Island, NY) and centrifuged at 500g for 20 minutes at 4°C. The pellet was resuspended in 0.75% BSA in HBSS and 100 μl droplets were spread on 42 mm coverslips and allowed to seed for 1 hour. Then, the coverslips were covered in iso-osmotic testing buffer and incubated overnight before testing. The iso-osmotic testing buffer was 15 mM HEPES, 10 mM NaCl, 140 mM KCl, 500 μM MgCl₂ and 100 nM CaCl₂. These ion concentrations are typical of those in a mammalian cell at equilibrium (143). For testing, the coverslips were mounted in the perfusion chamber, washed three times in iso-osmotic testing buffer and mounted on the microscope stage. To simulate the crowded environment of the cytoplasm, the buffer was exchanged for a 25% solution of 10 kDa dextran (Sigma-Aldrich, St. Louis, MO) in iso-osmotic testing buffer, allowed to equilibrate for 2 minutes and imaged in three dimensions using the confocal microscope. For the control condition, the fluid was then withdrawn from the chamber and replaced with an identical solution. For the high macromolecule condition, the fluid in the chamber was exchanged with a 34% dextran solution in iso-osmotic testing buffer. This condition models the increase in
macromolecular crowding that would arise from an abrupt 26% decrease in the cell volume, as is typical of chondrocytes subjected to 380 mOsm – 580mOsm hyper-osmotic step change (see Results). In the high ion condition, the fluid was exchanged with a 25% dextran solution in a high ion buffer consisting of 15 mM HEPES, 13.5 mM NaCl, 189 mM KCl, 676 μM MgCl$_2$ and 135 nM CaCl$_2$. These ion concentrations model the increase in ion concentration that would arise from an abrupt 26% decrease in the cell volume.

2.2.9 Image Analysis

Chondrocyte volume was determined using an edge detection algorithm applied to the DIC images as described previously (144). Briefly, the focal plane of the microscope was manipulated during the experiment to create a diffraction pattern in the form of white ring marking the edge of the cell (Figure 9). A custom-written application in PV-WAVE software (Visual Numerics, Inc., Houston, TX) was used to define an annular region around the cell containing the ring and apply Sobel edge detection and optimization algorithms to calculate a closed curve representing the edge of the cell. This allowed direct measurement of the cross-sectional area from which volume was inferred by assuming the cell to be spherical. The cross-sectional area, perimeter length and contour ratio of the nucleus were determined from the fluorescent images using Otsu segmentation and the Image Analysis Toolbox in Matlab. The contour ratio of a shape is defined as $4\pi \times \text{area} / \text{perimeter}^2$ (45, 55). The nucleus volume was also
determined from the cross sectional area by approximating the shape to be a sphere. For isolated nuclei, raw stacked images were deconvolved using commercial deconvolution software Huygens (Scientific Volume Imaging, Amsterdam, the Netherlands). The deconvolved image was exported to Matlab and thresholded using the Iterative Self Organizing Data algorithm (145) to determine the volume.

2.3 Results

Cell volume was found to be linearly related to inverse normalized osmolality throughout the domain tested, indicating that chondrocytes obey the Boyle Van’t Hoff relation for the volumetric response of an ideal osmometer. The osmotically active volume fraction of the chondrocyte was determined from a linear fit to be 67% (R²=0.97).

Nucleus volume was also sensitive to osmolality but in a non-linear manner. The nucleus volume increased linearly with inverse normalized osmolality in the hyper-osmotic range. In the hypo-osmotic range, the slope of the relationship decreased continuously, suggesting an upper limit to nuclear expansion. The key features of the relationship between nucleus volume and osmolality were found to be the same regardless of whether it was measured using fluorescent microscopy or a/LCI (Figure 6A). For the sake of comparison, the Ponder’s plot data was separated into four cases representing the two different structures, the cell and the nucleus (as measured by fluorescence microscopy), and two different ranges, hyper-osmotic and hypo-osmotic.
In each case, the data was fit to a straight line to allow comparisons of the osmotic sensitivity to be made (Figure 6B). A two factor ANCOVA revealed a main effect of structure (cell vs. nucleus), no main effect of osmotic range and a significant interaction between structure and osmotic range. The osmotic sensitivity of the cell did not change significantly between the hyper- and hypo-osmotic ranges but the sensitivity of the nucleus did change. The nucleus was more sensitive than the cell in the hyper-osmotic range and less so in the hypo-osmotic range. The nucleus volume response in the hyper-osmotic range was highly linear \((R^2 = 0.992)\) and the y-intercept of the linear fit was 0.204. This finding implies that the solid volume fraction of the nucleus is 20.4\%. 
Figure 6: (A) The Ponder’s relation (inverse osmolarity vs. normalized volume) was found to be linear for the cell but nonlinear for the nucleus. The non-linearity of the response of the nucleus was independent of the techniques used to measure nuclear size (i.e., fluorescent microscopy or angle resolved low coherence interferometry (a/LCI)). For nucleus by a/LCI, n = 11. For cell and nucleus by microscopy measurements, n is as per table 1. Error bars represent one standard error. V/Viso = volume / iso-osmotic volume, Ciso/C = iso-osmotic osmolarity / osmolarity.

(B) Values of the slope of the Ponder’s plot calculated for the cell and the nucleus (as measured by fluorescence microscopy) in the hyper- and hypo-osmotic range (error
bars denote the 95% confidence interval). Bars with different letters are significantly
different. The observed differences are protected by a two way ANCOVA, which
returns a significant main effect of structure (cell versus nucleus), no significant main
effect of osmotic range (hyper-osmotic versus hypo-osmotic) and a significant
interaction between structure and osmotic range. Slopes were considered different to
statistically significant degree if the 95% confidence intervals of the slopes do not
overlap. Bars labeled with different letters are statistically significantly different to
one another.

An existing mathematical model describing osmotic swelling of gels (21) was
modified to introduce a membrane representing the nuclear lamina (see Methods). This
model was fit to the hypoosmotic volume data as measured by confocal microscopy to
estimate the properties of the nuclear membrane. The optimal fit was obtained when the
dilational modulus of the nuclear envelope was 0.023N/m and the nuclear envelope
became taut at an extracellular osmolarity of 321 mOsm (Figure 7). The $r^2$ value of the fit
was 0.9998. The model was optimized from a range of initial values to ensure the
uniqueness of the reported optimal result. The optimization was repeated for several
values of the $K$, the bulk modulus of the nucleus. It was found that the optimal value of
the membrane stiffness scaled with the bulk modulus over four orders of magnitude
from 100 Pa to 1 MPa. However, the optimal value of the osmolarity at which the
nuclear membrane became taut remained the same across this range.
Figure 7: Observed nuclear volume data from 480 mOsm to 200mOsm along with the model for the same osmotic range with best-fit membrane properties and bulk modulus of 10 kPa.

Extracellular osmolarity influenced nucleus shape (Figure 8). The contour ratio increased linearly with inverse normalized osmolarity in the hyper-osmotic range, indicating that the shape was becoming smoother. The slope of the relationship decreased in the hypo-osmotic range, appearing to approach a limit. This behavior was similar to the behavior of the relationship between nucleus volume and inverse normalized osmolarity.
Figure 8: In the nucleus, the relationship between osmolarity and contour ratio followed a similar trend to the relationship between osmolarity and volume at equilibrium. Error bars represent one standard error.

The relationship of shape and size to osmolarity was also apparent qualitatively (Figure 9). Cell shape was approximately circular (i.e., spherical) at every osmolarity, except for small distortions at the peak value. The size of the cell increased continuously with decreasing osmolarity throughout the range shown. The irregular shape of the nucleus at elevated osmolarity became approximately circular and the nucleus expanded as the osmolarity decreased.
Figure 9: Typical images of a single cell (top) and its nucleus (bottom) during equilibrium experiments at a range of osmolarities. The number above each image pair indicates the osmolarity in mOsm. The number below each pair of images indicates the inverse normalized osmolarity.

The transient changes in the cross-sectional area of the cell and nucleus changed monotonically with time, showing negligible evidence of volume recovery (Figure 10), as evidenced by the fact that an exponential expression with a single time constant fit the data well. In the cell, the time constant for volume decrease in response to hyper-osmotic stress was lower than that for volume increase with hypo-osmotic stress. Other differences between time constants were not significant at 95% confidence (Table 1).
Figure 10: Cell and nucleus cross sectional area transients showed rapid first order dynamics with no evidence of recovery. (A) The means and standard errors of the cell transient response along with the best fit of Eq. 13 and the 95% confidence interval ($n = 46$ and 45 for hypo-osmotic and hyper-osmotic tests respectively). (B) The means and standard errors of the nucleus transient response along with the best fit of Eq. 13 and the 95% confidence interval ($n = 39$ for both the hyper-osmotic and the hypo-osmotic tests). Note that means are presented for illustrative purposes only and the actual fit was to the whole population of data points rather than to their means.
Table 1: Time constants in seconds along with 95% confidence intervals for best fits to transient responses

<table>
<thead>
<tr>
<th></th>
<th>Hypo-osmotic</th>
<th>Hyper-osmotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell</td>
<td>9.838 ± 1.013 (n = 46)</td>
<td>5.670 ± 0.939 (n = 45)</td>
</tr>
<tr>
<td>Nucleus</td>
<td>7.800 ± 3.924 (n = 39)</td>
<td>7.245 ± 1.788 (n = 39)</td>
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The rate of change in nucleus shape was lower than that of nucleus size. The transient of the contour ratio required 120 seconds to reach its peak value. As a result, the perimeter length of the nucleus initially increases rapidly under hypo-osmotic loading to a peak at about 15 seconds before decaying steadily for the rest of the experiment (Figure 11) as the shape evolves to accommodate the constant cross sectional area within a shorter perimeter.
Isolated nuclei contracted in response to increased macromolecule concentration but not in response to increased ion concentration. A highly significant (p<0.001) reduction of 9% in the volume of isolated nuclei occurred when they were subjected to an increase in macromolecule concentration consistent with observed hyper-osmotic shrinkage of the cell. However, a similar increase in ion concentration caused no detectable change in the volume of the isolated nuclei (Figure 12). The macromolecules used were 10 kDa dextrans, which are several orders of magnitude larger than ions but still small enough to pass through the pores in the nuclear envelope. This was confirmed using rhodamine-labeled dextrans.
2.4 Discussion

The findings of our study indicate that the equilibrium osmotic response of chondrocytes matches that of an ideal osmometer, as described by the Boyle Van’t Hoff relationship, and the transient results show negligible recovery over time. Therefore, the equilibrium and transient responses of the cells suggest that they behave in a passive manner under these experimental conditions. The transient changes in cross-sectional

Figure 12: Isolated nuclei contracted in response to increased macromolecule concentration but not in response to increased ion concentration, n = 100 – 126, error bars represent one s. d., * indicates statistically significant difference. “Hyper ions” denotes elevated ion concentration. “Hyper macro” denotes elevated macro molecule concentration (see Methods for details).
area of the nucleus also showed negligible recovery. However, under these same conditions, the equilibrium volumetric response of the nucleus to changes in osmolarity, as measured by two independent methods, was found to be highly non-linear. The mechanisms involved in this non-linear physicochemical behavior are not completely understood, but changes in the shape of the nucleus were found to parallel changes in the size of the nucleus under equilibrium conditions, suggesting that the two are interdependent. By contrast, shape change lagged size change in transient experiments. Further experiments suggest that the mechanism of nuclear morphology change is not cytoskeletal force transmission or concentration of intracellular ions but concentration of intracellular macromolecules.

In a previous study of nuclear size in chondrocytes subjected to a single step change of 200 mOsm, nuclei changed less than cells in the hypo-osmotic case but the changes were not significantly different in the hyper-osmotic case (30). These findings are in agreement with the results of the present study, where we found that the slope of the Ponder’s plot (i.e. the volume response) was only 0.24 for the nuclei while it was 0.66 for the cells in the hypo-osmotic range. However, in contrast to the previous results, we found a difference between the cell and nucleus in the hyper-osmotic range, where the slope of the Ponder’s plot was 0.67 for the cells and 0.8 for the nuclei. The difference between the cell and nucleus responses was greater in the hypo-osmotic range than in
the hyper-osmotic range (Figure 6B), supporting the asymmetry seen in the previous study (30).

The hyper-osmotic response of the nucleus on a Ponder’s plot was found to be linear with a y-intercept of 20.4%, suggesting that this is the solid volume fraction of the nucleus. This agrees with the findings of Rowat et al., who reported that micropipette aspiration of nuclei reduced their volume but it was difficult to reduce the nuclear volume by more than 70% (40). Despite the linearity of the response, the nucleus does not have the semi-permeable membrane characteristic of an ideal osmometer. The nuclear envelope is punctuated by pores that render it permeable to small ions (22).

There was a significant reduction in the volume of isolated nuclei loaded with increased concentrations of macromolecules but no significant change in the volume of isolated nuclei loaded with increased concentrations of ions (Figure 12). This suggests that the nucleus dilates because the macromolecular concentration in the cytoplasm changes when the cell volume changes. These findings are consistent with other reports in the literature. Albiez et al. reported that nuclear architecture was altered by hyper-osmotic loading with saline but that the effect was abolished by permeabilization of the cell membrane (64). Richter et al. reported that changes in ion concentration did not influence the nuclei of permeabilized cells but changes in macromolecule concentration did (66).
Macromolecules may exert an osmotic stress even on systems such as the nucleus that are not surrounded by a semi-permable membrane. Size exclusion of large molecules from small tortuous spaces in a gel creates dilute regions that are osmotically pressurized. The size exclusion effect is quantified by the partition coefficient, the ratio between solute concentrations inside and outside the gel. If the partition coefficient is less than one, the gel is osmotically pressurized. Experimental evidence for this phenomenon in alginate beads along with a theoretical analysis was provided by Albro et al.(21). They found that the partition coefficient of a solute molecule is a function of its size, since larger molecules are excluded from a larger fraction of the pores in the gel. Monovalent ions are small enough to enter even the smallest pores so that no osmotic response is expected for osmotic loading with saline in this model. On the other hand, large molecules such as 10 kDa dextrans are expected to have a partition coefficient substantially less than unity and exert substantial osmotic pressure. This is indeed the case for isolated chondrocyte nuclei (Figure 12). The increase in macromolecular concentration was intended to model the change in the cytoplasm of a cell subject to an extracellular osmolarity of 580 mOsm. However, the reduction in the volume of the isolated nuclei was only 9% whereas the reduction of volume for nuclei inside cells subjected to 580 mOsm was 28%. This may be because the solution used to load isolated nuclei contained only macromolecules of 10 kDa. The cytoplasm contains larger molecules that may have smaller partition coefficients with the nucleoplasm and hence
exert a larger osmotic load. Also, the cytoplasm contains molecules too large to pass through the nuclear pores that exert a conventional osmotic pressure on the nucleus. Hence, it is unsurprising that the volume reduction in isolated nuclei is less than for nuclei in situ.

The model presented by Albro et al. (21) predicts a linear relationship between osmolality and volumetric strain so the Ponder’s plot of the model output (in which osmolality is inverted) has a continuously declining slope. However, the decline in the slope is not sharp enough to fit the abrupt plateau in the observed volume data in the present study. This characteristic, combined with the observed correlation between shape and volume, motivated a modification of the model to include a membrane tension term to model the mechanical integrity of the nuclear lamina (see Materials and Methods). This model allowed estimation of the stiffness of the nuclear lamina along with the osmolarity at which the nuclear lamina becomes taut. The value determined for the stiffness of the nuclear lamina depends heavily on the value used for the bulk modulus of the nucleus, a property that can only be estimated from the available literature. A more accurate value for this property would increase the power of this approach to non-invasively determine the mechanical properties of the nuclear membrane. The osmolarity at which the nuclear lamina becomes taut, 321 mOsm, is not sensitive to the value of the bulk modulus. It is significant that this value lies below the osmolarity experienced by chondrocytes (380 mOsm), suggesting that physiologic
changes in cartilage osmolarity (secondary to mechanical loading) would result in changes in nucleus size in situ.

Nucleus shape changed more slowly than the nucleus size under hypo-osmotic load (Figure 11). The mechanism of this behavior is unknown but may result from inhomogeneity in the viscoelasticity of the nucleus. Previous studies suggest that the nucleoplasm creeps more rapidly than the lamina (31) so it is possible that nuclear morphology is determined initially by the nucleoplasm but thereafter is increasingly influenced by the lamina as the stiffness of the nucleoplasm decays. Since the lamina acts mechanically as a membrane, its influence is to move the nucleus towards a smoother shape that minimizes surface area and the length of the perimeter of the cross-section. The changing geometry of the nucleus may reflect the redistribution of strain between these two structures as their stiffnesses evolve over time. Further work is necessary to fully understand this phenomenon.

The transient response of the cell cross-sectional area was significantly slower in the hypo-osmotic condition than in the hyper-osmotic condition (see Table 2). While an equivalent change in osmolarity was applied in both conditions (100 mOsm), it is important to note that the volume change predicted by the Boyle Van’t Hoff relation is related to the reciprocal of osmolarity. In this case, the hypo-osmotic condition is a 35% increase in the reciprocal of osmolarity while the hyper-osmotic condition is a 21% decrease. The hypo-osmotic response may equilibrate more slowly because it represents
a larger effective change. Also, artificial disruption of the F-actin cytoskeleton has been shown to slow volume change in both hypo- and hyper-osmotically loaded chondrocytes (30). Hypo-osmotic loading disrupts the F-actin cytoskeleton in untreated chondrocytes but hyper-osmotic loading does not (9, 10). Thus, cytoskeletal disruption and remodeling may also play a role in the observed difference in the time constants.

This study relies on measurement of volume inferred from the cross-sectional area of a cell or nucleus in a two dimensional image for the transient and equilibrium experiments on nuclei within intact cells. Therefore, an important assumption in the interpretation of this data is that the cell or nucleus in question is a sphere. In this study, chondrocytes are observed to be quite spherical, but nuclei are often spheroidal and may even have more complicated shapes as indicated in the discussion of evolving contour ratios. For this reason, measured values were normalized to the iso-osmotic condition rather than presented as absolute values. Exact knowledge of the three-dimensional shape is required to relate cross-sectional area to absolute volume but the relationship between area dilatation and volume dilatation is less sensitive. The ratio of the reference and deformed volume for an isotropic deformation field is equal to the ratio of the reference and deformed cross-sectional areas raised to the power of 1.5, regardless of geometry. Isotropic deformations were assumed for the cell and nucleus in these experiments. To validate this approach, three dimensional confocal images were taken of stained nuclei in intact chondrocytes at three different osmolarities and used to
calculate volume. Then, the three dimensional images were projected on to a two
dimensional plane and volumes were estimated from the projected area. As expected,
the absolute volumes deduced from the two methods were different but the normalized
results were identical (data not shown). This procedure allowed us to take multiple
measurements of the same nucleus using two dimensional imaging without the
photobleaching associated with repeated three dimensional imaging. In contrast to
nuclei inside intact cells, isolated nuclei sit directly on the glass coverslip so they are
constrained on one side. In this case, it was considered appropriate to image the volume
directly using stacked confocal imaging and design the experiment to rely on only one
pair of stacked images.

The physiological implications of changes in nuclear morphology are not fully
understood, but they are observed in association with a number of important biological
phenomena. Genome organization influences a host of fundamental biological
processes (61, 146) and is presumably altered by changes in nucleus morphology. There
is evidence that changes in nuclear shape and volume may alter nucleo-cytoplasmic
transport. For example, nuclear permeability is increased in flattened cells as compared
to rounded cells, and increased tension in the nuclear envelope is one mechanism that
has been proposed for this phenomenon (147). A rough nuclear perimeter is observed in
cases where lamin is defective due to a genetic disease such as Hutchinson-Gilford
progeria syndrome (HGPS) (55), supporting the idea that lamin-dependent membrane
mechanics in the nuclear cortex maintain the conventional smooth shape of the eukaryotic nucleus. Despite the devastating consequences of the disease, the nuclei of HGPS cells are not more fragile than those of healthy cells but they do exhibit reduced lamin mobility and altered lamin organization (48). This finding suggests that the nuclear lamina is much more than a passive structural element and that reorganization of the lamina under load is a sensitive and important mechanotransduction process.

The smoothness of the nuclear perimeter also deteriorates in normal aging (56), increasing the relevance of these changes to degenerative diseases such as osteoarthritis. While both normal aging and HGPS reduce the capacity of the nucleus to repair DNA, it is not known what connection, if any, the physical changes associated with these conditions have to DNA repair. However, it is worth noting that exposure to a hyper-osmotic environment, which is shown here to produce a rough nuclear perimeter, also inhibits DNA repair (148). The role of cause and effect may be interchangeable between biological and physical phenomena in some cases. For example, cell shrinkage and chromatin condensation are traditionally viewed as effects of apoptosis but hyper-osmotic shrinkage sensitizes hepatocytes towards CD95 ligand-induced apoptosis (149).

In summary, the findings of this study show that nucleus morphology is osmotically sensitive and the relationship between morphology and osmolarity is significantly non-linear, in contrast to the behavior of the cell. The nuclear response does not depend on integrity of the actin cytoskeleton. Isolated nuclei contract in
respond to physiologic changes in macromolecule concentration but not in response to similar changes in the concentration of common intracellular ions, suggesting that osmotic pressure arises from size exclusion of large solute molecules rather than membrane confinement of ions. These results on passive cells also offer a valuable baseline for studies that seek to interpret physical changes that occur during active cell processes.
3 Buckling kinematics of the osmotically-loaded chondrocyte nucleus

3.1 Introduction

Lamins A and C support the nuclear envelope and give the nucleus structural integrity. They are also involved in a surprising number of important biological processes including premature aging disorders (53), normal aging (56), DNA repair (150), differentiation (47) and mechanotransduction (54). Most adult mammalian cells express lamins A and C at high levels but expression is low in stem cells (47), cancer cells (151) and neutrophils (152). Low lamin expression renders the nucleus deformable (45), allowing these cells to more easily invade tissue. These diverse and important functions stimulate great interest in phenotypes associated with lamin mutations or loss of lamin expression, particularly changes in the mechanics of the nuclear lamina. However, the presence of the nucleoplasm underneath the nuclear lamina confounds attempts to measure its force-displacement relationship in situ. Studies of lamina mechanics have employed harsh chemical treatments to swell the nucleus and separate the lamina from the nucleoplasm (39, 153). While these data are valuable, a method for measuring the mechanics of the nuclear lamina in situ despite of the presence of the nucleoplasm would represent a significant advance.

Buckling theory provides a potential solution to this problem. When a stiff, slender structure is subjected to steadily increasing compressive load, a point is reached at which deformation in bending can accommodate the load with less strain energy than
continued compressive deformation. At this point, the existing equilibrium becomes unstable and the geometry abruptly shifts into bending. When this happens, the structure is said to have buckled. The process can be modeled quantitatively by calculating the strain energy associated with each possible mode of deformation and finding the point at which the strain energy in bending becomes less than the strain energy in compression for a given set of boundary conditions. The simplest example of bucking is a column compressed between two pin joints. At a critical load, the column will buckle into a half sine wave shape (Figure 13 A and C). In theory, any one of an infinite series of sine waves of increasing frequency satisfies the boundary conditions for the post buckled shape but in practice only the lowest frequency shape occurs because it has lowest bending strain energy.

The situation becomes more nuanced when the column is supported on an elastic foundation. There are now two sources of strain energy: the bending of the column and the deformation of the underlying elastic foundation. Low frequency shapes create little bending in the beam but impose a large amount of displacement that compresses or stretches the foundation. Higher frequency shapes deform the foundation less but bend the column more (Figure 13 B and D). Therefore, the buckled shape of a column on an elastic foundation is not necessarily a half sine wave. For a soft column on a stiff foundation, the lowest energy geometry will involve a high frequency of bending and little lateral displacement. For a stiff column on a soft foundation, the buckled shape
will have a lower frequency and larger displacements. Therefore, it is possible in theory
to determine the stiffness of the column relative to the foundation without measuring
any loads by observing the buckled shape. This is a fundamental and powerful
departure from the traditional mechanical approach of determining stiffness by
comparing a known load to a known
displacement.

Figure 13: (A) A pinned-pinned column under compressive load. (B) A pinned-pinned column supported by an elastic foundation distributed along its length. (C) The unsupported column will buckle into a half-sine wave geometry when the load reaches a critical value. (D) The supported column will buckle into a higher frequency shape. The number of ripples in the buckled shape will depend on the bending stiffness of the column relative to the stiffness of the elastic foundation.

The same qualitative behavior applies to structures with more complicated
shapes that consist of a thin, stiff element resting on a softer continuum, although exact
analytical solutions quickly become impossible. In general, the frequency of ripples in
the post-buckled shape depends on the relative stiffnesses of the slender structure and
the underlying foundation (154). This principal is frequently employed in nature to
generate patterned structures. For example, human fingerprints form because the skin
of the finger tip grows faster than the flesh beneath it. This creates a compressive stress
in the skin that generates the high frequency, closely spaced ripples that make up finger
prints (155).

The chondrocyte nucleus can be modeled as a spheroidal substrate-film
structure, in which the film represents the lamina and the substrate represents the
nucleoplasm (Figure 14). A recent study used numerical methods to analyze buckling in
this model for various values of the aspect ratio of the spheroid, the thickness of the film
and stiffness of the film relative to the substrate (156). Compressive stress arises in the
film due to a mismatch in the area of the film and the surface area of the substrate
because either the substrate shrinks or the film expands. The compressive stress in the
film is greatest along the shortest circumference of the spheroid. The consequence of
this is that ripples form parallel to the long axis of the spheroid first (Figure 15). In
keeping with the general trend, thick, stiff films are resistant to bending and buckle into
shapes with a small number of deep ripples while thin, soft films buckle into shapes
with a larger number of shallow ripples.
Figure 14: Cut-away view of the spheroidal substrate-film model. The substrate represents the soft nucleoplasm that forms the core of the spheroid. The film represents the lamina, which forms a stiff shell encasing the spheroid.

Figure 15: A spheroid with ripples running parallel to the major axis. Buckling-induced ripples in spheroidal substrate-film structures tend to run parallel to the major axis in this manner because the compressive stress in the film is greatest along the shortest circumference of the spheroid. This image is illustrative and is not a quantitatively-determined buckled mode shape.

This model could, in principal, be used to determine the stiffness of the nuclear lamina relative to the nucleoplasm. A substrate-film model could be created from three
dimensional light microscopy images of the chondrocyte nucleus in an unbuckled configuration. Then, a hyper-osmotic stress could be used to shrink the nucleus and the nucleus could be imaged again to capture its post-buckled shape. The model would be run iteratively to identify the relative film stiffness that generated the post-buckled geometry most closely matching the nucleus geometry observed under hyper-osmotic load. This relative stiffness could be converted to an absolute value using existing literature on the mechanical properties of the nucleoplasm (33). The primary practical obstacle to this experiment is optical resolution. Conventional confocal microscopy has limited resolution in the z-direction, making the detailed three dimensional images required by this approach unattainable. However, emerging super-resolution microscopy techniques, most notably structured illumination microscopy (157-159), may soon overcome this obstacle. Imaging hurdles aside, the analysis depends on the fundamental assumption that the geometry of the chondrocyte nucleus after hyper-osmotic load is that of a post-buckled spheroidal substrate-film structure. This assumption must be tested before the inverse modeling study described above can be justified. The first goal of this study is to identify a hallmark of spheroidal substrate-film buckling that can be measured in two dimensional images and demonstrate its occurrence in the hyper-osmotically loaded chondrocyte nucleus.

The kinematics of a buckled spheroidal substrate-film structure follow well defined patterns. As noted above, these structures tend to buckle longitudinally.
Longitudinal buckling means that the ripples in the buckled shape run between the poles of the spheroid (the ends of the major axis) and reach their greatest depth at the equator (the shortest circumference) (Figure 15). This tendency for the greatest displacements to occur near the equator is henceforth referred to as equatorial bias in the displacement field. The hypothesis of this study is that displacement of the nucleus perimeter under hyper-osmotic stress exhibits equatorial bias. Once this hypothesis is proven, we accept that the osmotically loaded shape is a buckled shape and attempt to deduce lamina stiffness from the number of ripples in the buckled shape.

3.2 Methods

3.2.1 Osmotic loading of chondrocytes

Porcine chondrocytes were isolated and seeded on 42 mm glass coverslips as described in 2.2.2. They were incubated with a solution of 0.5 μM Syto 13 in iso-osmotic DMEM for 20 minutes to label the nuclei. A single two dimensional image consisting of a DIC channel and a syto 13 fluorescence channel was taken of the sample at 380 mOsm. Then, the media was exchanged for identical media and allowed to equilibrate for 3 minutes before the imaging was repeated to provide a control image. Then, the media was exchanged for media at 580 mOsm and allowed to equilibrate for a further 3 minutes before the another image was taken.
3.2.2 Measurement of equatorial bias

Images of individual nuclei before and after application of hyperosmotic load were thresholded in Matlab (The MathWorks, Inc., Natick, MA) using the adaptive ISODATA algorithm (145). A pre-existing function called the signature function (160) was applied to each image to compute the outline of the nucleus in polar co-ordinates and these two outlines were transformed to collocate their centroids and align their major axes (Figure 16). The radial displacement of points along the nuclear perimeter due to hyper-osmotic stress was computed by taking the difference between the two signature traces. The equatorial bias hypothesis was examined by testing for statistical significance in the correlation between the radial displacement of a point under hyper-osmotic stress and the angular displacement of that point from the major axis of the nuclear cross section. The equatorial bias hypothesis only applies to cross sections that lie close to the plane of the major axis. These cross sections are distinguished by the fact that they are more elliptical on average while sections perpendicular to the plane of the major axis are more circular (Figure 17). For this reason, the data was culled to exclude images of nuclei that had an aspect ratio of less than 1.1 before the application of hyper-osmotic load.
Figure 16: Process for determining radial displacements. (A) The image of the nucleus at 380 mOsm is thresholded automatically to produce the white outline. (B) The process is repeated with the image of the same nucleus after equilibration to 580 mOsm. (C) The two outlines are re-expressed in polar co-ordinates and realigned so that their major axes lie along the horizontal. The radial displacement is the radial distance between the outline at 380 mOsm (blue) and the outline at 580 mOsm (red).

Figure 17: A cross section of a spheroid taken close to the plane of the major axis is elliptical. This distinguishes it from a cross section taken at or near a plane perpendicular to the major axis, which is more circular. When considering a selection of cross-sections of spheroids that are oriented randomly in space (as are the nuclei of
chondrocytes), it is possible to select for cross sections close to the plane of the major axis by selecting cross sections that are substantially greater than 1.

3.2.3 Determination of stiffness from buckled mode shape

The buckled shape is characterized by the number of longitudinal ripples, n (Figure 18). The value of n can best be determined from cross sectional images taken close to the equatorial plane of the spheroid. The cross section of a spheroid is circular at the equatorial plane so cross sections close to the equatorial plane were identified for the purposes of this study as those having an aspect ratio less than 1.1. Contours were extracted from the two dimensional images before and after the application of osmotic load. Since the contours were approximately circular in these data, it was no longer feasible to align the contours using their major axes. Instead, these contours were aligned using an iterative closest point technique (161). This calculation yielded a vector of displacements between each point on the perimeter of the initial cross sectional image and the corresponding point on the cross sectional image taken after osmotic loading. This vector is henceforth referred to as the perimeter displacements. The two cross sections were scaled to the same area so that these perimeter displacements would reflect only the change in shape due to osmotic load. The perimeter displacement was negative when the contour of the nucleus after osmotic load lay inside the initial contour and vice versa. Hence, ripples induced by osmotic stress could be detected as peaks in the perimeter displacement vector (Figure 19).
Figure 18: Qualitative representations of buckled mode shapes. Panels A, B and C are surface renderings of a spheroid with 3, 4 and 5 ripples respectively. Panels D, E and F are cross sections of the surfaces in A, B and C, respectively.
Figure 19: Typical data set for buckled mode shape calculation. (A) First, the round cross section of the nucleus before hyper-osmotic loading (blue filled shape) is aligned with the contour of the same cross section after hyper-osmotic loading (red curve). (B) The perimeter displacements along the circumference of the nucleus. Positive values indicate that the hyper-osmotic contour lies outside the iso-osmotic contour and vice versa. This data set has 4 peaks in the perimeter displacement curve (the peaks at the beginning and end combine to form a single peak because the data is cyclical) so the number of ripples is 4 for this data set.

No two contours are identical and small amounts of perimeter displacement were observed even in iso-osmotic control experiments. Before the hyper-osmotic data could be analyzed, it was necessary to establish how much of the observed perimeter displacement was due to osmotic loading. To this end, the iso-osmotic controls were used to establish the level of perimeter displacement due to causes other than osmotic stress. The mean absolute perimeter displacement was calculated for 35 iso-osmotic controls and the 90th percentile value was taken as the lower bound for mean absolute perimeter displacements in hyper-osmotically loaded data that would be considered
true measures of shape change due hyper-osmotic stress. 35 hyper-osmotically data sets were analyzed. The number of ripples in each data set was computed by counting zero crossings in the perimeter displacement curve.

The number of ripples in the buckled shape depends on the ratio between film stiffness and substrate stiffness. Unfortunately, there is no analytical solution for the case of a spheroidal substrate-film structure so there is no equation that can be used to deduce mechanical properties from the number of ripples. As the aspect ratio of a spheroid approaches infinity, the shape approaches a cylinder. This is useful because the case of a cylindrical film with an elastic substrate has been solved analytically (note that, in the cylindrical model, the elastic substrate forms a core inside the film as it does in the spheroidal model). This solution can be used to approximate the behavior of the spheroid at large aspect ratios. In fact, the buckled geometry of the structure depends more on the thickness of the film (relative to the radius) and its stiffness (relative to the foundation) than it does on the aspect ratio. This means that the cylindrical solution yields excellent estimates of the spheroidal response for aspect ratios as low as 1.3 (162).

The following is a summary of the analytical solution for buckling of a cylindrical film with an elastic substrate that was presented by Yin et al. (162). The solution employs a plane strain assumption along with several other assumptions familiar from the mechanics of materials. Deformations are assumed to be small and plane sections are assumed to remain plane. The circumferential displacement is
denoted \( v \) and the radial displacement is denoted \( w \). Buckling is induced by a pressure \( p \) acting normal to the outer surface of the cylinder. The elasticity of the foundation is represented as a pressure that varies linearly with the radial displacement i.e. \( p = Kw \) where \( K \) is the foundation stiffness. This is the Winkler approximation, in which the foundation is assumed to be a series of one dimensional springs among which there is no lateral interaction. The solution follows the conventional approach for analysis of buckling problems. First, an expression for the potential energy of the structure is formed. For a cylinder of height, \( H \):

\[
\Pi = \int_0^{2\pi} \left[ \frac{1}{2} N \varepsilon a + \frac{1}{2} M \kappa a + \frac{1}{2} Kw^2 a + \frac{1}{2} p \left( w + (a + w)e - \beta \nu \right) \right] a d\theta
\]

(3.1)

where \( N \) is the circumferential force in the cylinder wall, \( \varepsilon \) is the stretching strain, \( M \) is the bending moment, \( \kappa \) is the curvature, \( a \) is the radius, \( e \) is the circumferential strain at the centroid \( (e = (dv/d\theta + w)/a) \), \( \beta \) is the rotation \( (\beta = (dw/d\theta - v)/a) \) and other terms have the definitions already given. The four terms in the integrand represent, in order, the strain energy due to stretching, the strain energy due to bending, the strain energy due to deformation of the foundation and potential energy due to the work done by the pressure as it compresses the cylinder. The relationship between applied load and deformation is found by setting the first variation of the potential energy equal to zero because the system is at equilibrium when the potential energy is minimized with respect to all degrees of freedom. This returns two equations relating load to deformation, one for the radial degree of freedom and one for the circumferential degree.
of freedom. One definition of the buckling load is the minimum load at which the system is at equilibrium in its initial configuration and also in another configuration infinitesimally displaced from the initial configuration. This condition is what allows the system to change its configuration while still minimizing potential energy i.e. to buckle. The perturbation technique is used to solve for this load. The perturbation of the displacements $v$ and $w$ have the form $v_1 = V \sin n \theta$ and $w_1 = W \cos n \theta$ because the cylindrical geometry imposes a requirement for periodicity. The parameter $n$ is the number of ripples in the buckled shape. Substituting the perturbed parameters into the equilibrium condition yields a pair of equations involving the load, the number of ripples and the properties of the cylinder. These expressions can be manipulated to yield the following expression relating the bifurcation load, $p_n$, to the number of ripples.

Note that subscript $f$ refers to the film and subscript $s$ refers to the substrate.

$$p_n = \frac{\overline{E}_f I (1 + \overline{K}) \left( n^2 - 1 \right)^2 + \overline{K} \left( 1 + A a^2 / I \right)}{a^3 \left[ n^2 - 1 + \overline{K} - \left( \overline{K} + \overline{K}^2 \right) / n^2 \right]}$$

(3.2)

where $A$ is the cross sectional area, $I$ is the second moment of area, $\overline{K} = K a^2 / \overline{E}_f A$ is the dimensionless foundation stiffness and $\overline{E}_f = E_f (1 - \nu_s^2)$ where $\nu_s$ is the substrate Poisson’s ratio. The critical load is determined by finding the value of $n$ that minimizes the bifurcation load, $n_{cr}$. However, this expression cannot be solved in closed form and must be further simplified by making certain reasonable assumptions. If the radius of
the film is much greater than its thickness, then \( I / \left( Aa^2 \right) = t^2 / \left( 12a^2 \right) = 1 \) and the terms associated with \( \overline{K} \) can be omitted from the denominator of (3.2) when \( n > 2 \) and \( \overline{K} < n \).

The reduced form of (3.2) can be used to deduce the following solution for the number of ripples:

\[
\Scr = a \left( \frac{K}{\overline{E} I} \right)^{\frac{1}{3}} = \left( \frac{a}{t} \right)^{\frac{2}{3}} \left( \frac{12\overline{E}_s}{\overline{E}} \right)^{\frac{1}{4}}
\]  

(3.3)

where \( \overline{E}_s = E_s / (1 - 2\nu_s) (1 + \nu_s) \). This definition of the stiffness related the one dimensional approximation used in this analysis to the conventional definition of stiffness in continuum mechanics as the Young’s modulus. This equation is used to compute the relative stiffness of the nuclear lamina.

### 3.3 Results

#### 3.3.1 Radial displacements of elliptical sections are biased towards the equator

Radial displacement of points along the perimeter of the nucleus under hyper-osmotic stress is positively correlated with the angular position of the point with respect to the major axis of the cross section. This implies that points near the equator of the nucleus move large distances as the nucleus changes shape while points near the poles move relatively little.
Figure 20: The radial displacement of the nuclear perimeter under hyper-osmotic stress is positively correlated with angular position with respect to the major axis. Data points represent 360 points tracked on the perimeters of each of 17 cells. The slope is highly significantly different from zero (p < 0.001).

Linear regression is the most appropriate statistical tool to test the hypothesis that radial displacement of a point on the nuclear perimeter increases as the angle between the point and the major axis increases. However, one potential criticism of this approach is that the number of points tracked along the perimeter of each nucleus was chosen arbitrarily during the image analysis process so the number of points in the correlation test (and hence its statistical significance) is not a strictly experimental
parameter. Unfortunately, there is no rigorous way to set a limit for the maximum number of points that should be tracked. However, it is possible to set a minimum. The most conservative approach in terms of the number of degrees of freedom is to track only four points in each nucleus, one at each end of the minor axis and one at each end of the major axis (Figure 21). A t-test is then used to compare the radial displacement of the polar points (i.e. the points on the major axis) with the radial displacement of the equatorial points (i.e. the points on the minor axis). This test confirms that the radial displacement of points at the equator is greater than that for points at the poles (Figure 21).

Figure 21: Minimal representation of radial displacement of nuclear perimeter after hyper-osmotic stress. The blue outline is before the application of stress and the red outline is after. The black arrows are the polar displacements and the white arrows are the equatorial displacements.
3.3.2 Analysis of buckled shape

Of the 35 hyper-osmotic loading data sets, 13 met the criteria for inclusion in the
mode shape calculation (aspect ratio < 1.1 and mean absolute perimeter displacement >
90th percentile value from iso-osmotic data). The median number of ripples was 3 and
the mean was 3.38. The median value of 3 is substituted into equation (3.3) to determine
the stiffness of the lamina. Other parameters in the equation are determined from
analysis of this data set or from the literature. The mean nucleus radius, a, observed in
this study is 3.34 μm. Electron microscopy shows that the thickness of the nuclear
lamina in the articular chondrocytes is, at most, 30 nm (163). The substrate modulus (i.e.
the modulus of the nucleoplasm) is assumed to equal a value measured from HeLa cells at 167 Pa (this value is modified from the reported value of 250 Pa because the report assumed the nucleoplasm was incompressible while this analysis assumes $\nu_s = 0$) (33). Substituting in these values and solving for $E_f$ returns a value of 34.1 MPa as the modulus of the nuclear lamina.

### 3.4 Discussion

Cell nuclei are often described as being either smooth or irregularly shaped. Irregular nucleus shape is an important phenotype associated with cancer (164), premature aging disorders (48) and normal aging (56). However, it is not clear how these irregular shapes are formed. It is possible that a complex shape arises from an irreducibly complex and random network of local forces that act at various points on the nucleus. Such forces might be applied by tensed actin cables, for example. However, it is also possible that complex shapes arise from the simple, repeatable and quantitatively tractable physics of buckling, which superimposes undulations on the initial geometry. A theory for the evolution of a post-buckled shape was recently presented that describes how multiple, evenly distributed undulations can collapse into a single, large invagination under certain conditions (165). This expands the range of geometric features that can be modeled as buckling phenomena. In this study, we observed equatorial bias in the displacement of the nuclear perimeter under hyper-osmotic stress. This is a hallmark of spheroidal substrate-film buckling.
The observation of buckling mechanics in the nucleus creates an opportunity to address a long standing and important problem in nuclear mechanics: in situ measurement of lamina properties. These are difficult to measure because the force-deflection response is influenced by the underlying nucleoplasm. Inverse modeling could be applied to three dimensional images of the nucleus before and after buckling to estimate its mechanical properties. However, this approach is currently frustrated by the limited resolution of confocal microscopy. We chose to execute a two dimensional analog to this experiment as a proof of concept and determined a value of 34.1 MPa as the modulus of the nuclear lamina. The membrane stiffness of the lamina in a tensile loading experiment has been reported to be 24 mN/m (39). The membrane stiffness is the product of the modulus and the thickness. For the assumed thickness of 30 nm, this leads to a lamina modulus of 0.8 MPa calculated from tensile testing, much less than the value calculated here from buckling theory. The discrepancy dissipates if the thickness is increased because membrane stiffness scales with t while bending stiffness scales with $t^3$. The discrepancy disappears when the thickness is assumed to be 196 nm, more than the lamina thickness observed in electron microscopy images. However, these images also show a thick dense layer of heterochromatin attached to the interior face of the lamina (163). It is possible that this heterochromatin layer contributes to the bending stiffness of the nuclear lamina and increases its effective thickness. This implies that the
bending stiffness of the nuclear lamina may change with changes in chromatin condensation, even if lamin expression remains constant.

The modulus measurements derived from buckling theory in this study are subject to limitations and serve primarily as a proof of concept. Two dimensional images provide a limited view of the true geometry of the hyper-osmotically loaded nucleus and assumptions are necessary to make conclusions. This approach to mechanical testing of the nuclear lamina requires accurate three dimensional images to reach its full potential. With super-resolution techniques entering the mainstream, it will soon be possible to measure the three dimensional, buckled geometries of wild type and lamin deficient cells and so get in situ data on lamina properties from a trivial osmotic loading experiment.
4 Hyper-osmotic stress accelerates transport between the cytoplasm and the nucleus

4.1 Introduction

Many important processes in cell biology involve transport across the nuclear envelope. RNA must be exported from the nucleus to the cytoplasm before it can be translated into protein. Building blocks of the genome, such as histone proteins, are manufactured in the cytoplasm and must be imported into the nucleus rapidly when new DNA is synthesized. A host of other bioactive molecules traffic between the cytoplasm and the nucleus. The cell controls signal transduction, cell cycling and RNA processing, along with many other processes, through finely tuned orchestration of this nucleocytoplasmic traffic (166). For example, IL-1 α acts via an endogenous signaling pathway in addition to the more widely studied secretory pathway, and activation of this pathway involves active transport of IL-1 α from the cytoplasm to the nucleus (167). IL-1β also shuttles between the cytoplasm and the nucleus, although this is exclusively due to passive diffusion (168).

All traffic between the cytoplasm and the nucleus passes through nuclear pore complexes (NPCs). NPCs are complex molecular machines that span the nuclear envelope. They consist of multiple proteins known as nucleoporins which self-assemble into a toroidal structure (169) with 8-fold symmetry and a channel at its center (Figure 3). Experimental observations and numerical models indicate that the NPC has the capacity to bend and stretch considerably (170, 171), dilating if necessary to allow...
passage of particularly large cargo (172). While some peripheral nucleoporins exchange freely between the NPC and the cytoplasm or nucleoplasm, many of the core proteins remain in place from the time the NPC is assembled until it is disassembled. Since NPC turnover occurs during mitosis, the core proteins of NPCs in post-mitotic cells such as chondrocytes remain in place throughout adult life (173).

Unfolded domains of phenylalanine-glycine (FG) repeats extend from the walls of the NPC channel, forming a sieve that blocks passage of molecules larger than ~40 kDa (174). However, molecules exceeding this size can pass through the nuclear pore complex if they are attached to nuclear transport receptors. Nuclear transport receptors bind weakly to the FG repeats obstructing the channel so they can travel through it by facilitated diffusion. The exact structure formed by the FG repeat domains remains a topic of active investigation. The ‘selective gate’ model (175) proposes that the FG repeat domains extend into the pore space with minimal interaction among themselves, forming bristles in a polymer brush that occupies the channel. The ‘selective phase’ model (176) proposes that the unfolded FG repeat domains are crosslinked by hydrophobic interactions into a gel that plugs the NPC (177). Nuclear transport receptors can compete with the crosslinking bonds and, in doing so, dissolve into the gel. Regardless of the mechanism, the result is that the nuclear envelope functions as a selective sieve, allowing passage of all small solutes but allowing large macromolecules to pass only if they are bound to the nuclear transport receptors. The Ran GTP/GDP
cycle governs the interactions between nuclear transport receptors and their cargo. Ran is maintained in a GTP bound form in the nucleus and a GDP bound form in the cytoplasm. This imposes directionality on nuclear transport of large macromolecules. Nuclear transport receptors can function as importins or exportins, depending on which form of Ran causes them to bind or disassociate from their cargo (166). Since this process consumes energy, Ran binding can function as a pump driving transport through the NPC against a concentration gradient (178).

The most commonly used tool for measurements of intracellular diffusion is fluorescence recovery after photobleaching (FRAP). In FRAP experiments, the diffusing solute of interest is tagged with a fluorescent molecule, typically fluorescein. A focused laser beam is used to bleach a small region of interest (ROI) in the fluorescent field. As the solute moves randomly via diffusion, bleached molecules in the ROI leave and unbleached molecules in the vicinity enter. Over time, the cumulative effect of this process is equilibration of the bleached region with its environment. The diffusion coefficient can be deduced from the rate constant of fluorescent recovery (179). FRAP experiments show that the crowded environment of the cell slows diffusion of macromolecules by a factor of about 4, regardless of whether measurements are taken in the nucleus or the cytoplasm (180). Cytoplasmic diffusion slows as extracellular osmolality increases due to increased frequency of collisions with cytoplasmic solutes that become more crowded as water is drawn out of the cell (181). The rate of passive
nucleocytoplasmic transport has also been measured using photobleaching (174). Diffusion across the nuclear envelope is much slower than diffusion in either the nucleus or the cytoplasm. This means that the intranuclear concentration rises uniformly when there is a flux of solute into the nucleus with negligible concentration gradient between the periphery and the center (174). This simplifies mathematical modeling considerably (see Methods).

Osmotic stress may alter transport to the intranuclear space via a geometric mechanism. The nucleus equilibrated with the cytoplasm more quickly in calcium signaling experiments when the nucleus switched from a smooth shape to a convoluted shape because the increased surface area to volume ratio in the convoluted morphology allowed more influx per unit volume (182). This change allowed the nucleus to respond to an important 5 Hz calcium signal in its convoluted shape that it could not respond to in its smooth shape, leading directly to changes in gene expression. Chondrocyte nuclei assume smooth shapes under hypo-osmotic stress and smaller, more convoluted shapes under hyper-osmotic stress (Figure 8). This may change the rate of transport into the nucleus by altering its surface to volume ratio.

It is also possible that the permeability of the nuclear envelope itself is sensitive to osmotic stress. The nuclear lamina may experience tensile or compressive stress as the nucleoplasm swells and shrinks under osmotic stress. NPCs appear to enlarge when tension is applied to the nuclear lamina (147). The diameter of NPCs released from the
nuclear envelope exceeds the in situ diameter, suggesting that NPCs in situ are compressed by the surrounding nuclear envelope in a manner that shrinks them substantially (171). Finally, the NPC may be directly affected by osmotic stress. The repulsive force exerted by the FG-repeat domains falls when they collapse (175).

Macromolecular crowding increases in cells under hyper-osmotic stress. This increases the collapsing entropic force acting on extended polymers (183) inside the cell and may be sufficient to increase the permeability of the NPC.

We hypothesized that nucleocytoplasmic transport would change in articular chondrocytes subjected to osmotic stress. Our mathematical model relies on the assumption, stated above, that the rate of diffusion across the nuclear envelope is much smaller than the rate of diffusion in either the nucleoplasm or the cytoplasm, regardless of osmolality. While reports exist in the literature of diffusion measurements in the cytoplasm at various osmolalities (180), no such data is available on intranuclear diffusion. Therefore, we measured intranuclear diffusion at a range of osmolalities to ensure that the assumption of rapid intranuclear diffusion would not be violated during the FRAP experiment. Scanning microphotolysis (184), an alternative fluorescent assay of diffusion (see Methods), was used to measure intranuclear diffusion coefficients.
4.2 Methods

4.2.1 Cell isolation and culture

Porcine chondrocytes were isolated and cultured on glass coverslips for microscopic imaging as described in section 2.2.2.

4.2.2 Loading dextrans by electroporation

Fluorescein-tagged dextrans were loaded into chondrocytes by electroporation. Electroporation is the application of short pulses of intense electric field to cells in order to permeabilize the cell membrane for a brief period (185). This allows impermeable molecules in the extracellular solution (10 kDa fluorescein-labeled dextrans in this case) to enter the cells. A custom-built microporator device (built by Geoffrey Erickson) was used to apply the electric field (186). The microporator device consisted of a pair of electrodes embedded in a plastic block (the ‘foot’) that was mounted on a rail so that its vertical position could be controlled precisely using a micrometer (Figure 23). A 5mm X 5mm recess was machined into the bottom face of the foot and electrodes were positioned along two parallel sides of this recess (Figure 23B). A coverslip coated with cells for electroporation was placed on a flat platform below the foot and 20 μl of a 25 mg/ml solution of 10 kDa fluorescein-tagged dextrans was pipetted on to the center of the coverslip. The foot of the microporator was lowered on to the coverslip so that the recess sealed against it to create a chamber surrounding the cells. An electric potential of 170 V was applied to the electrodes for two pulses of 50 ms separated by an interval of
5 s using a BTX Electro Square Porator ECM 830 (Harvard Apparatus, Holliston, MA) as a voltage source. The coverslip was immersed in feed media immediately after electroporation and incubated for 10 minutes at 37 °C before staining.

Figure 23: The microporator device. (A) The device consists of electrodes embedded in a plastic foot mounted on a linear bearing over a platform. The voltage source is not pictured. (B) Detailed schematic of the microporator foot. The foot seals against the coverslip to create a chamber full of dextran solution surrounding the cells on the coverslip. A voltage pulse between the electrodes temporarily permeabilizes the cells, allowing dextrans to enter.

4.2.3 Nuclear SCAMP experiments

Scanning microphotolysis (SCAMP) involves continuously scanning and imaging a single line of pixels in a field of diffusing solute at high laser power (184). The
fluorescent intensity during scanning is determined by the competing effects of bleaching, which lowers the intensity, and diffusion, which raises the intensity by bringing unbleached solute molecules into the ROI. A mathematical model incorporating the point spread function of the lens is used to model the evolution of the intensity for various values of the bleaching constant, k, and the diffusion coefficient of the fluorescent solute, D. This model is optimized against the actual evolution of the intensity profile in experimental data to determine D. One advantage of this model is that it bleaches a small region so it is appropriate for measuring diffusion in small compartments. Also, SCAMP employs the fastest imaging mode available on the confocal microscope so it can accurately measure diffusion that happens too quickly to be captured in two dimensional line-scanning images.

In this study, dextran loaded cells were stained with a 1 μM solution of Syto 82 (Molecular Probes, Eugene, OR), an orange nucleic acid stain, for 10 minutes at 37 °C to allow visualization of the nucleus. The coverslip was mounted in a perfusion chamber (PeCon GmbH, Kornhalde, Germany) and washed three times in phenol-free DMEM with 1.5% HEPES and an osmolality of 380 mOsm (all cell culture reagents were supplied by Gibco, Grand Island, NY). The chamber was mounted on the confocal stage at room temperature. The media in the chamber was exchanged twice using syringe pumps with media that had been adjusted using salt / deionized water to osmolalities at 6 values ranging between 255 and 612 mOsm. The osmolality of each solution was
verified with a freezing point osmometer (Precision Systems Inc., Natick, MA). The sample was allowed to equilibrate to the new media for 2 minutes before data was collected. An image of the nucleus was collected via excitation of the Syto 82 stain with the 543 nm laser and collection of light emitted between 570 and 585 nm. All images in this study and in the following FRAP study were acquired using a 100x, 1.3 numerical aperture oil lens. The nuclear image was used to position a ROI consisting of ten 0.12 μm pixels in a line at the approximate centroid of the nucleus. This line was excited with the 488 nm laser at high power (75% of maximum) and emitted light was collected through a 505 nm high pass filter. The line was imaged continuously 45 times and each scan took 767 μs. Two such sequences were taken for each sample so that the first one could be discarded to eliminate distortion of the results due to bleaching of bound, immobile dextrans. Diffusion coefficient values were obtained for each data set by fitting the data with a pre-existing computational model (coded by Holly Leddy and Susan Christensen) in Matlab (The Mathworks, Natick, MA). The model also returned an $R^2$ value as a measure of the quality of the fit. Diffusion coefficients were rejected as unreliable if the associated $R^2$ value was less than 0.9.

4.2.4 Nuclear FRAP experiments

Chondrocytes were cultured, loaded with dextrans, stained and mounted in a perfusion chamber on a confocal microscope as described in section 4.2.3. Cells with bright nuclear staining and abundant dextran loading were located for testing. The
media in the chamber was exchanged with experimental media that had been adjusted to 280, 380 or 480 mOsm and the cells were allowed to equilibrate for two minutes before any data was collected. In all cases, the media used was phenol-free DMEM high glucose and the osmolality was adjusted using deionized water or salt and verified using a freezing point osmometer. A circle 2.8 μm in diameter was used as the bleaching ROI in all experiments. This value was chosen because it was half the average equivalent diameter of the chondrocyte nucleus. The ROI used for quantifying recovery was the outline of the nucleus, as determined from the Syto 82 image. The goal of this arrangement was to limit signal loss during the bleaching pulse, since there is a limited reservoir of fluorescent dextran in the cytoplasm. Also, it is impossible to create a bleaching ROI that exactly matches the contour of the nucleus, particularly in light of the three dimensional shape of the nucleus and the bleaching PSF. Bleaching the whole nucleus inevitably leads to bleaching of a region of cytoplasm near the edge, which in turn introduces rapid, osmotically sensitive recovery that is not reflective of nucleocytoplasmic transport but rather reflects diffusion within the cytoplasm. Shrinking the bleaching ROI within the nucleus diminished this source of error. It is worth noting that the bleached region observed after photobleaching typically resembles the shape of the nucleus more than the shape of the specified ROI, suggesting that intranuclear diffusion is sufficiently rapid to spread the bleaching effect throughout the nucleus almost instantaneously so that observed recovery is due primarily to
nucleocytoplasmic transport (Figure 20). This approach is similar to the approach used by Luheshi et al. in tracking nuclear import of IL-1β (168). The timing of the bleaching experiment was as follows: the first 10 frames were recorded at 0.5 second intervals. The following 20 frames were recorded at 2.5 second intervals. The bleach pulse occurred between frames 3 and 4 and extended that interval to 0.6 seconds. Each image was 256 X 256 pixels with a pixel size of 0.07 μm.

![Figure 24: Bleaching dynamics of the nucleus. (A) Before the bleaching pulse, dextran fluorescence is distributed evenly throughout the nucleus. (B) The red staining represents the nucleus, the blue ring is the ROI that will be targeted by the bleaching pulse. (C) The first image after the bleaching pulse shows a bleached region that resembles the nucleus more than the specified ROI, indicating that rapid nucleoplasmic diffusion spreads the effect of the bleaching pulse throughout the nucleus very quickly so that most of the observed recovery will be due to nucleocytoplasmic transport.](image)

4.2.5 Mathematical modeling of FRAP experiments

This mathematical model describes the diffusion of labeled dextran molecules from the cytoplasm into the nucleus after the bleach pulse. It is assumed that the bleach pulse reduces the concentration of fluorescent dextran within the nucleus to zero while
leaving the concentration in the cytoplasm at its existing level, $C_0$. In practice, $C_0$ is equivalent to the difference between the concentration in the nucleus and the cytoplasm at the instant after bleaching. The cell is represented as two compartments: the nucleus, with volume $V_n$ and fluorescent dextran concentration $C_n$, and the cytoplasm, with volume $V_c$ and fluorescent dextran concentration $C_c$. The volume of each compartment is constant through the experiment since the cell has already equilibrated to the extracellular osmolality. However, the concentration in each compartment varies with time as fluorescent dextrans diffuse from the cytoplasm into the nucleus across the nuclear envelope. The nuclear envelope is represented in the model as a membrane with surface area $S$ and permeability $P$ that separates the two compartments. A quasi-steady state approach is taken (188), meaning that the accumulation of solute within the membrane and the residence time of solute within the membrane is neglected. The flux is defined as the mass crossing a unit area of the nuclear envelope per unit time and the permeability of the membrane is defined as the flux per unit concentration difference across the membrane.
Figure 25: Theoretical model for fluorescence recovery in the bleached nucleus. Compartment c represents the cytoplasm, compartment n represents the nucleus. The grey dashed line represents the nuclear envelope separating the two, which has a permeability P and a surface area S.

The governing equation is a mass balance stating that the mass entering the nucleus per unit time is equal to the mass of solute crossing the membrane per unit time. Hence:

\[ V_n \frac{dC_n}{dt} = PS(C_c - C_n) \]  \hspace{1cm} (4.1)

The two concentrations are related by another mass balance stating that the rate at which solute leaves the cytoplasm is equal to the rate at which solute enters the nucleus. Hence:

\[ V_n \frac{dC_n}{dt} = -V_c \frac{dC_c}{dt} \]  \hspace{1cm} (4.2)

Integrating this relationship gives:
\[ C_c = -\frac{V_n}{V_c} C_n + K \quad (4.3) \]

where K is the constant of integration. The initial conditions, \( C_n = 0, C_c = C_0 \), are applied to evaluate K as being equal to \( C_0 \). Substituting this into (4.1) yields a governing equation in terms of \( C_n \) alone.

\[ \frac{dC_n}{dt} = \frac{PS}{V_n} \left[ C_0 - RC_n - C_n \right] \quad (4.4) \]

where \( R \) is the ratio of nuclear volume to cytoplasmic volume, \( V_n/V_c \). Solving this first order differential equation yields the following solution:

\[ C_n(t) = \frac{C_0}{R + 1} \left[ 1 - e^{-kt} \right] \quad \text{where } k = \frac{PS(R + 1)}{V_n} \quad (4.5) \]

The amplitude of the recovery decreases as the ratio \( R \) increases. This is intuitive because the larger the nucleus is relative to the cytoplasm, the more it drains the cytoplasm down to its own concentration instead of rising up to the concentration of the cytoplasm (4.4). This solution follows the solution provided by Peters (174), who applied the same assumptions to a less biofidelic system that reflected the different focus of that study. In the Peters solution, the rate constant of the recovery \( k \) is equal to \( PS/V_n \). This is because that model system was treated to create an effectively infinite cytoplasmic volume around the bleached nucleus. The solution presented here approaches the Peters solution as the cytoplasmic volume approaches infinity and the ratio \( R \) approaches zero, indicating that the two results are consistent. Interestingly, this
solution states that the rate of recovery is faster when the nucleus is larger relative to the cell. Cells in which the nucleus dominates the intracellular volume experience little recovery of fluorescence and yield spurious data due to the low amplitude of the exponential recovery trace. To eliminate this potential source of error, data points were rejected if the ratio of the nuclear to cytoplasmic volume was greater than 1. This criterion led to the rejection of two data points from a total population of 70, one from the 280 mOsm group and one from the 480 mOsm group. In practice, the recovery curve is observed to have two time constants, a fast time constant reflecting diffusion within the nucleoplasm or the cytoplasm and a slow time constant reflecting nucleocytoplasmic (174). A variant of the model with two time constants was fit to experimental data using the trust-region-reflective algorithm in Matlab to determine the rate constant of nucleocytoplasmic transport, k. The fits were very good with an average $R^2$ of 0.998.

![Figure 26: Parametric behavior of fluorescence recovery model.](image)
The second half of (4.5) states that \( k \), the rate constant of equilibration of the nucleus to the cytoplasm after photobleaching, is related to \( P \), the permeability of the nuclear envelope, by two geometric ratios: \( R \), the ratio of the nuclear volume to cytoplasmic volume and \( S/V \), the ratio of nuclear surface area to nuclear volume. The experiment generates two dimensional images of the fluorescently-labeled nucleus and the cell, which is labeled by cytoplasmic dextrans. However, bleaching prohibits quantitatively accurate three dimensional imaging of the nucleus in the context of this experiment. Therefore, these geometric ratios must be estimated from two dimensional images. The ratio of nucleus volume to cytoplasmic volume was determined by measuring the area of the nucleus and the cytoplasm directly from Syto 82 and dextran staining respectively and assuming:

\[
\frac{V_n}{V_c} = \left( \frac{A_n}{A_c} \right)^{\frac{3}{2}}
\]

(4.6)

This amounts to the assumption that the area ratio in the imaging plane is not on average, greater or less than the area ratio out of the imaging plane. Since chondrocytes nuclei are not oriented with respect to the coverslip in monolayer culture, there is no reason to believe that this assumption is violated. In the case of the surface area to volume ratio of the nucleus, Peters [Peters, 1984 #509] assumed that the nucleus was a sphere and applied the following formula:

\[
\frac{S}{V} = \frac{3}{R}
\]

(4.7)
However, in this study, the nuclei are osmotically loaded and the shape becomes more convoluted as the osmolality increases (Figure 8). Hence, the determination of the surface to volume ratio based on a constant assumed geometry may create biasing errors. An alternative is to estimate surface area based on the perimeter length of the nucleus. Surface area is in general proportional to the square of the perimeter of the cross section. Similarly, volume is proportional to the cross sectional area raised to the power of 1.5. However, the constants of proportionality depend on the three dimensional shape and cannot be determined a priori. This leads to the following expression:

\[
\frac{S}{V} = F \frac{L^2}{A^2}
\]  

where \( L \) is the perimeter length, \( A \) is the cross sectional area and \( F \) is a shape factor that must be determined for each three dimensional geometry. Comparison of standard formulae for the volume and surface area reveal that, for example, \( F = 0.375 \) in the case of a vertically-oriented cube and \( F = 0.423 \) in the case of a sphere. High resolution three dimensional confocal images of chondrocyte nuclei were taken in a separate experiment so that the shape factor \( F \) could be computed for the case of the chondrocyte nucleus. Chondrocytes were stained with Syto 13 and imaged at optimal sampling rate at 380 mOsm and again at 501 mOsm. The images were deconvolved with a measured PSF acquired using the same parameters in a commercial software.
package (SVI Huygens 3D Deconvolution software, Scientific Volume Imaging B. V., Hilversum, the Netherlands). Five images acquired at 380 mOsm were used as a training data set. The actual surface area to volume ratio was determined from the 3D images and compared the value determined from cross sectional images using (4.8) with $F=1$. The actual ratio was divided by the ratio computed from cross sections to determine the true value of $F$ as 0.4059. The predictive ability of this approach was then tested by calculating the surface area to volume ratios using the same $F$ for a group of 10 other nuclei, first at 380 mOsm and then at 580 mOsm. These results were compared with results obtained using Peters’ formula (equation (4.7)). Equation (4.8) gave a smaller average error in the hyper-osmotic case but not in the iso-osmotic case. However, the mean absolute error was smaller using (4.8) in both cases. Hence, equation (4.8) was used in all remaining calculations. Combining both test cases, this method returned errors with a mean of -1.5% and a mean magnitude of 7.3%.

4.3 Results

4.3.1 Intranuclear diffusion measurements

No significant variation of the intranuclear diffusion coefficient, $D$, with osmolality was evident in the results of the nuclear SCAMP experiments (Figure 27). It is possible that a variation occurred that the experiment was not sensitive enough to detect. 95% confidence intervals were computed for two populations of measurements
as a measure of sensitivity. The 7 measurements taken at the lowest osmolalities tested had a 95% confidence interval ranging from 9.90 $\mu$m$^2$/s to 16.67 $\mu$m$^2$/s. The 8 measurements made at the highest osmolalities tested had a 95% confidence interval ranging from 9.28 $\mu$m$^2$/s to 15.49 $\mu$m$^2$/s. These results suggest that a change of more than 13% from the low osmolality condition across the range tested would have been detectable, at least in this comparison. If all the measurements of $D$ are assumed to come from a single population, the mean is 13.67 $\mu$m$^2$/s and the 95% confidence interval is 12.47 to 14.87 $\mu$m$^2$/s. For comparison, 20 kDa dextrans in the nuclei of HeLa cells diffuse at 10.8 $\mu$m$^2$/s (189). This agrees well with our results since our dextrans are smaller and hence are expected to diffuse somewhat more quickly. 10 kDa dextrans diffuse at 75.7 $\mu$m$^2$/s in solution (174) and this value is reduced by a factor of 0.6 to 0.15 in the cytoplasm, depending on the osmolality (180). From these data, we can conclude that the cytoplasmic diffusion is faster than or at least equal to nucleoplasmic diffusion.

Peters proposed the following criterion for the application of the instantaneous, intracompartmental diffusion assumption that underpins the applied mathematical model:

$$D \geq 10 \, r \, P$$  \hspace{1cm} (4.9)

Substituting the lower bound of the intranuclear diffusion for $D$ in this expression and entering the observed value of $r$, the average nuclear radius, as 3.09 $\mu$m
leads to an upper bound of 0.404 μm/s as the maximum nuclear envelope permeability for which the mathematical model is valid.

Figure 27: The intranuclear diffusion D does not vary with osmolality in the chondrocyte

4.3.2 Nucleocytoplasmic transport measurements

The rate of nucleocytoplasmic transport, k, was significantly affected by extracellular osmolality (one way ANOVA, p < 0.05). There was no significant change between 280 and 380 mOsm but there was a significant change between 380 mOsm and
480 mOsm (Fisher’s LSD test, p < 0.05), with an increase in average k indicating that the nucleus equilibrates with the cytoplasm more quickly under hyper-osmotic stress.

Figure 28: The rate of nucleocytoplasmic transport increases under hyper-osmotic stress. (n ≥ 22, error bars = 1 standard deviation, * indicates statistically significant difference)

The hypothesis that the permeability of the nuclear envelope was osmotically sensitive was also examined to determine if the observed change in nucleocytoplasmic transport was due to geometric effects or a change in the intrinsic properties of the membrane. The permeability showed no significant sensitivity to osmolality. At 380 mOsm, the mean permeability is 0.0501 μm/s and the 95% confidence interval is 0.0463 to 0.0539 μm/s. This is almost one order of magnitude less than the maximum threshold
of $0.404 \mu m/s$ established by the intranuclear diffusion measurements, indicating that the assumptions of the mathematical model are valid.

The surface to volume ratio of the nucleus rises steadily with rising osmolality as the nucleus shrinks and assumes a more convoluted shape (Figure 29). There is a significant difference between the observed values at 280 and 480 mOsm (one way ANOVA followed by Fisher’s LSD test, $p < 0.05$). There is no significant change in $R$, the ratio of nuclear to cytoplasmic volume.

Figure 29: The surface to volume ratio rises with rising osmolality. $n \geq 22$, error bars = 1 standard deviation, * indicates statistically significant difference.
4.4 Discussion

We found that nucleocytoplasmic transport was osmotically insensitive but intranuclear diffusion was not. The finding that intranuclear diffusion has no or only slight sensitivity to osmolality is surprising (Figure 27). Seksek et al. reported that cytoplasmic diffusion fell approximately 4-fold between 150 mOsm and 450 mOsm in Madin-Darby canine kidney (MDCK) cells (180). They attributed this decrease to increased macromolecular crowding in osmotically-shrunken cells. Such a large change would have been detected had it occurred in the nucleus in this experiment. The difference in the response may be related to the difference in the microstructure of the two compartments. The nucleoplasm resembles an open cell foam, with regions of dense chromatin separated and interdigitated by a network of pores and channels known as the inter-chromatin domain (24). The interchromatin domain enlarges under hyper-osmotic stress as the chromatin condenses (64). Large intranuclear particles move preferentially along these channels (190). Enlarging these channels may accelerate the motion of large molecules within the nucleus even as condensation of chromatin retards it. This would explain why the diffusion coefficient D is less osmotically sensitive in the nucleoplasm than it has been reported to be in the cytoplasm.

The rate of nucleocytoplasmic transport, k, increases under hyper-osmotic stress (Figure 28). This appears to be a geometric effect since there is no significant change observed in the permeability of the membrane, P. Two geometric parameters influence
the rate of nucleocytoplasmic transport: the surface to volume ratio, S/V of the nucleus and the ratio of nuclear to cytoplasmic volume, R. Of these, only the surface to volume ratio shows a significant effect of osmolality (Figure 29). This partially explains the observed increase in k although the shape and proportion of the k vs. osmolality curve are different to the S/V vs. osmolality curve, despite the fact that k and S/V are linearly related in the model. This suggests that the observed changes in k are only partially explained by the change in S/V and that other effects also play a role, possibly including subtle changes in P and R that are beneath the sensitivity threshold of this experiment.

The geometric mechanism for acceleration of nucleocytoplasmic transport by hyper-osmotic stress is important because the rate at which the nucleus equilibrates to the cytoplasm is increased even though the rate of transport across the nuclear envelope is constant. It is the effective transport, as determined from the rising concentration of solute in the contracted nucleus, which has changed. Since the shrunken nucleus contains the same genome as the unloaded nucleus, this effective change is as functionally important as a true change in the properties of the envelope and more general in its influence. All solutes see the same nuclear volume, implying that they will all see this effective acceleration of transport, regardless of how they interact with the nuclear envelope so the same trend is expected in active and passive transport. In light of this, geometric change in the nucleus under hyper-osmotic stress assumes new
significance as a mechanism for accelerating all processes that depend on equilibration of the genome to a cytoplasmic signal.

There is evidence that the rate of nucleocytoplasmic transport is the metronome of metabolism. The number of nuclear pore complexes in a cell correlates with metabolic activity (191). A comparison of different types of cerebellar neurons found that Purkinje cells, which have the highest levels of activity and protein synthesis, also have the highest nuclear pore density per unit volume (192). Testosterone treatment of castrated rats led to an increase in NPC density correlated with increased metabolism (193). The results of this study show that hyper-osmotic stress reproduces the effect of a temporary increase in the number of NPCs by accelerating nucleocytoplasmic transport. This would be a robust mechanism for upregulating protein synthesis in response to cartilage loading. It would also explain why synthesis is upregulated under cyclical loading but not under constant loading, since the change in nuclear morphology is expected to disappear under constant hyper-osmotic stress as the chondrocyte regulates its volume. Core proteins in the NPC remain in place during interphase and eventually accumulate oxidative damage in post-mitotic cells, leading to an increase in the permeability of the nuclear envelope (173). The results presented here suggest that this degenerative process mimics cartilage loading, creating a constant, aberrant, biosynthetic signal that may explain why transcription is generally upregulated in degenerate cartilage (194).
The NPC is a source of bioactive molecules in addition to being a conduit for them. Recently, a number of investigators have shown that a mobile sub-population of nucleoporins act as transcription factors that diffuse throughout the nucleus (195, 196). The key finding of this study is that the diffusion time for macromolecules between the nuclear envelope and the genome decreases under hyper-osmotic stress due to changes in nuclear geometry. This has consequences not just for the activity of cargo crossing the nuclear envelope but also for the activity of nucleoporins themselves. This study suggests that the net concentration of these transcription-promoting nucleoporins would increase under hyper-osmotic stress as the surface to volume ratio of the nucleus increases. In summary, the geometric changes induced by hyper-osmotic stress reduce the volume of the nucleus without reducing its surface area, increasing the intranuclear concentration of signals originating at the nuclear envelope.
5. The actin cytoskeleton modulates the osmotic sensitivity of the nucleus.

5.1 Introduction

The chondrocyte nucleus shrinks and wrinkles under hyper-osmotic stress. This accelerates nucleocytoplasmic transport and may render cell physiology sensitive to extracellular osmolality. Therefore, any process in the cell that constrains shrinkage of the nucleus may also blunt the osmotic sensitivity of the chondrocyte. The actin cytoskeleton forms a link in a chain of mechanical connections that extend from the extracellular matrix (ECM) to the nucleus and couples motion of the nucleus to motion of the ECM (127). A corollary to this observation is that, when the ECM is stationary, the same mechanical coupling holds the nucleus stationary. The chondrocyte is unusual in that it has little actin organization, particularly in the vicinity of the nucleus (9). ASCs, like most mesenchymal cells in monolayer culture, spread rapidly into extended flat geometries and bundle actin into thick, tensed cables (Figure 33). These cables extend throughout the cytoplasm and gather into a perinuclear cap that binds to the nucleus and determines its geometry (197) (Figure 34). In addition to providing an excellent model system for actin organization, ASCs are important in their own right because they represent a supply of human adult stem cells that reside in a relatively available and expendable tissue. This study examines the hypothesis that a stable, organized actin cytoskeleton constrains shrinkage of the nucleus under hyper-osmotic stress. The goal is
to demonstrate that disruption of actin amplifies hyper-osmotically induced contraction of the nucleus when perinuclear actin is present (i.e in the ASC) but has no effect when perinuclear actin is absent (i.e. in the chondrocyte).

Two different treatments are applied in this study to reduce actin constraints. The first is cytochalasin D treatment. Cytochalasin D inhibits polymerization of actin and disrupts the bundled cables that are characteristic of actin organization (Figure 33). It eliminates the putative actin constraint by breaking up the actin cables inside the cell. The second treatment is trichostatin A. Trichostatin A is a potent, universal inhibitor of a class of enzymes called histone deacetylases (75). These enzymes have many substrates in addition to histones and have been shown to affect microtubules [Hubbert, 2002 #634], actin [Zhang, 2007 #635] and focal adhesions [Tran, 2007 #699]. We observed that trichostatin A treatment decouples the cell from its substrate (see Results). Therefore, trichostatin A treatment reduces the constraining influence of actin by decoupling it from the substrate into which it is anchored.

These treatments also alter histone acetylation. Trichostatin A increases the acetylation of histones (75) while cytochalasin D decreases it (100). Biologically, histone acetylation generally promotes gene transcription (70). Physically, it causes chromatin to become less condensed (82). Acetyl groups bind to lysines in the histone tails, eliminating exposed positive charges that would otherwise neutralize the negative charges in the DNA wrapped around the histones. This increases self-repulsion of the
chromatin fiber, leading to a more extended chromatin conformation. Chromatin conformation also responds to osmotic stress (64), becoming more compact as extracellular osmolality increases. Therefore, a second hypothesis of this study is that these treatments will affect how chromatin conformation responds to osmotic stress.

5.2 Methods

5.2.1 Osmotic loading of cytochalasin D treated chondrocytes

Chondrocytes were isolated and seeded on glass coverslips as described in section 2.2.2. Cytochalasin D-treated chondrocytes were incubated with 2 μM cytochalasin D in DMEM with 10% FBS for 3 hours before testing while untreated chondrocytes were not. Both sets of chondrocytes were stained for 30 minutes with 0.5 μM Syto 13 (Molecular Probes, Eugene, OR) to fluorescently stain the nuclei. For testing, the coverslip was mounted in the perfusion chamber, washed three times in 380 mOsm media and mounted on the microscope stage. A single two-dimensional fluorescent image was taken before any fluid exchange to serve as a reference. Then, the 380 mOsm media in the chamber was exchanged with fresh 380 mOsm media and a second image was taken to serve as an iso-osmotic control. Finally, the 380 mOsm media was exchanged with 580 mOsm media and a third image was taken. Two minutes was allowed for equilibration after each exchange before an image was taken.
5.2.2 Osmotic loading of trichostatin A treated chondrocytes

Chondrocytes were isolated and seeded on glass coverslips as described in section 2.2.2. Trichostatin A-treated chondrocytes were incubated with 1 μM trichostatin A (Sigma-Aldrich, St. Louis, MO) in DMEM with 10% FBS for 1 hour before testing while untreated chondrocytes were not. Both sets of chondrocytes were stained for 30 minutes with 0.5 μM Syto 13 (Molecular Probes, Eugene, OR) to fluorescently stain the nuclei. For testing, the coverslip was mounted in the perfusion chamber, washed three times in 380 mOsm media and mounted on the microscope stage. All samples were first imaged in three dimensions using an optical slice thickness of 1 μm and a step size between images of 0.5μm with an in-plane pixel size of 0.12 μm. For iso-osmotic controls, the chamber media was exchanged for identical media. For hyper-osmotic controls, the chamber media was exchanged for media that had been adjusted to 549 mOsm by addition of saline. In both cases, the same sample was then imaged in three dimensions again. The three dimensional stack images were projected vertically in post-processing to create a two dimensional image that was insensitive to focusing artifacts.
5.2.3 ASC Isolation and Culture

Human ASCs were obtained from liposuction waste from 7 non-smoking, non-diabetic female subjects. Cells were plated on 225cm² flasks at 8,000 cells/cm² in expansion medium consisting of DMEM-F12 (Gibco, Grand Island, NY), 10% FBS (Atlas Biologicals, Ft. Collins, CO), 1% penicillin-streptomycin-fungizone (Gibco, Grand Island, NY), 0.25 ng/ml TGF-β1 (R&D Systems, Minneapolis, IN), 5 ng/ml epidermal growth factor (Roche Diagnostics, Indianapolis, IN) and 1 ng/ml fibroblast growth factor (Roche Diagnostics). The cells were incubated at 37 °C and 5% CO₂. The media was exchanged every 48 hours and the cells were passaged when they reached 90% confluence. Passaging consisted of lifting the cells with 0.05% trypsin and replating them at 8,000 cells/cm². The cells in this study were passaged 4 times and then stored at -140 °C. They were thawed four days before the experiment and passaged once before being used in an experiment.

5.2.4 ASC Stretching

Elastic silicone membranes (SMI, Saginaw, MI) were coated on one side with a suspension of 2 µm diameter red fluorescent microspheres (Invitrogen Corporation, Carlsbad, CA) in silicone adhesive (General Electric, Waterford, NY) and UV sterilized. Then, the opposite side was incubated at 4 °C overnight in a 40 μg/ml solution of human
plasma fibronectin (Sigma-Aldrich, St. Louis, MO) in PBS. Passage 5 ASCs were seeded on the fibronectin coated side of the membranes at 14000 cells/cm² overnight in expansion media (see 5.2.3). Control samples were incubated for 30 minutes at 37 °C in DMEM:F12 with 5 μM Syto 82 and 1.5 μM MitoTracker Deep Red 633 (Invitrogen Corporation, Carlsbad, CA). Trichostatin A treated samples were incubated under the same conditions in the same solution with the addition of 5 μM Trichostatin A. For testing, each end of the sample was gripped in a clamp and the clamps were mounted in a custom-built apparatus (built by Maureen Upton {Upton, 2008 #714}) on the stage of the confocal microscope. One clamp was fixed at a stationary point and another was mounted on a linear bearing so that its position could be precisely controlled using a micrometer. When mounted, the membrane was suspended in a chamber of media above a coverslip with the cells on the inferior side and the beads on the superior side (Figure 30). The membrane was imaged three times before stretch was applied: once to visualize the nuclei, once to visualize the mitochondrial staining and once to image the fluorescent spheres. The membranes were then stretched to a nominal end-to-end strain of 12% and imaged again. A custom-written Matlab code (written by Chris Gilchrist {Gilchrist, 2007 #715}) was used to determine the strain in the nuclei, the cytoplasm and silicone membrane by applying texture correlation to the images of the nuclei, mitochondria and fluorescent spheres, respectively. In texture correlation, landmarks are specified in an initial grey scale image and located in a subsequent deformed image.
by an optimization process that compares local intensity variations with the intensity variation around the landmark in the initial image. This yields a matrix of landmark displacements from which Lagrangian strain can be calculated [Gilchrist, 2007 #715]. In this study, the strain transfer ratio of the cytoplasm was defined as the ratio between the maximum principal strain in the substrate and the maximum principal strain in the cytoplasm. An analogous ratio was defined relating nuclear strain to substrate strain.

Figure 30: Cell stretching apparatus.

5.2.5 H2B-GFP Transfection

The H2B-GFP plasmid was a generous gift from the laboratory of Geoff Wahl (198) via the Addgene plasmid repository. The supplied stab culture was expanded using conventional techniques and the plasmid was isolated using a Plasmid Maxi kit.
(Qiagen, Germantown, MA). The isolated plasmid was sequenced at the Duke University DNA Analysis Facility to confirm that it was a 100% match to the sequence of the H2B-GFP plasmid. The Nucleofector device (Amaxa, Gaithersburg, MD) was used to electroporate the plasmid into suspended ASCs using a reagent kit supplied by the manufacturer. Cells were incubated in warm calcium-free medium for 10 minutes after electroporation and then transferred to a 225 cm$^2$ tissue culture plastic flask and incubated in expansion media for 24 hours to allow them to recover. The cells were then trypsinized and spread on glass coverslips in 200 μl droplets at a density of 10 000 cells/ml. The coverslips were incubated in expansion media for 24 hours to allow the cells to attach and spread before testing. Cytochalasin D treated cells were incubated with 0.5 μM cytochalasin D in DMEM-F12 for 1 hour immediately before testing. Trichostatin A treated cells were treated with 1μM trichostatin A for 1 hour immediately before testing (82). Testing for each coverslip was concluded less than 30 minutes after the samples were removed from the incubator.

5.2.6 ASC Microscopic Imaging

Images were obtained through an inverted fluorescent microscope (Axiovert 100M, Carl Zeiss) with a C-Apochromat, 63x, water immersion, 1.2-NA objective lens. H2B-GFP fluorescence was excited by an argon-ion laser (488 nm) at 25% power and the stimulated emission was collected through a 505 – 550 nm filter on an 8-bit intensity
Images of either 512 X 512 or 1024 X 1024 pixels (depending on the size of the nucleus) were recorded with a scale length of 0.05 μm per pixel in the imaging plane at vertical increments of 0.15 μm. These intervals were selected to maximize the available resolution after deconvolution according to the recommendations of the deconvolution software manufacturers. The deconvolution software used was SVI Huygens 3D Deconvolution software (Scientific Volume Imaging B. V., Hilversum, the Netherlands), a proprietary 3D deconvolution package designed for confocal and widefield microscopy images. The point spread function used in the deconvolution was acquired on the same scope by using the same settings to image 0.2 μm diameter fluorescent Tetraspeck beads (Invitrogen Corporation, Carlsbad, CA). These images were post-processed to determine the point spread function of the system using the SVI software. Experimental images were deconvolved using this measured point spread function to produce optimal resolution images of the nucleus and genome architecture.

5.2.7 Hyper-osmotic loading of ASCs

A glass coverslip was mounted in a custom-made perfusion chamber and washed three times with iso-osmotic (309 mOsm) DMEM-F12. The chamber was mounted on an automated stage and attached to a pair of perfusion pumps. The locations of three target cells were recorded using the automated stage and each nucleus was imaged in three dimensions. Then, the media was exchanged twice using the
perfusion pumps. For hyper-osmotic tests, the new media was DMEM-F12 with sodium chloride added to increase the osmolarity to 521 mOsm. Three minutes was allowed for the cells to equilibrate to the new solution and then another three dimensional image was taken of each cell.

5.2.8 Nuclear Volume Measurement

H2B-GFP fluorescence creates an image of the nucleus punctuated by voids. These voids were removed using the Image Analysis Toolbox in Matlab (The MathWorks, Inc., Natick, MA) by dilation and filling of the image followed by erosion to reverse the effect of dilation. An isosurface was then determined from the filled-in three dimensional image using a threshold determined automatically using the ISODATA (iterative self organization of data) technique (145).

5.2.9 Chromatin Fraction Measurement

The chromatin fraction was determined by considering the pixels inside the nuclear volume in isolation from the rest of the image and automatically separating them into bright and dark populations using the ISODATA algorithm (145). The chromatin fraction was defined as the number of bright pixels divided by the total number of pixels.
5.2.10 Lacunarity Measurement

Lacunarity gives a measure of the size of holes in an image. The average intensity of all the pixels in the image is defined as the global average. A local average is defined as the average intensity of pixels in a square subregion of the image. This local average is evaluated at every possible location of the square subregion within the image. The lacunarity is the mean difference between the local average and the global average. If the square is much bigger than the feature size in the image, then the local and global average will be close together and the lacunarity is small. If the feature size is on the same order as the size of the square than the local average may be substantially higher or lower than the global average, depending on its location, and the lacunarity value becomes larger (Figure 31). The analysis is confined to the region of the image corresponding to the nucleus by defining a weighting matrix that weights local differences according to whether or not they lie within the nucleus before averaging them (199). This approach was extended to three dimensions and implemented in Matlab. A cube with a side length of 3 pixels was scanned over a three-dimensional image of the nucleus that was downsampled by a factor of 3 in the XY plane. These parameters were chosen because they offered a balance of reasonable computational efficiency and sufficient sensitivity to resolve statistically significant differences.
Figure 31: Lacunarity measures the size of voids in the image. (A) The voids are much smaller than the square that is being scanned across the image so the local average within the square is usually close to the global average and lacunarity is small. (B) In this image the voids are close to the size of the square that is being scanned across the image. In the current location, the mean within the square will be much less than the global mean. In general, the difference between local and global mean will be large so the lacunarity will be large.

5.2.11 Statistical Methods

Significant differences were determined using a Fisher’s Least Squared Difference test (p<0.05). A two-way ANOVA was conducted to ensure that there was significant effect of osmolarity and a significant interaction between treatment and osmolarity before the Fisher’s test was applied. Statistical calculations were made using Statistica (StatSoft, Tulsa, OK).
5.3 Results

5.3.1 Actin disruption does not modulate the osmotic response of the chondrocyte nucleus

Cytochalasin D treatment did not influence the volume response of the chondrocyte nucleus to hyper-osmotic loading. Upon transition from 380 mOsm media to 580 mOsm media, nuclear volume decreased by approximately 26%, regardless of pretreatment with cytochalasin D (Figure 32). A two-way factorial ANOVA revealed a main effect of osmolarity but no main effect of cytochalasin D treatment and no interaction between osmolarity and cytochalasin D treatment, despite the large number of samples (n ≥ 34). The 95% confidence intervals for the untreated and treated hyper-osmotically loaded populations are ± 3% (of initial volume) so any undetected effect of treatment is small. The reduction of volume at high osmolarity was highly significant (p<0.001).
Figure 32: Cytochalasin D treatment does not influence the effect of hyper-osmotic loading on nucleus volume in chondrocytes. The nuclear volume is reduced by approximately 26% in both the untreated and cytochalasin D treated case (n = 34 nuclei per group, error bars represent one s.d., different letters indicate statistically significant difference).

5.3.2 Actin disruption amplifies the osmotic response of the stem cell nucleus

Phalloidin stained images of control ASCs show that the actin cytoskeleton is organized into thick bundles. This organization is absent in cytochalasin D treated cells (Figure 33). The actin cytoskeleton was observed to arch upwards over the mid region of the cell where the nucleus is located (Figure 34 A and B). Long channels occurred in the apical surfaces of some of the nuclei, presumably caused by tension in actin bundles
arching over the nucleus (Figure 34 C and D). These images suggest that there is a tense, organized cap of actin surrounding the nucleus in untreated ASCs.

Figure 33: (A) Phalloidin staining of untreated ASCs shows bundling of contractile actin filaments. (B) Bundles are destroyed after treatment with cytochalasin D (0.5 μM for 1 hour)
Figure 34: Evidence for a perinuclear actin cap in ASCs. (A) Phalloidin staining of actin organization at the base of the cell. Actin fibers are in sharp focus along the cell periphery. However, they are dim and poorly focused in the center of the cell where the nucleus is located. (B) Another confocal cross section of the same cell taken 3.6 μm above the cross section in panel A. At this height, peripheral actin is no longer in focus but sharply focused actin fibers are visible at the center of the cell. Taken together, these images show that actin fibers arch up over the nucleus at the center of the cell to form a perinuclear actin cap. (C) An apical section of a H2B-GFP stained nucleus. In a minority of examples, channels were visible in the fluorescent images of the nucleus, presumably due to tense actin bundles lying on top of it. (D) A surface image of the nucleus in panel C, showing channels made by actin cables lying across the apical surface of the nucleus.

Before osmotic response was considered, the effect of cytochalasin D treatment on nuclear morphology and genome architecture was measured. Lacunarity increased from 0.21 without treatment to 0.25 with treatment (p<0.05), indicating that the chromatin distribution became more punctate, and aspect ratio increased from 0.31 to
0.41 (p<0.05), indicating that the nuclei became more rounded and less elongated.

Nuclear volume and the chromatin fraction were not significantly affected by cytochalasin D treatment. However, both were significantly affected by hyper-osmotic stress and this effect was dependent on pretreatment.

The volume of the ASC nucleus decreases under hyper-osmotic load and this effect is amplified when the cell is pre-treated with cytochalasin D (Figure 35). Chromatin compacts visibly under hyper-osmotic stress (Figure 36). This change in the architecture of the genome is reflected by a decrease in the chromatin fraction (Figure 37) and an increase in the lacunarity (Figure 38). Both effects are amplified by pretreatment with cytochalasin D.
Figure 35: Volume change in ASC nuclei after media exchange. $n \geq 17$ nuclei per group, error bars represent one standard deviation, bars labeled with different letters are significantly different ($p<0.05$).

Figure 36: Image of ASC stained with H2B-GFP (A) before and (B) after hyper-osmotic loading. Local variations in intensity in panel A are more pronounced at the same location in panel B, indicating chromatin compaction.
Figure 37: Chromatin fraction change in ASC nuclei after media exchange. n ≥ 17 nuclei per group, error bars = one standard deviation, groups labeled with different letters are significantly different (p<0.05).
5.3.3 Acetylase inhibition does not modulate the osmotic response of the chondrocyte nucleus

Trichostatin A treatment did not influence the volume response of the chondrocyte nucleus to hyper-osmotic loading. Upon transition from 380 mOsm media to 549 mOsm media, nuclear volume decreased by approximately 16%, regardless of pretreatment with trichostatin A (Figure 39). A two-way factorial ANOVA revealed a main effect of osmolarity but no main effect of trichostatin A treatment and no interaction between osmolarity and trichostatin A treatment, despite the large number of
samples used (n ≥ 69). The 95% confidence intervals for the untreated and treated hyper-osmotically loaded populations are ± 1% (of initial volume) so any undetected change due to treatment is negligibly small. The reduction of volume at high osmolarity was highly significant (p<0.001).

Figure 39: The effect of hyper-osmotic loading on nucleus volume in chondrocytes is not influenced by trichostatin A treatment. The nuclear volume is reduced by approximately 16% in both the untreated and cytochalasin D treated case (n ≥ 69 nuclei per group, error bars represent one s.d., different letters indicates statistically significant difference).

5.3.4 Acetylase inhibition decouples the stem cell from its substrate

The coupling between the ASC and its substrate was quantified in cell stretching experiments using the cytoplasmic strain transfer ratio (STRc). A value of 1 indicates
perfect coupling while value of 0 indicates complete decoupling. Similarly, the nuclear strain transfer ratio quantifies how coupled nuclear strains are to substrate strains.

There was a significant main effect of treatment and sub cellular location (cytoplasm vs. nucleus) on strain transfer ratio (two-way ANOVA, p<0.05). TRC fell from 0.83 to 0.69 after trichostatin A treatment (Fisher’s LSD, p < 0.05), indicating that the cell was less coupled to its substrate. This most likely reduced the strain transfer ratio of the nucleus, which fell from 0.53 to 0.44 (Fisher’s LSD, p = 0.07). The nucleus had a lower strain transfer ratio than the nucleus in both the treated and untreated cases, indicating that it is stiffer than the cytoplasm (Fisher’s LSD, p < 0.05).

Figure 40: Coupling between the ASC and the substrate is reduced by trichostatin A treatment. n≥36, *=p<0.05, error bars = 1 standard deviation.
5.3.5 Acetylase inhibition amplifies the osmotic response of the stem cell nucleus

Iso-osmotic controls were omitted in these experiments since it is clear from prior experiments that the stem cell nucleus responds to hyper-osmotic stress. Trichostatin A treatment did not change the initial volume, chromatin fraction or lacunarity in a statistically significant manner although there was a change in the aspect ratio that was almost statistically significant. The initial mean value of the aspect ratio in the untreated condition was 0.24 whereas in the treated condition it was 0.34 (p = 0.067), suggesting that the nucleus becomes taller and rounder after trichostatin A treatment. Trichostatin A did diminish the osmotically-induced change in the chromatin fraction, indicating that it made chromatin less sensitive to compaction by osmotic stress (Figure 41A). The treatment also diminished the osmotically-induced change in the lacunarity, although this trend did not meet the criterion for statistical significance due to the presence of three outlying data points. When Chauvenet’s criterion was applied (200), these outliers were excluded and the treatment effect became highly significant (p < 0.01). Finally, the osmotically-induced change in nuclear volume increased after trichostatin A treatment (Figure 41B).
Figure 41: Effect of Trichostatin A on the hyper-osmotic response of ASCs. (A) The hyper-osmotically induced change in chromatin fraction is diminished by pretreatment with Trichostatin A. (B) The hyper-osmotically induced change in volume is amplified by pre-treatment with Trichostatin A. n ≥ 17 cells per group, error bars = one standard deviation, * indicates p < 0.05.

5.4 Discussion

In cells with organized actin cytoskeletons, the nucleus is encased in a perinuclear cap of actin fibers (197). In this study, we treated ASCs, which have highly organized cytoskeletons (Figure 33), with agents that either disrupted the actin cytoskeleton or decoupled it from its substrate. These treatments amplified the contraction of the nucleus under hyper-osmotic stress (Figure 35, Figure 41B). Similar treatments had no effect on osmotically-induced contraction of the nucleus in chondrocytes (Figure 32, Figure 39), which do not have a perinuclear actin cap (9). Taken together, these findings imply that the perinuclear actin cap constrains osmotically induced contraction of the nucleus. It follows that perinuclear actin also
diminishes osmotically induced acceleration of nucleocytoplasmic transport and therefore diminishes any downstream mechanotransductive signaling this may cause.

It is possible that the absence of perinuclear actin in the chondrocyte is a specific adaptation to allow the nucleus to contract under hyper-osmotic stress. This implies that the development of an organized, bundled actin cytoskeleton in a chondrocyte would blunt its osmotic sensitivity. This may be an unexplored pathology of osteoarthritis (OA). Several investigators report a correlation between increased actin organization and OA. TGF-α, which is expressed in the early stages of OA, causes increased stress fiber formation in chondrocytes (201, 202). Chondrocytes from osteoarthritic cartilage are stiffer than normal chondrocytes, suggesting greater organization of F-actin (203, 204). Actin organization increases as chondrocytes dedifferentiate in monolayer culture, a process that mimics osteoarthritic changes. This increased actin organization correlates with the release of a mediator of cartilage catabolism called cathepsin B (205).

In light of this, a rational therapeutic strategy would be to treat with an agent that reduces the actin constraint on the nucleus in osteoarthritic chondrocytes. Cytochalasin D is an impractical candidate for this approach because it is toxic. However, trichostatin A has already shown therapeutic potential. It inhibits cartilage degradation in a ligament transection model of osteoarthritis in the rabbit (206). It also reduces production of nitric oxide and prostaglandin E2 in healthy human chondrocytes.
stimulated with IL-1 (77). Both trichostatin A and another HDAC inhibitor called sodium butyrate inhibited cartilage degradation in an IL-1 treated cartilage explant assay (207). Trichostatin A also mitigated inflammation in a collagen antibody model of rheumatoid arthritis in the mouse (78). The proposed constraint-release effect has the potential to be a novel mechanism of action for this emerging therapeutic agent.

The mechanism by which trichostatin A mechanically decouples the cell from its substrate is unknown and must be studied further if the therapeutic and pathologic hypotheses developed above are to be thoroughly addressed. Trichostatin A treatment increases total focal adhesion area in NIH 3T3 cells and mouse embryonic fibroblasts (208). This is surprising because it suggests that substrate coupling will be increased but, in this study, it is actually decreased (Figure 40). However, there are multiple processes that may affect coupling in addition to focal adhesion expansion, many of which are influenced by trichostatin A. HDAC6, a substrate of trichostatin A, promotes cell motility through action on the microtubule (83) and actin cytoskeletons (84). HDAC6 also encourages the formation of actin-rich ruffles at the leading edge of the cell (209). It is worth noting that focal adhesions can move within cells (210) and that such motion has been observed in cells subjected to cyclical stretch (211). The issue of mechanical decoupling of cells treated with trichostatin A has ramifications beyond osteoarthritis. Trichostatin A is in clinical trials as a cancer therapy and is thought to exercise its anti-cancer effect in part through inhibition of cell attachment and migration.
An explanation of how it decouples the cell from its substrate would be of interest to this community.

Cytochalasin D treatment amplified compaction of chromatin by hyper-osmotic stress (Figure 37, Figure 38) while trichostatin A diminished it (Figure 41). Cytochalasin D reduces histone acetylation (100) while trichostatin A increases it (75). Taken together, these results suggest that chromatin becomes more resistant to osmotically induced compaction as it becomes more acetylated. This is consistent with the observation that chromatin assumes a more extended conformation when treated with trichostatin A at constant osmolality (82). Condensation of chromatin blocks transcription factors from accessing the genes it encodes so hyperacetylation might be a viable mechanism for a cell to maintain chromatin in an accessible state under hyper-osmotic stress.

Alternatively, hypoacetylated chromatin could function as an osmotically sensitive sensor if appropriately configured. Osmotic loading of chondrocytes with chromatin labeling would provide insight into these possibilities. A DNA dye such as Hoechst might be employed in place of H2B-GFP transfection in this case since the plasmid is human and the preferred chondrocyte source is porcine.

In summary, treatments that reduce the constraining influence of the actin cytoskeleton increase the osmotic sensitivity of the nucleus in ASCs, which have an organized perinuclear actin cap. These treatments have no effect in chondrocytes, which have no such perinuclear actin cap. This suggests that perinuclear actin has the capacity
to constrain contraction of the nucleus under hyper-osmotic stress. This contraction may play a role in the transduction of compressive load into biosynthetic activity. If this is true, the increased actin organization observed in osteoarthritic chondrocytes becomes pathologically significant and treatments that reduce the perinuclear constraint may restore function. Trichostatin A is an example of a treatment that sensitizes the nucleus to osmotic stress without having devastating effects that preclude clinical use. In fact, it has already shown therapeutic promise in numerous studies. This study demonstrates that the methods presented can detect subtle changes in the osmotic sensitivity of genome architecture and nucleus morphology. The same methods could be applied to measure the loss, if any, of osmotic sensitivity in the nucleus as chondrocytes dedifferentiate in monolayer culture. Restoration of lost sensitivity by treatment with trichostatin A or a similar agent would be a first step in demonstrating the therapeutic strategy postulated here.
6 Conclusions

Mechanotransduction is the process by which cartilage loading stimulates the chondrocyte to synthesize and degrade extra-cellular matrix and sustain tissue homeostasis. In osteoarthritis, this process fails and chondrocytes degrade cartilage instead of maintaining it. The goal of therapy is to shift the chondrocyte from a degradative mode back to a homeostatic mode. Figure 42A shows a schematic of the situation as it was perceived at the start of this study. The blue arrows denote physical processes and the red arrows denote biological processes. It was assumed that physical signals move through a cascade of different phenomena until they finally elicit a biological response that results in loading-stimulated homeostasis. It was also assumed that once a biological response has been elicited, the physiology of homeostasis, degradation and therapy are exclusively biological and physical processes play no further role.
Figure 42: Schematic of mechanotransduction in health, sickness and treatment. Blue arrows represent physical processes and red arrows represent biological processes. (A) Cartilage compression triggers a series of physical changes in the chondrocyte environment. In healthy tissue, the chondrocyte responds with a combination of catabolic and anabolic activities that are balanced to achieve homeostasis. In osteoarthritic cartilage, these activities move out of balance and net degradation results. Therapeutic interventions aim to restore this lost balance. (B) Close-up of intracellular observations in this research that are relevant to mechanotransduction. Joint loading causes extracellular hyper-osmotic stress. This shrinks and wrinkles the nucleus, accelerating nucleocytoplasmic transport. However, this response is inhibited if the nucleus is constrained by perinuclear actin. This inhibition can be reversed by removing the actin constraint.

The arrow labeled ‘Physical signals’ in panel A represents a collection of processes that have been well-studied. Joint loading causes cartilage compression, which in turn leads to fluid pressurization, hyper-osmotic stress, streaming potentials and fluid flow in the chondrocyte environment. This study focused on hyper-osmotic stress because it has been shown to elicit a robust biological response relevant to
cartilage homeostasis (5-7). The initial goal of this research was to determine the physical processes that connect extracellular hyper-osmotic stress to the genome. We have learned that the chondrocyte nucleus shrinks and wrinkles under hyper-osmotic stress (Figure 8). It shrinks because hyper-osmotic stress draws water out of the cell, driving up the intracellular macromolecule concentration. Elevated macromolecule concentration creates osmotic pressure drawing water out of the nucleus (Figure 12) because macromolecules partition unevenly between the cytoplasm and the nucleus. The nucleus wrinkles because it behaves as a substrate/film structure and buckles as the substrate shrinks while the film tends to maintain constant surface area (Chapter 3). In addition, the chromatin within the nucleus condenses under extracellular hyper-osmotic stress (Figure 37, Figure 38 and Figure 41 A).

Surprisingly, we have also observed a collection of physical phenomena (see blue arrows below nucleus in Figure 42 B) that may influence homeostasis, degradation and therapy, all of which were initially assumed to be exclusively biological processes. The shrunken nucleus has less volume to dilute molecules crossing the nuclear envelope but the surface area remains constant so flux is not reduced. The result is an effective increase in nucleocytoplasmic transport (Figure 28) because concentrations rises and fall more quickly at the same level of flux in the smaller nucleus. By the same token, mRNAs produced inside the nucleus are closer to the nuclear envelope and so diffuse out into the cytoplasm more quickly. Diffusion in the nucleus remains approximately
constant despite the increased crowding in the cell (Figure 27), presumably because the channels in the interchromatin domain enlarge even as the chromatin around them condenses (Figure 38). Assuming biochemical processes in the cell remain constant, this acceleration of nucleocytoplasmic exchange may cause an acceleration of metabolism. This would be a simple and robust mechanism for matching metabolism to joint loading. The upregulation of metabolism would be transient because the chondrocyte has the capacity to regulate its volume and in doing so, restore the nucleus to its original configuration. If this model is valid, shrinkage of the nucleus under hyper-osmotic stress is essential to normal cartilage function and that any change that inhibited shrinkage of the nucleus would inhibit mechanotransduction. Perinuclear actin organization may have such an inhibitory effect. Nuclei surrounded by highly organized actin cytoskeletons shrink more under hyper-osmotic stress after actin disruption (Figure 35) but in chondrocytes, which lack perinuclear actin, this effect is not seen (Figure 32). This suggests that an agent that releases the nucleus from pathologic actin constraints would have a therapeutic effect. Trichostatin A decouples the cell from its environment (Figure 40), allowing the nucleus to contract more under hyper-osmotic stress (Figure 41B). Trichostatin A has potential as a clinical treatment because it is non-toxic. In fact, several other studies have shown that it has therapeutic effect in osteoarthritis.
The model described above states that biosynthesis can be synchronized with joint loading by a series of physical processes and need not rely on activation and deactivation of a biological process. This model is novel but it must be tested further and shown to accurately predict behavior before it can be mined for novel therapeutic strategies. The critical missing piece of evidence at present is the connection between accelerated nucleocytoplasmic transport and accelerated biosynthesis. Design of an experiment addressing this is frustrated by correlation between multiple phenomena induced by hyper-osmotic stress. Upregulation of gene expression and protein synthesis associated with cartilage homeostasis by hyper-osmotic stress has already been shown. However, it is difficult to demonstrate that this is due to shrinkage of the nucleus and not, for example, calcium signaling. One plausible solution to this is to take advantage of what has been learned about how to inhibit and amplify the shrinkage of the nucleus under hyper-osmotic stress. This knowledge can be used to create a group of experimental scenarios in which the nucleus shrinks to different degrees under osmotic stress. Correlation of biosynthesis with nuclear shrinkage across these different scenarios would support this connection in the model. This could be accomplished by repeating the study described in chapter 5, this time comparing primary chondrocytes with passaged chondrocytes instead of ASCs. Chondrocytes develop organized actin cytoskeletons as they spread and dedifferentiate in monolayer culture. The first hypothesis would be that nuclear contraction under hyper-osmotic stress dissipates with
increasing actin organization and is restored by trichostatin A and cytochalasin D, which disrupt the actin constraint in different ways. The second hypothesis would be that, in a parallel FRAP experiment, hyper-osmotic acceleration of nucleocytoplasmic transport would be lost and restored in the same scenarios. If these two hypotheses were proven, the last step would be to show in another parallel experiment that hyper-osmotically induced biosynthesis is lost and restored under the conditions predicted by the model.

If this model is found to be valid, it supports the hypothesis that trichostatin A could have a therapeutic effect in osteoarthritis due to its capacity to decouple the nucleus from the extracellular matrix. Trichostatin A is a potent pan-inhibitor of the HDAC class of enzymes. A more specific inhibitor would be preferred as it would reduce the likelihood of off-target effects. However, it is impossible to identify a more specific inhibitor until more is known about the mechanism by which trichostatin A decouples the nucleus from the substrate. Inhibition of HDAC 6 is likely to play an important role. HDAC 6 is an exclusively cytoplasmic HDAC that influences microtubules (83), actin (84) and focal adhesions(208). There are specific inhibitors of HDAC 6, the most widely studied of which is tubacin (215). Total internal reflectance fluorescence (TIRF) microscopy of focal adhesions in passaged chondrocytes after treatment with tubacin or trichostatin A would shed light on the mechanism of the observed decoupling and generate hypotheses about whether it can be reproduced in vivo.
References


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